

**SORPTIVE MINERALS
INSTITUTE
APPENDIX C**

MSHA-2023-001



SORPTIVE MINERALS INSTITUTE

February 11, 2014

Via electronic submission

OSHA Docket Office
Docket No. OSHA-2010-0034,
U.S. Department of Labor, Room N-2625,
200 Constitution Avenue, NW
Washington, DC 20210

Docket Number: OSHA-2010-0034

Re: Comment of the Sorptive Minerals Institute (SMI); Occupational Safety and Health Administration's Proposed Rule on Occupational Exposure to Respirable Crystalline Silica

We are writing on behalf of the Sorptive Minerals Institute ("SMI"), a Washington, DC-based trade association representing the manufacturers and marketers of absorbent clay products. We are pleased to have the opportunity to comment on the Occupational Safety and Health Administration's (OSHA or the Agency) Proposed Rule on Occupational Exposure to Respirable Crystalline Silica, 78 Federal Register 56274 -56504 (September 12, 2013) (Proposed Regulation or Proposal). We hope the Agency will thoughtfully consider these Comments, and the scientific studies and literature provided and referenced herein.

Sorptive clays mined and processed by SMI members are a specific subset of clays that formed tens of millions of years ago. The sorptive clays that are found in the United States, which exist in only a few geologic deposits in the country, have specific properties that render them extremely useful in a wide range of consumer products and commercial and industrial applications, including, for instance, clay-based pet litter, cosmetics, pharmaceuticals, animal feeds, specialized drilling muds and fluids used in oil, gas and water well drilling, and environmental sealants for landfills and sewage lagoons.

The mining and processing (consisting of drying and sizing) of sorptive clays falls within the regulatory responsibility of the Mining Safety and Health Administration (MSHA). MSHA, therefore, has regulatory authority over SMI mining operations and much of the industry's associated processing facilities. However, pursuant to a 1979 Interagency Agreement between MSHA and OSHA,¹ OSHA has regulatory authority and jurisdiction over a number of SMI member companies' processing facilities, such as blending, product assembly, and packaging facilities. Thus, while OSHA's Proposed Regulation would not apply to most SMI member

¹ See Interagency Agreement Between the Mine Safety and Health Administration, U.S. Department of Labor and the Occupational Safety and Health Administration, U.S. Department of Labor (March 29, 1979) (available at https://www.osha.gov/pls/oshaweb/owadisp.show_document?p_table=MOU&p_id=222).

operations, absent an explicit exclusion, it would apply to numerous SMI facilities. It is for this reason, as well as because MSHA will likely give great deference to OSHA's Risk Assessment and Health Effects Analysis and its regulatory conclusions, that SMI wishes to make OSHA aware of the most current scientific assessment of the effects of exposure to silica in sorptive clay materials. Additionally, SMI believes it is important to provide comment on this Proposal because the Global Harmonization Standard (GHS) adopted in OSHA's Hazard Communication Standard (29 C.F.R. §1910.1200) will require labeling based on the final silica standard.

I. INTRODUCTION

Because sorptive mineral clays contain silica (in both the crystalline form addressed in this Proposed Regulation as well as in amorphous forms that OSHA recognizes raise no industrial health issues) SMI has, since its inception, concerned itself with the question of health effects of exposures to its products. Founded in 1970, SMI has served as a leader in scientific research and the leading storehouse of scientific work, information, and studies relevant to issues associated with the use and handling of sorptive clays. Through its Technical Committee, SMI has been heavily involved in crystalline silica research for over 28 years. During that time, SMI has produced a large body of research that has been presented at scientific conferences in the United States, Europe, and South Africa, and has been published in peer-reviewed scientific journals. SMI has worked cooperatively with regulatory bodies in California and other jurisdictions to assist these agencies in developing an understanding of the relevant scientific data necessary to assess inhalation exposure issues related to sorptive clay products. In California, for instance, this work led to the issuance of a Safe Use Determination by the California Environmental Protection Agency's Office of Health Hazard Assessment for crystalline silica in sorptive mineral-based pet litter.²

While various forms of silica can be found in sorptive clay materials mined and processed by SMI members, *in all cases*, the silica exists in either an amorphous form (*ie.* opal), not proposed to be regulated by OSHA, or, in a crystalline form as geologically ancient quartz characterized by amorphous occluded surfaces. It does not occur either as pure quartz or quartz with freshly fractured surfaces.

OSHA's proposed Health Effects Analysis and Risk Assessment recognizes that various physical factors including, specifically, the aged nature of quartz and the occlusion of the surface of quartz, can and do play an important role in the toxicological potential of silica, and therefore to the risk to exposed workers. *See* 78 Fed. Reg. 56311. The Agency further recognizes that NIOSH concluded in 2002 that "further research is needed to associate specific surface characteristics that can affect toxicity with specific occupational exposure situations and consequent health risks to workers." *Id.* Notwithstanding these findings, and its explicit conclusion that "there is considerable evidence to support the hypothesis that surface activity of

² While California's Safe Use Determination relates to consumer exposures, the work supporting this determination is relevant to OSHA's understanding of the nature of exposures to silica in sorptive clays. Accordingly, we have included the Safe Use Determination and supporting materials herein. *See* http://www.oehha.org/prop65/CRNR_notices/safe_use/sud1.html (Attachment 1).

crystalline silica particles plays an important role in producing disease” OSHA nevertheless now plans to conclude that there is no basis for distinguishing between different types of crystalline silica exposures for purposes of setting regulatory limits. This conclusion rests on the fact that the Agency is aware of no precise quantitative assessment distinguishing the health effects for “aged quartz.”

SMI disagrees with OSHA’s conclusion that the lack of a precise quantitative assessment provides a reason for regulating geologically ancient quartz found in sorptive clays. A large body of evidence exists indicating that the physical characteristic of occlusion mediates the ability of geologically ancient quartz to react to its environment and mediates the potential for geologically ancient quartz to pose a health risk. In these Comments, SMI provides information on the nature and types of silica found in sorptive clays that demonstrates why exposures to these clays should be treated differently from silica exposures known to cause deleterious human health effects. We alert OSHA to published research in this area that was not considered when OSHA conducted its Health Effects Analysis and Risk Assessment. This recent research supports distinguishing between silica exposures, just as OSHA does for amorphous forms of silica, and warrants a revision to OSHA’s Health Effects Analysis recognizing that the geologically ancient silica used and maintained in that form throughout the processing of sorptive clay minerals does not pose the same hazard as crystalline silica that is the focus of OSHA’s regulation.

The following Comments provide a detailed explanation supporting SMI’s position.

II. OSHA’s Risk Assessment Should More Clearly Recognize The Distinction Between Silica In Sorptive Clays And Other Forms Of Silica Associated With Deleterious Health Impacts (Cristobalite; Tridymite; Freshly Fractured Quartz).

A. The Nature and Type of Silica in Sorptive Clays.

The sorptive clays mined and processed by SMI members were formed tens of millions of years ago, and are found in only a few clay deposits in the United States. None of these clays have been exposed to temperatures higher than about 150 degrees Celsius during or since their formation. Typical of these deposits is bentonite, consisting predominately of smectite minerals. A complete description of the geology and mineralogy of these deposits, along with a map showing their locations and a geologic time chart may be found in Section 5 of Attachment 1.

A portion of the silica contained within sorptive clays exists as opal, which is a non-crystalline silica hydrate. Opal does not have the organized structure or long-range periodicity required to be crystalline even though it may have short range domains of increased order that may resemble crystalline forms.³ Opal, along with other forms of amorphous, or non-crystalline silica, that are

³ SMI is concerned that, without proper analysis, Opal-CT, a form of non-crystalline silica common to sorptive clays, could be confused with cristobalite because of analytical difficulties. Given the high temperatures required to create cristobalite, this form of crystalline silica is not found with the quartz in clays created at lower temperatures. SMI therefore requests that OSHA explicitly recognize the analytical approach necessary to differentiate Opal-CT

prevalent in sorptive clays, are not covered by OSHA's Proposed Regulation.⁴ The exclusion of amorphous silica from OSHA's Proposal is consistent with the long recognized distinction between it and various crystalline forms of silica, and the absence of a health risk associated with exposure to amorphous silica.⁵

Sorptive clays do, however, naturally contain some amount of quartz, a crystalline form of silica which, when of respirable size, is subject to the Proposed Regulation. While a significant fraction of this quartz is non-respirable, a portion is of respirable size. It is OSHA's assessment of the health risk of this respirable quartz that is of concern to SMI. The concern centers on the fact that OSHA's Proposal and Risk Assessment do not clearly recognize the significance of the fact that the respirable quartz present in sorptive clays exists in a distinct occluded form that significantly mitigates the potential for deleterious health effects. In order for OSHA to conduct a meaningful and proper risk assessment of the potential health effects associated with exposure to silica present in sorptive clays, it is necessary to understand the unique chemical and physical characteristics of the silica present in these clays and to evaluate the most current scientific literature on this subject.

In fact, as these Comments describe more fully below, and consistent with OSHA's current regulatory differentiation of amorphous silica from crystalline silica, the available scientific data also support a regulatory distinction for the occluded quartz (a form of crystalline silica) found in sorptive mineral clays.

B. Quartz In Sorptive Clays Is Occluded In Form, And, Therefore Exists In An Inactive State With Limited Availability To Its Environment.

Crystalline silica is formed in clay deposits in a number of ways. One way is when metamorphic or igneous rocks containing quartz crystals are weathered, *in situ*, into clay deposits. Such deposits can retain the quartz crystals in the clay matrix and are termed "residual deposits." A second way occurs when weathering products containing quartz crystals are carried by rivers and streams and deposited at downstream locations. These deposits are called "sedimentary

from crystalline silica in its final rule. NIOSH procedures 7500 and 7601 must be conducted sequentially to differentiate opal-CT and cristobalite, and, when done so, readily recognize Opal-CT as an amorphous silica. Few analytical laboratories are aware of the ambiguity between opal and cristobalite and routinely fail to distinguish between these two silica forms.

⁴ As OSHA recognized in its Proposed Regulation, silica is one of the earth's most plentiful compounds. As a result, it is encountered routinely by all humans without harm. Accordingly, OSHA has not sought through its Proposed Regulation to regulate all forms of silica, or all instances where it is used in industry. Rather, OSHA seeks to amend its existing standards for occupational exposure to "respirable crystalline silica" The proposed regulation defines silica as "a compound composed of the elements silicon and oxygen (chemical formula SiO₂)" and limits itself to regulating crystalline silica, even though it expressly recognizes that silica "exists in crystalline and amorphous states." O. Creutzenberg, *et. al.*, Inflammatory Effects of Quartz Samples after Intratracheal installation in Rats, Presented at 2003 Society of Occupational Toxicologist Meeting.

⁵ In excluding amorphous silica, OSHA recognizes that not all forms of silica require worker protection, and that it is possible to regulate certain forms of silica without having to regulate all forms.

deposits.” A third way crystalline silica can occur in clay happens when volcanic ash is dissolved by salty water, either at ambient or elevated (hydrothermal) temperatures to form a saturated solution from which clay and other minerals, including quartz, precipitate. This type of *in situ* precipitated crystalline silica is termed “authigenic.”

Regardless of the variety of quartz contained in sorptive clays, it exists in a form notably different than other forms of silica that OSHA is proposing to regulate. This quartz has been aged over millions of years and has had eons to react to a variety of other materials in its environment, resulting in the development of geologically ancient, occluded surfaces that mediate the quartz (SiO₂) interaction with its environment.

For example, bentonite, the most common type of material mined and processed in the sorptive clay industry, is a clay-rich rock produced when rhyolitic volcanic ash dissolves in brackish or salt water and then precipitates out of solution (settling to the bottom of an ancient sea over long periods of time), and is composed predominately of the clay mineral montmorillonite along with a variety of other minerals including feldspar, mica, gypsum, opal (an amorphous form of silica) and quartz. All of these minerals were formed in place and at the same time (authigenically). The result of this process is a complex mixture of minerals which are in intimate contact with each other since they were formed during the early to mid-Cretaceous period about 90 to 110 million years ago. Bentonite deposits also can be eroded and carried long distances by rivers only to be deposited in another location. Deposits of this type from the Paleocene epoch created 56 to 65 million years ago contain commercial amounts of bentonite clays.

Quartz particles in bentonitic and other sorptive clay deposits have had millions of years to react with and come to equilibrium in their environments, and to develop surface occlusions consisting of “adventitious” ions such as aluminum, iron, sodium and others. The presence of these ions changes the surface of the particles so that it no longer has the SiO₂ structure that is integral to the quartz grain and is not itself quartz. In addition, clay also may coat the surface of these quartz particles. Even when the particles are separated from the clay matrix in which they were created or resided, they continue to retain their occlusions and clay coatings. These quartz particles and their surfaces are distinctly different from those forms of quartz that are known to cause human health problems.

An important study of the surface characteristics of silica in sorptive industry clays was published by Wendlandt *et al.* 2007. This study, which was not cited by OSHA, provides an important characterization of the crystalline silica found in sorptive clays. In this study the coatings of hundreds of individual quartz grains of different sizes and origins, all taken from bentonite, were meticulously studied. Findings from this study show:

- Montmorillonite clay surface coatings are ubiquitously present;
- The coatings are resistant to removal by using dispersants and acid;
- Montmorillonite coatings cover all external surfaces of quartz grains regardless of the size of the grain (2-38 µm);
- Processing appears to have no apparent effect on the quartz surface coatings.

These authors conclude that the presence of montmorillonite coatings has the potential to mitigate quartz toxicity in the lung.⁶

C. Processing Conducted In The Sorptive Clay Industry Does Not Alter The Occluded Nature Of The Quartz.

Certain types of manufacturing or processing operations, primarily high heat calcination and extreme crushing or grinding, can fundamentally change the nature of silica. For example, when either amorphous silica (unregulated) or ancient quartz (regulated), is heated to a sufficiently high temperature (in the range of 1470 degrees Celsius) it can convert to cristobalite, a biologically aggressive form of crystalline silica. Further, when quartz is crushed, reactive surfaces can be created, leading to significantly higher bioactivity.

OSHA has correctly identified in its Initial Regulatory Feasibility Analysis those “clay” industries where these types of processes occur: structural clay products; concrete products; glass products; pottery products. OSHA’s analysis in fact identifies typical operations conducted in these industries that can have this effect on silica, such as dry casting methods to form bricks and shapes; finishing cast shapes with grinders and saws; and charging or tending melting furnaces to form ceramic fibers.

The sorptive clay industry neither conducts operations like those identified by OSHA, nor does it otherwise process its clay materials in ways that alter the nature of the occluded quartz or amorphous silica found in these clays. In fact, sorptive clays are not suited for the types of manipulation that occur in the clay industries identified by OSHA.⁷

Sorptive clay products produced by SMI member companies undergo only simple processing steps to generate a finished product. The manufacturing process essentially consists of: (1) low temperature drying of the clay to remove excess water where the temperature of the clay does not exceed about 250 degrees Fahrenheit; and (2) simple product sizing. Accordingly, typical mining and processing of sorptive clays contrasts significantly with the more extreme processing of other clays and silica-containing materials in other industries, leaving the crystalline silica in sorptive clays in the same geologically ancient form that it has existed for eons.

⁶ In light of the similar way in which they formed, other sorptive clays, such as palygorskite (attapulgite) and certain opaline shales, are likely to have similar occlusions, and, therefore should be treated similarly from a regulatory perspective.

⁷ If sorptive clay minerals were to undergo high temperature heating, such as ceramic fusion processes, for example, the absorption and adsorption properties that render these clays so useful in their end product applications would be destroyed.

III. The Best Available Scientific Literature Shows Reduced Risk From Exposure To Occluded Quartz Found In Sorptive Clays And Demonstrates That Occluded Quartz Does Not Pose A Risk Similar To That Posed by Freshly Fractured Quartz.

OSHA correctly recognizes that the toxicity of crystalline silica is affected by its surface activity. In fact, OSHA's summary of its Health Effects Analysis and preliminary Quantitative Risk Assessment highlights extensive research showing that occluded and aged quartz altered silica toxicity and reduced disease risk. A definition of the term "aged quartz" is not found in OSHA's preamble to the proposed rule, however, in its 2010 Occupational Exposure to Respirable Crystalline Silica -- Review of Health Effects Literature and Preliminary Quantitative Risk Assessment (https://www.osha.gov/silica/Combined_Background.pdf) OSHA defines the difference between "freshly fractured" and "aged" silica as follows: "When crystalline silica is subjected to high energy forces, as occurs in work processes such as abrasive blasting, rock drilling, tunneling, stone carving and the production of silica flour, it is said to be "freshly fractured." Silica that is not fractured by the work process is said to be "aged." OSHA does not reference this definition when evaluating the physical factors that may influence the toxicity of crystalline silica (*see* 78Fed. Reg. 56310) in the preamble, however, rather, it cites three animal studies (Porter, 2005; Shoemaker 1995; and Vallyathan, 1995) which compare the toxicity of respirable, freshly fractured silica to that of respirable "aged" silica (i.e., silica that has been aged for 2 months). These studies uniformly state that the freshly fractured silica has significantly greater toxicity than the aged silica even though it had only been aged for 60 days. OSHA uses these studies to establish that aged silica still retains significant toxicity, even though it is at a lower level. In contrast to the aged silica used in these studies, the geologically ancient occluded quartz found in sorptive clays, is tens of millions of years old and has surfaces that have been in equilibrium with their environment throughout that time. Any reliance by OSHA on these animal studies to show that truly "aged" silica (silica that is geologically ancient and has not been fractured by the work process) presents a health hazard is unsubstantiated and unjustified.

Nowhere in the Proposed Rule does the Agency address exposures or risk in the sorptive minerals industry. The Agency's primary reference for clay in its Proposed Regulation is the Miller/Soutar and Love studies of brick making and pottery making operations. Both these studies showed lower than expected cases of silicosis, supporting the concept that silicon from clays has a different risk profile than other forms of silica in non-clay materials. However, brick and pottery clays are not representative of clay products produced by the sorptive minerals industry, which are not heated to high temperatures or fractured. As discussed below, the data that exists for SMI's industry does not support an association for lung cancer or silicosis with exposure. We urge OSHA to add additional discussion regarding silica exposures in the sorptive minerals industry, as summarized in this Comment, and to distinguish silica exposures in the sorptive minerals industry from other industries which have freshly fractured crystalline silica exposure, as well as industries that use and manipulate clays in a manner such as in brick and pottery making.

As recognized in OSHA's Risk Assessment, there is little doubt that the level and type of occlusion is of critical importance to assessing the toxicity of the material. Studies of silica have shown that activity is significantly diminished by aluminosilicates, carbon compounds, and certain metal ions that occlude the surface of the quartz particles changing their biological activity. See 78 Fed. Reg. 56310.

Miles, *et al.* (2008) conducted a robust characterization of the distinguishable physicochemical properties of freshly fractured quartz and quartz with occluded surfaces, which explains the differences in the toxicological response reported between these materials, as discussed further below, in Creutzenberg *et al.* (2008). The Miles 2008 study⁸ and Creutzenberg *et al.* (2008)⁹ studies, published together, form a unique and robust characterization of the quartz samples from bentonite clay and the toxicological potential of these materials. (Attachments 2 and 3) We request that the Agency assess and consider this work in preparing a final Health Effects Analysis and Risk Assessment and include them in its preamble to the final rule.

A. Relevant *In Vitro* And Animal Studies.

A number of important studies for assessing the health effects of exposure to occluded quartz in the sorptive materials industry were not considered by OSHA. One such study is Geh, *et al.* (2006), which was not cited in OSHA's preliminary Risk Assessment. In this study, human lung fibroblasts were exposed *in vitro* to relatively high concentrations of bentonite containing varying levels of quartz and with difference in the presence and types of transition metals. These authors found only very low levels of genotoxicity as measured by micronucleus assays. Untreated samples with less than 1 percent quartz showed no increase in micronucleus formation. A copy of Geh, *et al.* is attached (Attachment 4).

Consistent with the findings of Geh are two recent animal studies by Creutzenberg *et al.* (2003; 2008) (Attachments 5 and 3) that also have not been cited by OSHA in its Risk Assessment. These studies found that quartz with occluded surfaces was substantially less toxic than freshly ground crystalline silica in rats after intratracheal instillation with follow-up for up to 90 days. In these studies, significant effort was made to extract quartz from a bentonite clay without altering the occluded surface of the quartz particles (quartz isolate) by grinding or crushing. This occluded quartz was used to assess its impact on lung tissue, as compared to freshly fractured quartz. These single high-dose studies provide sound evidence that cytotoxicity and inflammation were significantly less severe in animals dosed with the occluded quartz isolate as compared to fresh crystalline silica.

Specifically, the crushed crystalline silica positive control (DQ-12) induced a persistent inflammatory responses and significant tissue damage, but the response to the occluded quartz

⁸ Miles *et al.* (2008). Physicochemical and Mineralogical Characterization of Test Materials used in 28-Day and 90-Day Intratracheal Instillation Toxicology Studies in Rats. *Inhalation Toxicology*, 20:981–993.

⁹ Creutzenberg *et al.* (2008). Toxicity of a Quartz with Occluded Surfaces a 90-Day Intratracheal Instillation Study in Rats. *Inhalation Toxicology*, 20:995–1008.

isolate, at the same dose, was modest and not progressive. Although these studies did observe an inflammatory and cytotoxic response at the high dose used, the findings show that the response for occluded quartz is much different and far less potent than that of fractured or crushed DQ-12 quartz.

Because the mode of action for silicosis and lung cancer from crystalline silica requires prolonged progressive inflammatory response, and quartz with occluded surfaces is less biologically reactive and produces lower inflammatory response in test animals, it is reasonable to postulate that exposures at the current respirable dust PEL are sufficient to protect workers from material impairment of health, including lung cancer and silicosis.¹⁰

OSHA's conclusion that exposure to aged quartz poses significant toxicity seems to be based entirely on studies of "aged quartz" that is nothing at all like the ancient quartz found in sorptive clays. The "aged quartz" analyzed in the three studies cited by OSHA for its proposition on toxicity was aged for only a period of a few weeks to months, rather than tens of millions of years, as is the case with ancient quartz found in sorptive minerals. While the "aged quartz" studies cited by OSHA may provide insight into the mode of action and risk potential for freshly fractured quartz, they provide no insight into the risk potential of geologically ancient occluded quartz in sorptive minerals.

B. Epidemiological Data Do Not Support An Association Between Silicosis And Exposure In The Sorptive Clay Industry.

1. Sorptive Clays Studies.

Exposure to workers in the sorptive clay industry has not been extensively studied due to the lack of observed health effects in the industry. However, one retrospective cohort study conducted by NIOSH (Waxweiler et al. 1988)¹¹ (Attachment 6) evaluated the mortality workers in a sorptive mineral mine and processing facility in South Georgia. Geometric mean exposures to total dust ranged from 1.15 to 15.9 mg/m³ with a quartz content of four to eight percent. This study retrospectively followed a cohort of 2,302 males, employed for at least 1 month, between 1940 and 1975 in an attapulgite mine and milling facility with follow-up through 1975.

A significant deficit of nonmalignant respiratory disease was observed (SMR =43, 90%CI: 23-76), and no excess nonmalignant respiratory disease, regardless of presumed dust exposure level, induction-latency period, or duration of employment. Although a significant excess in lung cancer was observed among whites, a significant deficit occurred among nonwhites. Further, although the number of whites was greater than the number of nonwhites in the cohort, the

¹⁰ OSHA's analysis suggests that animal studies would provide a good surrogate in the absence of actual epidemiological data, as the Agency's review of epidemiological studies of workers exposed to freshly fractured quartz show that workers experience the same cellular effects and pulmonary reactions as seen in animals exposed to freshly fractured quartz. 78 Fed. Reg. 56310.

¹¹ Waxweiler, *et al.* (1988) Am J Ind Med. 13:3-5-315.

number of nonwhites with high exposure jobs outnumbered the whites by more than fourfold. The SMRs for lung cancer overall were neither significantly elevated (SMR = 119), nor were they elevated for cohort members in the high exposure group (SMR = 105) or among those with longer latency (SMR = 100). These findings would suggest that the increase in lung cancer among whites is not associated with industry exposure, but some other cause. (Factors such as smoking habits may have been involved in these findings; however smoking data was not available for the cohort). While this study was not cited in the OSHA Risk Assessment; it is important because it provides a characterization for exposures in the sorptive materials industry. We recommend that this study be cited and that exposures in the industry be recognized in the final rule as not posing the same hazard as those in industries with reactive crystalline silica.

Several reviews of worker exposures to crystalline silica have recognized that the lack of silicosis risk among clay workers with exposure to clay dust, including exposures to Fullers earth, bentonite, montmorillonite, and attapulgite (ACGIH, 1991; USEPA 1996; IARC 2005). The World Health Organization's (WHO) International Agency for Research on Cancer (IARC) prepared a Monograph reviewing health effects for workers exposed to kaolin and bentonite (IARC 2005). This Monograph recognizes that studies of workers exposed to sorptive clays have not identified significant silicosis risk. With regard to bentonite, IARC (2005) concludes:

With regard to bentonite, comparable montmorillonite pneumoconiosis [similar to that induced by exposure to kaolin] has not been consistently reported. Based on its surface chemistry, lack of fibrogenicity in experimental systems and limited human findings, inhaled bentonite is likely to be less dangerous to humans than kaolin.

It is further noteworthy that IARC concluded that the risk posed by kaolin was approximately ten times less than that of quartz, and that standards should be protective of nuisance dusts. IARC's review of bentonite and kaolin supports the concept that exposure to these clays, even though they contain quartz, do not pose a risk of silicosis or lung cancer. Although exposures to these clays should be controlled, as with any nuisance dusts, to prevent pneumoconiosis and related dust exposures, it is not appropriate to assess these occluded quartz in clay using OSHA's Risk Assessment for crystalline silica exposure.

2. Studies Of Coal Mine Workers.

An industry historically under close scrutiny for health problems is bituminous coal mining. Bituminous coal contains approximately 1-6 percent silicon including about .1 to 1.0 percent geologically ancient quartz, and thus exposures in this industry include exposures to quartz. Crystalline silica in coal mine dust is mixed with other minerals (kaolin and illite) and carbon particles (coalmine dust). Research shows that the complex matrix of coal mine dust, as with other aged silica containing dusts, contributes to toxicity in a complex way that is not easily predictable (Donaldson et al 2001).¹² Nonetheless, data from the coal mining industry can be

¹² Donaldson *et al.* (2001). The Quartz Hazard: Effects of Surface and Matrix on Inflammogenic Activity. *J. Environ. Path. Toxicol. Onol.* 20(suppl. 1) 109-118.

extrapolated to assess the risk of silicosis and lung cancer from exposure to geologically ancient quartz that may be considered to be similar to the quartz in the sorptive materials industry. IARC Monograph 68 presents a detailed review of the toxicology and epidemiology literature¹³ relating to this industry. IARC concluded that there was inadequate evidence of carcinogenicity for exposure in bituminous coal mining even though the dust was expected to contain measurable amounts of quartz. In the large number of epidemiological studies reviewed, most did not observe an increased lung cancer risk with exposure to coal dust, and no evidence for exposure-response was observed. Similarly, silicosis is not reported in most studies even among coal mining cohorts with pneumoconiosis and restrictive lung disease.

The extensive mixed-dust epidemiological studies of coal mine workers do not show a direct role for crystalline silica in lung diseases even though exposure to quartz occurs. Rather, similar to the data for kaolin and bentonite, nonmalignant lung disease has been reported but typically does not include silicosis. Although data to clearly correlate quartz in coal mine dust to quartz in mined sorptive materials has not been established, the available data do suggest that the surfaces of crystalline silica in geologically ancient quartz is less reactive than that of freshly fractured silica particles and does not contribute to silicosis or lung cancer.

IV. OSHA's Silica Standard Cannot Be Applied To The Sorptive Minerals Industry Because It Has Not Conducted A Feasibility Analysis Of The Impact Of The Proposed Rule On The Industry.

Numerous SMI processing operations are covered by OSHA's Proposed Regulation. Yet OSHA has conducted neither an economic nor technological feasibility analysis of the impacts of its Proposal on the sorptive clay industry. It is undeniably the case that the Agency is required to conduct a proper feasibility analysis for impacted industries before promulgating a health standard under section 6(b) of the Occupational Safety and Health Act, and unless and until such an analysis is conducted, any application of this standard to SMI member operations would be impermissible and legally invalid. *See Color Pigment Manufacturers Association, Inc. v. Occupational Safety and Health Administration*, 16 F3d 1157 (11th Cir. 1994).

It is currently unclear whether it is technologically feasible for the covered SMI facilities to comply with the Proposed Regulation. SMI members' preliminary analysis suggests that engineering controls may not be available to consistently reduce exposures to the proposed levels of the PEL and Action Level set forth in OSHA's Proposal. SMI also notes that, to the extent technology is available to achieve these limits, the cost to the industry to install and implement this equipment would be extremely high, certainly approaching \$20 to \$30 million and likely to be at least \$1 to \$2 million per facility for initial capital expenditures. While these are extremely preliminary cost estimates, subject to revision as SMI members assess their operations and available control technologies, it seems clear that any cost-benefit analysis of this Proposed Regulation likely would not support its application to the industry, as there is no evidence

¹³ IARC (1997) Monograph on the Evaluation of Carcinogenic Risk to Humans. Silica, Some Silicates, Coal Dust and para-Aramid Fibrils. Volume 68. WHO, Lyon, France.

showing that silicosis is present in the sorptive minerals industry. Accordingly, it is expected that a cost-benefit analysis would show that compliance costs of rule implementation would greatly exceed benefits.

V. Ambient Levels Of Crystalline Silica Approach The Action Level Of 25 $\mu\text{g}/\text{m}^3$.

Crystalline silica exists in ambient air across the US at levels that represent a significant fraction of the proposed Action Level of 25 $\mu\text{g}/\text{m}^3$. For example, mean concentrations of respirable crystalline silica in 22 cities across the US range from 0.9 to 8 $\mu\text{g}/\text{m}^3$ (EPA 1996)¹⁴. At the upperbound, ambient crystalline silica, therefore, represents 32 percent of the proposed Action Level in certain areas of the country.

Levels of silicon in ambient air measured across California vary considerably, with the highest levels in more arid areas¹⁵. For example, levels in Calexico, California average 10-12 $\mu\text{g}/\text{m}^3$ with maximum values of approximately 17 to 28 $\mu\text{g}/\text{m}^3$, while average values in Richmond, California average 1.7-2.9 $\mu\text{g}/\text{m}^3$, with peaks of 4.4-13 $\mu\text{g}/\text{m}^3$. A fraction of silicon will be present as crystalline silica.¹⁶ Assuming the percentage of crystalline silica identified by a US Environmental Protection Agency (EPA) 1996 report, the California air data suggest that ambient crystalline silica may contribute significantly to measured levels that are regulated under the Proposed Rule and compared to both the proposed Action Level and PEL.

Establishing an Action Level and PEL at the levels proposed by OSHA would pose a hardship on employers in these areas because the high levels of crystalline silica in the ambient air would contribute significantly to employee exposures, significantly decreasing allowable industrially-generated incremental exposures to ensure compliance with the regulatory limits. The ability to comply with the PEL and Action Level for members of the sorptive minerals industry, which operates to a significant degree in arid areas of the US such as Wyoming and California, will be significantly affected by ambient crystalline silica.

VI. Conclusion.

SMI appreciates the opportunity to participate in OSHA's public comment process on the crystalline silica rulemaking, and encourages the Agency to review its Comments carefully and the referenced scientific materials thoroughly as it develops its final Health Effects Analysis and Quantitative Risk Assessment.

It is important to SMI and its membership to clarify their intent in submitting these Comments. SMI is not attempting to manufacture unfounded doubt, nor is it attempting to unnecessarily delay regulation of any substance that warrants regulation to ensure worker protection. We ask the Agency to accept that these Comments are submitted in full good faith, and with an objective

14 USEPA (1996). Ambient Levels and Noncancer Health Effects of Inhaled Crystalline and Amorphous Silica: Health Issue Assessment. ORD. Washington, DC. EPA/600/R-95/115.

15 <http://www.arb.ca.gov/adam/toxics/sitelists/sisites.html>

16 USEPA (1996) cites that approximately 30 percent of silicon in arid areas may exist as crystalline silica.

of providing the Agency with updated research that it has not referenced in its Proposed Regulation and which is relevant to the understanding of the geologically ancient quartz in sorptive clays and exposures in this industry.

As stated at the outset, OSHA's preamble to the Proposed Regulation explicitly recognizes that in 2002 NIOSH concluded that further research was needed to associate specific surface characteristics that can affect toxicity with specific occupational exposure situations and consequent health risk to workers. In fact, OSHA notes that NIOSH called for "*further research on the association of surface properties with specific work practices and health effects.*" (emphasis added) (78 Fed. Reg. 56310). OSHA, then, seems to recognize that NIOSH concluded over a decade ago that further study was needed on occluded quartz, presumably before regulation was warranted. The Agency has not identified any work since NIOSH's determination that additional study was necessary that furthers the understanding of the nature of exposures to geologically ancient silica.

OSHA conclusion that there have been no studies comparing fresh and aged silica is incorrect. The work discussed and referenced herein strongly suggests to the contrary, although perhaps does not definitively answer this question. Accordingly, we encourage OSHA to ask NIOSH to conduct further study on the health effects of occluded quartz exposure, and SMI commits to continue to identify, compile, and encourage further objective research in this area, regardless of outcome.

SMI is committed to ensure its workers are adequately protected. With over 80 years of operation without one known instance of silicosis among the industry's historic or current workforce, the industry has legitimate scientific and legal questions and concerns about OSHA's preliminary determination that occluded quartz ought to be regulated in the same manner as freshly fractured quartz and other forms of crystalline silica. Based on the current science, SMI questions whether this finding is meritorious, warranted, or valid.

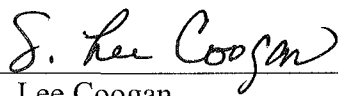
OSHA is not in a position to promulgate a standard that applies to the sorptive minerals industry at this time in any case, as it has not conducted the requisite feasibility analysis of OSHA-regulated operations conducted in this industry.

Accordingly, SMI encourages OSHA to defer application of the Proposed Rule to its industry. Pursuant to the Memorandum Agreement between MSHA and OSHA, the agencies are to develop compatible health standards, conducting joint rulemakings where appropriate. In this case, where both agencies have jurisdiction over portions of the sorptive minerals industry, it seems particularly appropriate for OSHA to defer application of this rule to this industry pending coordination with MSHA. This will provide time for an appropriate feasibility analysis to be conducted, and will allow the agencies to consider the recent studies that SMI includes in these Comments and that were not considered in OSHA's Proposal.

February 11, 2014
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We look forward to further dialog in this area with both OSHA and MSHA.

Sincerely,



S. Lee Coogan
Executive Director
Sorptive Minerals Institute

Sorptive Minerals Institute

Attachment 1

**Application for a Safe Use Determination
for the Presence of Crystalline Silica in
Sorptive Mineral-Based Pet Litters**

**Prepared by the
Sorptive Minerals Institute**

July 17, 1998

SUMMARY NARRATIVE: SECTION - 1

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UNIT OF MEASURE, SYMBOLS, AND TECHNICAL ABBREVIATIONS

USED IN TEXT

Å	angstrom
°C	degrees Celsius
C.I.	crystallinity index
Cu	copper
d-s spacing	X-ray diffraction unit in Å
DMF	dimethylformamide
DMPO	5,5-dimethyl-1-pyrroline-N-oxide
DNA	dioxyribonucleic acid
DPL	dipalmitoyl phosphatidylcholine
EDS	energy dispersive spectra
EDX	energy dispersive X-ray microanalysis spectroscopy
EPR	electron paramagnetic resonance
ESR	electron spin resonance (same as EPR)
°F	degrees Fahrenheit
g	gram
h	hour
KOH	potassium hydroxide
kV	kilovolts
lb	pound
µg	microgram
µm	micrometer
ma	milliamperes
mg	milligram
mL	milliliter
mm	millimeter
MIN-U-SIL	high-purity commercial quartz sand
min	minute
LEED	low energy electron diffraction
PEL	permissible exposure limit
PVC	polyvinyl chloride
RfC	reference concentration
RfS	reference standard
rpm	revolutions per minute
s.g.	specific gravity
SEM	scanning electron microscopy
TADC	tubular aerosol dust chamber
TLV-TWA	threshold limit value - time-weighted average
TSPP	trisodium polyphosphate
XPS	X-ray photoelectron spectroscopy
XRD	X-ray diffraction

ACRONYMNS IN TEXT

ACGIH	American Conference of Governmental Industrial Hygienists
ASTM / JCPDS	American Society for Testing Materials / Joint Committee on Powder Diffraction Standards
EPA	Environmental Protection Agency
IARC	International Agency for research on Cancer
JMTC	Johns Manville Technical Center
MSHA	Mine Safety and Health Administration
NIOSH	National Institute of Occupational Safety and Health
NIST	National Institute of Standards and Technology
OEHHA	Office of Environmental Health Hazard Assessment
OSHA	Occupational Safety and Health Administration
SMI	Sorptive Minerals Institute
SUD	Safe Use Determination

SUMMARY NARRATIVE

1.0 INTRODUCTION

Silica, crystalline (airborne particles of respirable size) is listed in the California Safe Drinking Water and Toxic Enforcement Act of 1986 (the "Act") as a chemical known to the State of California to cause cancer. Many sorptive mineral-based pet litters (hereafter referred to as "pet litter") may contain minor amounts of crystalline silica in the form of quartz. The amounts of crystalline silica resulting in consumer exposure during the normal use of pet litter poses no hazard. The Sorptive Minerals Institute (SMI), on behalf of its member companies, requests that the State of California Office of Environmental Health Hazard Assessment (OEHHA) grant a Safe Use Determination (SUD) for the presence of crystalline silica in pet litters.

The SMI is presenting a comprehensive technical document showing that the normal use of pet litters does not pose a cancer risk to the consumer. The salient points are outlined in this narrative summary. A more detailed discussion and bibliography is presented in the Reference Section. The supporting scientific evidence appears in the attached Appendices. This presentation will show that, during the normal use of pet litter,

- Consumer exposure to respirable quartz is well below any guidelines presently recognized in the United States
- The nature of the surfaces of the quartz particles in pet litter *is not that of the biologically active crystalline quartz that results from some industrial processes*

2.0 BACKGROUND

2.1 SMI INTERACTION WITH OEHHA

This proposal for a SUD continues the productive interaction between the SMI and the State of California dating from the 1980's. When California first considered listing crystalline silica as a carcinogen, the SMI suggested listing only airborne particles of respirable size. During the public comment session, the SMI provided extensive scientific information to support its suggestion⁷³. The Science Advisory Panel adopted this suggestion, and thus avoided unnecessary regulation of a common mineral.

At that time, SMI developed a protocol to measure consumer exposure to respirable quartz during the normal use of pet litter. The SMI submitted its protocol to Dr. Steven A. Book, Science Advisor to the Secretary, Health and Welfare Agency, State of California on March 8, 1989⁷⁴. Dr. Book stated that the procedures appeared to be straightforward in terms of their practical, scientific approach⁷⁵.

3.0 COMPONENTS OF SMI INVESTIGATION

The SMI used a multifaceted approach in determining the safety of pet litters. Special emphasis was given to comparing the variety of quartz present in pet litters with well-known quartz exposures where no silica-related lung diseases exist. This effort required the development of techniques to determine the consumer exposure to both types of pet litter dusts. It also necessitated the development of new investigative procedures in separating and characterizing the mineralogy of quartz and the other components in the dusts. To assist in this effort, the SMI enlisted the expertise of a number of highly qualified research scientists in this area.

- Dr. Karen Vetrano of TRC Environmental Corporation conducted an exposure determination of the actual amount of dust and respirable quartz encountered during the normal use of pet litter by the consumer.
- A characterization of the quartz and other minerals in the bulk fraction, the $\leq 10\mu\text{m}$ fraction, and the consumer-respirable fraction of the pet litters was performed by Dr. Robert Hamilton of the Johns Manville Technical Center.
- Dr. Michael Hochella and Dr. Donald Rimstidt of Virginia Polytechnic Institute and State University performed a characterization and comparison of the undisturbed surfaces and the recently fractured surfaces produced by some industrial processes.
- A determination and definition of non-crystalline silica and hydrous silica phases that naturally occur in pet litter and may be confused with crystalline silica was conducted by Dr. Deane Smith of the Pennsylvania State University.
- Dr. Smith also performed a review of the properties of quartz that determine or enhance its biological activity.

4.0 PET LITTER

The idea of providing a medium for the sanitary collection of waste materials from indoor pets has been utilized since the end of the last century. Large-scale commercialization and marketing of pet litter began in the 1950s. Since that time the development of safe, effective, and inexpensive pet litter has contributed significantly to the growing popularity of companion animals such as cats.

Today, an amazingly wide variety of pet litter products are being produced. Among these products, mineral-based pet litters have become particularly attractive because they are effective at absorbing liquid wastes and do not provide nutrients for bacterial growth. This is especially true of the clays used in pet litter because their properties can act to delay the formation of unpleasant odors resulting from the fermentation of urea in animal urine. The recent introduction of the new clay-based clumping litters has had a significant impact on the litter market. The ability of these products to cause urine and feces to be localized, allowing easy and frequent removal, has enabled them to capture a large share of the pet litter market. As a result of these characteristics the current popularity of pet litter shows no sign of abating. Additional details of the properties of pet litter, as well as a description of the manufacturing processes (Figures 1 & 2) used to produce them, may be found in Reference Section 2.

5.0 GEOLOGY – OCCURRENCE AND MINERALOGY

The materials used in pet litter in the United States are obtained from only a few clay deposits. All of these deposits are sedimentary in origin and range from 1.6 million to 110 million years of age. None of these materials has been exposed to temperatures higher than 150°C, either during formation or after deposition. The total quartz content of the clays mined from these deposits ranges from non-detectable to approximately 9%. Principal among these deposits are:

- The Monterey formation of California
- The diatomite deposits of central Oregon
- The Porters Creek formation of the Mississippi valley
- The Twiggs fullers earth of the southeastern United States
- The Meigs fullers earth of the southeastern United States
- The Wyoming-type sodium bentonite deposits
- The calcium bentonite deposits of north central Florida
- The fullers earth deposits of eastern Virginia

A complete description of the geology and mineralogy of these deposits, along with a map showing their locations (Figure 3) and a geologic time chart (Figure 4) may be found in Reference Section 2.

6.0 REGULATION AND COMPLIANCE

6.1 REGULATION OF QUARTZ DUST

Despite a large amount of scientific research over many years, quartz and other natural silica phases remain an enigma, especially with respect to their bioactivity. This fact was affirmed recently when IARC determined that quartz is not always carcinogenic²³. It has only been during the past few decades that research has shown the positive link between quartz particles inhaled immediately after fracturing and acute lung damage. In contrast, the United States Environmental Protection Agency (EPA), in a recent comprehensive study of ambient dusts, has shown the exposure to dusts containing crystalline silica from farming and wind blown dusts is not a problem²⁷.

Despite these recent findings, regulations continue to be based upon research that employed pure, single crystal, recently fractured grains of quartz of high-temperature origin. This practice has had the effect of erroneously indicting all quartz particles, including polycrystalline chert and grains whose surfaces are occluded by aluminosilicate minerals and/or opaline silica, such as clay or non-crystalline silica, as being biologically aggressive.

6.2 METHODS OF DETERMINING COMPLIANCE

Monitoring the air for respirable quartz particles and determining their concentration provides the principal method for regulating health hazards associated with the inhalation of quartz dust.

Several federal regulatory agencies, such as OSHA and MSHA, have prescribed procedures for the use of such monitors. Two principal analytical methods are available to quantify the amount of crystalline silica gathered by air monitors. These are X-ray diffraction and infrared spectroscopic techniques. X-ray diffraction is the preferred method because it can identify, as well as quantify, the polymorphs of crystalline silica that are present in monitor samples. Identification and quantification requires substantial skill and experience, however. Smith recently discussed the problems encountered in using X-ray diffraction methods to detect and quantify quartz in samples²¹. He noted that analytical laboratories must exercise great care in making X-ray diffraction determinations of quartz and other polymorphs of crystalline silica if they are to produce accurate results.

7.0 THE NATURE OF SILICA

The following discussion outlines the complexity of a seemingly simple substance, crystalline silica. Crystalline silica can vary widely in its degree of crystallinity and in its surface properties. The variation in these properties can have a profound effect on biological activity.

7.1 TYPES OF SILICA

Crystalline silica, including quartz, is virtually everywhere in the environment. After feldspar, quartz is the most common mineral in the crust of the earth. Quartz is the principal component in most beach sand and is a major constituent of most soils⁷⁶. Crystalline silica, particularly in the form of quartz, also occurs naturally in the atmosphere with ambient levels up to 1.9 micrograms per cubic meter found in the United States²⁷.

Despite its simple chemical formula, silica (SiO_2) is an exceedingly complex substance¹¹ which exists in both non-crystalline and crystalline forms. The seemingly simple Si – O bond that, thus far, has defied adequate understanding concerning its ionic or covalent nature illustrates an example of this complexity^{12,13}. Under normal pressures, the basic building block of silica minerals is a silicon atom surrounded tetrahedrally by four oxygen atoms. These tetrahedra have many possible arrangements in space. Some are random or semi-random and cannot build up the three-dimensional periodicity required of a crystalline structure. Others are in periodic arrangements, which give rise to various crystal structures. These are classified as amorphous or paracrystalline.

7.1.1 Crystalline Silica

The various crystalline configurations exhibited by silica are termed polymorphs¹⁴. The most common polymorphs are quartz, cristobalite, and tridymite. The phase diagram in Figure 5 concisely illustrates the pressure and temperature relationship between the various forms of crystalline silica¹⁵. The only polymorph of crystalline silica occurring in pet litters produced by SMI member companies is quartz^{64,65}. As illustrated in the previously mentioned phase diagram, quartz forms at temperatures well below that of tridymite and cristobalite.

7.1.2 Variety of Quartz

Quartz can possess many different degrees of crystallinity. This fact has long been recognized by petrographic optical microscopists. X-ray diffraction techniques that are now the method of choice for determining the crystalline forms of silica, can also detect differences in crystallinity. Figure 6 shows X-ray diffraction patterns for several varieties of quartz, illustrating their differences. Over 20 years ago, Murata and Norman¹⁶ determined that a group of five diffraction peaks are diagnostic of the degree of crystallinity (Figure 7). They devised a crystallinity index, also known as the “Murata ratio”, that quantifies the degree of crystallinity and distinguishes among the different varieties of quartz. This index can differentiate between single crystal quartz formed at high temperatures and polycrystalline, low-temperature quartz having a lower degree of crystal ordering. Unfortunately, investigators concerned with the regulation of crystalline silica rarely use these techniques.

Several varieties of quartz can be found in the geological deposits used for pet litter. One variety comprises single crystal grains that are derived from igneous or metamorphic rocks and have a high degree of crystal ordering. These grains were transported to the deposit, during its formation, by wind or water and are called “detrital”. Some clay deposits form by weathering of preexisting rocks. The quartz grains that remain after weathering destroys the original rock, *in situ*, are called “residual” grains. They can also have a high degree of crystal ordering. A third variety can form after the original deposition of the clay or its parent minerals. This variety results from precipitation of silica dissolved in the pore water contained in the sediment. These grains are called “authigenic” and normally have a low degree of crystal order.

7.1.3 Amorphous and Non-Crystalline Silica

Many naturally occurring forms of silica do not have long range periodicity and are not crystalline. These include the hydrous silica forms opal-A, opal-CT and opal-C. Opal-CT occurs in most pet litters. Unfortunately, much confusion has existed regarding the nature of these opal forms. Smith recently made an exhaustive survey of the scientific literature and developed a straightforward nomenclature and classification¹⁷. He defined opal-CT as a non-crystalline form of hydrated silica. This he termed “paracrystalline,” indicating that it has limited short-range ordering of six-member Si—O rings in a disordered matrix. The chemistry of opal also distinguishes it from anhydrous crystalline silica. Paracrystallinity connotes a lack of true crystallinity, and implies only partial ordering in a disordered matrix. *Non-crystalline forms of silica are not considered carcinogenic by IARC, which lists them in class 3.*

Opal-CT produces a few X-ray scattering effects falling in the same areas as some of the X-ray diffraction lines of cristobalite. Cristobalite is a biologically aggressive form of crystalline silica formed under conditions of high temperature and pressure (Figure 5). This similarity has often caused opal to be confused with cristobalite. To avoid this confusion, Miles¹⁸ combined two existing NIOSH procedures, 7500¹⁹ and 7601²⁰ to enable the positive differentiation of opal-CT and cristobalite. Smith⁶⁹ found that very few analytical laboratories are aware of the ambiguity between opal and cristobalite in the literature. He also found that these laboratories routinely fail to distinguish between cristobalite, which is regulated, and opal, which is not.

8.0 NATURE OF THE SURFACE OF QUARTZ

The reactivity of solid materials is often surface dependent. Therefore, understanding the surface properties of quartz is essential for interpreting its biological activity^{35,36,37}.

8.1 FRESHLY FRACTURED VERSUS AGED SURFACES

The surfaces of geologically aged quartz particles have reached equilibrium with their environment and have little or no chemical reactivity. Breaking open a grain of quartz, however, exposes fresh, chemically reactive surfaces. These surfaces have broken chemical bonds that are not easily satisfied within the structure of the quartz crystal because of the rigidity of that structure. Spectroscopic measurements have readily detected these free radicals. Chemical changes induced by ambient moisture or water immediately begin to reduce the reactivity of these surfaces. Eventually a new, chemically stable surface results, exhibiting little, if any reactivity.

Fubini³⁸ and Castranova³⁹ have measured the nature and abundance of the free radicals produced by fracturing quartz grains (Figure 8 & 9). They have also studied the implication of free radicals in biological activity. As the number of free radicals declines, even over as short a period of time as two months, the ability to cause biological damage drops to a very low level.

The inability to generate free radicals results from hydration of the fractured surface. Under ambient conditions the broken bonds resulting from the fracture, and their attendant surface structure begin to hydroxylate in a stepwise fashion leading to a fully hydrated surface (Figure 10). Although this surface is slightly soluble and will slowly dissolve in water, hydroxylation of the quartz surface proceeds at the same rate, regenerating the hydrated surface. As a result, the ability of a fractured surface to generate and/or present free radicals to either biologic systems or to the environment rapidly vanishes and never reappears.

8.2 OCCLUSION OF QUARTZ PARTICLES

Over geologic time, quartz grains in the environment can experience other surface changes. These changes include adsorption of adventitious material such as aluminum ions, magnesium ions, and carbon compounds. Aluminosilicate materials, as well as amorphous hydrated regions can also develop on these surfaces. In some deposits the quartz grains may become intimately cmeshed in clay or opal, forming agglomerates. All of these changes act to further reduce the biological activity of the quartz surfaces. Significantly, these concepts help to explain why exposures to some types of quartz do not lead to silica-related lung disease.

9.0 BIOLOGICAL ACTIVITY OF QUARTZ

Silica-related lung disorders are not pandemic in the general population despite the ubiquitous presence of silica in the environment. Even in dusty and wind-blown arid regions, silicotic problems are isolated and rare²⁷. Silica-related lung diseases are problems in the workplaces that employ practices that fracture quartz.

9.1 EPIDEMIOLOGICAL EVIDENCE

9.1.1 Epidemiological Study in the Sorptive Minerals Industry

Normally, epidemiological studies target industries that show unusually high incidences of exposure-related diseases. No such studies have targeted the sorptive minerals industry. One study did examine the possible health effects of minerals other than quartz. This study, by Zumwalde²⁸ at NIOSH, investigated the mortality of workers in a sorptive mineral mine and processing facility in south Georgia. Although the clay contained five or more percent quartz, no significant increase in standard mortality rates due to lung cancer or other lung disorders was evident in the workers. The processing of this clay did not involve aggressive crushing, thereby precluding the presence of biologically active fractured grains.

9.1.2 Epidemiological Studies in Coal Mine Workers

Perhaps the closest parallel to the nature of the quartz exposures in the sorptive minerals industry is found in bituminous coal mining. Both types of materials contain minor amounts of geologically aged quartz. Further, epidemiological studies of exposure to dust from these materials do not commonly show silica-related lung disorders.

The extensive mixed-dust epidemiological studies of American^{29,30}, British³¹, and European³² coal mine workers do not always show a direct role for crystalline silica in lung diseases. The data included in these studies show that the surface properties of the quartz present are remarkably similar to those of the quartz found to be present in pet litter. These coal studies are discussed extensively by Castranova, Vallyathan, and Wallace³⁷. The studies show a general correlation between lung disease and coal rank (the carbon-to-hydrogen ratio), rather than quartz content.

In bituminous coal the quartz grains can have coatings of aluminosilicates. Additionally, bituminous coal is soft enough to break around the included quartz grains rather than fracturing them. However, metamorphism through geologic conditions of temperature and pressure can induce changes in the coal beyond merely reducing the amount of volatiles. In anthracite (hard coal), a highly altered "high rank" coal, the coatings on the quartz grains are minimal or absent. Anthracite coal is also sufficiently hard so that quartz grains may fracture when the coal is broken. As a consequence, the quartz in such high rank coals exhibits far greater biological aggressiveness than quartz from lower rank coals.

This situation of quartz in bituminous coal is similar to that of quartz in the minerals used in pet litter. In both instances the grains have old occluded and/or coated surfaces and are not broken during processing. As a result, the bituminous coal experience lends support to the concept that the quartz found in pet litter does not pose a human health hazard.

9.1.3 Other Studies

Some studies have purported to find aggressive biological activity in geologically aged quartz. A study by Holland⁵¹ subjected animals to low dose response, long-term inhalation experiments using raw and spent domestic oil shale. This study showed a positive response, however the preparation of the shale samples completely compromised the experiment. The samples were ground to $\leq 5\mu\text{m}$ prior to being introduced to the animals thus producing abundant freshly fractured quartz surfaces. As a result, this study merely confirmed that freshly fractured quartz is indeed biologically aggressive. Nevertheless, this study eloquently speaks to the point that researchers must fully understand the nature of the raw materials that they use and the effect that the experimental methods may have on these materials if the results they obtain are to be useful.

9.2 CONSIDERATIONS OF THE BIOLOGICAL ACTIVITY OF QUARTZ

A number of well-qualified investigators have published excellent surveys on the mechanism of the biological effects of crystalline silica. Interestingly, all of these studies involve freshly fractured quartz or cristobalite. Some researchers have begun to investigate the effect of aged or occluded quartz, although the extreme difficulty of separating the grains from the mineral matrix in which they occur without altering their surface makes such studies unusually challenging.

The body has several different pathways to clear inhaled particles, depending on the site of their deposition in the respiratory tract^{44,45}. Most particles are sufficiently large so that they are deposited prior to penetrating deeply into the lung. These particles are rapidly eliminated from the body through the action of a variety of mechanical bodily processes. Very small particles, $\leq 3\mu\text{m}$ in size, may penetrate deeply into the alveolar region of the lung, however. These particles are removed by alveolar macrophages after being phagocytosed. Clearance of these particles from the lung may take weeks or months. Where the particle load in the lung is high, macrophages become engorged, impairing their ability to phagocytose and migrate. This results in the death of the macrophage with the subsequent release of the particles and an expansion of the inflammatory process⁴⁶. In these cases, particles can persist in the lung indefinitely.

Evidence that varieties of quartz differ in biological activity suggests that many factors are at work. Animals do have many protective strategies, and these factors require examination of the whole animal and not reliance on *in vitro* investigations. The unexpectedly low incidence of silica-related lung diseases in bituminous coal mineworkers has led to several hypotheses concerning the reaction of organisms to quartz. Of particular interest is the role of the aluminosilicate^{57,58} occlusions on quartz grains in altering macrophage response to quartz and protecting these cells from premature death.

The variability of quartz as illustrated by the facts found in the existing literature shows the difficulty, if not impossibility, of assessing a single cancer potency value for quartz.

10.0 SMI RESEARCH PROGRAM

SMI initiated three research programs in an effort to ascertain the safety of pet litter use. These were:

- establishing the potential consumer exposure to respirable quartz from pet litter use
- establishing the properties of the surfaces of geologically aged quartz grains
- establishing the properties of quartz in pet litter

10.1 POTENTIAL FOR EXPOSURE TO RESPIRABLE QUARTZ FROM PET LITTER

Pet litter is a granular product and contains a very small amount of $\leq 10\mu\text{m}$ respirable size particles. In order for these particles to become inhaled they must first be launched into the air. The activities associated with normal pet litter use, such as pouring or scooping, do not cause significant quantities of these particles to become airborne.

10.1.1 Objective

To develop carefully constructed laboratory experiments that would duplicate the exposure to respirable pet litter dusts and the respirable particles it contains during the normal use of pet litter. TRC Environmental Corporation⁶⁶ was retained to develop and conduct such experiments to determine the amount of respirable dust that a consumer might inhale during the normal use of pet litter and to establish the nature of that dust.

10.1.2 Materials

The samples used in these experiments were purchased by TRC Environmental Corporation at retail grocery, pet or mass merchandise stores. A detailed characterization of the respirable mineral components collected is described in Appendix III.

10.1.3 Methods

For conventional litters, the experimental methodology involved pouring litter from the litter package into a litter box. For clumping litters, the experiment again involved pouring litter from a litter package, then creating and scooping clumps, and finally replenishing the litter in the litter box by re-pouring. Both sets of experiments were conducted in a small, enclosed space in order to contain any dust that was produced and allow it to be sampled. Air monitors calibrated to capture $\leq 10\mu\text{m}$ particles sampled the dust generated during each set of activities. The height of the litter package duplicated the typical positions used by a consumer. The air monitors were located at the position of the breathing area of the consumer. A more detailed description of the experimental methodology and setup is given in APPENDIX III and in the report of Dr. Karen Vetrano⁶⁶.

10.1.4 Experimental Results

The calculated 8-hour TLV – TWA for quartz in the dust encountered when pouring the conventional pet litters listed in Table 4 ranged from $2.07 \times 10^{-4} \text{ mg/m}^3$ to $27.66 \times 10^{-4} \text{ mg/m}^3$. The calculated 8-hour TLV – TWA for quartz encountered when pouring and scooping clumping pet litter ranged from $2.83 \times 10^{-4} \text{ mg/m}^3$ to $23.8 \times 10^{-4} \text{ mg/m}^3$. The letters used in Tables 4, 5, 6 and 7 are used to designate the pet litter products in order to maintain company confidentiality.

It is noteworthy that, in all cases, the calculated TLV – TWA's listed for both the conventional and the clumping litters were well below the ACGIH TLV – TWA of 0.1 mg/m^3 . In fact, they range from 0.2 to 3.6% of the ACGIH TLV – TWA. These actual values demonstrate that the consumer's exposure to respirable quartz during normal pet litter use is extremely small. Exposures to such small levels of quartz should not pose a burden to the lungs.

10.2 STUDIES OF THE SURFACE PROPERTIES OF GEOLOGICALLY AGED QUARTZ GRAINS

Quartz that is present in clay deposits is millions of years old and has correspondingly old surfaces. Little if any research has appeared in the literature that addresses the properties of these surfaces. Recognizing this, the SMI commissioned two preeminent researchers at Virginia Polytechnic Institute and State University to conduct quartz surface properties studies. Dr. Michael Hochella studied the surfaces of geologically aged quartz using instrumental methods⁶¹, while Dr. Donald Rimstidt studied them using dissolution techniques⁶².

10.2.1 Materials

Quartz is a minor component in the clay deposits used for pet litter and is intimately associated with other minerals making it difficult to separate without altering its surface characteristics. As an alternative these studies used geologically old quartz grains from sand/kaolin deposits. The grains could be easily separated and concentrated without alteration of their surface properties and they provided a good model for the quartz grains from the clay deposits used for pet litter. Additionally, a 480 million-year-old quartz crystal, known as a "Herkimer Diamond" was used to develop and validate the test methods because of its flat, uniform crystallographic surface. A sample of freshly fractured IOTA quartz (see Appendix I) with highly cleaned surfaces was also examined for comparison purposes.

10.2.2 Experimental Approach

These studies combined direct surface observation and dissolution techniques. The direct observation experiments included the use of LEED, a method that can study the crystallography of a surface, and XPS, a method that can probe to several nanometers beneath a surface. The dissolution experiments were used to obtain data on the chemical properties of surfaces by detecting changes in solubility over time.

10.2.3 Experimental Results – Instrumental Study

The LEED experiment was conducted only on the Herkimer Diamond due to its uniform surface. The experiment revealed no diffraction pattern in the as-received condition. This result indicated that an adventitious layer composed of adsorbed materials, including carbon compounds, was present on the crystal surface to a thickness of at least a nonometer (10 Å). A diffraction pattern indicative of quartz began to be apparent only after vigorous cleaning of the crystal surface to remove these contaminants.

The XPS data also showed that the surfaces of the grains were not pure quartz, but rather had an adventitious layer up to 10 Å in thickness. Additionally, these data showed that the chemical environment of the oxygen atoms at the surface of the crystal were not that of pure quartz. In contrast, a significantly smaller amount of foreign material was found to be present on the freshly fractured surface of a grain that was deliberately broken for this experiment.

These data show that geologically aged surfaces are chemically not those of crystalline silica due to the presence of an adventitious layer of foreign material. These data further show that geologically aged quartz surfaces do not exhibit any of the crystallinity expected of quartz unless vigorously cleaned and, even then, only a small amount of the expected order is observed.

10.2.4 Experimental Results – Dissolution Study

In his summary report Rimstidt has shown that amorphous silica is more soluble than quartz across a range of temperatures (Figure 12a). He has also shown that the solution of geologically aged quartz proceeds through an initial period of rapid dissolution followed by much slower dissolution (Figure 12b). The data indicate that this phenomenon results from the presence of a discontinuous surficial layer of amorphous material that has formed on a naturally hydrated silanol layer. The amorphous surface coating was shown to dissolve at rates comparable to amorphous silica until the stable silanol layer covering the entire quartz surface was reached, at which point the rate of dissolution significantly decreased.

These data from the instrumental and dissolution studies support the argument that quartz particles that exist in the ambient environment, and that have not been subjected to harsh treatment, have surface coatings that prevent the formation of bio-chemically reactive surfaces. This is in marked contrast to the lack of surface coatings on the bio-chemically reactive surfaces known to be present in freshly fractured quartz particles.

10.3 THE NATURE OF QUARTZ IN PET LITTER

The nature of the quartz present in pet litter must be correctly defined in order to establish the type and degree of hazard, if any, that it may pose to consumer health. Only those quartz particles of respirable size ($\leq 10\mu\text{m}$) have the potential to be inhaled deeply enough into the lungs

to cause health problems. Despite this, laws and regulations are often established which do not take these facts into account.

10.3.1 Objectives

The objective was to thoroughly characterize the nature of the quartz in both bulk pet litter and in respirable size pet litter dust in order to establish whether any differences exist between these materials. Dr. Robert Hamilton^{64,65} was retained to perform a comprehensive study to characterize the quartz in the deposits represented in the exposure assessment work. Because the nature of quartz may vary with particle size, the study tested several size distributions.

10.3.2 Materials

Two groups of samples purchased directly from retail grocery, pet supply or mass merchandising stores were used in these experiments. The first group of samples included several hundred pounds each of products representing the absorbent clays used in pet litter. These samples included products containing clays from the Monterey formation, the Oregon diatomite, the Porters Creek formation, the Twiggs fullers earth, the Meigs fullers earth, and Wyoming bentonite. A second group of samples included products representing clays of the Monterey and Porters Creek formations and Wyoming bentonite. A summary of the properties of the sample materials used in these experiments may be found in Appendix II.

10.3.3 Methods

The experimental method used for the bulk pet litter tests employed wet sieving and heavy liquid-size fractionation techniques to separate the various sizes of quartz particles. The proper characterization of the $\leq 10\mu\text{m}$ (respirable) material required the use of a tubular aerosol dust chamber (TADC), however. This was necessary because normal air monitor collectors were not able to capture a sufficient amount of quartz dust for analysis due to the very small amount of dust generated by pouring.

The TADC extracted the $\leq 10\mu\text{m}$ particles directly from the screened <100 -mesh fraction of the bulk pet litter. Although small, the amount of quartz dust captured using this method was sufficient for this study. When compared on a percentage basis the amount of $\leq 10\mu\text{m}$ quartz found in the dust generated by the TADC process was equal to or slightly higher than the total percentage quartz concentration found in the bulk litter samples. It was also found to be approximately four times greater than the $\leq 10\mu\text{m}$ quartz fraction that was found in the monitor samples from the consumer pouring tests.

Further review of the TADC method revealed that the high respirable quartz levels observed in the TADC samples were an artifact of the method. This method uses glass beads to keep the particles fluidized in the chamber. As these beads circulate in the moving airstream inside the chamber they tend to abrade and degrade the litter particle agglomerations. This results in a higher number of individual $\leq 10\mu\text{m}$ quartz particles than existed in the <100 -mesh fraction of the bulk material. As a result, the values obtained from the TADC samples overestimate the amount

of respirable quartz making them very conservative indicators of the quartz concentration found in pet litter dust.

Chemical, x-ray diffraction, and electron microscopy techniques were used to define the nature of the samples obtained in each study. A more detailed description of the experimental methods used in this testing may be found in Appendix II.

10.3.4 Experimental Results

Hamilton found that, with the exception of the Wyoming bentonite, the quartz in all of the litter samples studied was exclusively of the single crystal variety with the crystallinity indices above 4.0. This is consistent with the detrital origin of these materials. The sodium bentonite studied was found to contain both detrital and authigenic quartz (crystallinity index below 4.0). It is noteworthy that these low crystallinity index ("poorly crystallized") quartz appears almost exclusively in the sieved fines (<325 mesh), air monitor respirable fractions, and $\leq 10\mu\text{m}$ heavy liquid separation fractions. This indicates that, for bentonite, a sizeable portion of the respirable size quartz particles will be of the type least likely to cause human health problems.

None of the samples were found to contain any respirable quartz that was similar to the freshly ground quartz used in *in vivo* or *vitro* experiments. Further, none of the samples were found to contain fractured particles of the type produced by abrasive blasting, dry drilling or grinding and explosive blasting. Electron micrographs show that the quartz grains typically appear in agglomerates of clay and/or opal. Electron micrographs shown in Figures 15 through 20 illustrate the nature of the grains of quartz from these experiments. These liberated grains are all either rounded or sub-angular. In many cases, the quartz appears as sub-angular particles enclosed in grains of non-crystalline opal, or in composite grains of clay and quartz. Figure 17f clearly shows the typical small quartz crystals encased in opal.

Energy dispersive X-ray microanalysis spectroscopy (EDS) measurements showed that the surfaces of all quartz grains that were observed were not pure silica but, rather, contained measurable amounts of aluminum and often magnesium. These elements are typically present in the materials from which clays are formed.

Table 2 summarizes the quartz concentration test results for each of the major materials used for the production of pet litter. These data shows that the amount of potentially respirable ($\leq 10\mu\text{m}$) quartz in the bulk litter materials is extremely small, ranging from 0.0006% to 0.0053% of the total litter on a weight basis. It also shows that the amount of respirable ($\leq 10\mu\text{m}$) quartz collected is very small, representing less than 2.5% of the respirable size dust collected from the consumer pouring tests and less than 10% of the $\leq 10\mu\text{m}$ dust collected in the TADC study.

Tables 6 & 7 summarize the respirable quartz concentration results from the simulated consumer exposure testing for each product. The data shows that the maximum respirable quartz captured in these tests represents less than 0.0000000475 % of the total amount of litter poured during each test, on a weight basis. As a result, these tests show that a consumer would inhale no more than 176 μg (0.000176g) of respirable quartz from the use of 100 pounds of litter (100 lbs. x

$453.6\text{g/lb} \times 0.00000000475 (4.75 \times 10^{-8} \% \text{ as above}) \times [13.9 \text{ L/min (high avg. human respiration rate)} \div 1.7 \text{ L/min (monitor rate)}]$. This confirms that only an extraordinary small fraction of the potentially respirable quartz contained in the litter granules actually becomes airborne during normal pet litter use by the consumer. It also demonstrates that consumer exposure to respirable quartz from the normal use of pet litter is exceptionally low and should pose no health hazard.

11.0 SUMMARY

The Sorptive Minerals Institute undertook a research program to consider the human health risk associated with the normal use of pet litter products. The results of this research have shown:

- **Consumer exposure to respirable quartz from pet litter during normal use is extremely low.**

Carefully modeled, conservative exposure testing has shown that consumer exposure to respirable quartz when using conventional pet litter of the SMI companies is only 0.2 to 2.8 percent of the established ACGIH-TLV. The exposure when using clumping pet litter is only 0.2 to 3.6 percent of the established ACGIH-TLV.

- **The only crystalline silica found in pet litter is quartz.**

Pet litter products may contain an abundance of amorphous hydrated silica in the form of opal. Problems in differentiating this type of material from the biologically aggressive, high temperature silica polymorph, cristobalite, have led to the incorrect determination that pet litter contains cristobalite. Pet litter is dried to remove excess moisture at temperatures far below those required to produce high-temperature silica polymorphs such as cristobalite. Proper application of available research methods and cited literature reveals that quartz is the only crystalline silica form present in pet litter.

- **According to the literature, freshly fractured quartz has a high degree of biological activity.**

The freshly fractured quartz surface has been found to have a high degree of chemical reactivity due to the presence of broken, unsatisfied chemical bonds. These broken bonds can produce highly reactive chemical free radicals that are known to cause problems in biological systems.

- **Pet litter does not contain fractured quartz.**

The process of manufacturing pet litter granules does not involve the use of any grinding or milling processes that fracture the types of quartz grains found in pet litter. Neither does the consumer fracture the grains during the normal use of pet litter.

- **The quartz found in pet litter has very low biological reactivity.**

The surfaces of the quartz particles found in pet litter are very old, ranging from 1.6 to 110 million years. Any freshly fractured surfaces, which can generate free radicals, disappeared long ago through reaction with water and other environmental constituents. The surfaces are also occluded with aluminosilicates and other adventitious material, which further reduces the reactivity of the surfaces. The nature of the quartz in pet litter is similar to that found in soft coal mining where silica-related lung diseases are minimal or absent.

1. *Although exposure to respirable dust collected from pet litters contains quartz, as erroneously determined by standard NIOSH methods 7500 and 7601, it is:*
 - *Exceedingly small, and well below any established ACGIH-TLV for an 8-hour worker exposure; and*
 - *The quartz content, as quantitatively determined, is not indicative of the biological reactivity of the quartz surfaces.*
1. *Consumer exposure to biologically active respirable quartz from the normal use of conventional and/or clumping pet litter is non-existent.*

In summary, when reviewing the safety of pet litter products for the purposes of Proposition 65, OEHHA should take into account the views of California's "qualified experts", the Science Advisory Panel (SAP). Crystalline silica of respirable size was listed as a carcinogen after the SAP reviewed toxicological data for silica in its public meeting on September 16, 1988. At that meeting, the SAP clearly recognized that the various forms of silica varied considerably with regard to biological activity and that carcinogenicity was strongly dependent upon morphology. At the public hearing, SAP member, Dr. W.L. Spengler stated:

"This is a substance whose ability to cause neoplasia is strongly dependent on physical characteristics or particle morphology. There appears to be a rather narrow range of morphological characteristics of silica significant in biological activity and a very narrow range of persons who would appear to be at risk to this particular substance." ("Transcript, September 16, 1988, p. 188).

During the same meeting, panel members Dr. Bruce Ames, Dr. D. Warner North and Dr. Spengler recommended that the agency should consider the different forms of silica that were used in underlying studies of carcinogenicity and incorporate that information into risk assessment decisions (SAP, Transcript, pp. 220-221).

The Sorptive Minerals Institute believes that the information summarized in this narrative and detailed in the attached Reference Section and Appendices proves that the use of pet litter does not pose a human health hazard and, therefore, that this application for a Safe Use Determination should be granted.

REFERENCE SECTION

1.0 INTRODUCTION

This Reference Section contains the detailed basis for the synopsis presented in the preceding narrative summary.

1.1 METHODOLOGY SUMMARY

- An exposure determination of the actual amount of dust and respirable quartz encountered by the consumer during the normal use of pet litter
- A characterization of quartz and other minerals in the bulk fraction and a $\leq 10\mu\text{m}$ fraction of pet litter
- A characterization of the properties of the surface of the quartz particles in both fractions of pet litter
- An integrated determination of the properties of the surfaces of freshly fractured and geologically aged quartz
- A determination and definition of non-crystalline silica and hydrous silica phases that naturally occur in pet litter, which may be confused with crystalline silica
- A thorough review of the surface properties which affect the biological activity of quartz

1.2 EVALUATION

An evaluation of the scientific studies detailed in this document leads to the following conclusions:

- Consumer exposure to this non-aggressive quartz during normal use of pet litter is well below any guidelines presently recognized in the United States
- The only form of crystalline silica detected in pet litter is quartz with a biologically non-aggressive surface
- The quartz found in pet litter is geologically old and has biologically non-aggressive surfaces

2.0 PET LITTER

2.1 PROPERTIES

Conventional pet litters are typically composed of granules of clay, approximately 4 mesh (4.76mm) to approximately 25 mesh (0.71mm) in size. They have the ability to absorb from 50% to 150% of their weight in liquids such as urine. The bulk density of conventional litters ranges from approximately 25 pounds per cubic foot to approximately 50 pounds per cubic foot. Clumping pet litters are typically composed of smaller granules of clay, often 8 mesh (2.00mm) to 60 mesh (0.25mm). They have the ability to absorb from 50% to 100% of their weight in

liquids such as urine. They also have the ability to agglomerate quickly upon wetting with urine to form dense, durable clumps that resist the scratching of the animal in the litter. These clumps remain firm after drying enabling easy removal of the waste from the litter box. The bulk density of clumping litters ranges from approximately 30 pounds per cubic foot to 70 pounds per cubic foot.

The clay minerals that will meet the above criteria are not widespread. Most common clays are unacceptable as pet litter. Clays that function well as pet litters occur only in geographically restricted sedimentary deposits.

2.2 PROCESSING

The clays used for pet litter are typically found in shallow deposits and are mined using surface mining methods. After mining producers return each mined-out area to a condition equal to or better than its original condition by grading, restoring topsoil, planting grass and trees, and even creating recreational lakes.

Trucks haul the crude clays to the processing plant where a shredder breaks the large chunks of crude clay into smaller pieces. The clay then goes into dryers where it is heated to less than 300°F (less than 150°C) to drive off water. Gentle granulation reduces the particle size of the clay to the granules. These granules are then passed over screens to recover the sizes required for specific products. Dedusting, and the addition of other ingredients such as colorants and fragrances follow. The finished product is then packaged in paper or plastic bags, jugs or cartons. After palletizing, the product leaves the plant in trucks or railcars. Figures 1 and 2 illustrate the general methods used for producing conventional and clumping pet litter.

3.0 GEOLOGY – OCCURRENCE AND MINERALOGY

The six sorptive minerals studied in this investigation are from deposits highlighted in Figure 3. Additional deposits used by SMI members are also highlighted.

All of these deposits are sedimentary in origin and millions of years old. None of these materials have experienced temperatures higher than 150° C either during formation or after deposition. The key to the nomenclature of geological ages used in these descriptions appears in Figure 4. The basic semi-quantitative mineralogy of the sorptive mineral-based pet litter products marketed from these deposits, as determined by X-ray diffraction techniques, appears in Table I. It is important to note that the values given in Table I are semi-quantitative due to the inability of

the X-ray diffraction technique to directly measure amorphous material, such as opal. As a result, the amounts of the crystalline mineral constituents present in a sample are overestimated making these values very conservative.

Monterey formation

The Monterey formation, of Miocene age, is up to 1,000 meters in thickness, and is predominantly a diatomite altered under pressure and low-temperatures to a variety of siliceous

rock types^{1, 2}. Opal-CT, a non-crystalline form of hydrated silica, is very common in the formation. The change from diatomite, which is composed of opal-A to opal-CT occurred at low temperatures, probably 50° C to 100° C, over geologic time.

The major mineral component of the Monterey formation is opal. Appearing in minor amounts are feldspar, mica and smectite, with possible traces of dolomite. Quartz accounts for approximately 7.0 percent of the Monterey material.

Diatomite deposits of central Oregon

The Oregon diatomite, found in the Christmas Valley area of Lake County, Oregon, is of lower Pliocene age, and results from the accumulation of siliceous shells of microscopic algae in a fresh water lake. The material consists largely of the partially broken diatomite frustules composed of amorphous opal A and clay³.

The major components are diatom frustules, which produce an X-ray diffraction pattern of an opaline amorphous material. Also present in minor amounts are calcite, dolomite, feldspar, and smectite. Quartz is not detected.

Porters Creek formation

The Porters Creek formation, part of the Midway group of lower Paleocene age, has a middle unit consisting of claystone. The regions of the claystone that contain opal function as a sorptive material. Outcrops of the sorptive claystone appear along a narrow arc in the lower Mississippi Valley (Figure 3). The Porters Creek formation resulted from the deposition of sediments in a shallow sea, often in the environment of deltas⁴.

The major components are opal and smectite. Also present in minor amounts are feldspar, mica, and in trace amounts clinoptilolite and pyrite. Quartz accounts for approximately 8.5 percent of the Porters Creek material.

Twiggs fullers earth

The Twiggs fullers earth lies above kaolin deposits located in central Georgia and is part of the Barnwell group of upper Eocene age⁵. It consists largely of clays and opal. The origin appears to be the result of sedimentation in lagoons near the sea.

The major components are opal and smectite. Also present is a minor amount of kaolinite. Quartz accounts for approximately 5.3 percent of the Twiggs material.

Meigs fullers earth

The Meigs fullers earth, of middle Miocene age, occurs in a narrow band extending from the Florida-Georgia line generally north through Thomas County, Georgia. The deposit formed in a narrow seaway that crossed from the Gulf of Mexico to the Atlantic Ocean. The sources of the material for the clay were siliceous materials derived from the Piedmont and carbonates from the Florida platform. As the land rose, the seaway dried up and the sediments altered to clays⁶.

The major components are smectite and palygorskite, with locally abundant opal. Merkl also reports minor amounts of apatite, kaolinite and sepiolite. Quartz accounts for approximately 5.3 percent of the Meigs material.

Wyoming bentonite

Wyoming bentonite, of middle Cretaceous age, describes several related deposits occurring in Wyoming, northeastern South Dakota, and Montana^{7, 8}. Wyoming bentonite occurs in the Frontier, Mowry, and Thermopolis Shale formations. The clay results from alteration of rhyolitic volcanic ash that fell into a shallow sea. As used here, the term "bentonite" is restricted to a geologic deposit consisting of smectite clay minerals derived from the alteration of volcanic ash.

The major component is sodium montmorillonite, a smectite clay mineral. Also present are mica and opal. Minor amounts of calcite and feldspar. Gypsum may be present locally. Quartz accounts for approximately 2.7 percent of Wyoming bentonite.

Calcium smectite deposits of north central Florida

The calcium smectite deposits found in northern Marion County Florida occurs in the Hawthorne formation of Miocene age. These deposits are of sedimentary origin.

The major mineral component is calcium montmorillonite. Also present are opal-CT and minor amounts of apatite, feldspar, and illite. Quartz accounts for less than 10% of the north central Florida smectite deposits⁹.

Fullers earth deposits of eastern Virginia

The eastern Virginia fullers earth deposit is in the Calvert formation, middle to lower Miocene, of the upper Pliocene to lower Miocene Chesapeake Group. These sedimentary sequences are predominantly olive-gray in color and intermittently clayey and silty. The unit ranges from 0 to 600 feet in thickness¹⁰.

The major components are calcium smectite clay and opal-CT, with minor amounts of accessory minerals. The quartz content is approximately 9%.

4.0 NATURE OF SILICA IN PET LITTERS

4.1 TYPES OF SILICA

"Silica," is in actuality, a complex group of substances¹¹. Some types of silica are crystalline others are non-crystalline. The silica tetrahedron, a silicon ion surrounded tetrahedrally by four oxygen ions, provides the basic building block. The arrangement of these tetrahedra in space can be orderly thus producing various crystal structures. The arrangement can also be random or partially random and thus, be incapable of producing a periodic crystalline structure. A further complexity is the nature of the Si-O bond itself. Its ionic or covalent nature is not fully understood^{12, 13}. Some forms silica also have essential water constitution. These forms are not "silica" in the strictest sense, but hydrated silica.

4.1.1 Crystalline Silica

The various crystalline configurations exhibited by silica are termed polymorphs¹⁴. The most common polymorphs are quartz, cristobalite, and tridymite. The phase diagram (Figure 5) concisely illustrates the relationships among various forms of crystalline silica¹⁵. The only polymorph of crystalline silica occurring in pet litters produced by SMI member companies is quartz. Quartz forms at temperatures well below that of tridymite and cristobalite, as seen at the left side of the phase diagram.

Petrographic microscopists have long recognized that quartz possesses many degrees of crystallinity. The conventional X-ray diffraction patterns for several quartz varieties appear in Figure 6. These varieties include a National Institute of Standards and Technology ("NIST") quartz, a commercial tripoli, and a chert nodule commonly found in limestone. The NIST quartz standard consists of well-ordered single crystal fragments of the better-crystallized high-temperature variety. Tripoli and chert are the poorly crystalline, less ordered, authigenic, low-temperature precipitate varieties commonly appearing in sedimentary rocks. A cursory examination by X-ray investigators with little mineralogical background might conclude that the crystallinity of these three samples would be either identical or not relevant.

Over 20 years ago Murata and Norman¹⁶ carefully examined X-ray diffraction patterns of quartz samples and discovered that a group of five diffraction peaks are diagnostic of the crystallinity. These peaks, appearing at $68^\circ 2\theta$ when using copper $K\alpha$ radiation are termed the "68° quintuplet." Figure 7a shows the 68° quintuplet for a series of crystallinities for different varieties of quartz. The quartz varieties with the best degree of crystallinity are from high-temperature single crystal grains. Those with the poorest degree of crystallinity are from low temperature, polycrystalline quartz grains. Well-ordered quartz reveals five resolved, distinct X-ray diffraction peaks. Poorly crystallized quartz is defined by increasingly broader and unresolved maxima. Murata and Norman devised a crystallinity index, graphically illustrated in Figure 7b. The crystallinity index, sometimes termed the "Murata ratio," ranges from a normalized 10 for well crystallized varieties to less than 1 for the poorest crystallized, polycrystalline, low temperature material. Asterisks in Figure 6 denote the quintuplet peaks. The dissimilarity of the crystallinity is readily apparent between the well-resolved quartz standard and the tripoli and chert varieties.

Quartz found in the geological deposits used for pet litter can include several varieties. One variety comprises single crystal grains that ultimately derive from igneous or metamorphic rocks and have high crystallinity. Wind or water brings these grains into the deposit during its formation. Such grains are termed "detrital." Some clay deposits form by weathering of preexisting rocks. The quartz grains that remain after weathering destroys the original rock, *in situ*, are "residual" grains. They also can have a high crystallinity. A third variety can form after the original deposition of the clay or its precursors. This variety results from precipitation of silica from the pore water contained in the sediment. These are "authigenic" grains, and normally have low crystallinity. Examples of this variety of low crystallinity quartz are chert and chalcedony.

4.1.2 Amorphous and Non-Crystalline Silica

Many naturally occurring forms of silica do not have long range periodicity and are not crystalline. These forms include the opals, which are hydrous forms of silica. These forms are usually specified as opal-A, opal-CT, and opal-C. Opal-CT occurs in most pet litters. Unfortunately, much confusion has existed regarding the nature of these opal forms. Smith recently made an exhaustive survey of the scientific literature and developed a straightforward nomenclature and classification¹⁷. He defined opal-CT as a non-crystalline form of hydrated silica. This he termed "paracrystalline," indicating that it has limited short-range ordering of six-member Si—O rings in a disordered matrix. Paracrystallinity connotes a lack of true crystallinity, and implies only partial ordering in a disordered matrix. Non-crystalline forms of silica are not considered carcinogenic by IARC, which lists them in class 3.

Opal-CT produces a few X-ray scattering effects occurring in areas near to some of the X-ray diffraction lines of cristobalite, a biologically aggressive form of crystalline silica. This similarity has often caused opal to be confused with cristobalite. To avoid this confusion, Miles¹⁸ combined two NIOSH procedures, 7500¹⁹ and 7601²⁰, to enable the positive differentiation of opal-CT and cristobalite. This combined procedure involves the processing of samples in phosphoric acid prior to x-ray analysis to dissolve and remove the amorphous and non-crystalline phases such as opal. This results in concentration of the insoluble crystalline components such as cristobalite, tridymite and quartz. Smith found that very few analytical laboratories are aware of the ambiguity between opal and cristobalite in the literature and routinely fails to distinguish between the regulated cristobalite and the non-regulated opal.

5.0 REGULATION AND COMPLIANCE

5.1 REGULATION OF QUARTZ DUST

The link between high exposures to industrially generated quartz dust and human lung disease has been known since antiquity. Development of standards to control exposure to quartz containing dust began in the United States in 1928 for granite workers and other similar industries. ACGIH began developing standards in 1946, and continues to update them. OSHA and MSHA have developed standards similar to those of the ACGIH.

During the past few decades research has shown that the quartz dust that causes lung damage is the result of processes that break the quartz into freshly fractured particles that are then immediately respired. These processes include blasting in mines, sawing quartz-containing rocks, concrete and bricks, dry drilling in siliceous rocks, abrasive blasting and cleaning using quartz sand, and grinding quartz sand from foundry castings. Conversely, exposures to quartz dust contained in non-fractured quartz such as that generated by farmers during plowing appears to be far less of a problem. Quartz is a minor component of pet litter. The type of quartz found in pet litter has geologically aged surfaces and often exhibits a broad range of crystallinity. The processing of the clay for pet litter does not produce freshly fractured surfaces.

Certain pet litters contain bentonite with both well-crystallized, single crystal quartz and poorly crystallized chalcedonic quartz. As Table 3 shows, the crystallinity indices range from 3.5 to 8.7. The amorphous solubility characteristics of single crystal lota quartz, finely pulverized below 1µm, suggest a relationship with polycrystalline, poorly crystallized chalcedonic quartz. The lota material has lost most of its crystalline character and approaches the amorphous condition brought about by a combination of particle size diminution and lattice distortion induced by mechanical grinding. IARC has classified amorphous silica in Group 3, and not in regulated Group 1. A method that can determine such silica varieties combines the concentration techniques of NIOSH procedure 7601, coupled with heavy liquid techniques that separate the lighter poorly crystalline quartz from the heavier single crystal varieties. Additional solubility measurements can confirm if the chalcedonic quartz indeed has true amorphous characteristics, and thereby does not fall under crystalline silica regulations.

Current crystalline silica-monitoring programs fail to acknowledge the influence of crystallinity on biological processes. Both the regulators and the biological experimenters, seemingly unaware of the potential influence of crystallinity, have limited *in vitro* and *in vivo* experiments to pure and non-contaminated, crushed high-crystallinity quartz such as MIN-U-SIL. This omission erroneously indicts all quartz varieties as being biologically aggressive. Furthermore, this preoccupation with single crystal fragments totally ignores the preponderance of low-dose exposures such as those encountered with pet litter or with commingled well-crystallized quartz and poorly crystallized chalcedony. This conclusion validates the IARC contention in 1997 that quartz is not always carcinogenic even in industrial exposures.

5.2 METHODS OF DETERMINING COMPLIANCE

Monitoring the air for respirable quartz particles and determining their concentration provides the principal method for regulating health hazards associated with respired quartz. Several federal regulatory agencies, such as OSHA and MSHA, have prescribed procedures for the use of such monitors. Two principal analytical methods are available to quantify the amount of crystalline silica gathered by air monitors: X-ray diffraction and infrared spectroscopy. X-ray diffraction is the preferred method because it can identify as well as quantify the polymorphs of crystalline silica present. Identification and quantification requires substantial skill and experience, however. Smith recently discussed the problems encountered in using X-ray diffraction methods to detect and quantify quartz in samples²¹. He noted that analytical laboratories must exercise great care in X-ray diffraction determinations of quartz and other polymorphs of crystalline silica if they are to produce accurate results.

5.3 IMPORTANCE OF THE TYPE OF QUARTZ USED IN BIOLOGICAL STUDIES

Biological studies, either *in vivo* or *in vitro*, often provide the data on which regulators base their decisions. The literature on the subject is vast.

The health effects of crystalline silica, which are associated with the non-malignant disease silicosis, have long attracted scientific interest. The IARC decisions of 1987 and 1997 regarding the carcinogenicity of quartz have intensified the need to understand the implications of quartz

on health^{22 23}. The relationships are not always clear-cut^{24 25 26}. Confounding effects, methodological issues, and the complexity of silica itself show the need for extreme care in assessing data.

The earlier discussion stressed the differing degrees of crystal perfection in quartz and the great difference in quartz surfaces. Consequently, any study of the biological effects of silica must define and consider the particular properties of crystalline silica use under consideration. Laboratory experiments designed to elucidate environmental or industrial exposures must use the type of crystalline silica involved in such exposures. Researchers involved with inhalation experiments must crush quartz to obtain sufficient respirable material. The properties of crushed quartz are quite different from those found in environmental exposures involving quartz with non-fractured surfaces. Consequently, the results of such experiments will have little bearing on predicting the health effects in such environmental exposures. Few investigators have studied the nature of the surface of quartz used in *in vitro*, *in vivo*, or epidemiological experiments. The interactions between biological fluids and tissue occur at the surface of the quartz. If the surfaces of two quartz samples are dissimilar, then it is quite likely that the biological reactivity will be different. Regulation of quartz exposure should consider the nature of the surface of quartz, not merely the presence of quartz.

6.0 EPIDEMIOLOGICAL EVIDENCE

The epidemiology of quartz does not show a clear-cut relationship between exposure and the onset of silica-induced lung disease. In many industrial exposures silica-induced lung diseases are either absent or much less common than the amount of exposure would suggest.

6.1 ENVIRONMENTAL EPIDEMIOLOGY

For a substance so ubiquitous in the environment, reports of silica-related lung disease are virtually absent outside of industrial exposures. Most of the suspected cases involve arid, dusty regions of the world. The EPA has provided an excellent summary of possible environmental exposures leading to silica-induced lung diseases²⁷.

6.2 EPIDEMIOLOGY OF A SORPTIVE MINERAL PLANT

Epidemiological studies generally target industries that show unusually high incidences of exposure-related diseases. Only one such study exists in mines related to the types of minerals used in pet litter. That was a study of the possible health effects of attapulgite, not quartz. This study, by Zumwalde and coworkers²⁸, investigated the mortality of attapulgite workers in south Georgia. Attapulgite clay contains five or more percent quartz. Nevertheless, no significant increase in standard mortality rates due to lung cancer or other lung disorders was evident.

6.3 EPIDEMIOLOGY IN COAL WORKERS

The closest epidemiological parallel to the quartz encountered in pet litter may be bituminous coal mining. Both bituminous coal and the sorptive minerals used in pet litter commonly have

small amounts of quartz. As Zumwalde showed, silica related lung diseases are absent in the sorptive mineral processing plant. Similarly, in bituminous coal operations, lung disorders are not a function of the content of quartz in the dust, but of the total exposure to dust. The surface properties of quartz in these coal mines have parallels with the surface properties of quartz found in pet litter.

Extensive studies of American²⁹, British³⁰⁻³¹, and German³² coal mine workers show that the onset of pneumoconiosis and progressive massive fibrosis is a function of the coal rank rather than the quartz content. Coal rank is the carbon to hydrogen ratio. The hydrogen content is a measure of the volatiles in the coal. Metamorphic changes in the coal induced by geological heat and pressure can reduce the volatiles and induce other changes in the coal. These changes can result in the aluminosilicate coatings of quartz in bituminous coal disappearing, thereby producing the high rank coal, anthracite. Castranova, Vallyathan, and Wallace have discussed these changes in detail.

The experience in Pennsylvania underscores these observations. Silica-induced lung diseases occur in the high rank anthracite fields in the eastern part of that state. In the Johnstown area, the coal is more bituminous and of lower rank. While it may have more quartz than the anthracite, silica-induced lung diseases are less common in the Johnstown area. In the Pittsburgh area, the coal is bituminous and of low rank, but with a higher amounts of quartz than the anthracite. In this area, lung disorders are not a function of the quartz content, but of total dust exposure. Apparently, the aluminosilicate coatings occluding the surface of the quartz grains in the bituminous coal reduces the biological activity of the quartz. The metamorphic changes that raised the rank of the anthracite are also reduced or eliminated by the aluminosilicate occlusions on the quartz grains. A section below discusses the mechanism of these coatings in affecting biological activity.

6.4 EPIDEMIOLOGY IN AGGRESSIVE INDUSTRIAL PROCESSES

The evidence shows that aggressive biological activity of quartz is related to freshly fractured surfaces. Silica-related lung diseases most frequently occur in those industrial processes where the surfaces are fractured and the particles are immediately respired. These processes include blasting siliceous rocks in mines, dry drilling and sawing siliceous rocks, concrete and bricks, and abrasive blasting with quartz sand. None of these processes are encountered in the production of pet litter.

6.5 STUDIES OF OIL SHALES

In the 1980's, oil shales promised to be a major source of petroleum. As a point of due diligence, studies were done to assess the possible health risks associated with the mining and processing of the oil shale, as well as the disposal of the copious processed, retorted wastes. These studies included long term, low-dose inhalation by experimental animals of raw and retorted oil shale³³. The animals did show extensive silica-related lung diseases, however the preparation of the dust required aggressive crushing and ball milling, thus producing preponderantly freshly fractured grains of quartz. As a result, the data are not that of dusts

containing quartz with undisturbed surfaces, but quartz from processes that produce biologically aggressive quartz. Consequently, the data are not relevant to discussions of dusts containing quartz with old, undisturbed surfaces.

7.0 THEORIES ON THE BIOLOGICAL ACTIVITY OF QUARTZ

7.1 IMPORTANCE OF THE SURFACE

The surface of a solid, as well as its internal structure, can very often determine its chemical activity in a system³⁴. Epidemiological studies show this fact is true for quartz. In many cases, the biological activity of a dust is not related to the quartz content. It is the surface of quartz that is the principal factor in its biological activity. Understanding this surface is absolutely essential in interpreting epidemiological data and assessing any risks. The types of quartz surfaces most often considered are freshly fractured, silanol covered, and occluded. The freshly fractured surface appears to be by far the most biologically aggressive. The occluded surface appears to be the least aggressive.

7.2 IMPORTANCE OF FRESHLY FRACTURED VERSUS AGED SURFACES

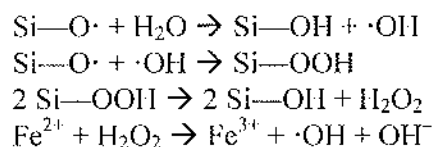
7.2.1 Generation of Free Radicals by Freshly Fractured Surfaces

Several investigators have studied the surface properties of quartz in an effort to understand its biological activity^{35 36 37}. Quartz is a stable substance with a low solubility. However, breaking open a grain of quartz will reveal a fresh, chemically reactive surface. This surface has unsatisfied or "dangling" chemical bonds that are not easily satisfied because of the rigidity of the crystal structure of the grain. This reactive surface can generate chemical free radicals that are biologically very aggressive. Chemical changes that occur rapidly over time reduce the activity of this surface, eventually creating a new, chemically stable surface. This aged surface has a structure and chemical composition that is different from that of the internal portion of the quartz grain. As a result of this process, the surface no longer has the ability to generate biologically aggressive free radicals.

The extreme importance of free radicals in biological activity has encouraged extensive investigations of quartz surfaces using electron spin resonance (ESR) spectroscopy, also termed electron paramagnetic resonance (EPR) spectroscopy. Fubini³⁸, Castranova³⁹ and others have described the effects of fracturing quartz on the ability of the surface to generate radicals. Fubini listed the possible species on a broken quartz surface, and showed evidence of both $\equiv\text{Si}\cdot$ E' centers and $\equiv\text{Si}-\text{O}\cdot$ radicals on freshly fractured surfaces. Figures 8a and b illustrate the spectra obtained by Fubini. The concentration of radicals depends on the length of time since fracturing. Figure 8c shows the significant increase in radicals on freshly ground quartz compared to the same sample ground several years ago.

Castranova has made an extensive study of the nature of the radicals generated by the surface and the implications to biological activity. He proposed the generation of the radicals through the breaking of Si-O bonds (Figure 9a), and also demonstrated their presence through ESR

spectroscopy (Figure 9b). Castranova also studied the method by which these radicals can generate hydroxyl radicals through a Fenton-type reaction with ambient water. The reaction progresses by the following scheme:



Castranova offers experimental evidence for the hydroxyl radicals by using DMPO (5,5-dimethyl-1-pyrroline-N-oxide) as a trap, and detecting and measuring the DMPO -- $\cdot\text{OH}$ adduct by ESR. Unground quartz has little if any ability to generate hydroxyl radicals, but ground quartz has a significant ability to do so (Figure 10a). The ability of a freshly ground quartz sample to generate hydroxyl radicals follows first order kinetics, with a half-life of $T_{1/2} = 1$ day (Figure 10b).

7.2.2 Biological Damage Caused by Free Radicals from Freshly Fractured Surfaces

Castranova also has shown the importance of the freshness of the fracture on biological activity. Grinding pure IOTA quartz in an air jet mill will produce a strong ESR signal (Figure 11a). Aging the sample for only two months will produce a significantly weaker signal (Figure 11a). Inhalation experiments with male Fischer 344 rats employed the quartz in inhalation experiments. One set of rats experienced the quartz immediately after milling; another set of rats experienced the quartz two months after milling. Examination of the rats provided information on cellular yield from bronchoalveolar lavage, as well as protein and phospholipid in the acellular lavage fluid. Nitric oxide synthase-dependent CL as well as zymosan-stimulated CL generated from alveolar macrophages from the lungs was also studied (Figure 11b-e). Castranova concludes "...inhalation exposure of rats to freshly milled α quartz causes significantly greater cytotoxicity and inflammation than exposure to an equal concentration of aged dust of the same particle size and composition. Cytotoxicity is demonstrated by elevation of red blood cells and protein in the air spaces. Inflammation is demonstrated by the enhanced recruitment of leukocytes, increased potentiation of oxidant release from alveolar macrophages, and greater lipidosis. Nitric oxide accounts for a portion of the increased number of oxidant species generated from silica-exposed alveolar macrophages. This increased oxidant load with freshly milled silica versus aged silica may account in part for the increased risk of silicosis in workers such as sand blasters, rock drillers, and silica flour millers, who are exposed to freshly fractured silica particles." The decline in two months of the biological activity is substantial, but some remains. The likelihood of troublesome activity remaining after hundreds or thousands or millions of years is remote. A depiction of the surface of quartz through other techniques can give a basis for this contention.

7.2.3 Reduction of Free Radical Generation by Hydration

Depicting the surface of quartz as it relates to chemical activity poses substantial problems. Solubility measurements can give insight into the nature of the surface. Dove⁴⁰ and Rimstidt and

Dove⁴¹ have described and discussed the solubility of quartz in great detail. Rimstidt⁴², in his summary report, has shown that amorphous silica is more soluble than quartz over a range of temperatures (Figure 12a). His studies of solution of a grain of quartz show a distinct break in the solution curve. The first part of the curve shows rapid dissolution and the second shows a much slower dissolution (Figure 12b). Two phenomena could account for the initial rapid dissolution: the rapid dissolution of an amorphous surficial coating or the dissolution of sharp points and edges of quartz. Figure 13a shows the increased solubility of these points and edges to be related to decreasing radii of curvature. That is to say, broadly rounded surfaces are less soluble than points. The lifetimes of these sharp points and edges is short (Figure 13b). The much slower rate is the gradual solution of the broad surface, described immediately below.

The dissolution of the freshly fractured surface shows a stepwise development from the fresh surface to a fully hydrated surface. The dangling bonds and their attending surface structures are unstable and begin to hydroxylate under ambient conditions. Hydroxylation continues until the surface is composed primarily of Si-OH or "silanol" groups. The hydroxylated surface adsorbs water and the adsorbed water film above three monolayers (see Figure 14) exhibits bulk water properties to biological tissue according to Parks⁴³. The dissolution of the silica surface by water continues slowly. It progresses through reaction of water molecules with the hydroxylated surface. The reaction simply replaces one silanol layer with another. The structure of the freshly fractured surface with the attendant free radicals does not reappear.

Studies involving direct observation of the surface combined with other measurements to elucidate the surface are rare in the literature. A study specially done for this effort will appear in a later section.

7.2.4 Reduction of Biological Activity with Aging

As noted above, Castranova and his associates have been active in understanding how quartz surfaces interact with biological systems. They measured the changes in the surface of freshly fractured quartz by ESR spectroscopy. They coupled the spectroscopic study with animal studies using freshly jet-milled standard IOTA quartz immediately after milling and after two months aging. The freshly milled quartz had significantly greater concentration of free radicals, and the decline in biological response lay in the decline in the number of free radicals.

7.3 CLEARANCE OF PARTICULATES FROM THE LUNG

The body has several different pathways to clear inhaled particles, depending on the site of their deposition in the respiratory tract^{44 45}. Larger particles with a mean aerodynamic diameter of $>10\mu\text{m}$, deposit in the nasal passages. Sneezing or blowing typically eliminates those that deposit near the front of the non-ciliated epithelium. Those particles deposited in the posterior region of the nasal passages on the mucus-lined ciliated epithelium are swept to the nasopharynx and swallowed. Swallowed particles are eliminated via the gastrointestinal tract. Smaller particles, $3\mu\text{m}$ to $10\mu\text{m}$, impact in the tracheobronchial region and are swept by mucociliary action toward the oral cavity. There they are either expectorated or swallowed. The clearance rate of particles from these two regions is constant and rapid, with half-lives of hours to days.

Particles of 0.5 to 3 μ m may reach the alveolar region of the lung. Here, particles will be phagocytosed by alveolar macrophages, which then migrate to two locations. One is the ciliated epithelium and then to the oral cavity via mucociliary action. The other is the lymphatic system and eventually to the gastrointestinal tract. Clearance from the alveolar region of the lung can take weeks to months. In circumstances where the particle load in the lung is very high, macrophages become engorged, impairing their ability to phagocytose and migrate. This overload results in the death of the macrophage with the subsequent release of the particles and an expansion of the inflammatory process⁴⁶. In these cases, particles can persist in the lung indefinitely.

7.4 THEORIES OF MECHANISM OF BIOLOGICAL DAMAGE

7.4.1 Inflammation

Theories describing silica-induced lung diseases will, in most instances, first consider the inflammatory response to quartz. Inhalation of crystalline silica at inflammatory levels leads to the recruitment and activation of phagocytic cells, and subsequent release of mediators including reactive oxygen species. These inflammatory cell oxidants have two effects that may contribute to the formation of tumors. First, they may cause damage to DNA resulting in mutations. Second, they increase cell proliferation, providing a means for fixing and perpetuating the mutation. Borm and Driscoll⁴⁷ have shown that oxidants produced by lung cells as a result of intratracheal dosing with crystalline silica can cause mutations in rat lung epithelial cells. An additional mechanism whereby crystalline silica may cause lung damage is by direct toxicity to DNA. Two studies have shown that crystalline silica can interact directly with DNA, *in vitro*, causing strand breaks^{48 49}.

The importance of this mechanism in the human disease response remains undefined. Crystalline silica is not very active in most of the standard assays for mutagenic *in vitro* genotoxic effect, supporting the notion that direct damage to DNA may be of secondary importance. Checkoway⁵⁰, Holland⁵¹, Lippmann⁵², and McDonald^{53 54} have published excellent surveys on the biological activity of crystalline silica. All of these studies, however, involve freshly fractured quartz or cristobalite.

The surface of pure quartz that has hydroxylated will contain silanol, Si—OH, groups⁵⁵. These groups have far less activity than free radicals, yet because of their potential acidity, they will have some biological activity. Some investigators have associated hydrogen bonds between the silanol surface and biological membranes, with the loss of membrane integrity.

7.4.2 Damage to Macrophages

Macrophages that have phagocytized a bacterium, for example, can generate a variety of biological chemicals to destroy it. When a macrophage phagocytizes an inorganic particle, the macrophage will secrete factors promoting fibroblast proliferation and collagen synthesis. Ultimately, when engorged with reactive mineral particles, the macrophage can die, and release even more biologically active chemicals.

Fubini and coworkers extensively discussed the probable fate of macrophages that engulfed silica particles. They emphasize that "... surface radicals may play a specific role in the fibrogenic response to silica ..." They did note that fibrogenic response is possible from milled silica stored for several years. For a laboratory experiment, several years is a long time, but it is the barest fraction of geologic time. The grains of quartz found in pet litters have surfaces millions of years old, with enough geologic time and weathering for all the free radical activity to have vanished.

Vallyathan has discussed the fate of macrophages that have engulfed mineral particles, including quartz, bentonite (smectite) and kaolin⁵⁶. Although all three minerals showed *in vitro* response, only in quartz was there a progressive granulomatous and fibrotic response *in vivo*. He concluded that *in vitro* responses are not good predictors of fibrogenicity.

7.4.3 Protection of Macrophages

The lack of silica-related lung diseases among bituminous coal workers suggest that some protective mechanism is at work in the lung. The difference in *in vitro* and *in vivo* results of mineral dusts indicate that extracellular factors are involved. In fact, a particle penetrating deep into the lung will first come in contact with pulmonary surfactant, particularly dipalmitoyl phosphatidylcholine (DPL). These surfactants can coat the particle. Consequently, the macrophage initially will encounter the surfactant and not the mineral surface⁵⁷.

Wallace has shown that thin coatings of aluminosilicate clays cover the surfaces of quartz from bituminous coal mines. He has also shown that the pulmonary surfactants will not remove these coatings⁵⁸. Other work has studied the attack of phospholipase A₂, an enzyme associated with cellular membranes and lysosomes, on the DPL layer on both kaolin clay and quartz. The DPL disappears more rapidly on quartz than on kaolin⁵⁹. Both the structure and the chemistry of the kaolin may be responsible. Attachment of the DPL to quartz appears to be through interaction with the trimethylammonium groups⁶⁰. The trimethylammonium groups are probably involved with attachment to kaolin, but interaction of the phosphate portion of the DPL molecule can also interact with the aluminol groups of the kaolin.

A complete understanding of the protection of the macrophages by coatings of clay awaits more research. Nevertheless, the clay coatings and other aluminosilicate adventitious material on the surface of quartz grains, and the adsorption of pulmonary surfactants appear to provide protection for macrophages.

The amount of quartz respired when using pet litter is so small that the normal clearance mechanisms will expel the majority of it prior to its reaching the alveoli, thereby eliminating its ability to engorge the macrophages. In geologically aged samples, the surface has lost its ability to generate free radicals, which are biologically aggressive. In addition, the surfaces are occluded with adventitious material, which further minimizes the biological activity. Consequently, it is unlikely that the quartz will cause the death of the macrophages, with the attendant expulsion of inflammatory agents.

8.0 SMI RESEARCH PROGRAM

SMI initiated several research programs to ascertain the safety of pet litter.

8.1 RESEARCH PROGRAM OBJECTIVES

The objectives of the research program were as follows:

- establishing consumer exposure to quartz during the normal use of pet litter
- determining the nature of the surfaces of geologically aged quartz grains
- establishing the properties of quartz in pet litter

8.2 STUDIES ON THE SURFACE OF GEOLOGICALLY AGED QUARTZ GRAINS

The quartz that is present in clay deposits has geologically aged surfaces. Little if any research has appeared that addresses the properties of these surfaces. Consequently, the SMI commissioned two studies at the Virginia Polytechnic Institute and State University. Dr. Michael Hochella studied the surfaces of geologically aged quartz by instrumental methods⁶¹, while Dr. Donald Rimstidt studied them using solution techniques⁶². Both researchers are preeminent in their fields.

8.2.1 Materials

Quartz is a minor component in clay deposits used for pet litter, and is intimately associated with other minerals. Removal of individual grains requires techniques that would significantly alter their surfaces. Rounded, geologically aged quartz grains are present in certain sand/kaolin clay deposits. Their separation, classification, and concentration is through water beneficiation techniques, which does not alter the mineral surfaces. This study used these quartz grains as good models for the quartz grains from clay deposits used for pet litter. The pioneering aspect of the direct observation studies did require examination of at least one quartz particle that had flat surfaces to maximize results. The particle chosen was a quartz crystal of Ordovician age (480 million years old), known as a "Herkimer diamond." For comparison, a sample of IOTA quartz, with a freshly fractured, highly cleaned surface was included.

8.2.2 Experimental Approach

The work combined direct surface observation and dissolution techniques. Appendix I gives detailed descriptions of the experiments. The direct observation included x-ray photoelectron spectroscopy (XPS). This method can probe a sample to several nanometers below the surface. The purpose of the dissolution experiments was to detect any surface characteristics that differ from bulk quartz. It also included low energy electron diffraction (LEED), a method that can study the crystallography of a surface.

Dissolution experiments detect changes in solubility with time to examine chemical properties of a surface. Treatment of the data by kinetic and thermodynamic techniques can postulate the character of the surface being dissolved.

8.3 EXPERIMENTAL RESULTS AND DISCUSSION

8.3.1 Instrumental Study

The LEED experiment, done only on the Herkimer “diamond” because of its flatness, showed no diffraction pattern in the as-received condition. This result indicated adventitious material at least 10 Å thick. Only by very vigorous cleaning, did even a meager pattern appear.

The XPS data showed that the surfaces of the grains are not pure quartz, but have “adventitious” materials, that is, a layer up to a nanometer in thickness of adsorbed materials, including carbon-containing compounds. The data also show that the surroundings of the oxygen atoms at the surface are not that of pure quartz. A grain deliberately broken for this experiment showed a much scantier presence of foreign material.

The data show that the geologically aged surfaces are not those of crystalline silica. An adventitious layer of foreign material resides on the surface. The surface of the Herkimer “diamond” does not show crystallinity unless vigorously cleaned. Even after vigorous cleaning, the surface shows only meager order.

8.3.2 Dissolution Study

The dissolution data for the geologically aged quartz showed an initial somewhat fast dissolution followed by a much slower dissolution. Two explanations for this behavior are numerous sharp edges and points, or the presence of amorphous silica. Kinetic arguments show that sharp edges and points are not the reason and are absent. The explanation is presence of amorphous material. Calculations show that if this material is in a continuous layer it is 0.45 Å “thick.” Because such a thickness is a physical impossibility, the data indicate that the layer is discontinuous.

The data indicate the grains have discontinuous amorphous coatings on a naturally hydrated silanol layer. The amorphous surface, resulting from precipitation phenomena, dissolved at rates comparable to amorphous silica until reaching the stable silanol covering the entire quartz surface. This silanol layer is slightly soluble in water and biological fluids, thereby replicating itself so that the detrimental dangling free radical sites are prohibited from developing. Additionally, Rimstidt’s data strengthens the argument that freshly fractured quartz particles produced during aggressive quartz processing schemes are pathogenic. In comparison, the quartz particle existing in the ambient environment, not subjected to harsh treatment, are benign. Quartz grains produced by industrial processing that does not freshly fracture those grains, should also be biologically inactive. Quartz grains, also in conjunction with its passivated surface, may also develop coatings of clay⁶³ or amorphous materials, such as opal.

9.0 CHARACTER OF AND EXPOSURE TO RESPIRABLE QUARTZ IN PET LITTER

Pet litter is a granular product. Particles of respirable size, $\leq 10\mu\text{m}$, comprise a very small fraction. These particles must be launched into the air to be respired. Pouring or scooping can launch only a small fraction of the particles of respirable size into the air, certainly not all of them at once. A carefully executed experiment can model consumer use of pet litter and measure the amount of respired dust encountered by the consumer.

9.1 GENERAL OBJECTIVES

The objectives were to develop and carry out methodologies both to determine the amount of respirable dust breathed during the normal use of pet litter, and to establish the nature of the quartz present in the dust. The nature of the surfaces of quartz present in pet litter dust appears in an earlier section of this presentation.

9.2 NATURE AND DISTRIBUTION OF QUARTZ IN PET LITTER

The nature of airborne particles of respirable size produced during the normal use of pet litter requires thorough study because these are the particles that can penetrate most deeply into the lung. Of particular importance during such studies is the thorough characterization of the type and nature of the quartz particles present. These are the particles that have the most potential to cause health problems. Characterization of the quartz present in the respirable pet litter dust poses some unusual problems, however. For example, the amount of respirable dust generated by using the pet litter is very little and the amount of respirable quartz is small. Consequently, the amount of dust collected by typical air monitors is much too small to yield a sufficient amount of quartz for proper characterization. The use of a tubular aerosol dust chamber (hereafter termed "TADC") successfully addresses this shortfall. Although this approach does not duplicate the launching of respirable dust by pouring, it does supply a sufficient amount of $\leq 10\mu\text{m}$ particles for proper mineralogical characterization.

In addition to understanding the properties of the quartz present in respirable pet litter dust, the understanding of the type and nature of the quartz present in the bulk litter product is also necessary. The reason is that regulators tend to base conclusions about the potential health hazards of a product on the total quartz present rather than on the respirable fraction. The respirable fraction may be much different in both character and amount.

9.2.1 General Approach

The protocol used in this analysis was composed of three principal elements:

1. Determination of the nature and quantity of the quartz in all size fractions in bulk pet litter. This effort used wet size separation, chemical, x-ray diffraction, and electron microscopy techniques
2. Determination of the nature and quantity of potentially respirable quartz in pet litter. This effort used a tubular aerosol dust chamber for collection of $\leq 10\mu\text{m}$ fraction.
3. Determination of consumer exposure during the normal use of pet litter. This method used a method to duplicate consumer use.

The portion of these studies that is largely mineralogical in nature appears in the reports of Dr. Robert D. Hamilton at the Johns Manville Technical Center^{64 65}. The portion of these studies dealing primarily with respirable dust and quartz encountered by the consumer appears in reports by Dr. Karen M. Vetrano of TRC Environmental Corporation⁶⁶.

9.2.2 Materials

Two groups of pet litter products were tested as part of these experiments. All of the products were purchased directly from the shelves of retail grocery stores, pet stores or mass merchandisers. These included products representing clays from the Monterey formation, the Oregon diatomite, the Porters Creek formation, the Twiggs fullers earth, the Meigs fullers earth, and the Wyoming bentonite. This group of products was purchased in January 1997. Testing of this group of samples included mineralogical examination of $\leq 10\mu\text{m}$ fractions obtained from the TADC.

The second group of samples included products representing clays of the Monterey, Porters Creek, and Wyoming bentonite. These samples were obtained directly from the shelves at retail grocery stores, pet supply stores and mass merchandisers. The purpose of the second group of samples was to compare the nature of the respirable dust collected by the tubular aerosol dust chamber with the respirable dust and quartz collected by the air monitors in the consumer exposure test.

9.2.3 Methods

This study comprised several principal steps, namely –

- establishing the distribution of silica phases in the bulk material, including distribution of varieties with differing degrees of crystallinity
- establishing the presence and quantity of respirable quartz in the dust using a tubular aerosol dust chamber; and
- comparison of respirable quartz from consumer-use tests and tubular aerosol dust chamber tests.

Bulk analysis

In order to completely characterize and understand the quartz found in pet litter, the Hamilton study used the procedures described in Appendix II. Each bulk product was separated by wet sieving into a +325 mesh grit fraction ($> 44\mu\text{m}$) and a finer component ($< 44\mu\text{m}$). The grit was further fractionated in a heavy liquid media of 2.52 specific gravity to separate the single crystal from polycrystalline varieties of quartz. Each specific gravity fraction was subsequently examined by x-ray diffraction for its crystallinity index, confirming the concentration of polycrystalline quartz varieties in the lower density fractions and the single crystal varieties in the higher density fractions.

Total respirable dust analysis

The water process above concentrated the quartz for mineralogical analysis, but did not directly address the respirable quartz existing in the dry, as-received pet litter. Pouring pet litter will

launch only part of the respirable fraction. Nevertheless, the amount of potentially available quartz in a pet litter is important for general discussions. An air elution technique using the tubular aerosol dust chamber was found to separate effectively the $\leq 10\mu\text{m}$ fraction sample from the bulk pet litter. The individual mineral components thus liberated were in sufficient quantity for further physical, chemical, and mineralogical examination. Appendix II-2 gives details of the device.

Consumer use respirable dust analysis

A key question is whether the $\leq 10\mu\text{m}$ quartz obtained by the tubular aerosol dust chamber is identical to the respirable fraction collected in the pouring experiments. The typical user of pet litter employs similar methods of filling the litter box. Consequently, closely controlled laboratory tests can duplicate these conditions to determine the amount of crystalline silica respired during use. The tests, carried out by Dr. Karen M. Vetrano of TRC Environmental Corporation, are similar to the one examined by Dr. Book in 1989. A full description of the method used to duplicate the normal use of pet litter by the consumer is included in Dr. Vetrano's Final Report, which is included in Appendix III.

Concentration techniques

The crystalline and non-crystalline silica components of each product were concentrated in the grit fraction for further evaluation. Each grit fraction was subjected to the phosphoric acid digestion described in the NIOSH Analytical Method 7601. The non-crystalline silica hydrates, such as opal, and the silicate minerals are particularly soluble in this phosphoric acid digestion process. The single crystal and polycrystalline varieties of quartz are digestion resistant. In addition, this is a valid method for distinguishing opal from crystalline silica phases.

9.2.4 Experimental Results

Hamilton's work is summarized in Tables 2 and 3, listing both the quartz content of the dust and the characteristics of the grit and fine fractions. A description of the individual samples appears Appendix II, with a summary below.

9.2.5 Discussion of Results

With the exception of sodium bentonite, quartz is exclusively the single crystal variety with crystallinity indices above 4.0. This is because the quartz is largely detrital in origin in these sedimentary deposits. The sodium bentonite studied was found to consist of both detrital and authigenic quartz (below 4.0 crystallinity index). It is noteworthy that these low crystallinity index species appear in the fines, air monitor respirable fractions, and $\leq 10\mu\text{m}$ fractions. In no case do the quartz grains appear fractured or broken. In all instances, the energy dispersive X-ray microanalysis spectroscopy (EDS) measurements show that the grains, or at least their surfaces, are not pure silica but possess measurable amounts of aluminum, and often magnesium.

The amount of $\leq 10\mu\text{m}$ quartz generated by the TADC does not correlate with that of the bulk material. The quantity, however, of the $\leq 10\mu\text{m}$ bulk quartz fraction generated by this approach is extremely small (Table 2). One sample did not contain quartz in any size fraction. The total

amount of $\leq 10\mu\text{m}$ quartz in the remaining eight samples ranges from 0.0006 and 0.0050 percent. In comparison, the total bulk quartz for these eight samples listed range from 5.3 to 9.5 percent. The total quartz content, therefore, gives no insight as to the potentially respirable quartz present. Particle size, aggressive handling and other factors control the number of respirable quartz particles present.

The quartz content of the respirable dust collected by pouring is less than the $\leq 10\mu\text{m}$ material captured by the TACD. The TADC produced approximately four times greater quartz content in the $\leq 10\mu\text{m}$ dust fraction, as did pouring. The TADC includes glass beads to fluidize the pet litter granules in the instrument. These beads also act as abrasers, releasing more quartz from the larger agglomerates constituting the granules. Further, the TADC produces a potentially different distribution of quartz dust in the respirable range. Nevertheless, the tubular aerosol dust chamber provides a maximum content of potentially respirable quartz in the dust. Therefore, the percent of respirable quartz as measured by the TADC (Table 1) overestimates the amount of respirable quartz encountered.

9.2.6 Individual Sample Descriptions

None of the samples show respirable quartz similar to freshly ground quartz particles used in *in vitro* or *in vivo* experiments. Also, none of the samples show fractured particles as encountered in abrasive blasting, dry drilling or grinding, and explosive blasting. Electron micrographs shown in Figures 15 through 20 illustrate the nature of the grains of quartz from these experiments. These liberated grains are all either rounded or sub-angular. In many cases, the quartz appears as sub-angular particles enclosed in grains of non-crystalline opal, or in composite grains of clay and quartz. Figure 17f clearly shows the typical small quartz crystals encased in opal. The quartz grains in pet litters do not have pure silica surfaces, but surfaces containing aluminum and other adventitious ions. Table 1 provides typical quartz contents of these samples as determined by X-ray diffraction.

Monterey formation

The Monterey formation originally contained detrital quartz, abundant amorphous diatoms, and other non-crystalline silica hydrates. Post depositional alteration of the biogenic silica indurated the formation, entrapping the detrital quartz in a non-crystalline siliceous groundmass. The quartz particles are grains 0.05 mm or smaller, that is, silt-size. In the pet litter, these silt-size particles are enmeshed much larger agglomerates. The grit fraction contains silicified particles with few truly distinct quartz particles. Therefore, although the total quartz content may be 7 percent, the potentially respirable quartz content is only 0.005 percent. Electron micrographs appear in Figure 15.

Oregon diatomite

The Oregon diatomite has no detectable quartz. The mineral grains appearing among the diatom pieces are principally feldspar and dolomite. Apparently the site of deposition was too alkaline for quartz to form from silica derived from the diatoms. Electron micrographs appear in Figure 16.

Porters Creek formation

The Porters Creek formation contains small composite grains of clay and quartz silicified with non-crystalline opal and free grains of quartz. None of the angular grains in the Porters Creek formation or the other formations covered here have the sharp points or edges to damage tissue. Rimstidt has shown that such sharp points and edges are not stable in an aqueous environment.

Examination of the grit shows the heavy fraction consists largely of angular and sub-angular quartz. Although the total quartz content may be 9.5 percent, the potentially respirable quartz content is only 0.005 percent. Electron micrographs appear in Figure 17.

Twiggs fullers earth

The Twiggs fullers earth contains small grains of quartz that appear largely in composite grains of quartz and clay silicified with non-crystalline opal. The heavy fraction of the grit shows angular individual quartz grains. The lighter fraction largely is siliceous clay grains with no angular quartz. Although the total quartz content may be 5.3 percent, the potentially respirable quartz content is only 0.002 percent. Electron micrographs appear in Figure 18.

Meigs fullers earth

The Meigs fullers earth contains small free grains of quartz with fewer small angular quartz grains in partially silicified clay composite grains. The independent quartz grains are partially rounded with frosted and pitted surfaces. The heavier grit fraction consists principally of sub-rounded quartz grains. The lighter fraction is the composite clay and quartz grains. Although the total quartz content may be 8.6 percent quartz, the potentially respirable quartz content is only 0.0007 percent. Electron micrographs appear in Figure 19.

Wyoming bentonite

The Wyoming bentonite studied contains both detrital and low-crystallinity authigenic quartz, and has experienced little or no silicification. One sample contains composite grains consisting of an intergrowth of opal, low crystallinity chalcedonic quartz, and clay. These phases formed together from the original volcanic ash as it altered in the sea. The other sample contains largely detrital quartz grains and little or no chalcedony. Although the total quartz content may be up to 6.7 percent, the potentially respirable quartz content is only 0.003 percent or less. Electron micrographs appear in Figure 20.

9.3 EXPOSURE TO RESPIRABLE QUARTZ

9.3.1 Methods

A set of carefully constructed experiments was developed to establish the upper limit of exposure by modeling the conditions of normal consumer use. The experimental design took care to afford "conservative" estimates, that is, estimates that would not underestimate but if anything, would overestimate the exposure in actual customer use of the pet litter. The pouring and the

scooping tests were performed under strict laboratory conditions, which, in all probability, actually overstated the consumer exposure to respirable quartz. Dr. Karen Vetrano of TRC Environmental Corporation performed the experiments.

For conventional litters, the experiment involved pouring litter from a box in a small, enclosed space. For clumping litters, the experiment again involved pouring from a jug, creating and scooping clumps, and replenishment of the litter by re-pouring. Air monitors calibrated to capture $\leq 10\mu\text{m}$ particles sampled the dust during the pouring. The height of the box or jug duplicated the typical positions used by a consumer. The air monitors were located at the position of the breathing area of the consumer. The experimental setup is detailed in Dr. Vetrano's Final Report, which is included in APPENDIX III.

The air monitor filters that captured the $\leq 10\mu\text{m}$ dust were weighed before and after use to determine the weight of the dust. From the airflow rate and the running time of the monitor, airflow through the apparatus in cubic meters per minute (m^3/min) was calculated. These measurements allowed establishing the amount of respirable dust and thus, the quantity of respirable dust in milligrams per cubic meter (mg/m^3). X-ray diffraction analysis of the dust established the percentage of quartz in the respirable dust, and consequently the respirable quartz concentration in mg/m^3 .

9.3.2 Permissible Exposure Limit for Quartz

The United States Environmental Protection Agency has suspended its development of a Reference Concentration (RfC) for quartz. The American Conference of Governmental Industrial Hygienists (ACGIH) prescribes a Threshold Limit Value-Time Weighted Average (TLV-TWA) for respirable quartz⁶⁷ for an 8-hour day of $0.1 \text{ mg}/\text{m}^3$. The occupational PEL for manufacturing and mine workers administered by the Occupational Safety and Health Administration (OSHA) and the Mine Safety and Health Administration (MSHA) closely approximates the TLV-TWA limit of $0.1 \text{ mg}/\text{m}^3$ per 8-hour workday. OSHA and MSHA used the following formula:

$$\text{PEL} = 10 \text{ mg}/\text{m}^3 \div (\% \text{SiO}_2 + 2)$$

where $\% \text{SiO}_2$ is the amount of respirable $\leq 10\mu\text{m}$ quartz collected on the personal air monitor, not of the total bulk quartz

At or below this value, these agencies have determined that an individual can work safely without developing silica-related lung diseases.

9.3.3 Results

Table 4 gives the calculated TLV-TWA for quartz in dust encountered while pouring conventional pet litter, and Table 5 gives the calculated TLV-TWA while pouring and scooping clumping pet litter. It is important to note that in those instances where no quartz was detected in a sample, the detection limit of the instrument was used. Letters designate the products in order to maintain company confidentiality.

9.3.4 Discussion of Results

In all cases, note that the calculated TWA was well below the ACGIH TLV (0.2% to 3.6% of the ACGIH TLV), indicating that the consumer's exposure to respirable quartz during pet litter-related activities is very small. These very small exposures will not pose a burden to the lung.

Figure 5 - Phase Diagram for SiO₂, after Klein and Hurlbut¹⁵.

Asterisks indicate the 68° 2 Θ quintuplet used in crystallinity index calculations.

**Figure 6. - X-ray Diffraction Patterns for Varieties of Quartz,
after Ampian and Virta⁶⁸.**

a = 68° 2 θ quintuplet for varieties of quartz
b = Calculation of the crystallinity index

**Figure 7. - Back Reflection Diffraction Peaks as a Function of Crystallinity,
after Murata and Norman¹⁶.**

- a = Electron spin resonance (ESR) spectrum of pure quartz ground in air, including overmodulation of wings to show details
- b = Detail of central portion of spectrum
- c = ESR signal of quartz ground several years ago, and after regrinding

**Figure 8. - ESR Spectrum of Free Radicals on Quartz Surfaces,
after Fubini, Giamello, Volante, and Bolis³⁸.**

a = Mechanism for generating surface radicals through breaking quartz

b = Presence of radicals demonstrated by electron spin resonance (ESR) spectroscopy in milled and unmilled quartz

**Figure 9. - Generation of Surface Radicals by Breaking Quartz,
after Castranova³⁷.**

- a = Absence of hydroxyl radicals on aged quartz surfaces and presence of hydroxyl radicals on freshly fractured surfaces (with DMPO).
- b = Decay of ability of quartz to generate hydroxyl radicals with time.

**Figure 10. - Generation of Hydroxyl Radicals on Quartz Surfaces,
after Castranova³⁷.**

- a = Electron spin resonance (ESR) signal from quartz immediately after jet milling and two months later
b = Cellular yield from brachioalveolar lavage from rats exposed to air, fresh and aged quartz

**Figure 11. - Biological Activity of Fresh and Aged Quartz,
after Castranova³⁷.**

- c = Protein and phospholipid in the acellular lavage fluid
- d = Nitric oxide synthase-dependent CL generated from alveolar macrophages
- e = Zymosan-stimulated CL generated from alveolar macrophages

**Figure 11 (concl.). - Biological Activity of Fresh and Aged Quartz,
after Castranova³⁷.**

**Figure 14. - Stepwise Hydroxylation of Freshly Fractured Quartz Surfaces,
after Dove and Rimstidt⁴⁰, from Parks⁴³.**

- a = Solubility of amorphous silica and quartz as a function of temperature
b = Solubility of quartz showing a two-step solution

**Figure 12. - Solubility of Silica,
after Rimstidt⁴³.**

- a = Solubility of quartz as a function of radius of curvature
b = Lifetime of sharp edges as a function of radius of curvature

**Figure 13. - Effect of Radius of Curvature on Solubility of Quartz,
after Rimstidt⁴³.**

a and b = Dust from tubular aerosol dust chamber;
c = Dust from pouring

Figure 15. - Electron Micrographs - Monterey Formation.

d = >2.5 μ m grit,
e = <2.5 μ m grit,
f = cemented grit cross section

Figure 15 (cont'd). - Electron Micrographs - Monterey Formation.

a = >2.5 μ m grit
b = <2.5 μ m grit
c = cemented grit cross section

Figure 16. - Electron Micrographs - Oregon Diatomite.

a and b = Dust from tubular aerosol dust chamber
c = Dust from pouring

Figure 17. - Electron Micrographs - Porters Creek Formation.

d = >2.5 μ m grit
e = <2.5 μ m grit,
f = cemented grit cross section

Figure 17 (cont'd). - Electron Micrographs - Porters Creek Formation.

a = >2.5 μ m grit
b = <2.5 μ m grit
c = cemented grit cross section

Figure 18. - Electron Micrographs - Twiggs Fullers Earth.

a = >2.5 μ m grit,
b = <2.5 μ m grit,
c = cemented grit cross section

Figure 19. - Electron Micrographs - Meigs Fullers Earth.

a and b dust from tubular aerosol dust chamber

Figure 20. - Electron Micrographs - Wyoming Bentonite.

c = >2.5 μ m grit,
d = <2.5 μ m grit,
e = cemented grit cross section

Figure 20 (cont'd). - Electron Micrographs - Wyoming Bentonite.

Table 2. - Quartz Content of Bulk Pet Litter, ≤10µm TADC Fraction and Consumer Pouring Test Dusts.

	Monterey Formation		Oregon Diatomite	Porters Creek Formation		Twiggs Fullers Earth	Meigs Fullers Earth	Wyoming Bentonite	
	#1	#2		#1	#2			#1	#2
Bulk									
Quartz %	5.3	7.2	n/d		9.5	5.3	8.6	6.7	2.7
C.I.	n/a	9.0	n/a		9.0	7.5	6.7	6.9	8
Dust									
TADC									
≤10µm									
Quartz %	7.5	7.1	n/d	5.3	9.8	7.5	9.6	6.9	6.7
C.I.	5.6	9.3	-	8.1	9.7	n/a	7.3	<1	10
Bulk									
≤10 µm dust									
Quartz %	0.0017	0.0050	n/d	0.0013	0.0053	0.0022	0.0007	0.0027	0.0006
Pouring Test									
Quartz %		1.71	n/d		2.44				n/d
C.I.		7.3	-	n/a	5.7	n/a	n/a	n/a	n/a

C.I. crystallinity index

n/d not detectable

n/a not available

TADC Tubular Aerosol Dust Chamber

Do not confuse the % quartz with the Permissible Exposure Limit (PEL)

Table 3. – Quartz Content of Grit and Fines Characteristics and Crystallinity Index.

	Monterey Formation		Oregon Diatomite	Porters Creek Formation	Twiggs Fullers Earth	Meigs Fullers Earth	Wyoming Bentonite	
	#1	#2	#1	#1	#1		#1	#2
Quartz Total								
%	6.7	7.2	0	9.5	5.3	8.6	6.7	2.7
C.I.		9.0	-	9	7.5	6.7	x	8
Grit								
Total	5	28.5	15.5	9.4	30.2	29.0	5.2	5.3
Quartz %	15.5	9.2	0	9.2	13.7	10.2	15.5	8.6
C.I.		9.8	-	7.4	8.9	7.2	7.6	8.7
>2.52 sp. Gr.								
Vol. %	56	5	5	50	15	5	56	60
Quartz %	15.5		0	n/a	n/a	n/a	n/a	n/a
C.I.	7.6		-	n/a	n/a	n/a	6.7	n/a
<2.52 sp. Gr.								
Vol. %	44.5	95	9.5	50	85	95	44	40
Quartz %	5.0		0	n/a	n/a	n/a	n/a	n/a
C.I.	x		-	n/a	n/a	n/a	7.0	n/a
Fines								
Total	94.5	71.5	84.5	95.6	69.8	71.0	94.8	94.7
Quartz %	5.0	4.8	0	8.5	5.1	6.4	5.0	2.3
C.I.	x	5.8	-	7.7	5.5	7.6	x	3.5
10 µm in 100 mesh								
Aerosol Chamber %	41.9		46.6	17.0	27.6	15.1	13.4	
Sedimentation %	63.0		78.7	77.3	78.3	64.4	83.6	

Grit = -325 mesh (>44µm) wet screened

Fines = -325 mesh (<44µm) wet screened

s. g. = specific gravity

C.I. = crystallinity index

#1 = samples of January 1997

#2 = samples of August 1997

X = not calculated (excessive interference)

n/a = not available

Table 4. - Quartz Exposure -- Conventional Litter.

Product ⁺	Respirable Dust Concentration mg / m ³	Quartz in Dust ¹ %	Respirable Quartz Concentration mg / m ³	Calculated 8 hour TLV- TWA* mg / m ³	Fraction of ACGIH TLV- TWA** %
A	0.338	4.7	0.016	3.33x10 ⁻⁴	0.3
B	1.837	1.8	0.034	6.59x10 ⁻⁴	0.7
C	2.250	5.9	0.133	27.66x10 ⁻⁴	2.8
D	3.033	1.0	0.030	6.32 x10 ⁻⁴	0.6
E	1.029	5.7	0.059	12.22 x10 ⁻⁴	1.2
F	1.006	5.2	0.052	10.90 x10 ⁻⁴	1.1
G	0.653	5.7	0.037	7.75 x10 ⁻⁴	0.8
H	3.536	2.5	0.088	18.42 x10 ⁻⁴	1.8
I	1.090	2.6	0.028	5.90 x10 ⁻⁴	0.6
J	0.664	3.2	0.021	4.43 x10 ⁻⁴	0.4
K	0.456	2.4	0.011	2.28 x10 ⁻⁴	0.2
L	0.085	11.7	0.099	2.07 x10 ⁻⁴	0.2

¹It is important to note that in those instances where no quartz was detected in a sample, the detection limit of the instrument was used.

* Calculation of 8 hour TWA = $(C_1T_1 + C_2T_2)/480$

Where C_1 = measured concentration of quartz, mg/m³

T_1 = exposure time to dust, taken to be 10 minutes

C_2 = ambient quartz concentration, assume $C_2 = 0$ away from litter

T_2 = exposure time, taken to be 470 minutes

** TLV = TWA for 8 hour day = 0.1 mg/m³

⁺ Letters are used to maintain product confidentiality

Table 5. - Quartz Exposure - Clumping Litter.

Product ⁻		Respirable Dust Concentration mg / m ³	Quartz in Dust %	Respirable Quartz Concentration mg / m ³	Calculated 8 hour TLV-TWA* mg / m ³	Fraction of ACGIH TLV-TWA** %
M	Pour	0.669	8.0	0.053	11.1 x10 ⁻⁴	1.1
	Clump Removal	3.098	5.6	0.174	36.1 x10 ⁻⁴	3.6
	Clump Removal/Replenish	1.565	7.3	0.114	23.8 x10 ⁻⁴	2.4
N	Pour	0.088	20.0	0.018	3.67 x10 ⁻⁴	0.4
	Clump Removal	0.573	3.7	0.021	4.42 x10 ⁻⁴	0.4
	Clump Removal/Replenish	0.896	5.4	0.049	10.1x10 ⁻⁴	1.0
O	Pour	1.105	5.1	0.056	11.7 x10 ⁻⁴	1.2
	Clump Removal	1.599	7.4	0.118	24.7x10 ⁻⁴	2.5
	Clump Removal/Replenish	2.462	4.4	0.109	22.6x10 ⁻⁴	2.3
P	Pour	0.516	12.4	0.064	13.3x10 ⁻⁴	1.3
	Clump Removal	2.397	7.0	0.108	35.0x10 ⁻⁴	3.5
	Clump Removal/Replenish	0.715	11.3	0.081	16.8x10 ⁻⁴	1.7
Q	Pour	0.192	4.5	0.009	1.80x10 ⁻⁴	0.2
	Clump Removal	1.074	1.6	0.067	3.58x10 ⁻⁴	0.4
	Clump Removal/Replenish	1.299	3.7	0.048	10.0x10 ⁻⁴	1.0
R	Pour	0.276	9.5	0.026	5.46x10 ⁻⁴	0.5
	Clump Removal	4.518	1.2	0.056	11.3x10 ⁻⁴	1.1
	Clump Removal/Replenish	1.677	3.0	0.050	10.5x10 ⁻⁴	1.1
S	Pour	0.109	13.3	0.015	3.02x10 ⁻⁴	0.3
	Clump Removal	0.365	10.8	0.039	8.21x10 ⁻⁴	0.8
	Clump Removal/Replenish	0.114	18.3	0.021	4.36x10 ⁻⁴	0.4
T	Pour	0.109	21.7	0.024	4.93x10 ⁻⁴	0.5
	Clump Removal	1.732	2.3	0.040	8.30x10 ⁻⁴	0.8
	Clump Removal/Replenish	3.577	1.9	0.068	14.2x10 ⁻⁴	1.4
U	Pour	0.109	6.7	0.007	1.52x10 ⁻⁴	0.2
	Clump Removal	0.036	13.3	0.005	1.00x10 ⁻⁴	0.1
	Clump Removal/Replenish	0.116	11.7	0.014	2.83x10 ⁻⁴	0.3

* Calculation of 8 hour TWA = (C₁T₁ + C₂T₂)/480

Where C₁ = measured concentration of quartz, mg/m³

C₂ = ambient quartz concentration, assume C₂ = 0 away from litter

T₁ = exposure time to dust, taken to be 10 minutes

T₂ = exposure time, taken to be 470 minutes

** TLV – TWA for 8 hour day = 0.1 mg/m³

* Letters are used to maintain product confidentiality

Figure 1. - Manufacturing Process for Conventional Pet Litter.

Figure 2. - Manufacturing Process for Clumping Pet litter.

<u>Time Unit</u>	<u>Years In The Past</u>
CENOZOIC ERA	
Quaternary period	
Holocene epoch	Present to 10,000
Pleistocene epoch	10,000 to 1.6 million
Tertiary period	
Pliocene epoch	1.6 million to 5.3 million
Miocene epoch	5.3 million to 23.7 million
Oligocene epoch	23.7 million to 36 million
Eocene epoch	36 million to 57.8 million
Paleocene epoch	57.8 million to 66.4 million
MESOZOIC ERA	
Cretaceous period	66.4 million to 144 million
Jurassic period	144 million to 208 million
Triassic period	208 million to 245 million
PALEOZOIC ERA	
Permian period	245 million to 286 million
Pennsylvanian period	286 million to 320 million
Mississippian period	320 million to 360 million
Devonian period	360 million to 408 million
Silurian period	408 million to 438 million
Ordovician period	438 million to 505 million
Cambrian period	505 million to 570 million
PRECAMBRIAN time	570 million and older

Figure 4. - Geologic Column.

Table 1. - Pet Litter Mineralogy and Crystallinity Index.

Mineral	Monterey Formation	Oregon Diatomite	Porters Creek Formation	Twiggs Fullers Earth	Meigs Fullers Earth	Wyoming Bentonite
Smectite	+	+	++	++	++	++
Kaolinite	-	-	+	+	+	-
Palygorskite	-	-	-	-	++	-
Mica	+	-	+	-	-	+
Feldspar	+	+	+	-	-	+
Calcite	-	+	-	-	-	+
Dolomite	+	+	-	-	-	-
Gypsum	-	+	+	-	-	+
Opal	++	++	++	++	++	+
Clinoptilolite	-	-	+	-	-	-
Quartz	7.2	0	9.5	5.3	8.6	2.7
C.I.	9.0	-	9.0	7.5	6.7	8.0

++ >10%

+ <10%

C.I. crystallinity index

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APPENDIX I

Instrumental and Solution Studies of Quartz Surfaces

I-1.0 Materials

The materials used in these studies included –

- Set One
 - quartz grains, Ottawa sand, Illinois
 - quartz grains, Edgar Plastic Kaolin deposit
 - quartz crystal, Herkimer “diamond,” New York state
- Set Two
 - quartz grains, Lone, California
 - IOTA quartz, 191 μ m mass median diameter, >>99 pure, semiconductor grade Spruce Pine, Unimin Corporation

The IOTA quartz has experienced profound purification, including HCl and HF leaching¹

I-2.0 X-ray Photoelectron Spectroscopy (XPS)

I-2.1. Description of Analysis

XPS can provide surface compositional, oxidation state, and structural information of a surface. Analysis depths can range from a few to over 200 Å, depending on the sample and instrumental setup. In the technique, soft x-rays striking the sample can eject photoelectrons from valence and core levels of the surface and near-surface atoms. An electrostatic electron analyzer measures the kinetic energy E_K of the ejected photoelectrons. Photoelectrons that have now lost momentum leaving the sample have characteristic energies that can identify the element, determine the oxidation state, and sometimes structural details. The data appear as electron binding energy E_B versus signal intensity. The binding energy is simply the incident photon energy minus the kinetic energy.

I-2.2 Instrumental Parameters

These experiments used a Perkin-Elmer 5400 XPS instrument with Al $K\alpha$ radiation at a power between 200 and 300 watts. Base pressures were better than 5×10^{-8} torr. Survey scans were collected to 1000 eV binding energy using 1 eV steps of 50 ms per step counting time, and a detector pass energy of 89.454 eV. Collection time for survey scans was 5 minutes. High resolution narrow scans had a range of 20 eV with 0.1 eV of steps of 50 ms per step and a detector pass energy of 17.55 eV. Perkin-Elmer peak fitting routines were used on C peaks which used Shirley background correction and Gaussian-Lorentzian peak shapes.

I-2.3 Data Interpretation

Reactive mineral surfaces immediately begin to adsorb materials from the surrounding environment immediately upon fracturing. These surfaces can adsorb from the air O₂, H₂O, CO₂, hydrocarbons, N₂ and NO₂. This material forms the “adventitious” layer. The adventitious layer can passivate the surface quite rapidly. The C/Si ratio can give a measure of the thickness of the adventitious layer. The width of the O 1s peak gives further information on the adventitious layer. The wider the peak, the more types of environments the oxygen is in the surface region.

I-2.4 Results

The results^{2 3} appear in Table I-1. In Set One the quartz fracture surface is the cleanest surface, the C/Si value of 0.39 indicating a thin adventitious surface. The uncleaned Herkimer surface with a C/Si value of 4.27 is by far the dirtiest. The two sands were surprisingly clean, with an adventitious surface being about 10 Å thick. The width of the O 1s line gives further information. If the value for the fractured surface is a baseline, then the Herkimer has oxygen in many environments. Cleaning the Herkimer surface reduces the thickness of the adventitious layer. The O 1s values for quartz indicates that water and hydroxyls have modified the surface.

In Set Two, the data show that the lone sand is similar to the sands in Set One. The IOTA quartz particles are exceptionally clean. The C/Si value of 0.27 is remarkably low. The value of the O 1s peak is also remarkably narrow. Clearly, the IOTA samples are much different from the samples directly from geological deposits.

I-3 Low Energy Electron Diffraction (LEED)

I-3.1 Description of Analysis

LEED is very successful in the study of surface crystallography because it can yield qualitative and quantitative data on the first few monolayers only. The beam of electrons used usually is low energy, between 30 and 200 eV. The beam impinges perpendicularly on the surface. If the surface is atomically ordered, or crystalline, any primary, elastically backscattered electrons will undergo Bragg diffraction. The spatial distribution of these backscattered/diffracted electrons appears on a hemispherical fluorescent screen placed over the sample. Retarding grids repel inelastically backscattered electrons in order to provide a low background. For qualitative results, photographing and measuring the pattern on the screen allows calculation of the unit cell size and shape. Quantitative information leading to the atomic structure of the surface is more difficult. This calculation requires measuring diffracted beam intensities while varying the intensity of the primary beam. Unfortunately, such calculation is complex and can yield ambiguous results.

I-3.2 Instrumental Parameters

The LEED instrument used an Omicron UHV Compact Lab chamber.

I-3.3 Results

The LEED experiment was only on the Herkimer sample because the flatness of the crystal surface optimized the chance for useable data. On the as-received sample, and on the sample after gently washing, no LEED pattern appeared. The lack of a pattern suggested that the surface contained adventitious material at least 10 Å thick. Much more vigorous cleaning with high phosphate soap and sonication produced a surface that did give meager diffraction spots. Consequently, only vigorous cleaning could reduce the adventitious layer so that any pattern appeared.

I-4 Quartz Solubility Studies

I-4.1 Description of Analysis

Various forms of silica have differing solubilities. Amorphous and glassy silica is more soluble than quartz. Several investigations have noted that quartz grains immersed in solution at first dissolve at an anomalously high rate. Some workers have interpreted this data to mean an amorphous layer exists on the quartz. Unfortunately, attempts at direct measurement by instrumental means have heretofore lead to ambiguous results. Other, more subtle interpretations of the data can lead to a concept of the surface of quartz differing from a thick amorphous layer, and more importantly, from a highly crystalline surface. This experiment studied the rate of dissolution of a quartz grain from the clay deposit in Lone, California.

I-4.2 Experimental Conditions

The experiment consisted of eleven 30 ml. Teflon™ bottles each containing 10 g of sand and 10 mL deionized water. After weighing, the bottles were incubated at 65°C for various lengths of time in a constant temperature bath. The runs were mixed by inversion once every 24 hours. After the required incubation time, the bottle was reweighed to check for leakage. After removal of the top, approximately 5 mL of solution was decanted into a clean 30 mL polyethylene bottle. Silica concentration in each sample was determined using the molybdate blue colorimetric method.

I-4.3 Results

The results⁴ appear in Table I-2. All plots show a two step solution rate, with the exception of the sample of the intensely crushed IOTA quartz. The different shape and slope of the plot suggests a different nature of the surface of quartz that has experienced intense dry crushing.

I-4.4 Interpretation

Quartz dissolution shows a rapid initial rate that slows dramatically after some period of time. Four hypotheses attempt to explain this behavior and ultimately to explain the reactivity of quartz.

- The strained lattice/etch pit hypothesis suggests the faster dissolution rate arises from strain energy in the crystal caused by grinding and the resulting crystal defects dissolve faster. Unfortunately, a sufficient number of such defects and their resulting etch pits would have to cover the entire surface. They do not.
- The siloxane/silanol surface hypothesis proposes that freshly fractured surfaces have siloxane groups, some of which will hydrate to silanol groups. The hypothesis suggests that dissolution of the siloxane groups would dissolve faster, but supporting data are scanty.
- The surface free energy hypothesis considers that breaking a mineral requires energy to create the new surface. The total free energy is now the sum of the free energy of the bulk crystal plus the surface free energy. The radius of curvature of the grain would have to be less than $0.1\ \mu\text{m}$ and to equal that of amorphous silica, it should be $0.012\ \mu\text{m}$. Abundant grains all containing points with radii of curvature of $0.012\ \mu\text{m}$ is most unlikely.
- The amorphous silica hypothesis postulates that abrasion and deformations related to wetting and drying produces an amorphous layer. This hypothesis has considerable plausibility.

Although the amorphous silica hypothesis is plausible, the layer seemingly is non-continuous. The equation of Barnes and Rimstidt allowed a calculation of the leached layer thickness. The dissolution rate is similar to that of amorphous silica. The calculated thickness of the layer is $0.45\ \text{\AA}$ if evenly distributed on the surface. This thickness is less than one atom, an impossible interpretation. Consequently, the amorphous silica must be in localized areas on the surface.

Nevertheless, dissolution experiments of freshly fractured surfaces should not produce the same sort of dissolution plot. Fresh surfaces would have sharp points and edges with varying solution rates. Fresh surfaces would also have surfaces with broken bonds, which would also have different solution rates. The data show that the surface is relatively even, with localized areas of amorphous material. The data do not show evidence for surfaces with broken bonds, and with sharp edges, which would be much more biologically active.

Table I-1 X-ray Photoelectron Spectroscopy Data

<u>Sample</u>	<u>Surface C/Si Atomic Ratio</u>	<u>O 1s Peak FWHM (eV)</u>
First set		
Surface fractured for this experiment and exposed to air only 3 min	0.39	1.6
Herkimer surface, no pretreatment	4.27	2.9
Herkimer surface after detergent and methanol wash	0.97	2.2
Herkimer surface after additional rinsing in water	0.94	2.1
Feldspar Corp. sand with no pretreatment	0.85	2.5
Ottawa sand with no pretreatment	0.78	2.1
Second set		
Ilene, California, sand	1.24	2.2

FWHM full width of the peak at half the maximum height, measured in electron volts

Table 1-2. - Dissolution Experiment

Table 1-2. - Dissolution Experiment (con't)

Table 1-2. - Dissolution Experiment (concluded)

Aspects of the Nature of Quartz Surfaces

A Report to Dr. Lee Coogan, Sorptive Minerals Institute

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Executive Summary

This is a preliminary report which explores the chemistry and atomic structure of quartz surfaces. It contains a review and assessment of selected data available in the literature, as well as original compositional and structural data on four quartz samples with different geologic/sample preparation histories. Although additional work in this area is strongly advised (see the end of this report), it is apparent, at least to this author, that all quartz surfaces are not alike. Differences will affect how these surfaces interact with, and respond to, their environment.

Introduction

The following is a brief report on the general chemical and structural nature of quartz and other silica surfaces with some directed emphasis on biological activity. The report starts with two fundamental background sections on silica surface reactivity and the development of surface electrostatic charge, as well as the role that the surface atomic structure may play in the bioactivity of silica materials. Next, because surface analytical

techniques (XPS, LEED, RNRA) were used or reported as part of this study, I include very brief descriptions of these techniques so that the data which follows can be better interpreted and appreciated by the reader. Finally, the surface analytic data on quartz surfaces is presented. Conclusions can be drawn directly from these data as described at the end, along with suggestions for future work.

General Background

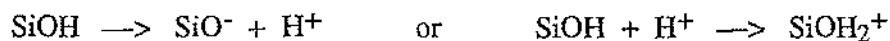
Fundamental Silica Surface Reactivity and Charge

It has been known for some time that all surfaces are electrically charged except in the special case where positive and negative species on the surface balance, resulting in a net neutrality. Surface charge may be an important factor to consider in the reactivity of mineral dust in biological systems. Here, we define and characterize the notion of surface reactivity and charge with regards to silica in contact with water.

When a fresh surface is exposed to ambient conditions, dangling bonds are quickly satisfied as species in the surrounding medium attach (bond) to these highly reactive sites. Infrared and ultraviolet photoelectron spectroscopies show that the first monolayer of water molecules on an oxide surface dissociate, that is, they break down into H^+ and OH^- . This means that for each siloxane bridge (Si-O-Si linkage) on the surface of silica, one reacting water molecule results in the formation of two silanol groups (Si-OH) according to the following reaction:



When such a surface is submersed in water, each silanol group can react in one of two ways:



The former reaction, in which a hydrogen is given off to solution, creates a negative surface site and decreases the pH of the surrounding solution. The latter reaction, in which a hydrogen in solution is sorbed, creates a positive surface site and increases the pH of the

surrounding solution. These reactions are dependent on the pH of the solution, and therefore there exists a pH (called pH_{crit}) where the surface will be neutral. At pH's lower than pH_{crit} , as the activity of H^+ increases, the latter reaction is favored and the surface becomes positive; at pH's higher than pH_{crit} , as the activity of H^+ decreases, the former reaction is favored and the surface takes on a negative charge. Therefore, there must be a pH for each solid such that, when it is introduced to water, no pH change occurs as both reactions are equally favored and the surface remains neutral. This special condition is called the point of zero charge (PZC), or to be more exact, the point of zero net proton charge (PZNPC). As one moves further away on either side of the PZNPC, the surface becomes more highly charged.

Evidence for bioactivity dependence on silica surface atomic structure

There have been several studies on the role of mineral surface atomic structure in biological interactions, especially with regards to silica dust and the pathogenesis of silicosis (*in vitro* studies: Stalder and Stober, 1965; Ottery and Gormley, 1978; Kozin, 1982; *in vivo* studies: King et al., 1953; Attygalle et al., 1956; Brieger and Gross, 1966, 1967; Pratt, 1983. Note: All references in this section are cited in Reviews in Mineralogy, vol. 28, 1993, p. 305). In these studies, cytotoxic and pathogenic observations have been made as a function of the silica polymorph used in this study. Although there are various apparent contradictions among these studies, in general quartz, tridymite, and cristobalite are more biologically active than stishovite and amorphous silica. Some of the problems in this area of research has been recently addressed by Wiessner et al. (1988). In this study, both *in vitro* (red blood cell lysis) and *in vivo* (lung fibrosis in laboratory mice) experiments were performed. All particles of the various silica polymorphs used in their study were carefully chosen to be in the same size range. BET surface area measurements were made to assure that each experiment utilized the same crystal surface area for direct comparison of results. Trace metal oxide contaminants were removed by an acid pretreatment. Wiessner et al. found that quartz, tridymite, and cristobalite were actively

lytic to red blood cells *in vitro* and were also active in mice lungs leading to inflammatory responses and eventually fibrosis. On the other hand, coesite was less active in its lytic capacity *in vitro* and gave negative fibrosis results *in vivo*. An inflammatory response was not even observed. These results lead Wiessner et al. to conclude that surface crystal structure does significantly contribute to biological activity, at least in this case. They postulate that the atomic packing density is essentially the only variable which is left to explain the difference reactivity of these minerals in their experiments. Quartz, cristobalite, and tridymite are stable under relatively low pressure, high temperature conditions, and their packing density is also relatively low. Coesite is a high pressure silica polymorph, and its packing density is relatively high. Therefore, their work suggests that the biological activity of silica increases as the atomic packing density of the structure (and therefore the surface) decreases.

A Brief Description of Techniques Used in This Study

X-ray Photoelectron Spectroscopy (XPS)

X-ray photoelectron spectroscopy was the first vacuum-based surface chemical analysis technique to be developed. Because it is relatively straightforward to collect and interpret the data, and because it provides surface compositional, oxidation state, and structural information, XPS has become the most widely used surface analytic tool today. Analysis depths range from a few to over 100 Å depending on the type of sample, the energy of the photoelectrons measured, and the instrumental set-up. Any surface that is compatible with moderate vacuums (as high as approximately 10^{-5} mbar) can be analyzed, and semi-quantitative near-surface compositions can usually be calculated easily. The latest XPS technology provides for superior energy resolution (which provides for additional interpretation of oxidation and structural state information) and smaller analysis areas. Newly developed "photoelectron microscopes" have already demonstrated 10 µm spatial

resolution, with the potential for micron resolution. Commercially available systems now routinely analyze areas smaller than a few hundred microns in diameter.

In the XPS technique, soft x-rays strike a surface, ejecting photoelectrons from valence and core levels of the surface and near-surface atoms. An electrostatic electron energy analyzer is used to accurately measure the kinetic energy, E_K , of the ejected photoelectrons. Photoelectrons which have not lost momentum on the way out of the sample have characteristic energies that can be used not only to identify the element from which they come, but also to determine the element's oxidation state and, in certain cases, structural details. X-ray photoelectron spectra are plotted as the electron binding energy E_B (the energy by which the electron was bound to its parent atom which, simply speaking, is the incident photon energy minus E_K) versus signal intensity. The probability (cross-section) for photoelectron ejection from H and He atoms at the photon energies used for XPS are extremely low, and these are the only elements that cannot be detected with the technique.

Low Energy Electron Diffraction (LEED)

LEED has long been the principal tool used for the study of surface crystallography. It can be used on both conducting and insulating surfaces, it is sensitive to the first few monolayers only, and it can be used in a qualitative or quantitative sense. In this technique, a low energy beam of electrons (usually between 30 and 200 eV in energy) impinges on (and is perpendicular to) a surface. Primary electrons which are elastically backscattered (that is backscattered with the same energy as the primary beam) will undergo Bragg diffraction if the surface is atomically ordered (i.e., crystalline). The spatial distribution of these backscattered/diffracted electrons is viewed on a hemispherical fluorescent screen situated over the sample, where retarding grids repel inelastically backscattered (lower energy) electrons to keep the background in the diffraction pattern low. For qualitative LEED, this pattern is photographed and measured, from which the unit cell size and shape can be determined. In quantitative LEED, the diffracted electron beam intensities must be

measured as the primary beam energy is varied. With this, it is possible to "solve" the atomic structure of the surface by coming up with a model which most closely accounts for the change in diffracted peak intensities versus primary beam energy. However, this is generally a complex and not always unambiguous task, especially for large surface cells.

Resonant Nuclear Reaction Analysis (RNRA)

Although RNRA has not generally been used to study the near surface chemistry of minerals, it has found a limited but important application in geochemistry. RNRA has been used to depth profile for hydrogen in the near-surface of minerals. This has been particularly useful in the study of mineral dissolution and weathering processes. This is despite the fact that the depth resolution is no better than several tens of Angstroms. Nevertheless, these studies were the first to show that silicates (specifically feldspars, olivines, and pyroxenes) can become protonated and/or hydrated to depths of several hundred Angstroms during dissolution reactions.

Hydrogen depth profiles can be obtained by taking advantage of the nuclear reaction $^{15}\text{N} + ^1\text{H} \rightarrow ^{12}\text{C} + ^4\text{He} + 4.43 \text{ MeV } \gamma$ (where γ represents a gamma-ray emission) which can be induced by bombarding the sample with high energy ^{15}N ions. As ^{15}N penetrates the sample to deeper and deeper levels, it loses energy at a predictable rate. When the ^{15}N energy reaches exactly 6.385 MeV (the resonant energy in this case), there is a dramatic increase in the probability of ^{15}N interaction with hydrogen, and the above reaction will proceed. At ^{15}N energies higher or lower than this, the reaction probability drops off sharply. Therefore, if a ^{15}N energy of greater than the resonant energy for this reaction is used, the nitrogen will get to a certain depth before it obtains the resonant energy, at which point easily detectable 4.43 MeV gamma-rays will be produced depending on the amount of H at that depth in the sample. In general, depth profiles are produced by varying the energy of the incoming ions and measuring the intensities of the characteristic gamma-rays which are emitted. From this, hydrogen depth profiles are constructed.

Results and Discussion

XPS

Six XPS data sets were collected on 4 different samples as shown in the table below. Surfaces analyzed included a freshly fractured single crystal quartz surface, a growth face of a quartz single crystal, and grain surfaces of two industrial sands. The spectra numbers in the table correspond to the labeling of the XPS data appended to the end of this report. All sample descriptions in the table are self-explanatory, except the "Herkimer" sample. This is a single crystal quartz specimen from Herkimer County, New York. These crystals, like the sample used in this study, are nearly perfect single crystals of exceptional clarity and purity. They are known in the trade as "Herkimer diamonds".

<u>Sample</u>	<u>Spectra No.</u>	<u>Surface C/Si Atomic Ratio</u>	<u>O 1s Peak FWHM (eV)</u>
fracture surface of quartz, exposed to air for only 3 min.	1	0.39	1.6
Herkimer surface, no pretreatment	2	4.27	2.9
Herkimer surface after detergent and methanol wash	3	0.97	2.2
Herkimer surface after additional rinsing in water	4	0.94	2.1
Feldspar Corp. sand with no pretreatment	5	0.85	2.5
Ottawa sand with no pretreatment	6	0.78	2.1

The C/Si atomic ratios give one a quantitative idea of the surface "cleanliness" of each of the samples at a glance. Mineral surface compositions are rarely representative of

the bulk. Only "unreactive" surfaces (such as a gold surface), or one created by fracturing in ultra-high vacuum, have the potential to be clean. Otherwise, mineral surfaces exposed to air or an aqueous solution immediately take up hydrogen, oxygen, usually carbon, and sometimes nitrogen (from O_2 , H_2O , CO_2 , hydrocarbons, and N_2 and/or NO_x in the air or solution). This is generally known as "adventitious" material. The build-up of these and other adventitious materials "passivates" the clean highly reactive surface very quickly. Excluding gross contamination, adventitious materials of the type mentioned above may not obtain a thickness of more than a few to several Angstroms. Generally, in this case, only the first one or two monolayers of adventitious material are considered to be important to the reactive nature of the mineral surface because these atoms/molecules are interacting directly with the top layer of atoms on the mineral.

Going back to the table, one immediately sees that the "cleanest" surface observed in our experiments was that of the quartz fracture surface, whose only chance for contamination was being in air for a few minutes. The C/Si ratio of 0.39 as measured by XPS, when taking into account the depth of analysis of this technique, translates into only approximately a few Angstroms of surface contamination. Within this extremely thin layer, the C is not coming from CO_2 (the most abundant C-containing molecule in the air), but from hydrocarbons in the air that are very reactive with the surface of the quartz. We know this from the energy of the C 1s XPS photoline. The uncleaned Herkimer quartz surface was by far the dirtiest of all surfaces tested, with adventitious surface species probably on the order of a few tens of Angstroms thick. The two industrial sands were surprisingly clean, considering that they also were analyzed without any pretreatment. The adventitious material on these surfaces is on the order of 10 Angstroms thick. They were even slightly cleaner than the laboratory washed Herkimer quartz crystal using two methods as described in the table.

The width of the oxygen 1s photoelectron peak measured at half height (FWHM means full width at half maximum), adds to the information about the adventitious material

on these surfaces. Generally speaking, the wider the peak, the more 'kinds' of oxygen are in the near surface region. The fracture surface oxygen peak width of 1.6 eV can be considered as a baseline, that is, the only oxygen that we are measuring is from the quartz itself plus a very small contribution from surface hydroxyls. The untreated Herkimer surface gives an oxygen peak of 2.9 eV, almost twice as broad. Therefore, oxygen on this surface is in many more environments than from the quartz itself. These other environments are found in the adventitious layer and include both physisorbed and chemisorbed H_2O and OH . Although the industrial sands are relatively clean, the oxygen peaks are relatively broad (especially for the Feldspar Corporation sand), indicating that water and hydroxyls have definitely modified these surfaces.

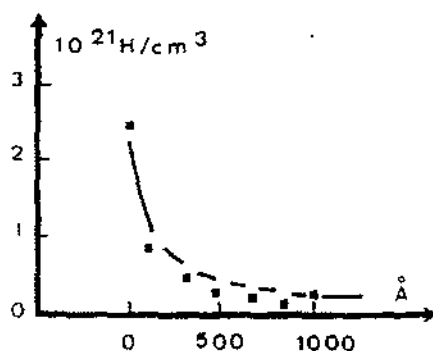
LEED

The same "Herkimer diamond" quartz crystal used in the XPS study above was degreased and water-washed, mounted on a stainless steel plate, and loaded into an Omicron ultra-high vacuum chamber for LEED analysis. We chose to try to obtain an electron diffraction pattern from a prism face on the quartz crystal because of its size (easier to 'hit' with the LEED electron beam) and its flatness. Although the LEED system was operating normally and surface charging was not a problem, a LEED electron diffraction pattern could not be obtained on the quartz prism face at any beam energy (energies between 100 and 1,000 eV were tried). We can draw one of two conclusions from this result. One possibility is that the surface of the quartz surface probed is not crystalline, but amorphous. The second possibility is that there remained too much adventitious material on this surface despite extensive washing (see XPS section above). The adventitious material is obviously amorphous, and although it may be only one nanometer or so thick, this may be enough to keep us from obtaining a pattern due to the extreme surface sensitivity of LEED. We have an ion sputtering system in our Omicron chamber to atomically clean sample surfaces, but it is well known that this ion bombardment would also destroy the crystallinity of the surface, making any following LEED measurements

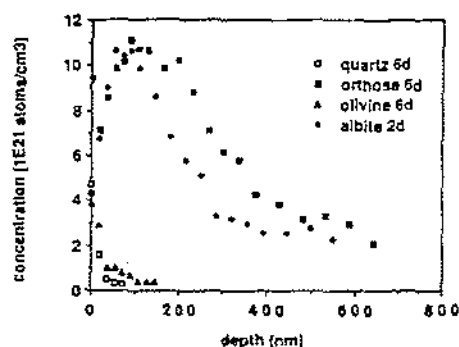
meaningless. My best professional judgment, from years of taking LEED patterns on mineral surfaces and studying their surface crystallinity, is that pristine quartz surfaces probably remain crystalline up to nearly the outermost layer of surface atoms. However, quartz surfaces that have been mechanically perturbed or chemically attacked will become amorphous to depths of several atomic layers to much deeper depending on the time and severity of the external interaction.

RNRA

To get a further idea of how quartz near-surfaces can be hydroxylated, we present here the RNRA data of Petit and others (from Chem. Geol. 76, 365, 1989; EPSL 93, 292, 1989). The following figure is a hydrogen depth profile for quartz subjected to aqueous solution at pH 5.6 and 100°C for 24 hours.



Considering that the RNRA technique only has a depth resolution of several 10's of Angstroms, the hydrogen concentration with depth drops off to near background levels at very shallow depths. In fact, even at 200°C in deionized water for up to 6 days, hydrogen penetration in quartz is dramatically less than in either orthoclase and albite as shown in the following figure.



Overall, the hydrogen enrichment in the near-surface region of quartz is vanishingly small, and the depth of penetration also very small, most likely smaller than any other silicate with the possible exception of other silica polymorphs.

Conclusions

Quartz surfaces that have been exposed to the ambient environment of the earth's surface have all been chemically and structurally modified. This modification is, especially by bulk chemical standards, subtle, but it is probably enough to affect quartz reactivity in measurable ways, especially in the early stages of reactions. Ample evidence shows that quartz surfaces have between a few and many monolayers of contamination (between one and several nanometers) of principally H₂O and hydrocarbons, and sometimes also sodium and nitrogen as well as other elements depending on the exact history of the sample. Trace amounts of hydrogen can also penetrate a few nanometers beneath quartz surfaces. In addition, it is the best estimation from this author that the upper few atomic layers of the quartz itself is actually non-crystalline due to interaction of the contaminants on these surfaces which affects surface atomic periodicity in a non-uniform way. Therefore, strictly speaking, when going into a quartz grain, one first encounters surface contaminants which overlie a monolayer or more of silica, which overlies true quartz which makes up the bulk. The actual thickness of the contaminant and silica layers will depend on the history of each particular sample.

Perhaps the most important message to take from this assessment is that not all quartz surfaces are "created" equal. The varying nature of quartz surfaces with different histories is expected to invoke a range of biological responses, especially in the early stages of exposure.

Suggestions for Future Work

Surface chemical and structural analyses must be performed on the prepared (pretreated) quartz particles that have been used by biologists for *in vivo* and/or *in vitro* testing. These results must be compared with similar surface analyses from quartz grains recovered from nature that are subject to inhalation. Ideally, this should be followed by *in vivo* and *in vitro* testing of freshly ground quartz dust, vs. laboratory-aged quartz dust, vs. quartz dust recovered from clay, soil and rock samples or deposits.

More LEED work will also be required in an attempt to confirm, and thereafter to quantify, the presence and thickness of amorphous silica on quartz grains as a function of grain history.

The actual samples that I wish to study in this phase of my work, which will achieve the goals stated just above, are already in my lab and include the following:

<u>Sample</u>	<u>Proposed Work</u>
IOTA Standard Quartz (used by Castranova in <i>in vivo</i> inhalation tests)	XPS
Milled IOTA Quartz (used by Castranova in <i>in vivo</i> inhalation tests)	XPS
Ione (California) sand	XPS
Herkimer quartz single crystal	LEED

Cost and Payment Terms

The cost of the surface analyses of these samples, along with a written report similar to this one, is priced at \$1,500. Payment must be made via check sent directly to me, but made payable to "Treasurer of Virginia Tech", after the work is complete and within 45 days of the receipt of the report by SMI.

Strongly Recommended Additional Future Work

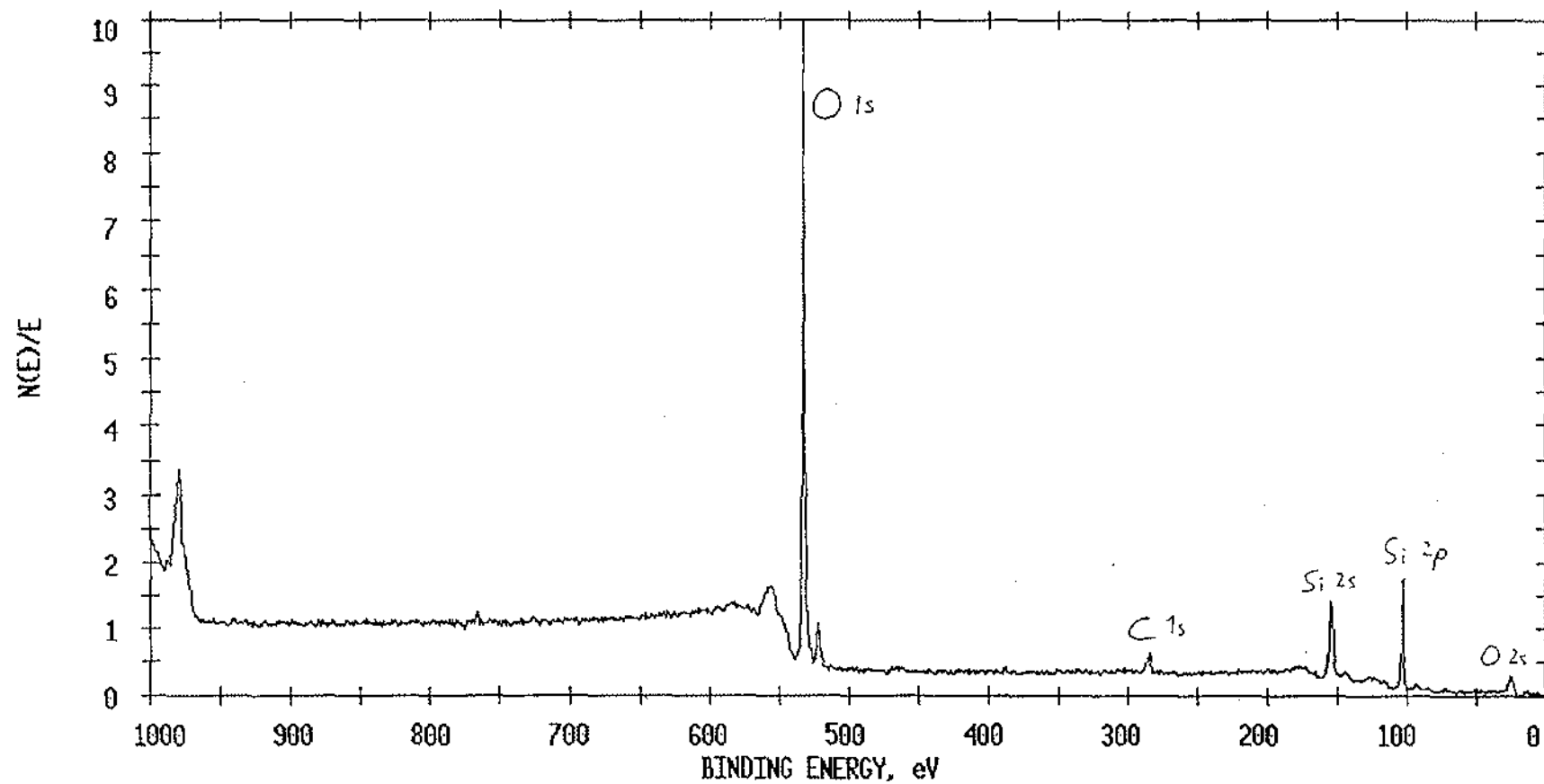
These analyses would be especially useful if they were accompanied by measurements of the chemical reactivity (in terms of dissolution kinetics) of quartz grain surfaces. Such a study could be carried out at Virginia Tech by Don Rimstidt, a professor of geochemistry in our department, who has 20 years experience in the study of silica solubility and dissolution kinetics. In this study, he would measure and compare the rates of dissolution of the surfaces of appropriate samples to show how differences in chemical reactivity relate to the sample's history and surface structure as characterized by our surface analytical methods. These results, in combination, will influence our views on the pathogenicity of silica dust.

Payment terms for this work, if requested, should be negotiated directly with Professor Rimstidt.

ESCA SURVEY 3/21/97 ANGLE= 45 deg ACQ TIME=3.75 min

FILE: mfh110 virgin quartz surface

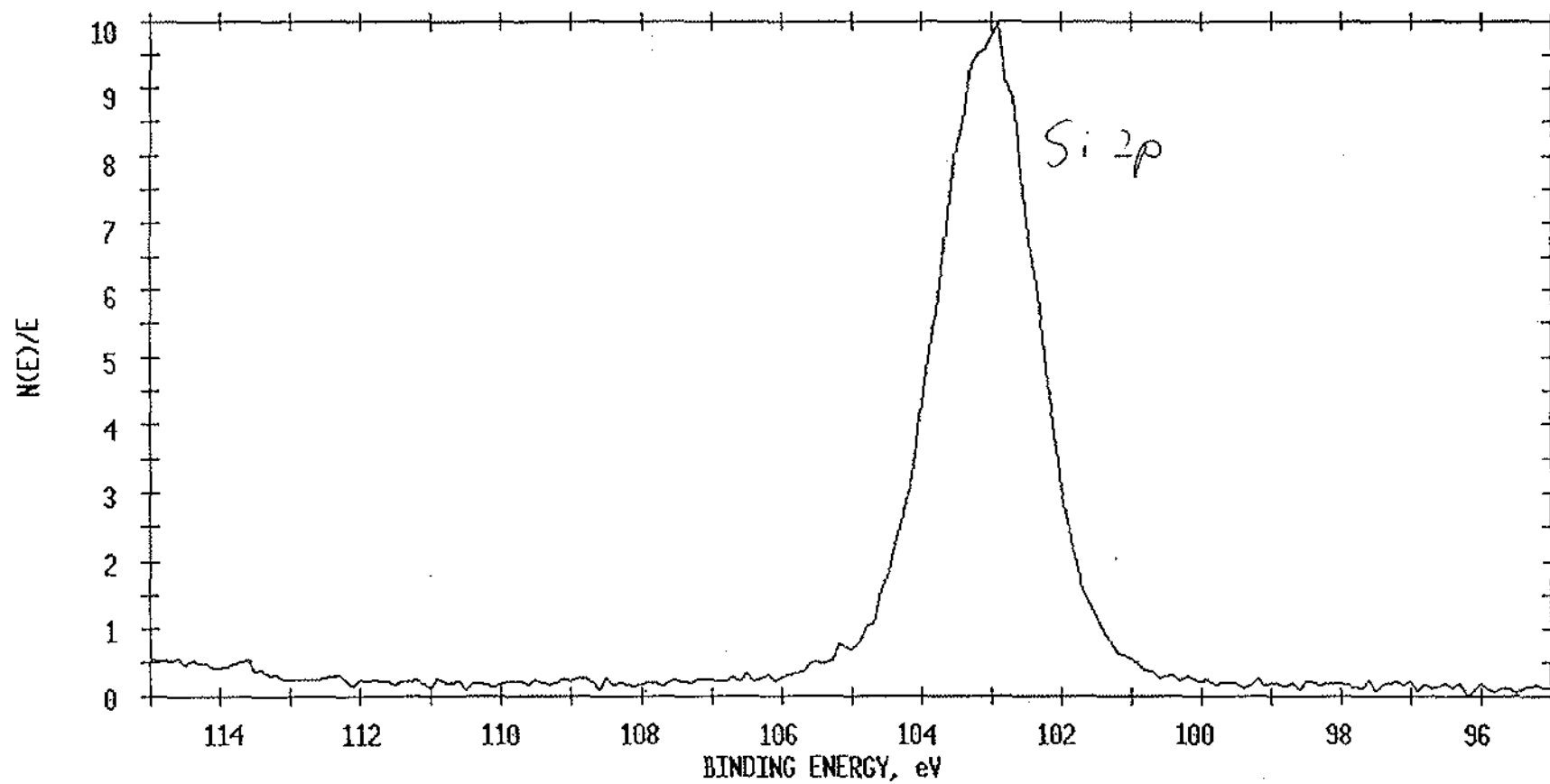
SCALE FACTOR= 6.550 k c/s, OFFSET= 0.458 k c/s PASS ENERGY= 44.750 eV Al 300 W



ESCA MULTIPLEX 3/21/97 EL=Si1 REG 1 ANGLE= 45 deg ACQ TIME=3.35 min

FILE: mfh111 virgin quartz surface

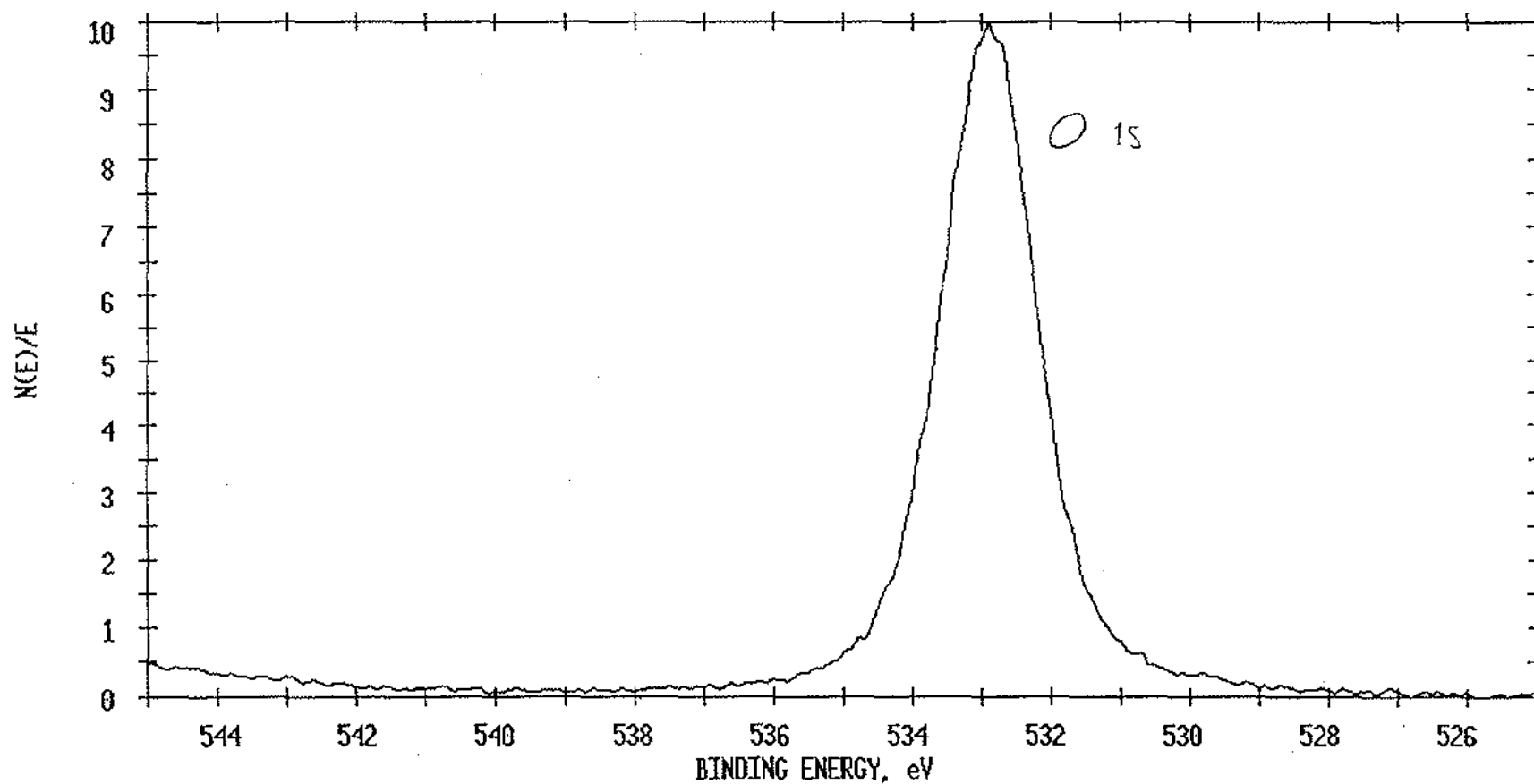
SCALE FACTOR= 0.438 k c/s, OFFSET= 0.305 k c/s PASS ENERGY= 17.900 eV Al 300 W



ESCA MULTIPLEX 3/21/97 EL=01 REG 2 ANGLE= 45 deg ACQ TIME=1.34 min

FILE: mfh111 virgin quartz surface

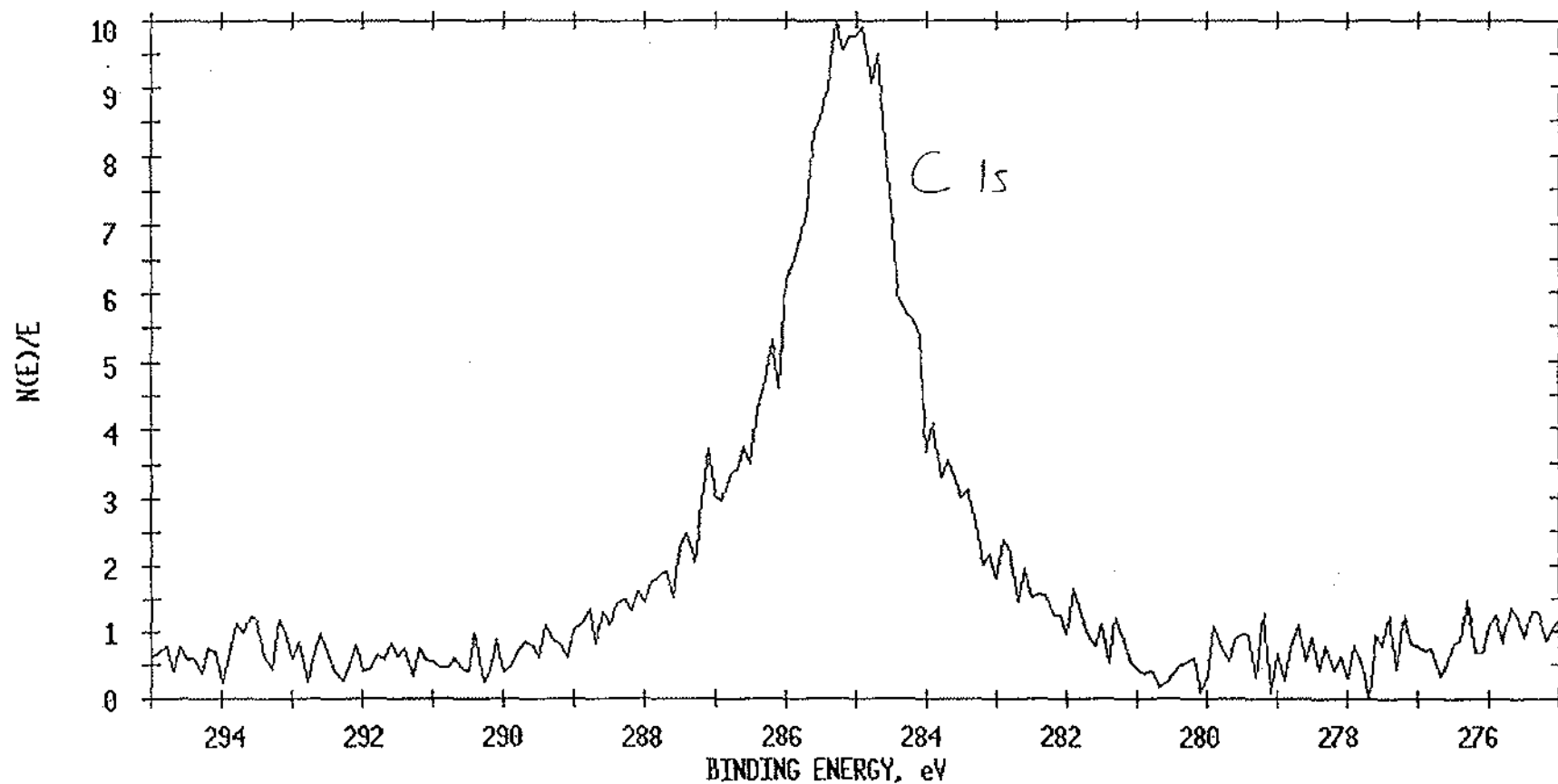
SCALE FACTOR= 2.482 k c/s, OFFSET= 1.160 k c/s PASS ENERGY= 17.900 eV Al 300 W



ESCA MULTIPLEX 3/21/97 EL=C1 REG 3 ANGLE= 45 deg ACO TIME=4.69 min

FILE: mfh111 virgin quartz surface

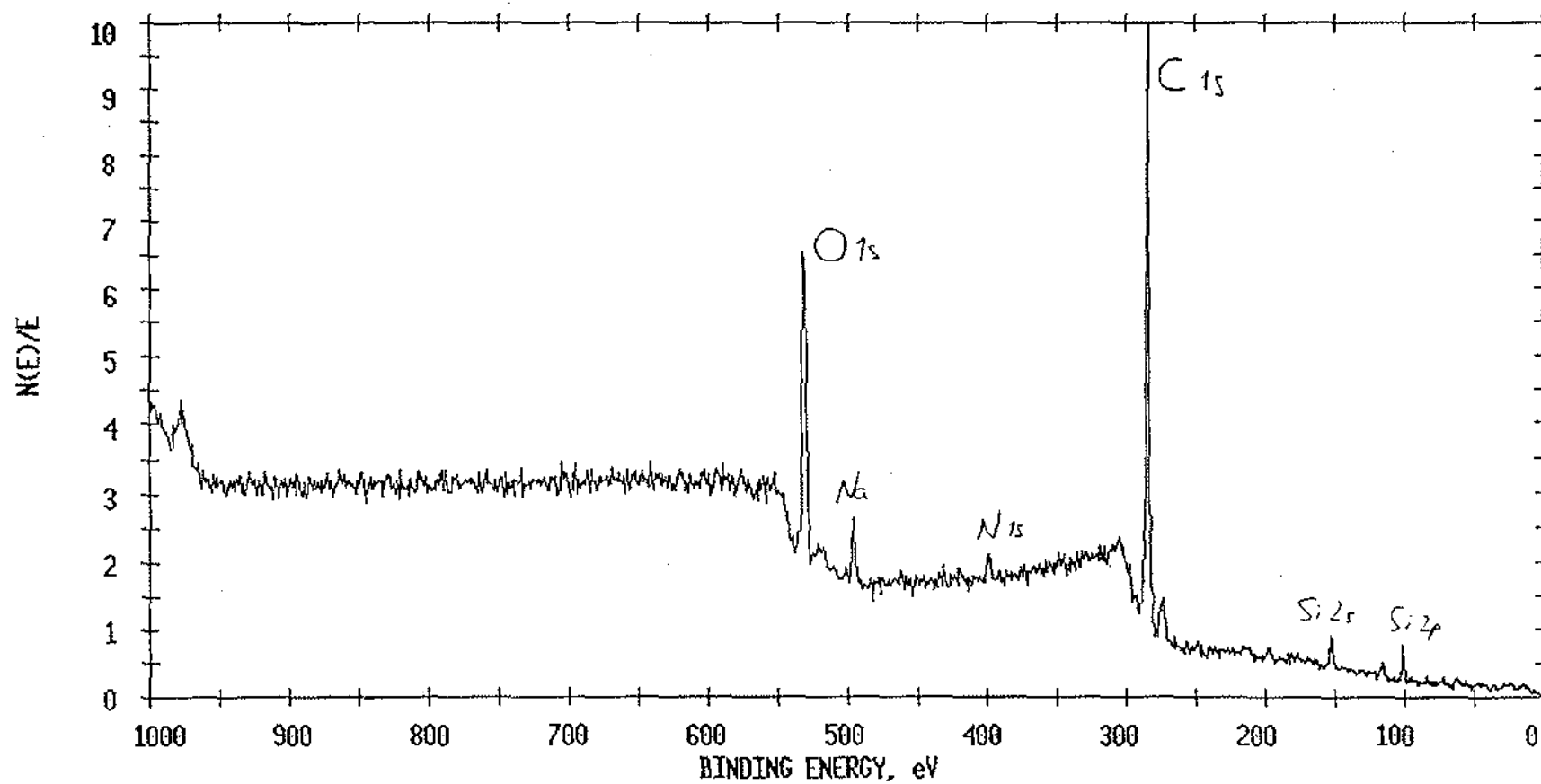
SCALE FACTOR= 0.086 k c/s, OFFSET= 0.831 k c/s PASS ENERGY= 17.900 eV Al 300 W



ESCA SURVEY 3/21/97 ANGLE= 45 deg ACQ TIME=3.75 min

FILE: mfh112 herkimer diamond, no sample pretreatment

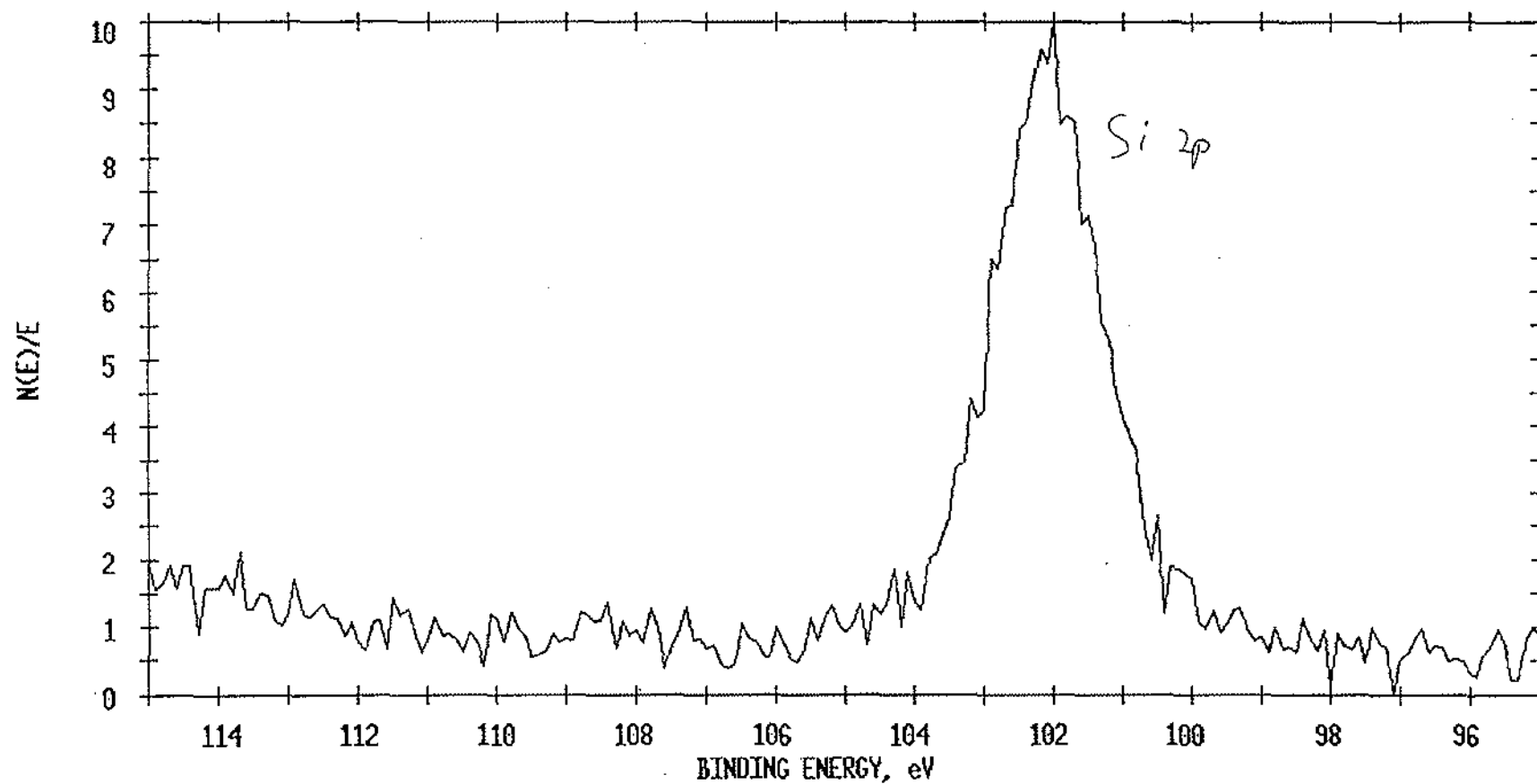
SCALE FACTOR= 1.716 k c/s, OFFSET= 0.276 k c/s PASS ENERGY= 44.750 eV Al 300 W



ESCA MULTIPLEX 3/21/97 EL=Si1 REG 1 ANGLE= 45 deg ACO TIME=5.28 min

FILE: mfh113 herkimer diamond, no sample pretreatment

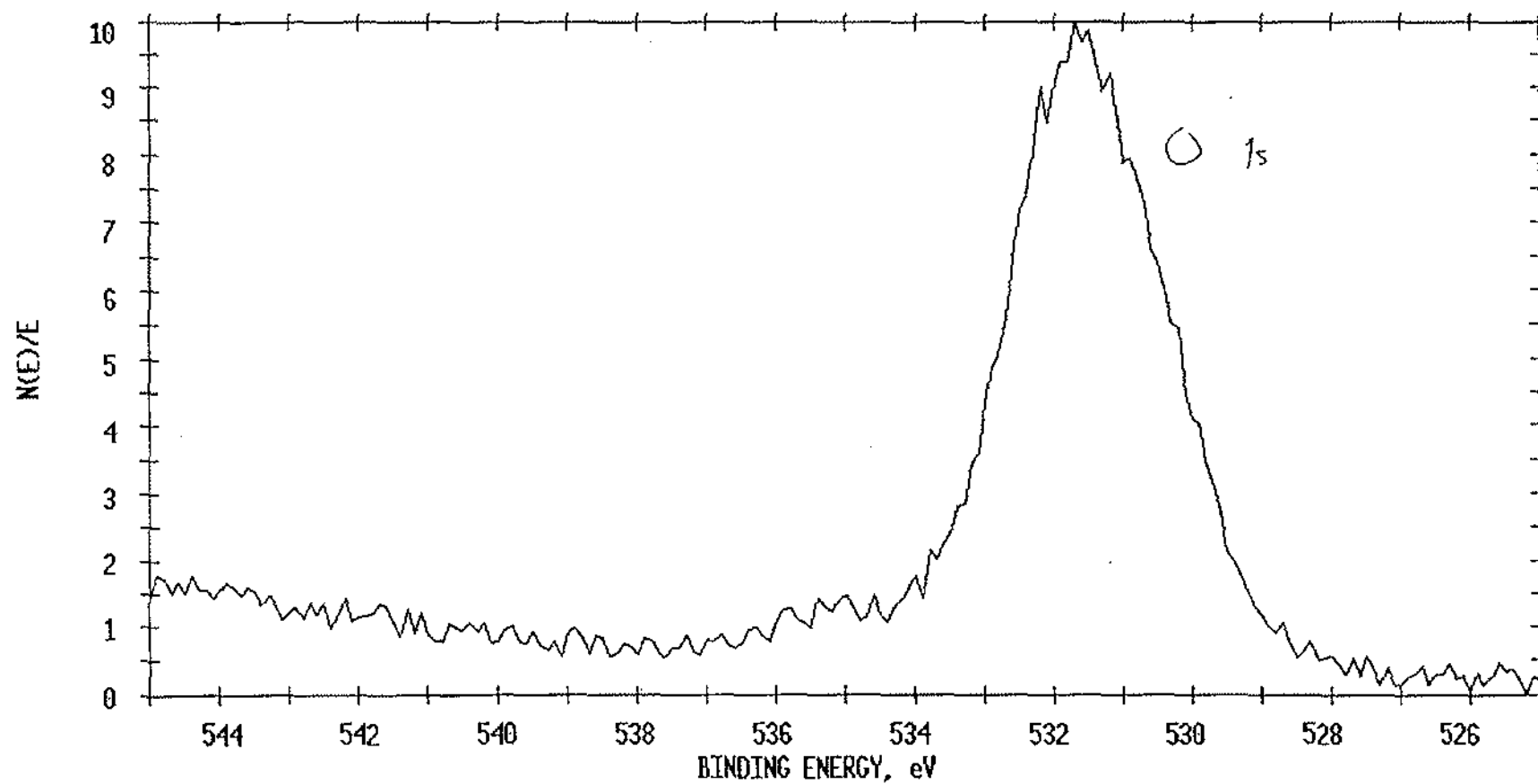
SCALE FACTOR= 0.051 k c/s, OFFSET= 0.204 k c/s PASS ENERGY= 17.900 eV Al 300 W



ESCA MULTIPLEX 3/21/97 EL=01 REG 2 ANGLE= 45 deg ACQ TIME=3.02 min

FILE: mfh113 herkimer diamond, no sample pretreatment

SCALE FACTOR= 0.312 k c/s, OFFSET= 1.163 k c/s PASS ENERGY= 17.900 eV Al 300 W

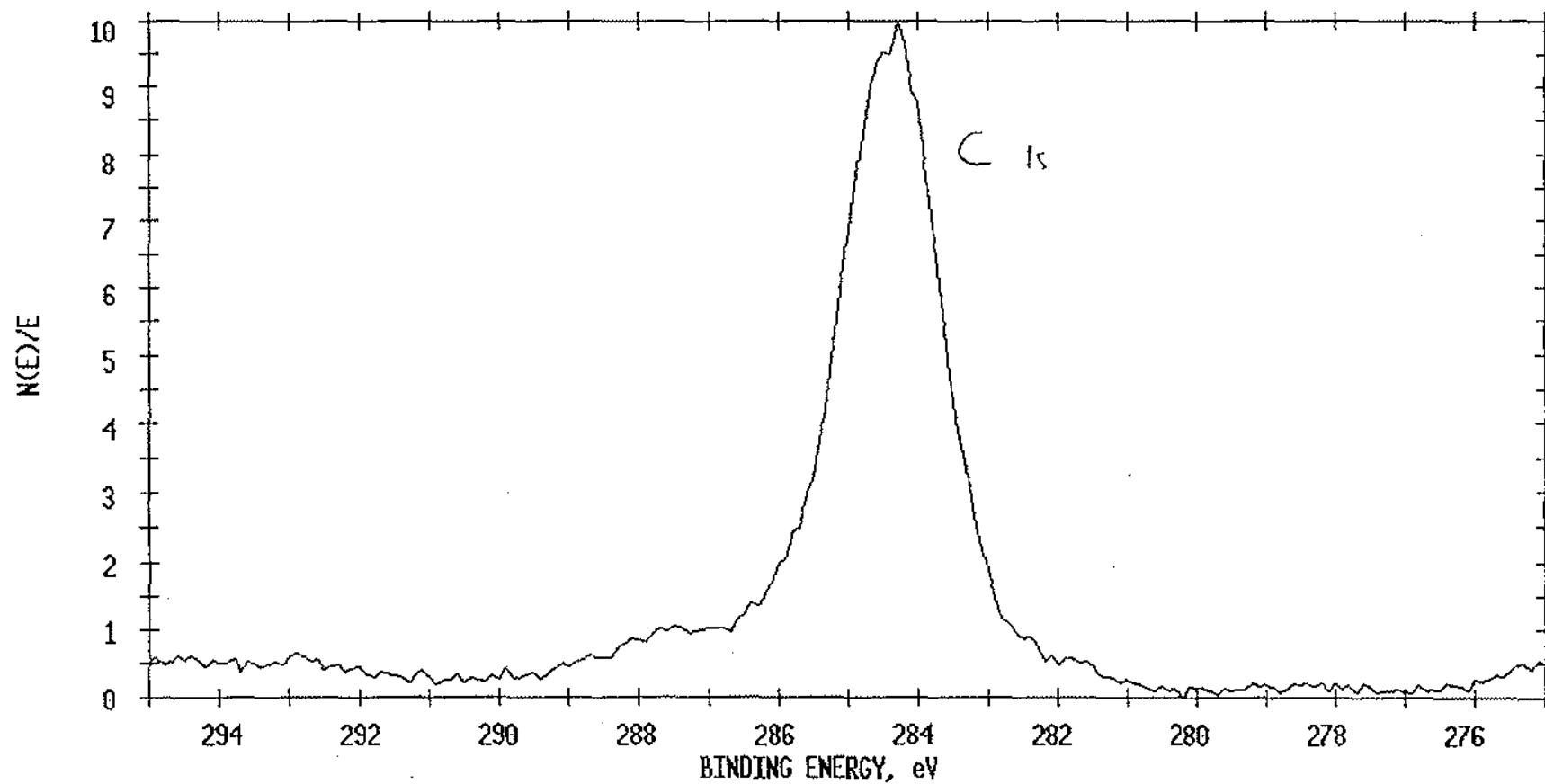


(1)

ESCA MULTIPLEX 3/21/97 EL=C1 REG 3 ANGLE= 45 deg ACQ TIME=1.51 min

FILE: mfh113 herkimer diamond, no sample pretreatment

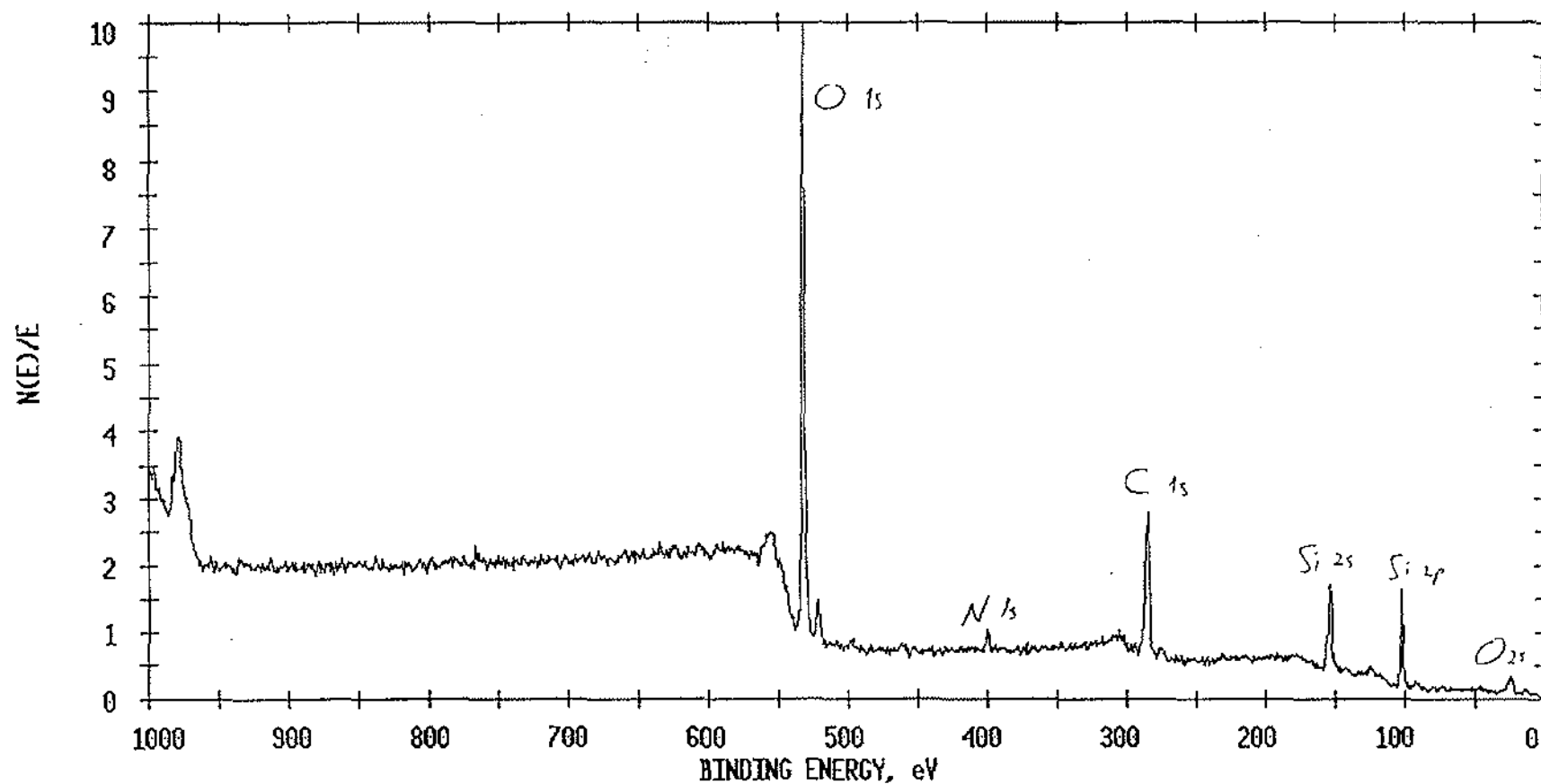
SCALE FACTOR= 0.721 k c/s, OFFSET= 0.573 k c/s PASS ENERGY= 17.900 eV Al 300 W



ESCA SURVEY 3/22/97 ANGLE= 45 deg ACQ TIME=3.75 min

FILE: mfh114 herkimer diamond after det/water, acet, meth wash

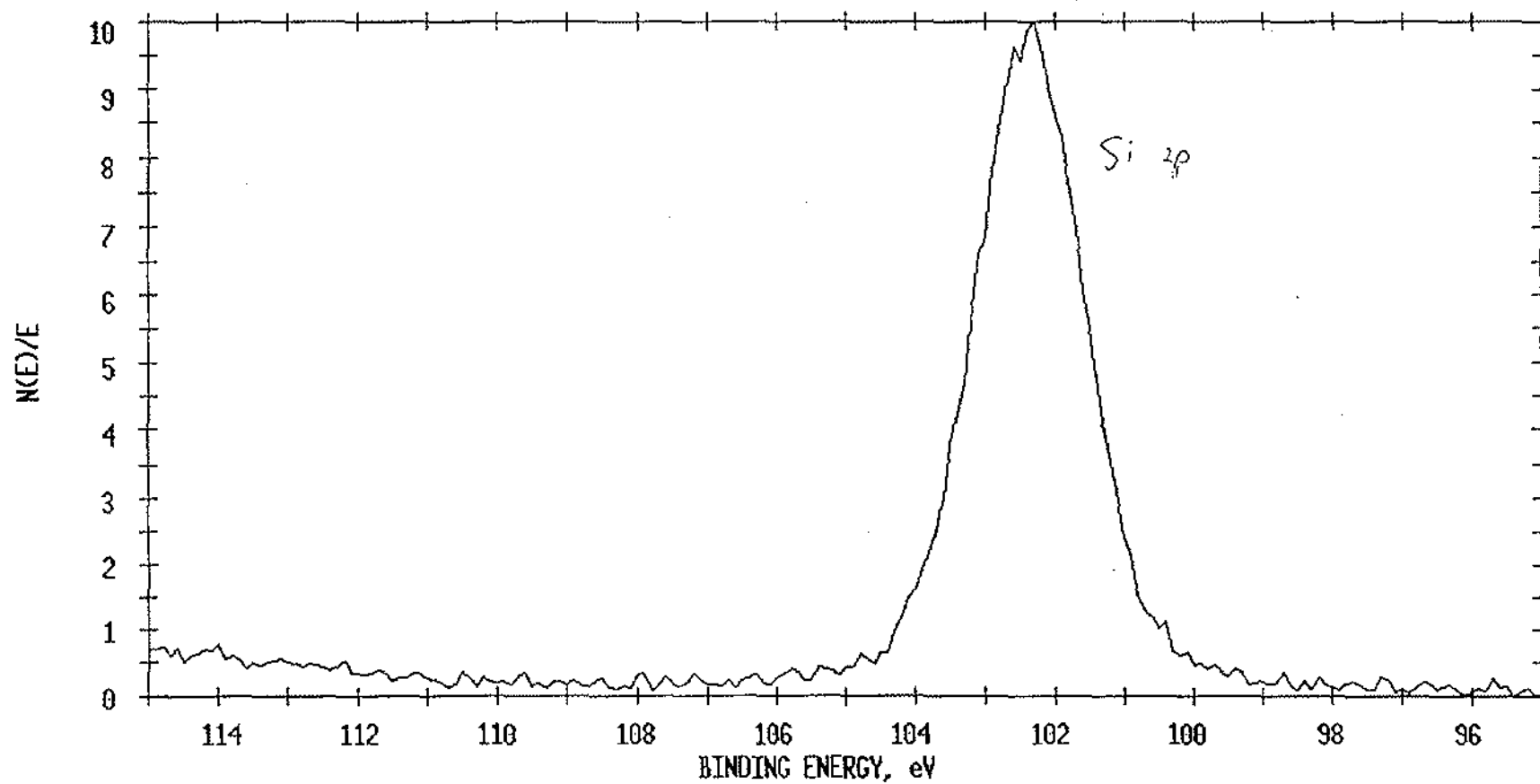
SCALE FACTOR= 3.132 k c/s, OFFSET= 0.378 k c/s PASS ENERGY= 44.750 eV Al 300 W



ESCA MULTIPLEX 3/22/97 EL=Si1 REG 1 ANGLE= 45 deg ACO TIME=3.77 min

FILE: mfh115 herkimer diamond after det/water, acet, meth wash

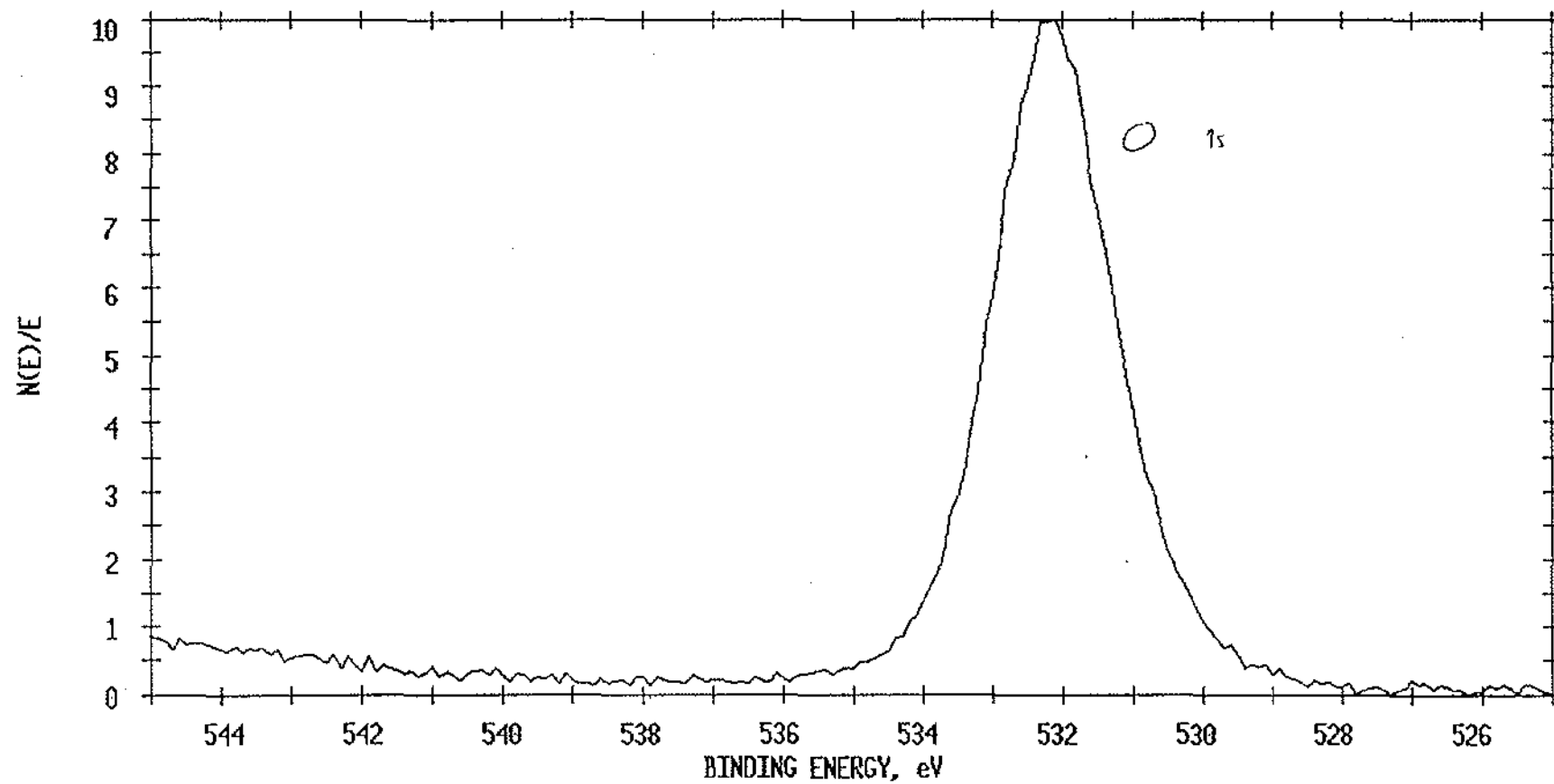
SCALE FACTOR= 0.231 k c/s, OFFSET= 0.299 k c/s PASS ENERGY= 17.900 eV Al 300 W



ESCA MULTIPLEX 3/22/97 EL=01 REG 2 ANGLE= 45 deg ACQ TIME=1.51 min

FILE: mfh115 herkimer diamond after det/water, acet, meth wash

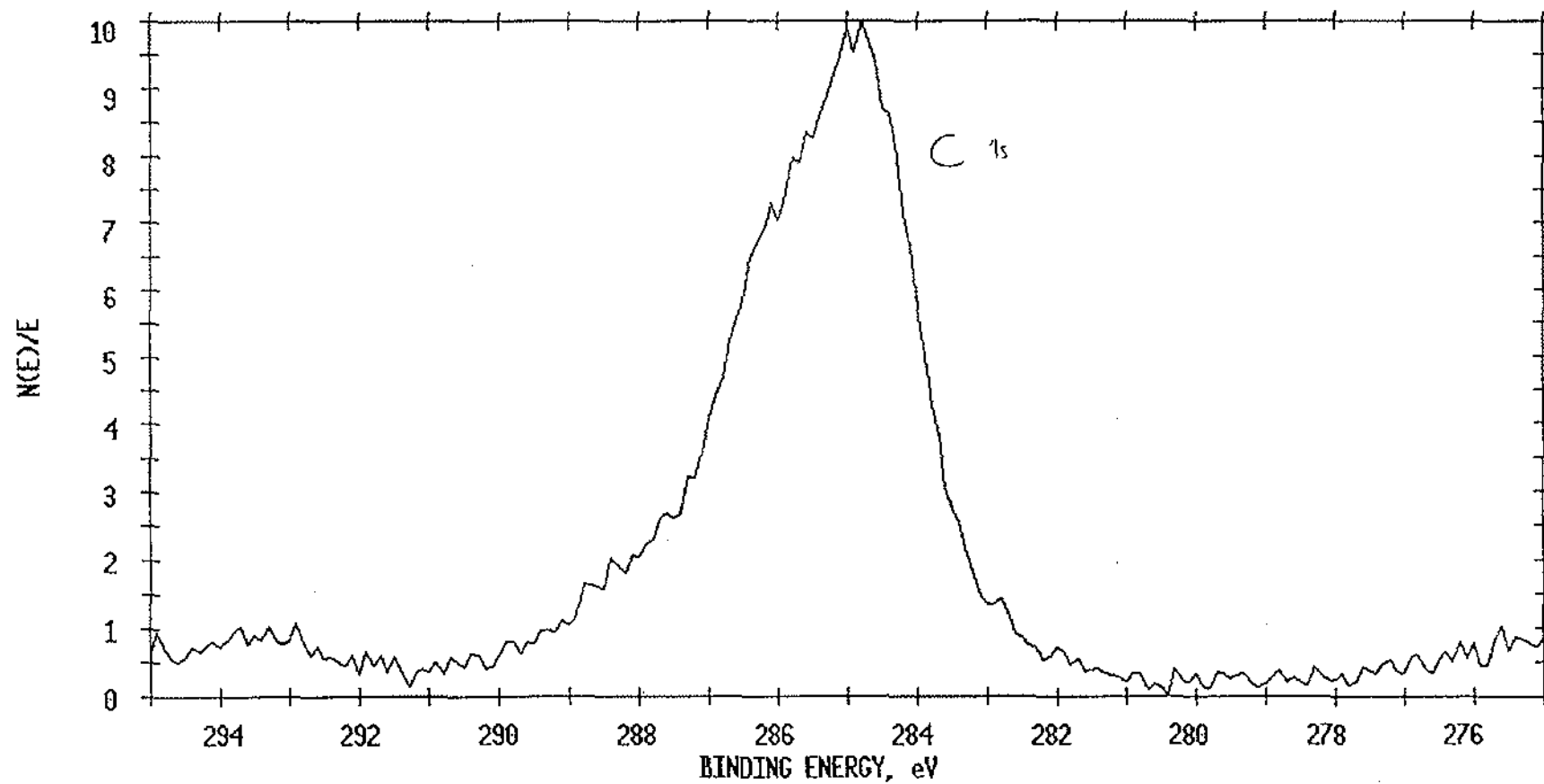
SCALE FACTOR= 1.168 k c/s, OFFSET= 1.049 k c/s PASS ENERGY= 17.900 eV Al 300 W



ESCA MULTIPLEX 3/22/97 EL=C1 REG 3 ANGLE= 45 deg ACQ TIME=4.52 min

FILE: mfh115 herkimer diamond after det/water, acet, meth wash

SCALE FACTOR= 0.246 k c/s, OFFSET= 0.770 k c/s PASS ENERGY= 17.900 eV Al 300 W

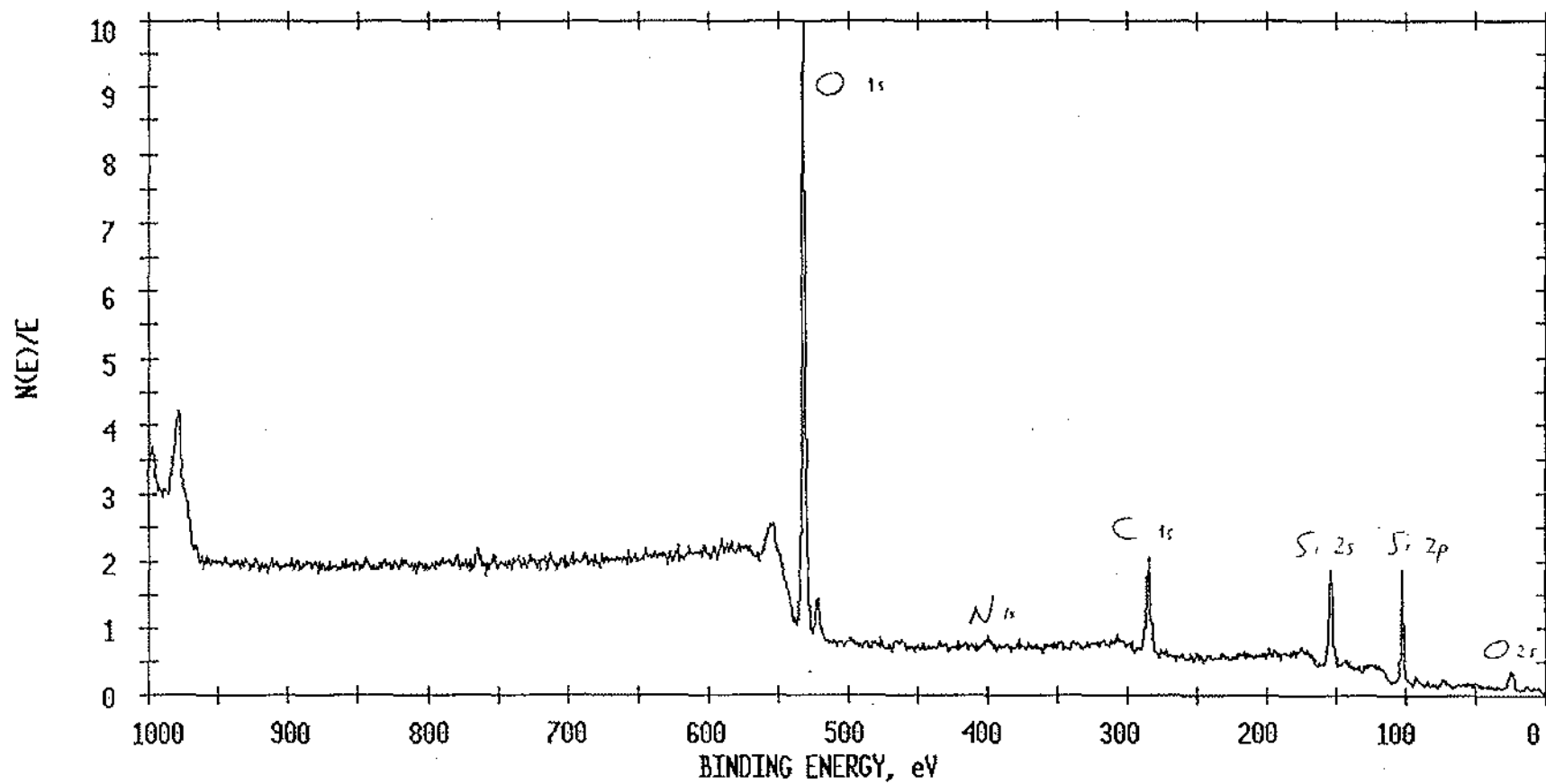


(3)

ESCA SURVEY 3/22/97 ANGLE= 45 deg ACQ TIME=3.75 min

FILE: mfh120 Herkimer diamond after det/water and 5 min water rinse

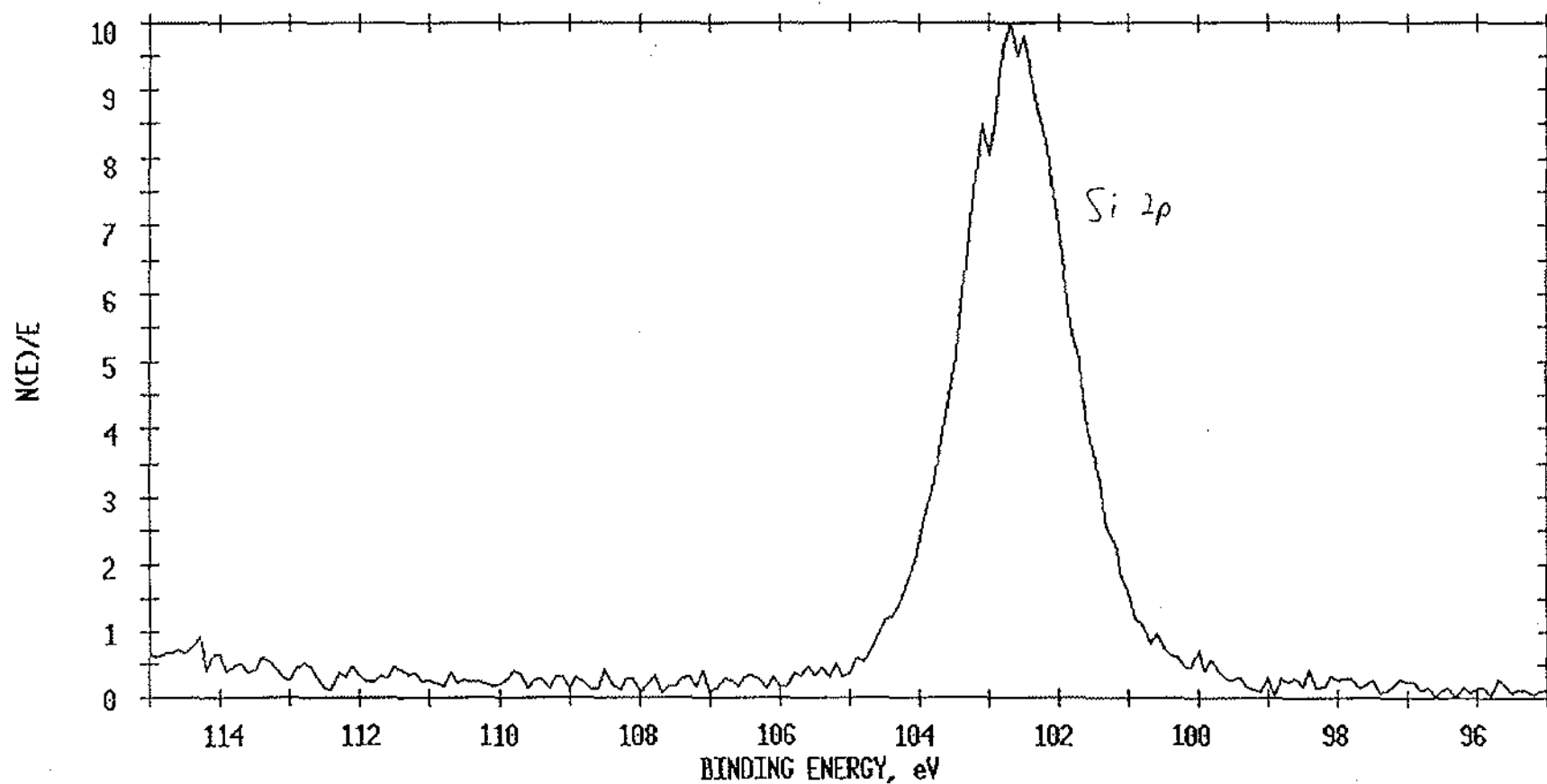
SCALE FACTOR= 3.127 k c/s, OFFSET= 0.324 k c/s PASS ENERGY= 44.750 eV Al 300 W



ESCA MULTIPLEX 3/22/97 EL=Si1 REG 1 ANGLE= 45 deg ACQ TIME=2.09 min

FILE: mfh121 Herkimer diamond after det/water and 5 min water rinse

SCALE FACTOR= 0.270 k c/s, OFFSET= 0.293 k c/s PASS ENERGY= 17.900 eV Al 300 W

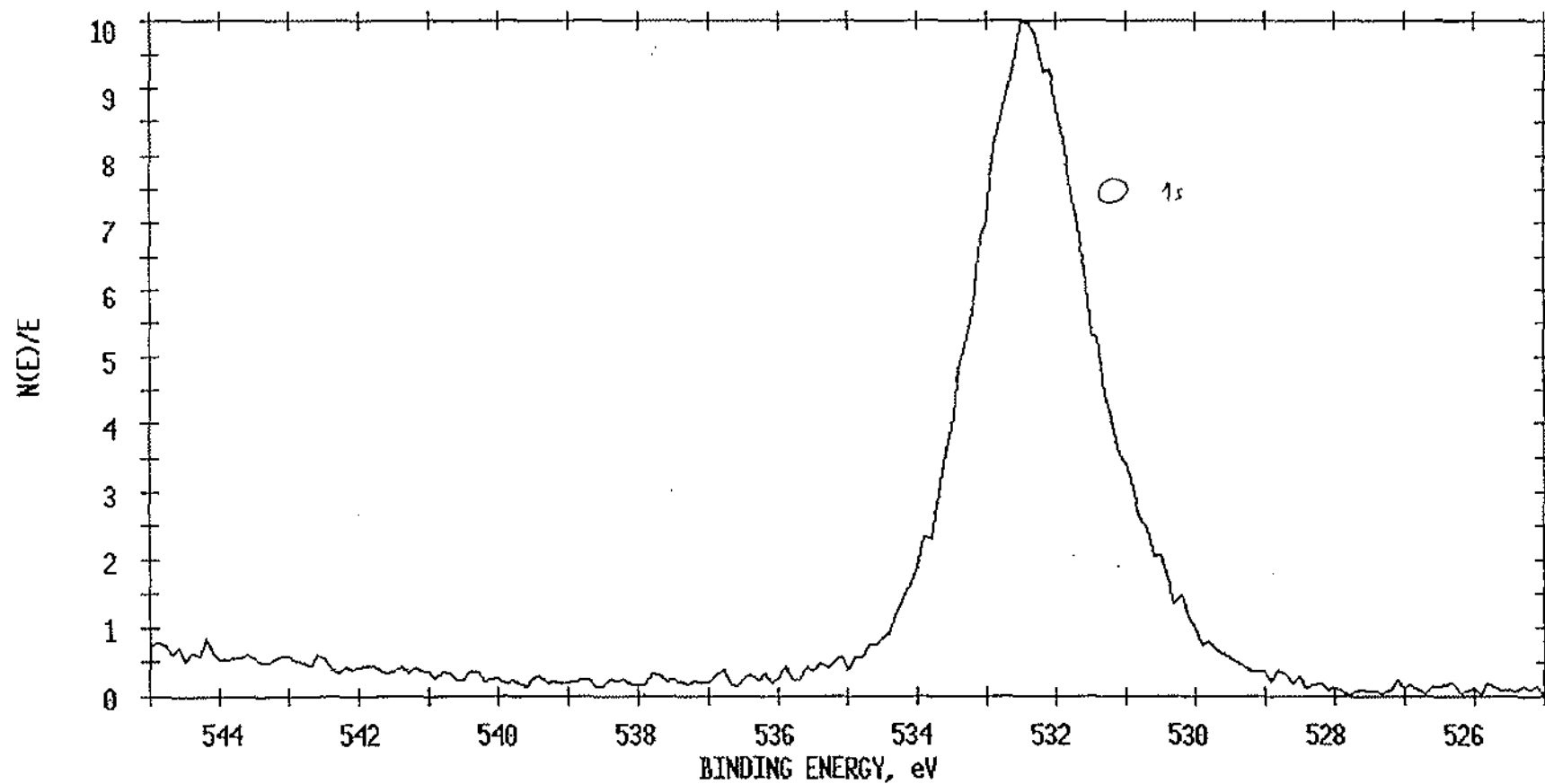


7

ESCA MULTIPLEX 3/22/97 EL=01 REG 2 ANGLE= 45 deg ACQ TIME=0.67 min

FILE: mfh121 Herkimer diamond after det/water and 5 min water rinse

SCALE FACTOR= 1.333 k c/s, OFFSET= 1.025 k c/s PASS ENERGY= 17.900 eV Al 300 W



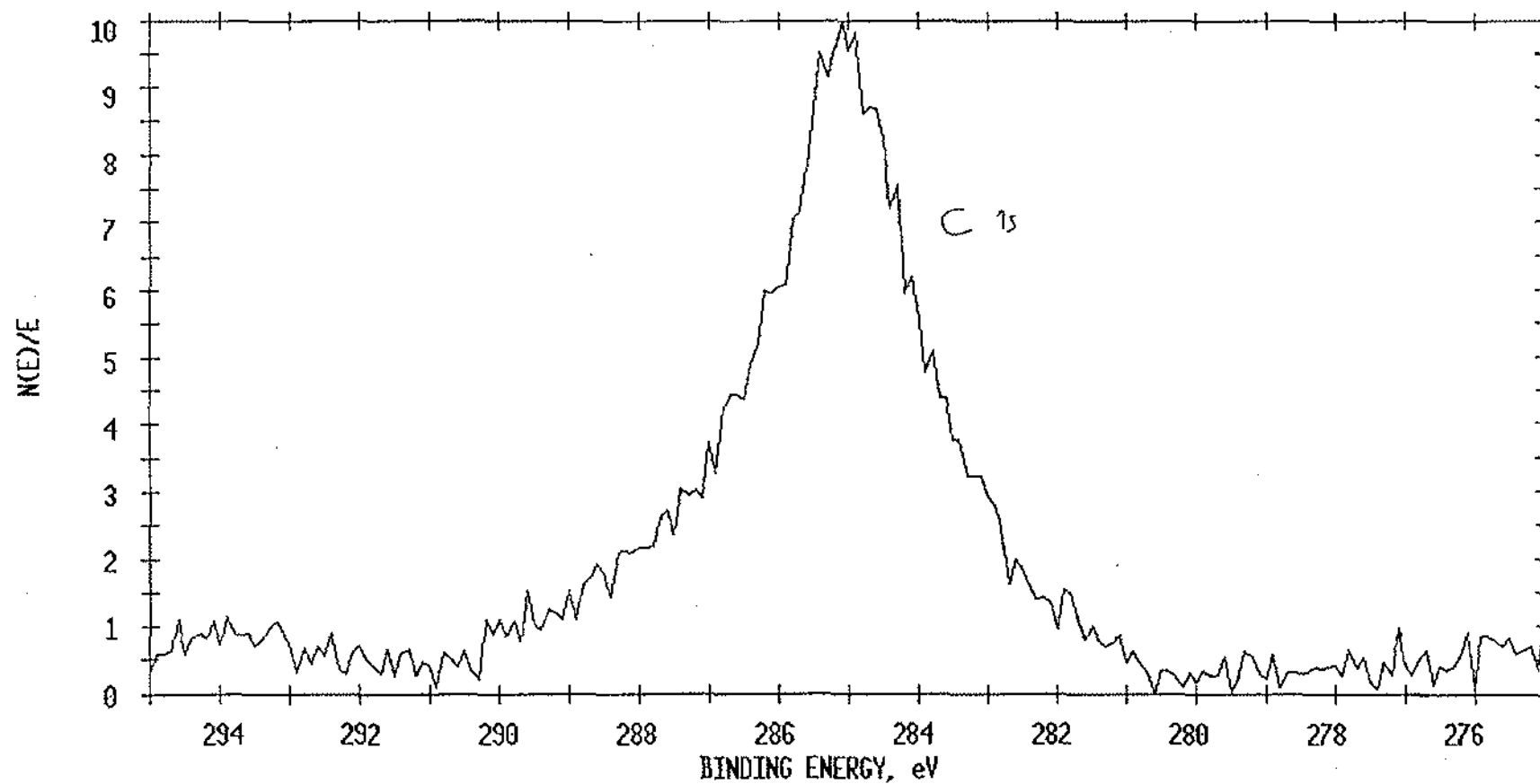
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5.0211

ESCA MULTIPLEX 3/22/97 EL=C1 REG 3 ANGLE= 45 deg ACQ TIME=2.01 min

FILE: mfh121 Herkimer diamond after det/water and 5 min water rinse

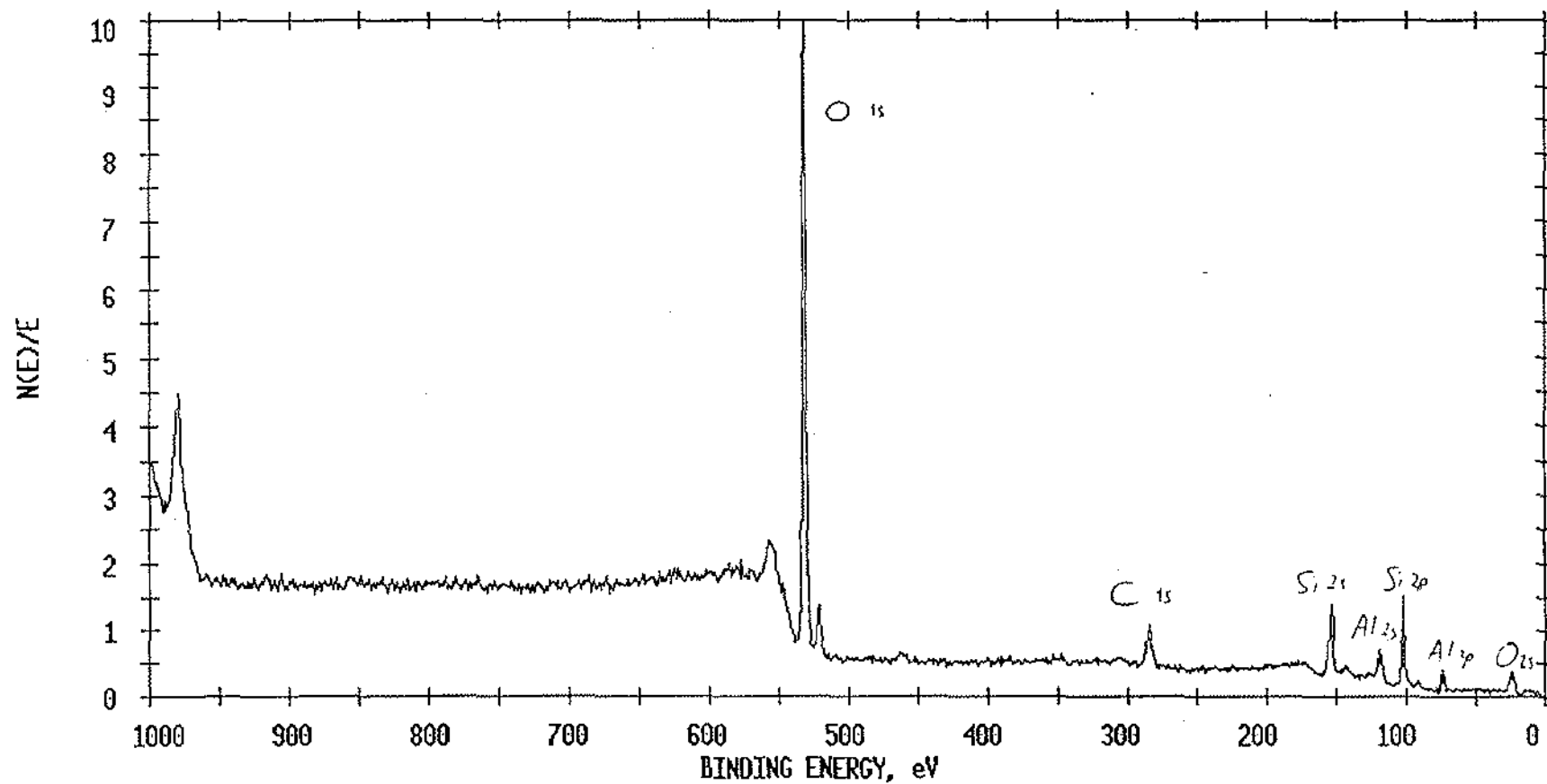
SCALE FACTOR= 0.163 k c/s, OFFSET= 0.753 k c/s PASS ENERGY= 17.900 eV Al 300 W



ESCA SURVEY 3/22/97 ANGLE= 45 deg ACQ TIME=3.75 min

FILE: mfh116 Feldspar Corp. sand, 50 mesh, no pretreatment

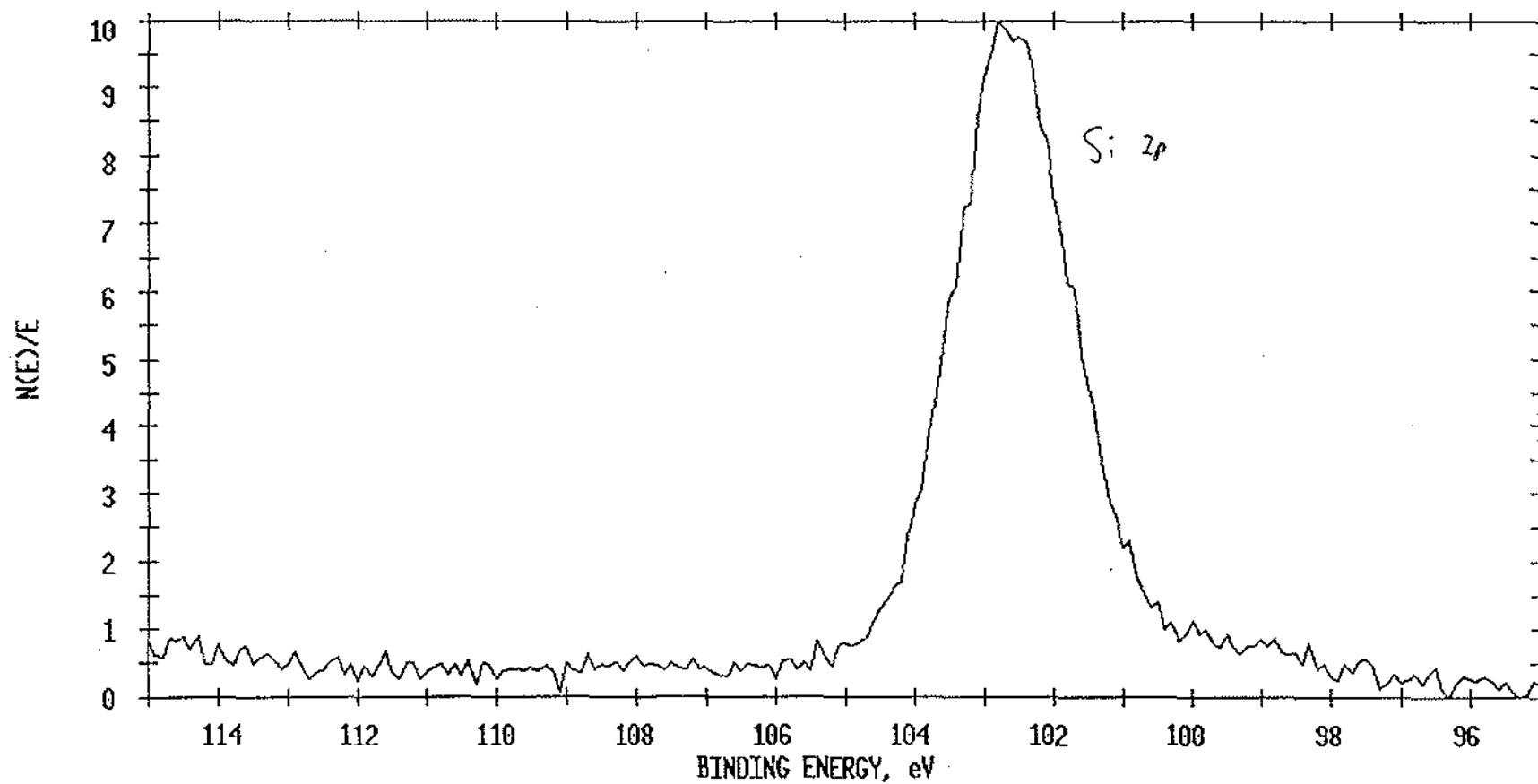
SCALE FACTOR= 2.899 k c/s, OFFSET= 0.284 k c/s PASS ENERGY= 44.750 eV Al 300 W



ESCA MULTIPLEX 3/22/97 EL=Si1 REG 1 ANGLE= 45 deg ACO TIME=3.77 min

FILE: mfh117 Feldspar Corp. sand, 50 mesh, no pretreatment

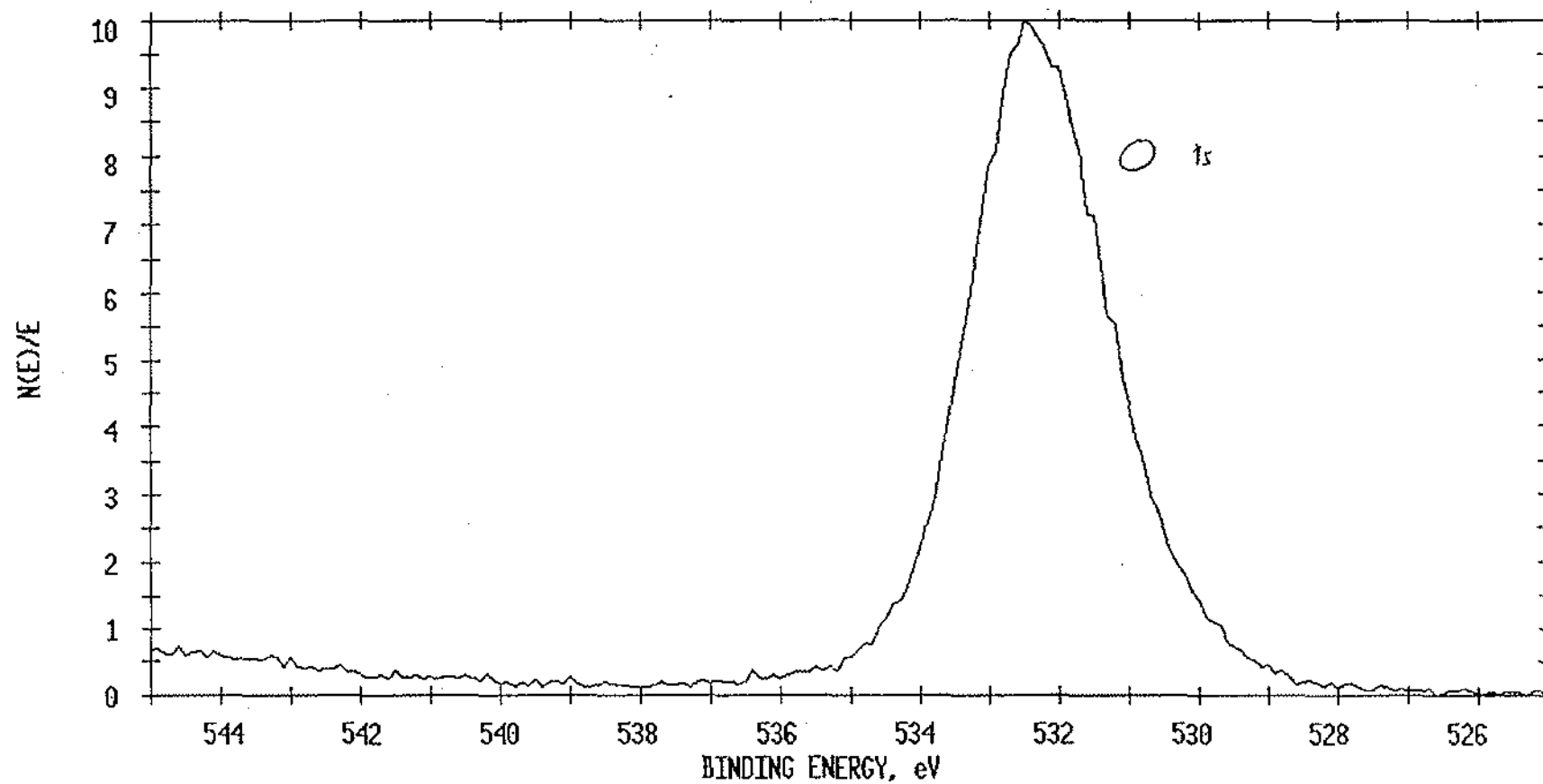
SCALE FACTOR= 0.147 k c/s, OFFSET= 0.220 k c/s PASS ENERGY= 17.900 eV Al 300 W



ESCA MULTIPLEX 3/22/97 EL=01 REG 2 ANGLE= 45 deg ACO TIME=1.51 min

FILE: mfh117 Feldspar Corp. sand, 50 mesh, no pretreatment

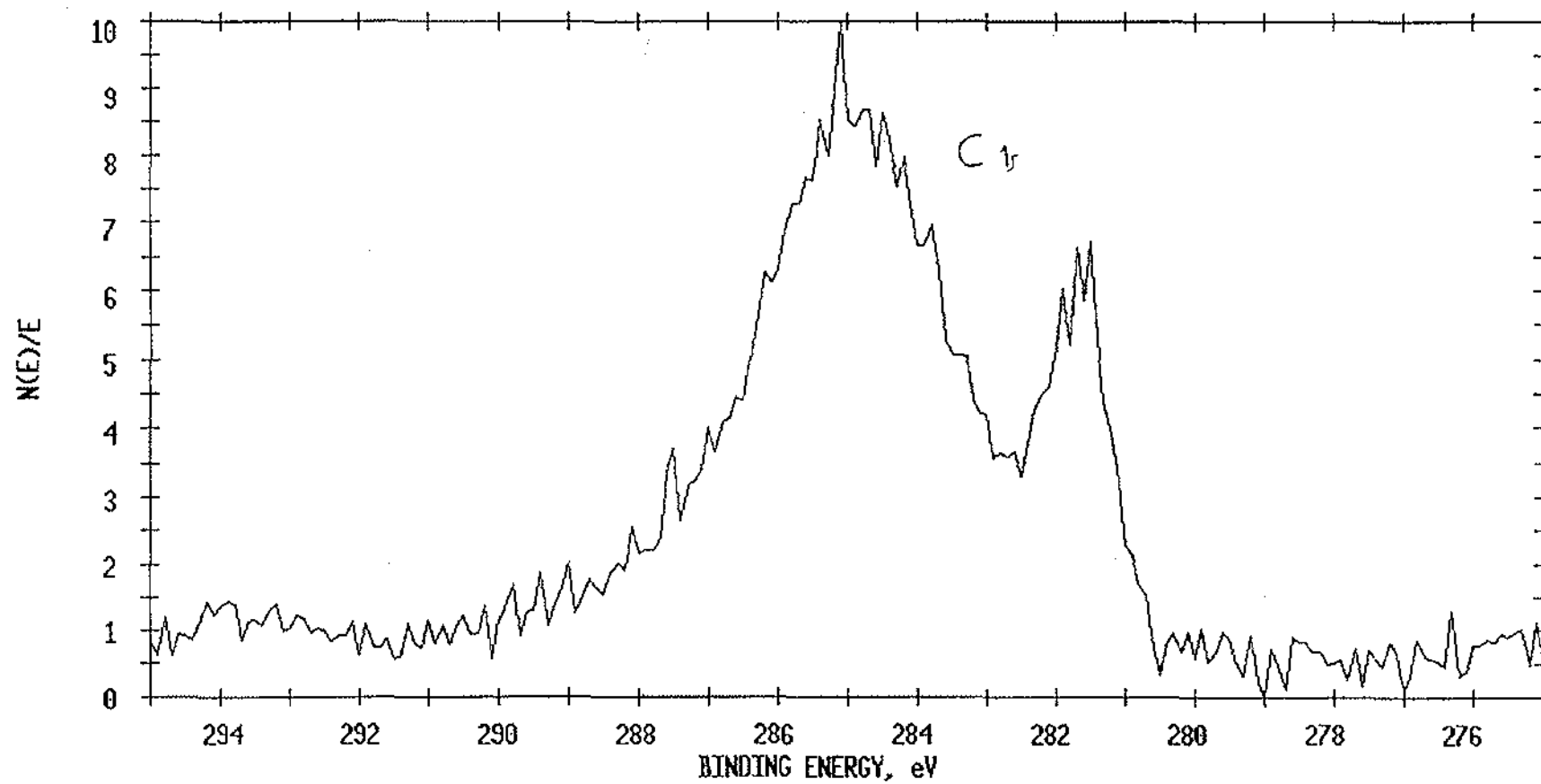
SCALE FACTOR= 1.014 k c/s, OFFSET= 0.749 k c/s PASS ENERGY= 17.900 eV Al 300 W



ESCA MULTIPLEX 3/22/97 EL=C1 REG 3 ANGLE= 45 deg ACQ TIME=4.52 min

FILE: mfh117 Feldspar Corp. sand, 50 mesh, no pretreatment

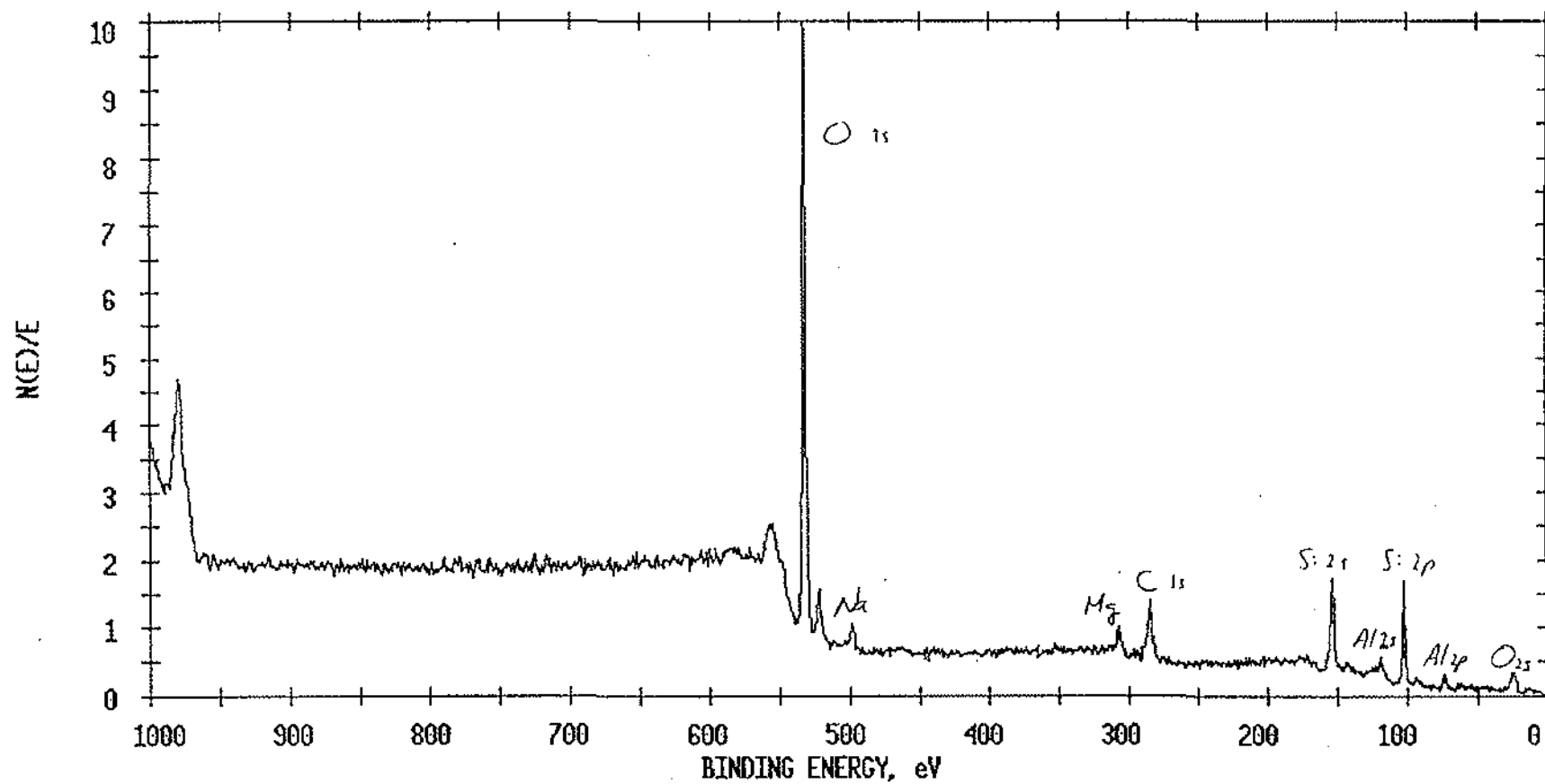
SCALE FACTOR= 0.077 k c/s, OFFSET= 0.479 k c/s PASS ENERGY= 17.900 eV Al 300 W



ESCA SURVEY 3/22/97 ANGLE= 45 deg ACQ TIME=3.75 min

FILE: mfh118 Ottawa sand, 60 mesh, no pretreatment

SCALE FACTOR= 2.573 k c/s, OFFSET= 0.302 k c/s PASS ENERGY= 44.750 eV Al 300 W

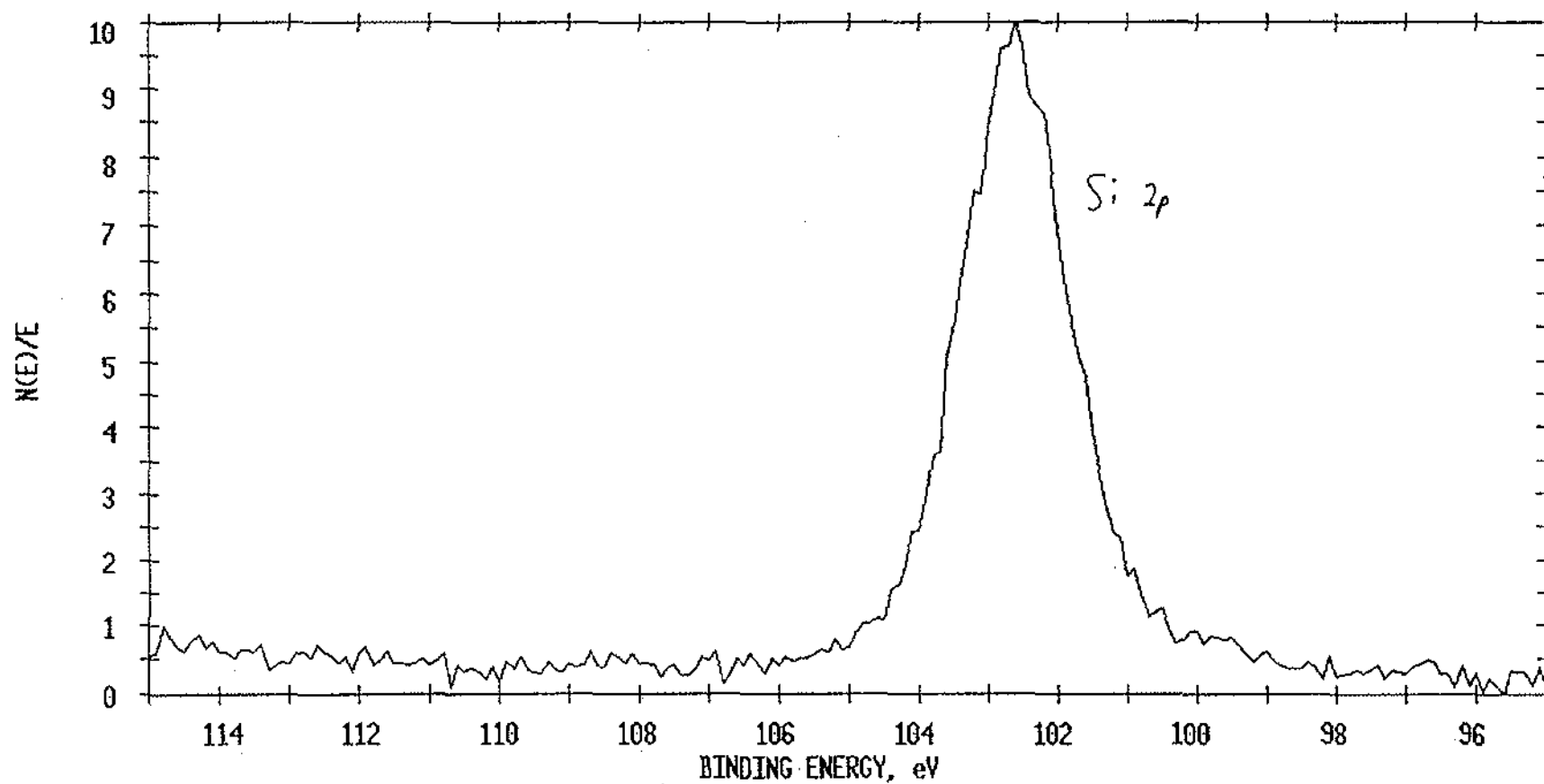


6

ESCA MULTIPLEX 3/22/97 EL=Si1 REG 1 ANGLE= 45 deg ACO TIME=2.43 min

FILE: mfh119 Ottawa sand, 60 mesh, no pretreatment

SCALE FACTOR= 0.170 k c/s, OFFSET= 0.197 k c/s PASS ENERGY= 17.900 eV Al 300 W

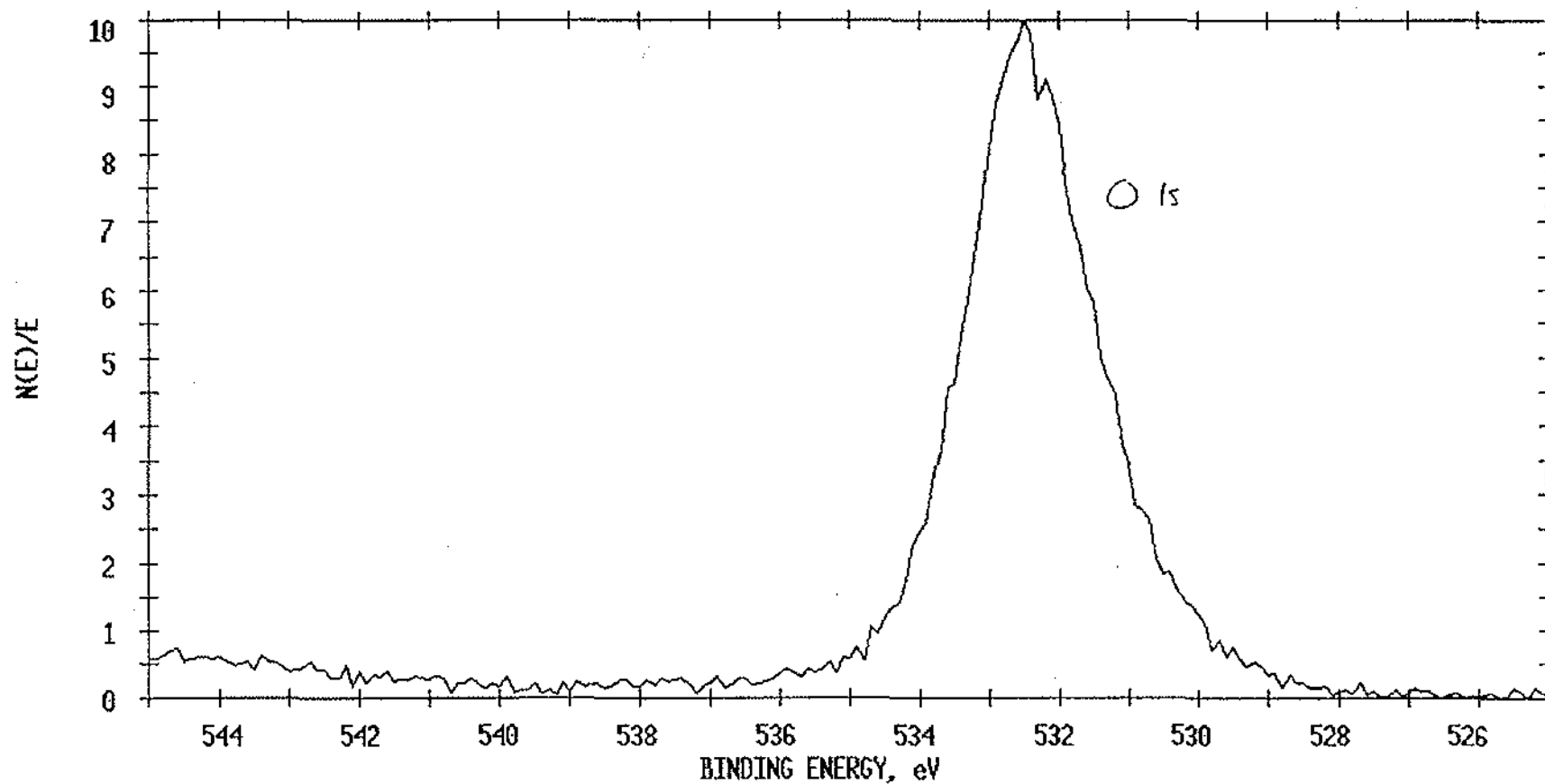


9

ESCA MULTIPLEX 3/22/97 EL=01 REG 2 ANGLE= 45 deg ACQ TIME=0.84 min

FILE: mfh119 Ottawa sand, 60 mesh, no pretreatment

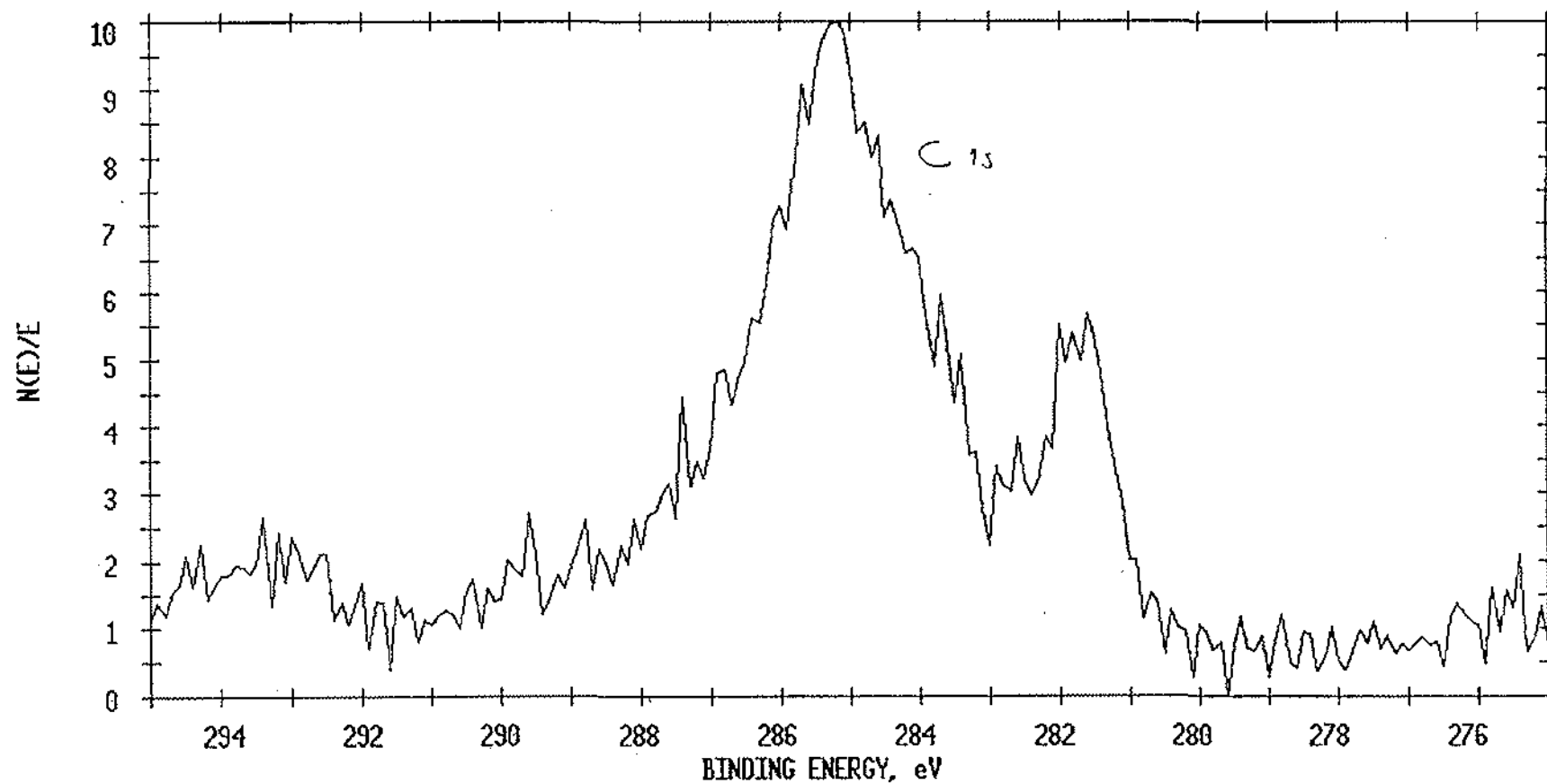
SCALE FACTOR= 0.986 k c/s, OFFSET= 0.816 k c/s PASS ENERGY= 17.900 eV Al 300 W



ESCA MULTIPLEX 3/22/97 EL=C1 REG 3 ANGLE= 45 deg ACQ TIME=2.51 min

FILE: mfh119 Ottawa sand, 60 mesh, no pretreatment

SCALE FACTOR= 0.091 k c/s, OFFSET= 0.475 k c/s PASS ENERGY= 17.900 eV Al 300 W



Curriculum Vitae of Michael F. Hochella, Jr.

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e-mail: hochella@vt.edu

Education:

- 8/77 to 8/81 - Ph.D., Stanford University, Department of Geology.
- 9/75 to 7/77 - M.S., Virginia Tech, Department of Geological Sciences.
- 9/71 to 6/75 - B.S., Virginia Tech, Department of Geological Sciences.

Employment:

- 3/97 to present - Professor, Department of Geological Sciences, Virginia Tech.
- 8/92 to 2/97 - Associate Professor, Department of Geological Sciences, Virginia Tech.
- 9/89 to 7/92 - Associate Professor (Research), Department of Geology, Stanford University.
- 11/83 to 8/89 - Acting Assistant Professor/Senior Research Associate, Department of Geology and Center for Materials Research, Stanford University.
- 9/81 to 11/83 - Senior scientist, Corning Glass Works, Corning, N.Y.

Honors:

- 39 invited talks in the last 6 years, 6 in Europe
- Fulbright Scholar, 1997-98, for study in Germany
- Vice President, The Geochemical Society (1998-1999)
- Fellow, Mineralogical Society of America (elected 1990)
- Member, Board of Directors, Geochemical Society (appointed 1995-1998)
- Former Member, Mineralogical Society of America Executive Council (1992-1994)
- Appointed MSA Lecturer for 1992/93 and 1993/94.
- Mineralogical Association of Canada Visiting Lecturer (1989/90)

Selected current professional and administrative activities:

General:

- Vice President, The Geochemical Society (1,700 members in 47 countries)
- Associate Editor, *Geochimica et Cosmochimica Acta*, 1991-present
- Cambridge University Press Series Editor, appointed 1993
- Editorial Board, CRC Press Series on Surface Science, appointed 1992
- Board Member, Geochemical Society, 1995-present
- 35 invited talks in the last 6 years, 5 in Europe

Virginia Tech:

- Chair, Graduate Students Affairs Committee (includes graduate admissions)
- Member, Departmental Executive and Planning Committees

Overall research funding:

- Total research funding since coming to Virginia Tech 4 years ago: **\$1,072,311.**
- Total research funding last 10 years (6 years at Stanford; 4 years at Va. Tech): **\$2,669,159.**

Previous funding sources: National Science Foundation, Gas Research Institute, US Department of Energy, US Geological Survey, American Chemical Society (Petroleum Research Fund), California Office of Competitive Technologies, Newmont Metallurgical, Chevron, Texaco, and cost sharing at Virginia Tech.

Current research grants (external only):

- Funding agency and dates: National Science Foundation, 1993-1997
Title of research: Studies in Mineral Surface Reactivity and Geochemistry to the Atomic Level
Amount awarded: **\$165,000**
- Funding agency and dates: Amer. Chemical Society (Petrol. Research Fund), 1997-1999
Title of research: Silicate Dissolution/Precipitation in the Presence of Confined Water Films
Amount awarded: **\$50,000**
- Funding agency and dates: National Science Foundation, 1996-1998
Title of research: Development of Scanning Tunneling Spectroscopy (STS) for Research in Mineralogy and Geochemistry
Amount awarded: **\$109,205**
- Funding agency and dates: National Science Foundation, 1996-1999
Title of research: Studies in the Fundamental Nature of Mineral Surface Reactivity
Amount awarded: **\$174,606**

General publication record:

- Total professional publications to date: **73**
- Peer reviewed publications to date: **66**
- Authored book chapters to date: **9**
- Books edited to date: **1**

Selected Publications:

- Hochella M.F., Jr., and White A.F. (1990) Mineral-water interface geochemistry: An overview. In: Mineral-Water Interface Geochemistry (M.F. Hochella, Jr. and A.F. White, eds.), Reviews in Mineralogy, 23, 1-16. Published by the Mineralogical Society of America.
- Hochella M.F., Jr. (1990) Atomic structure, microtopography, composition, and reactivity of mineral surfaces. In: Mineral-Water Interface Geochemistry (M.F. Hochella, Jr. and A.F. White, eds.), Reviews in Mineralogy, 23, 87-132. Published by Mineral. Soc. Amer.
- Eggleston C.M., and Hochella M.F., Jr. (1991) Scanning tunneling microscopy of galena (100) surface oxidation and sorption of aqueous gold. Science, 254, 983-986.
- Hochella M.F., Jr. (1993) Surface chemistry, structure, and reactivity of hazardous mineral dust. In: Health Effects of Mineral Dusts (G.D. Guthrie, Jr. and B.T. Mossman, eds.), Reviews in Mineralogy, 28, 275-308. Published by the Mineralogical Society of America.
- Hochella M.F., Jr. (1994) Mineral surfaces: Characterization methods, and their chemical, physical, and reactive nature. In Mineral Surfaces (D. Vaughan and R. Patrick, eds.), pp. 17-60. Chapman and Hall, London.
- Hochella M.F., Jr. and Banfield J. (1995) Chemical weathering of silicates in nature: A microscopic perspective with theoretical considerations. In: Chemical Weathering Rates of Silicate Minerals (A. White and S. Brantley, eds.), Reviews in Mineralogy, 31, 353-406.

December, 1997

Aspects of the Nature of Quartz Surfaces

Report 2

**A follow-up report to Lee Coogan, Bill Moll, Sark Ampien,
and the Sorptive Mineral Institute**

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Department of Geological Sciences
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Executive Summary

This is a follow-up report to the one prepared and submitted to SMI in October, 1997. In this study, I have looked at the surface chemistry of Ione sand from California, as well as IOTA standard and milled quartz (both of these IOTA quartz samples were used by Castranova in *in vivo* inhalation tests with rats) using X-ray photoelectron spectroscopy (XPS). I have also performed additional low energy electron diffraction (LEED) analysis of a Herkimer quartz single crystal. The Ione sand is expectedly "dirty" with a moderate amount of adventitious C on the grain surfaces. Besides Si and O (and C), XPS detected Na, Mg, Al, Ca, and Ti. These latter elements probably come from clays, feldspars, and titania that are contaminating the sand. Both samples of the IOTA sand are exceptionally clean, with very little C contamination (as low as I have ever seen it for quartz that has been exposed to air).

The LEED results on a carefully cleaned Herkimer quartz single crystal show a faint and partial diffraction pattern. This indicates that at least a significant portion of the top few monolayers of Si and O atoms on a quartz crystal face, and perhaps the whole surface, is crystalline. This is a remarkable result, and explains why dissolution experiments suggest that an amorphous surface layer on quartz is exceptionally thin or nonexistent. Better cleaning procedures will be needed to obtain more satisfactory (and useful) LEED patterns.

Recommended future work includes additional cleaning and LEED studies of quartz crystal growth faces, as well as atomic force microscope imaging of these surfaces.

Introduction

In my previous report to SMI in October of 1997, I discussed quartz surface contamination and crystallinity in relatively general terms. The purpose of this report is to quantify the surface composition of three sands of particular interest to SMI, and to attempt to clear up some uncertainties in the crystallinity of quartz surfaces.

The samples analyzed for this report, and the specific techniques used, are summarized in the following table:

<u>Sample</u>	<u>Work Performed</u>
IOTA Standard Quartz (used by Castranova in <i>in vivo</i> inhalation tests)	XPS
Milled IOTA Quartz (used by Castranova in <i>in vivo</i> inhalation tests)	XPS
Ione (California) sand	XPS
Herkimer quartz single crystal	LEED

The IOTA Standard and Milled Quartz samples were supplied to me from Sark Ampien who obtained them from Vince Castranova's research group. The IOTA Standard sample is the same sand used in the study presented at the Sixth International Meeting on the Toxicology of Natural and Man-Made Fibrous and Non-Fibrous Particles in Lake Placid, New York last year. The Milled IOTA sample is the standard sample that has been ground to submicron size. The Ione sample is a production sand from California, and the Herkimer sample is a euhedral clear quartz single crystal of hydrothermal origin from Herkimer, New York.

Experimental Methods

X-ray Photoelectron Spectroscopy (XPS)

The XPS instrument and procedures used in this study are exactly the same as described in my October, 1997 report to SML. Briefly, I used a Perkin-Elmer 5400 XPS instrument with Al K α X-radiation at a power of between 200 and 300 Watts. Base pressures were better than 5×10^{-8} Torr. Survey scans were collected to 1000 eV binding energy using 1 eV steps of 20 ms per step counting time, and a detector pass energy of 89.45 eV. Collection time for survey scans was 5 min. High resolution narrow scans had a range of 20 eV with 0.1 eV steps of 50 ms per step and a detector pass energy of 17.55 eV. Perkin-Elmer peak fitting routines were used on C peaks which utilized Shirley background correction and Gaussian-Lorentzian peak shapes.

The sands were mounted on sample stubs with colloidal carbon suspended in alcohol (DAG). In some cases (in this study, for the standard IOTA and Ione quartz), a signal from the DAG is obtained along with the sample. However, the carbon present on the sample surfaces can be easily distinguished from the carbon DAG. This is done by knowing the carbon line energies for the DAG and subtracting this contribution away from the carbon line collected from the sample. This is done using peak fitting, and the procedure is routine.

Low Energy Electron Diffraction (LEED)

The LEED done in this study was similar to that described in my October, 1997 study using a Omicron reverse view 4-grid LEED mounted on an Omicron UHV Compact Lab. However, there was one important difference. LEED is so surface sensitive that any surface contamination may preclude one from obtaining a surface diffraction pattern. Yet the purpose of doing LEED on quartz in this study was to see if a pattern could be obtained from a natural crystal growth face, by definition a surface that will have surface

contamination due to its prolonged exposure to the environment. Therefore, in order to have a chance at collecting a surface diffraction pattern, I cleaned the Herkimer quartz single crystal as described in the sample handling section below.

Sample Handling

The two IOTA sands and the Ione sand were analyzed with XPS with absolutely no sample pretreatment. Samples were simply removed from their plastic bags, mounted with DAG (see above) on an aluminum sample stub, and run. On the other hand, for reasons given above, the Herkimer quartz crystal was cleaned. First, the crystal was scrubbed in Sparkleen, a high phosphate soap used to clean laboratory glassware, and warm water. Next, the crystal was sonicated in this solution for 5 minutes with two water and soap changes. Then the sample was rinsed well in DI water and sonicated again (in DI water) for 5 minutes. Sonication was repeated two more times, first with ethanol, then again with DI water. After the last DI water rinse, the crystal was blown dry with a fast stream of nitrogen gas. The sample was then introduced into the vacuum of the LEED instrument within 5 minutes.

Results and Discussion

XPS

The Table below presents some of the most important XPS data collected from each of the three different samples. The spectra numbers in the table correspond to the labeling of the XPS data appended to the end of this report. All sample descriptions in the table are self-explanatory, and they are also detailed in the text above.

<u>Sample</u>	<u>Spectra No.</u>	<u>Surface C/Si Intensity Ratio</u>	<u>O 1s Peak Width (eV)</u>
Ione (California) sand	7	1.24	2.2
IOTA Standard	8	0.27	1.6
IOTA Milled	9	0.50	1.9

As described in the last report, the C/Si intensity ratios give a quantitative idea of the surface "cleanliness" of each of the samples at a glance. Mineral surface compositions are rarely representative of the bulk. Mineral surfaces exposed to air or an aqueous solution immediately take up hydrogen, oxygen, usually carbon, and sometimes nitrogen (from O₂, H₂O, CO₂, hydrocarbons, and N₂ and/or NO_x in the air or solution). This is generally known as "adventitious" material. The build-up of these and other adventitious materials "passivates" the clean highly reactive surface very quickly. Excluding gross contamination, adventitious materials of the type mentioned above usually do not obtain a thickness of more than a few to several Angstroms.

Based on this criteria, the Ione sand is relatively clean, and both of the IOTA sands are exceptionally clean. In fact, in the last report I measured the C/Si intensity ratio to be 0.39 on a piece of freshly fractured single crystal quartz that had only been exposed to air for a few minutes. The C/Si ratio of 0.27 on the IOTA standard sand is the lowest that I have ever measured on a surface that has been exposed to the air. The fact that the O1s peak width is 1.6 eV confirms that the surfaces of these grains are especially clean and pure (see previous report). I have never measured an oxygen peak width narrower than this on even an in-vacuum fractured quartz surface. The IOTA Milled sand has a C/Si ratio of 0.5, with a O1s peak width of 1.9 eV, so it is apparent that grinding IOTA sand activates the

surfaces a bit which adds to their adventitious layers, but these surfaces are still exceptionally clean and pure.

The survey scans for the IOTA samples, besides Si, O, and C (H cannot be detected, but it is certainly present on the surface), show only minor amounts of Ti. The Ti is probably in the form of small numbers of TiO_2 grains mixed in with the sand. No other elements are present in or on these samples down to about the 0.5% level (the detection limit of XPS).

The Ione sand has moderate amounts of adventitious material on its surfaces relative to what I have seen in the past. There is certainly nothing unusual about its make-up, including the small amounts of Na, Mg, Al, Ca, and Ti that are seen in the survey scan. These elements probably come from small amounts of clays, feldspars, and titanium oxides that are contaminating the sand.

No iron was observed on any of these surfaces using XPS. This may be important, as redox cycling of Fe on silicate surfaces in lungs has been suspected of free radical generation under physiological conditions.

LEED

The Herkimer quartz single crystal used for LEED was mounted on its side with metal straps so that one of its {hk0} prism faces was in a convenient position for LEED analysis. The sample was cleaned in place as described above, and then inserted into the UHV environment of the LEED apparatus. As the beam energy was increased, sample charging was observed on the diffraction screen which is typical and normal for an electron beam impinging on an insulating surface. As the beam energy was increased to over 60 eV, the charging went away and a stable background was obtained. During the next several minutes, various beam energies and sample positions were tried. On two instances, partial LEED patterns were obtained. The patterns showed no more than 2 or 3 diffraction spots, and they were quite weak. However, we were able to confirm that they were real

diffraction spots by watching them move as the sample was tilted. The sample was removed, recleaned, and re-inserted, but the results could not be improved. The sample was then heated to 300°C for 5 minutes in hopes of driving off any surface contamination, but again the results could not be improved. Subsequent XPS analysis of this surface, after an additional recleaning, showed that the C/Si ratio was about 0.8 which is relatively clean, but not as clean as quartz surfaces that have been exposed to air can be.

The fact that at least a partial LEED pattern was obtained on this Herkimer quartz crystal is very exciting. Almost certainly a complete, intense pattern could not be obtained because the surface contamination, although greatly reduced by our cleaning, was in excess of that required for such a pattern. Nevertheless, this is the first time (to my knowledge) that any LEED pattern has been obtained on a quartz surface. What this means is that the surface of this quartz crystal, even though it is a natural growth face that has been exposed to the environment for literally millions of years, is still crystalline. There is no possible way that even one diffraction spot would be visible if the top few monolayers of Si and O on the quartz surface were amorphous.

Conclusions and Future Work

Both the Standard and Milled IOTA samples have exceptionally clean surfaces with no additional elements present except very slight amounts (a few monolayers at the most) of H, C, and O. The minor amount of Ti is probably tied up in titanium oxide particles which makes up a very small part of the sample. The fact that the milled sample has more adventitious material on its surface than the standard sample, and that the oxygen 1s XPS line is a bit broader, is not surprising. These fresh fractured surfaces (Milled IOTA) would be expected to pick up a bit more from the atmosphere than the aged surfaces (Standard IOTA).

The LEED results from the {hk0} prism face of a quartz single crystal are exciting. They indicate that the near surface (or at least of significant portion of it laterally speaking)

is crystalline. This explains why Professor Rimstidt calculated a "disturbed" (amorphous) surface thickness to be on the order of a fraction of an Angstrom thick for the Ione sand if it was evenly distributed across the surface. It is remarkable to think of a material whose surface is so insoluble and well-structured that it remains crystalline even after millions of years of exposure to the environment near the Earth's surface.

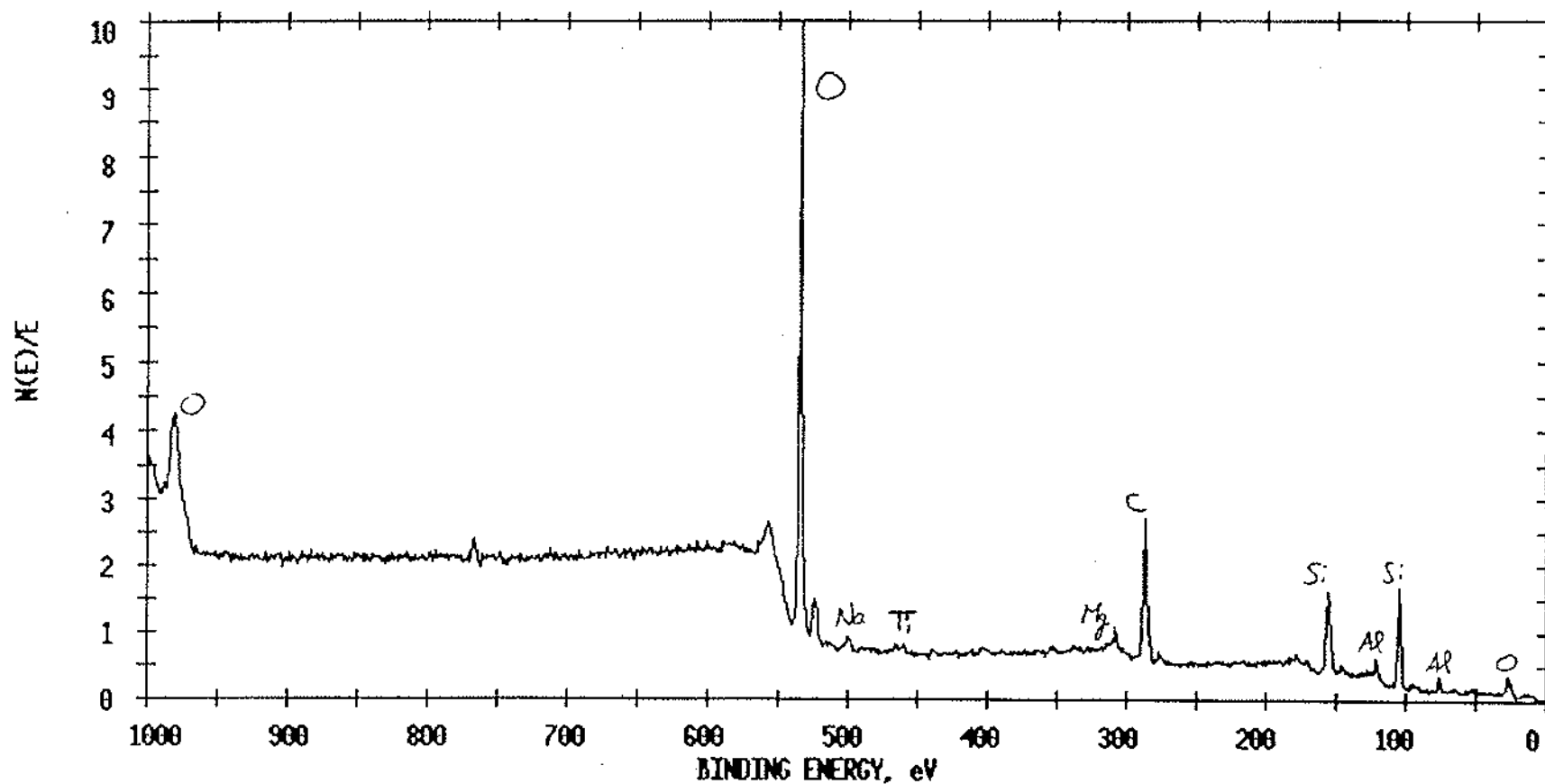
This study leads to two obvious directions to take in the future. First, more effective cleaning techniques should be found for the quartz surfaces used for LEED. The adventitious layers need to be further reduced before the LEED patterns can be clear and complete. If this can be accomplished, the LEED pattern can be quantified in terms of surface crystallographic symmetry and cell dimensions. Second, it would be interesting to perform high resolution atomic force microscopy (AFM) on the quartz surfaces used for LEED analysis to see whether dissolution features can be observed, and to generally quantify surface roughness on these natural growth faces. This information may help to interpret the LEED and XPS results.

7

ESCA SURVEY 11/9/87 ANGLE= 45 deg ACO TIME=6.67 min

FILE: wfh6 Ione, CA sand

SCALE FACTOR= 2.968 k c/s, OFFSET= 0.252 k c/s PASS ENERGY= 44.750 eV Al 300 W



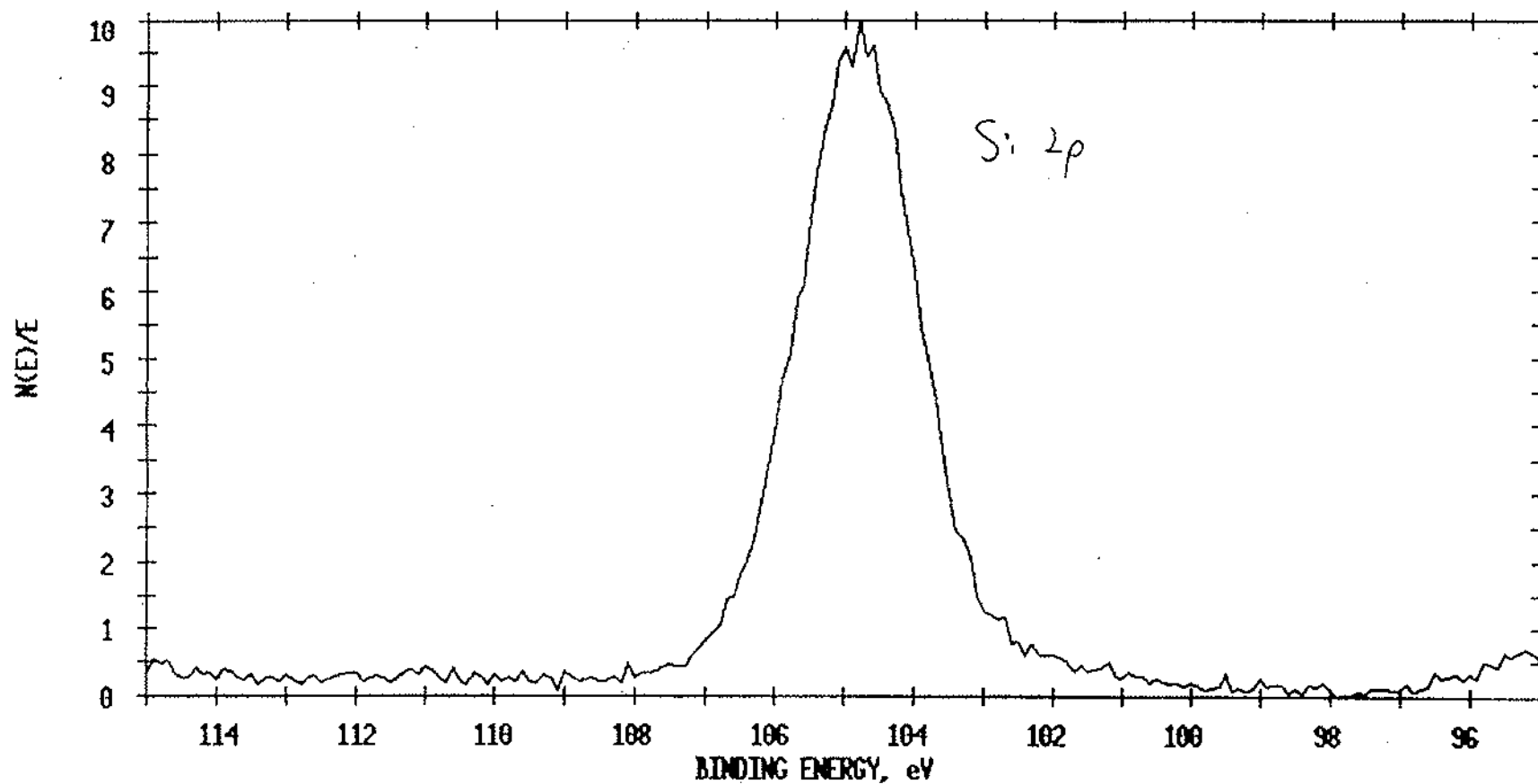
+

(7)

ESCA MULTIPLEX 11/9/87 EL=Si1 REG 1 ANGLE= 45 deg ACQ TIME=7.71 min

FILE: mfh7 Ione, CA sand

SCALE FACTOR= 0.167 k c/s, OFFSET= 0.253 k c/s PASS ENERGY= 17.900 eV Al 300 M



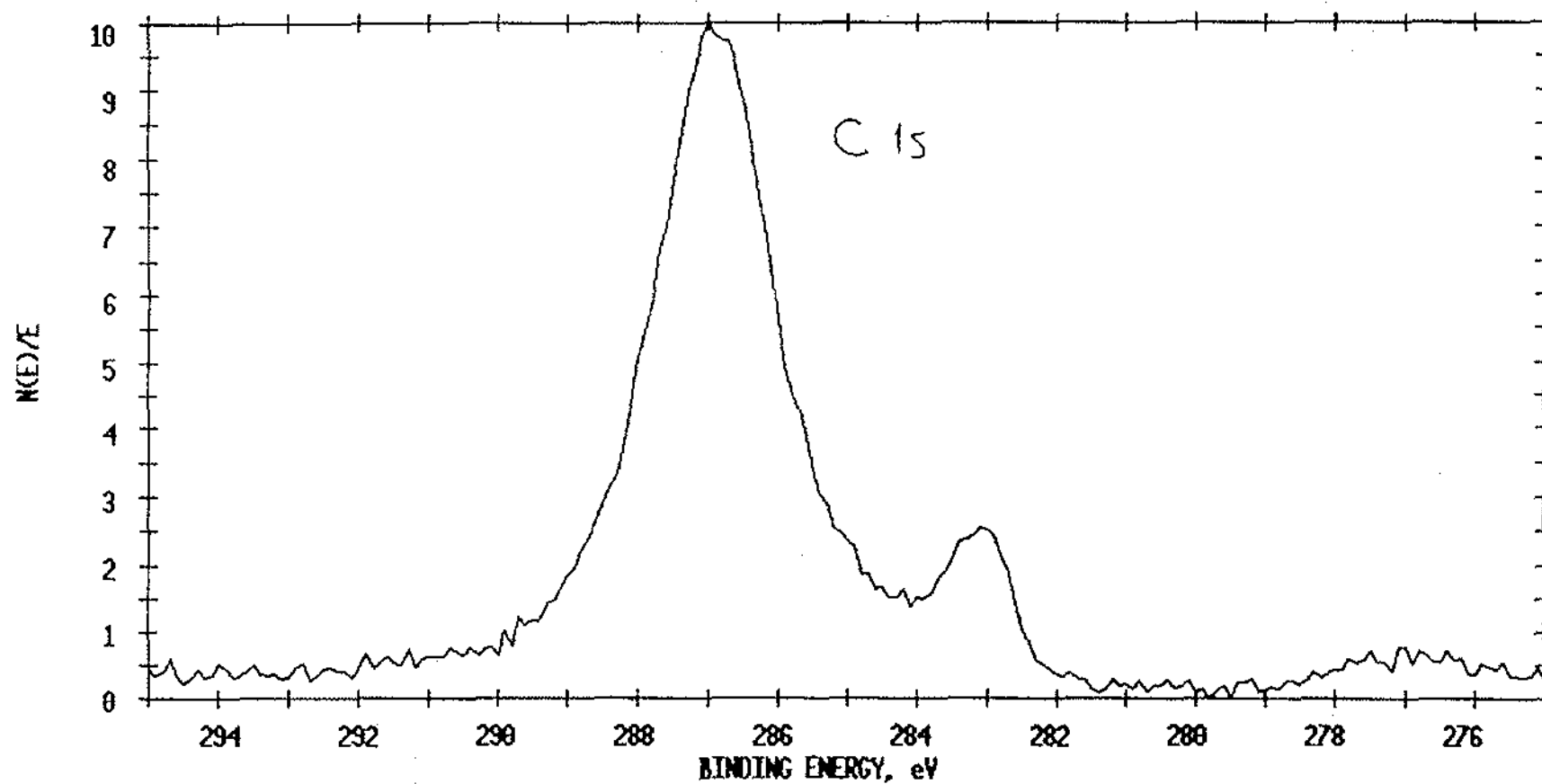
(+)

7

ESCA MULTIPLEX 11/9/87 EL=C1 REG 2 ANGLE= 45 deg ACQ TIME=7.71 min

FILE: nfh7 Ione, CA sand

SCALE FACTOR= 0.209 k c/s, OFFSET= 0.624 k c/s PASS ENERGY= 17.900 eV Al 300 W



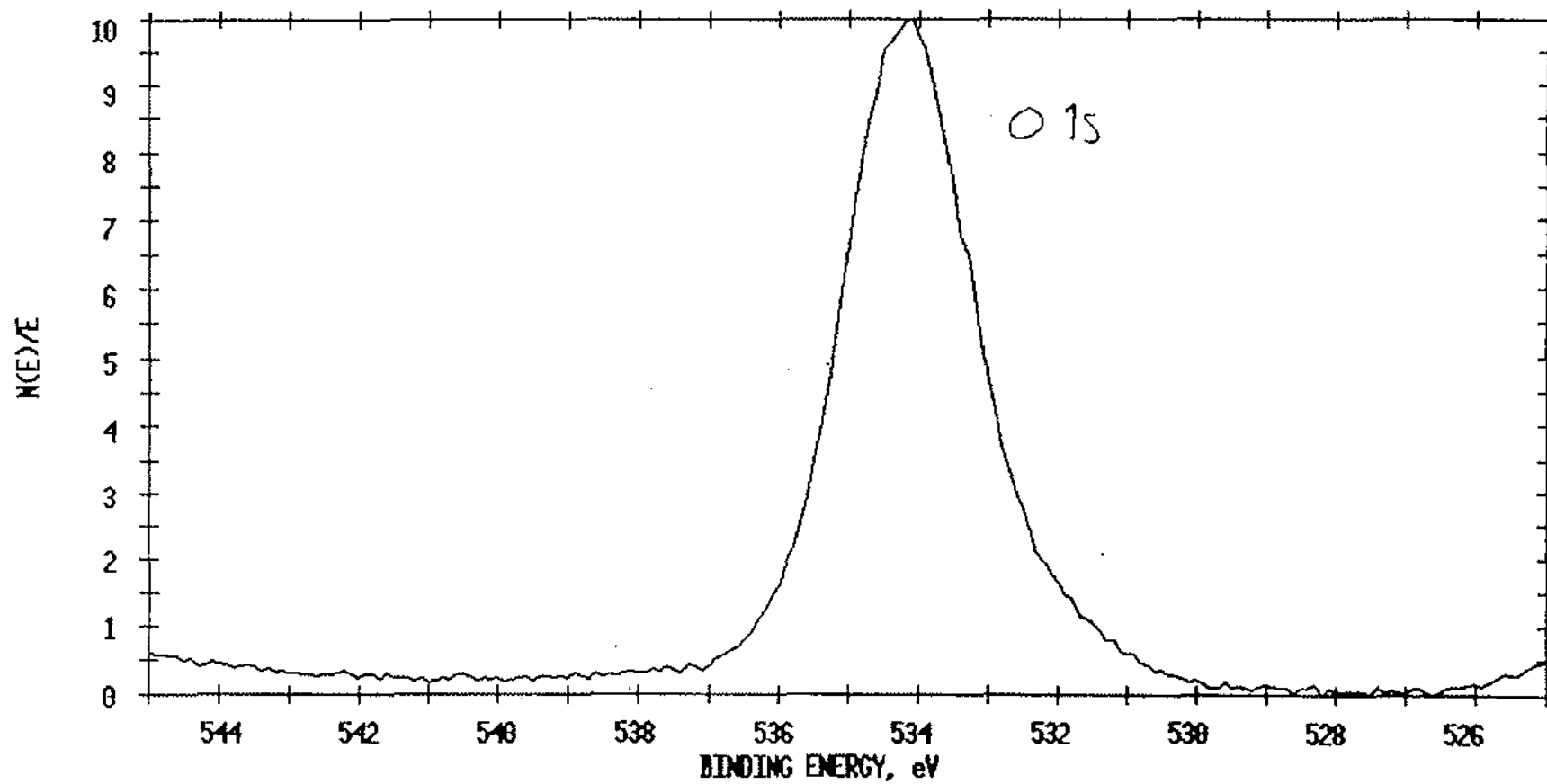
7

7

ESCA MULTIPLEX 11/9/87 EL=01 REG 3 ANGLE= 45 deg ACQ TIME=3.85 min

FILE: nfh7 Ione, CA sand

SCALE FACTOR= 0.976 k c/s, OFFSET= 0.965 k c/s PASS ENERGY= 17.900 eV Al 300 W

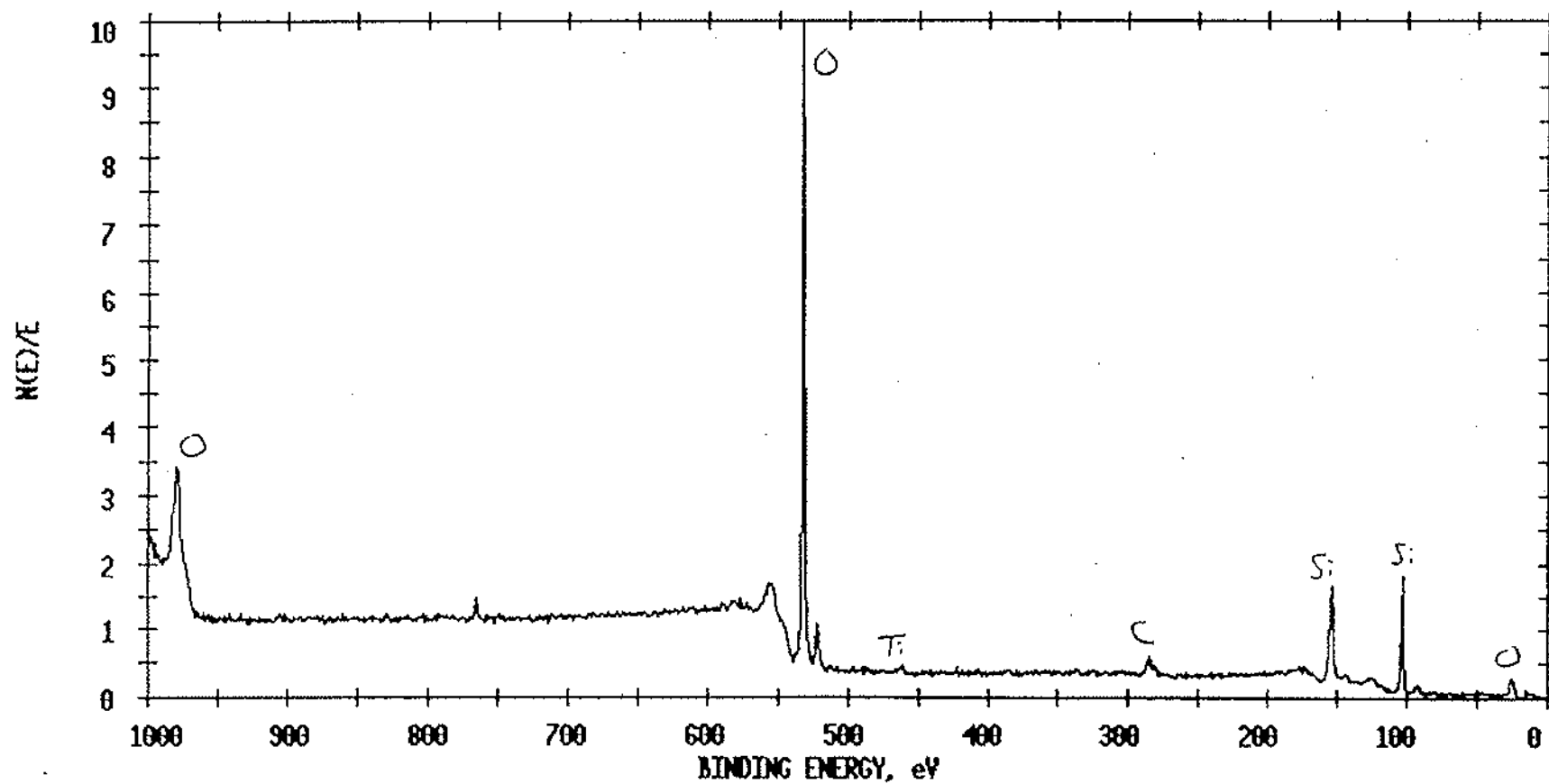


7

ESCA SURVEY 11/8/87 ANGLE= 45 deg ACQ TIME=3.75 min

FILE: mfh1 IOTA standard quartz

SCALE FACTOR= 5.291 k c/s, OFFSET= 0.320 k c/s PASS ENERGY= 44.750 eV Al 300 W

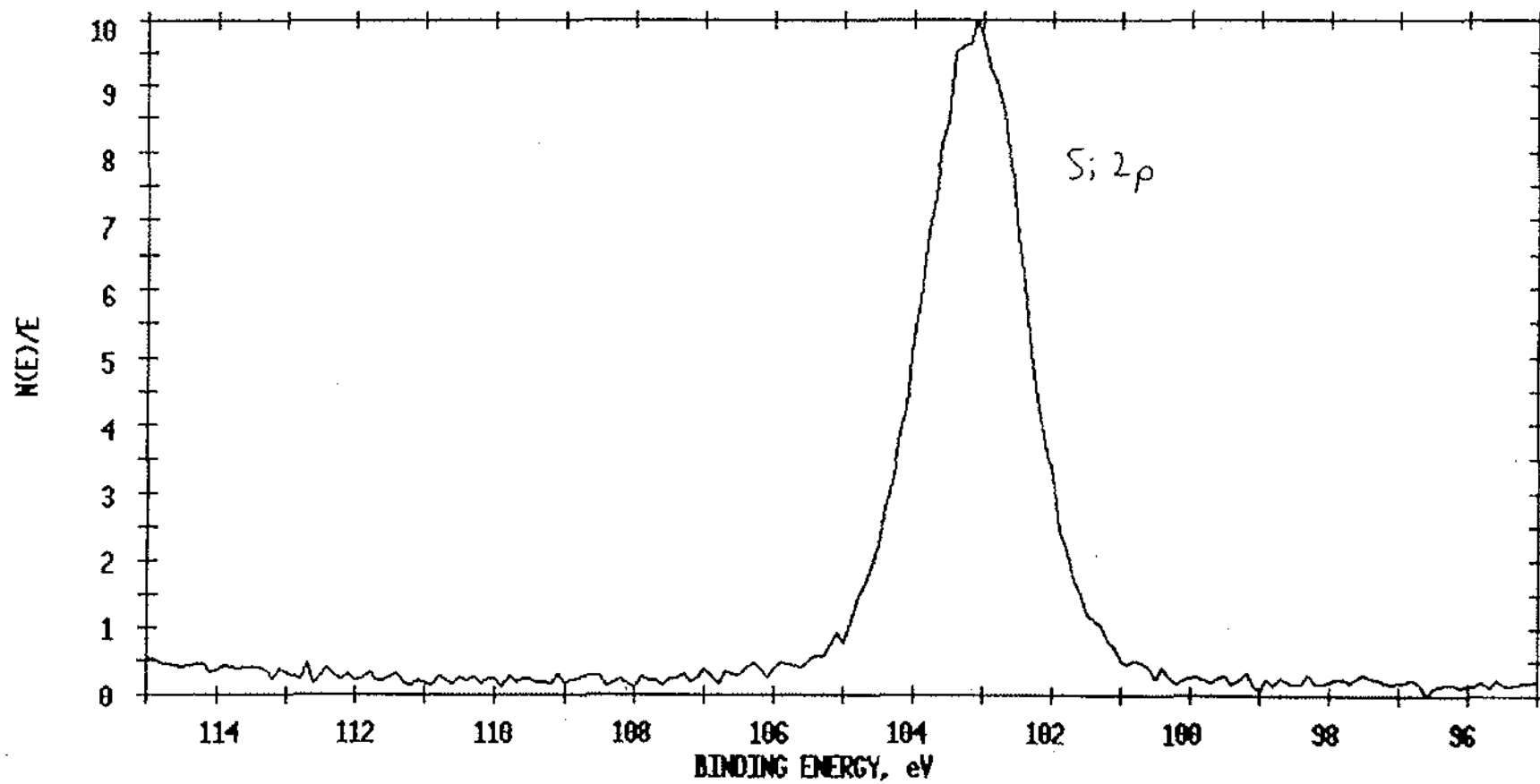


8

ESCA MULTIPLEX 11/8/87 EL=Si1 REG 1 ANGLE= 45 deg ACQ TIME=2.68 min

FILE: mfh3 IOTA standard quartz

SCALE FACTOR= 0.355 k c/s, OFFSET= 0.195 k c/s PASS ENERGY= 17.900 eV Al 300 W



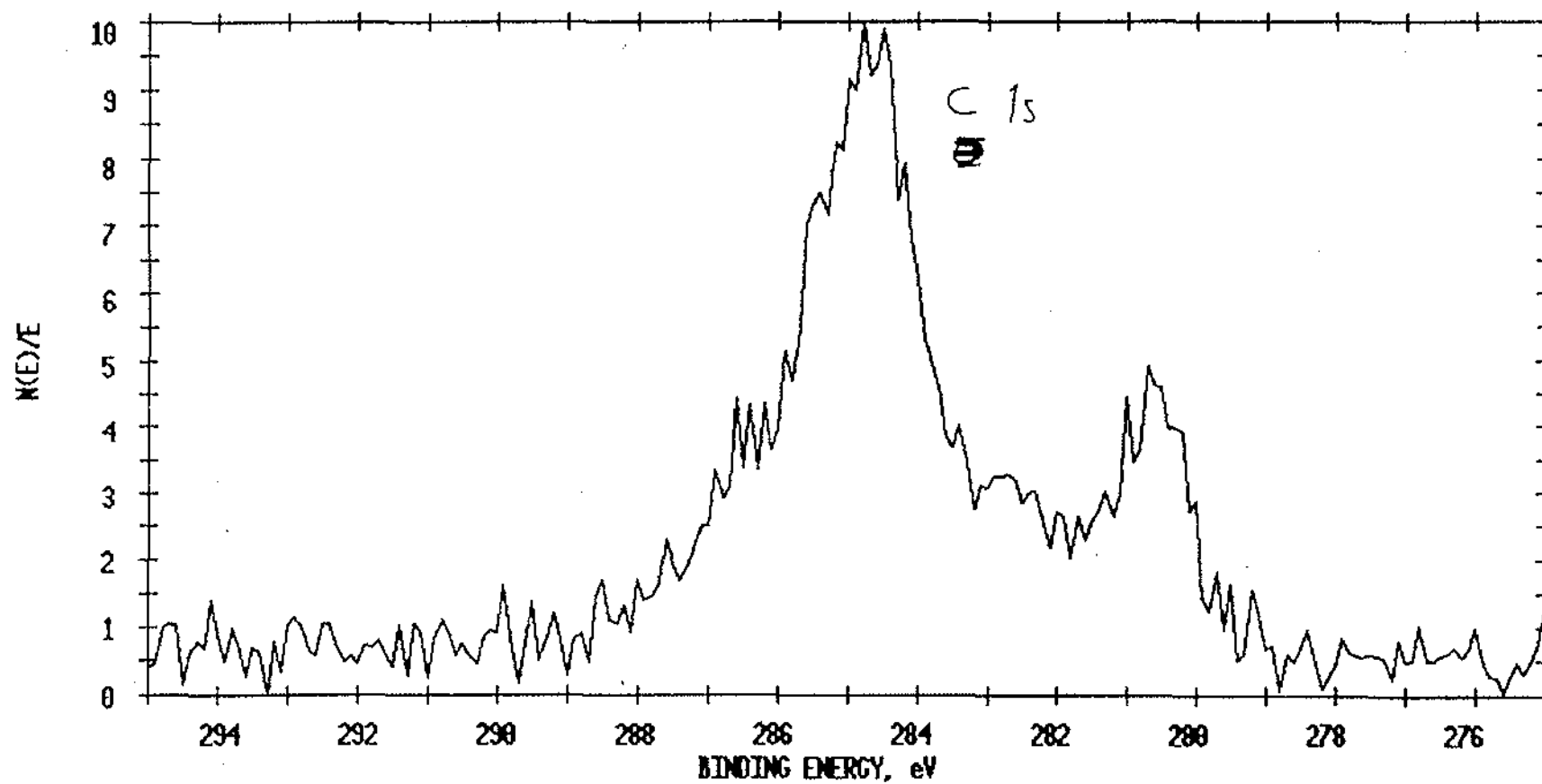
3

END

ESCA MULTIPLEX 11/8/87 EL=C1 REG 2 ANGLE= 45 deg ACQ TIME=2.68 min

FILE: mfh3 IOTA standard quartz

SCALE FACTOR= 0.004 k c/s. OFFSET= 0.647 k c/s PASS ENERGY= 17.900 eV Al 300 W



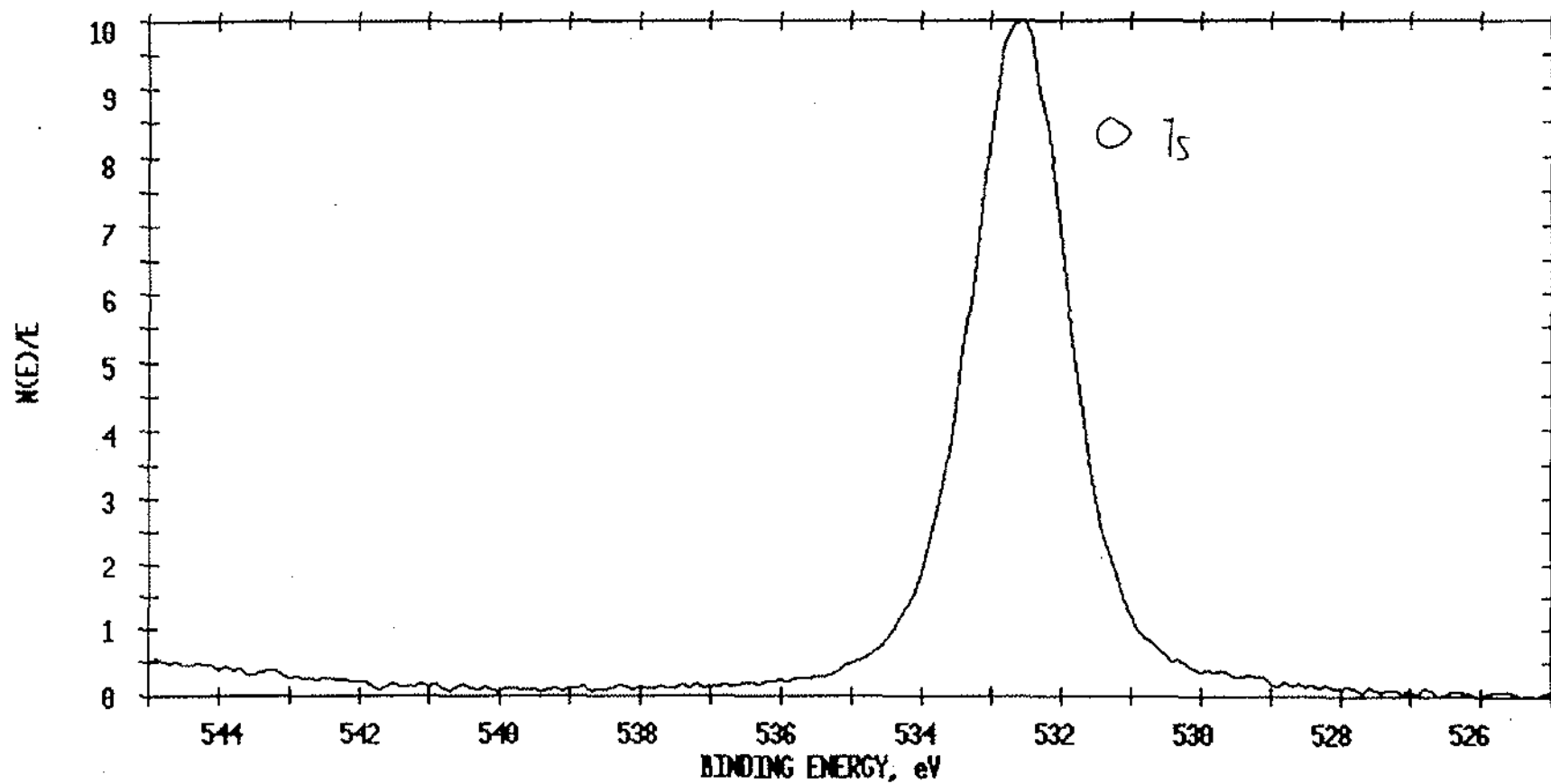
(C)

counts/eV/sec

ESCA MULTIPLEX 11/8/87 EL=01 REG 3 ANGLE= 45 deg ACQ TIME=1.34 min

FILE: mfh3 IOTA standard quartz

SCALE FACTOR= 1.862 k c/s, OFFSET= 0.908 k c/s PASS ENERGY= 17.900 eV Al 300 W

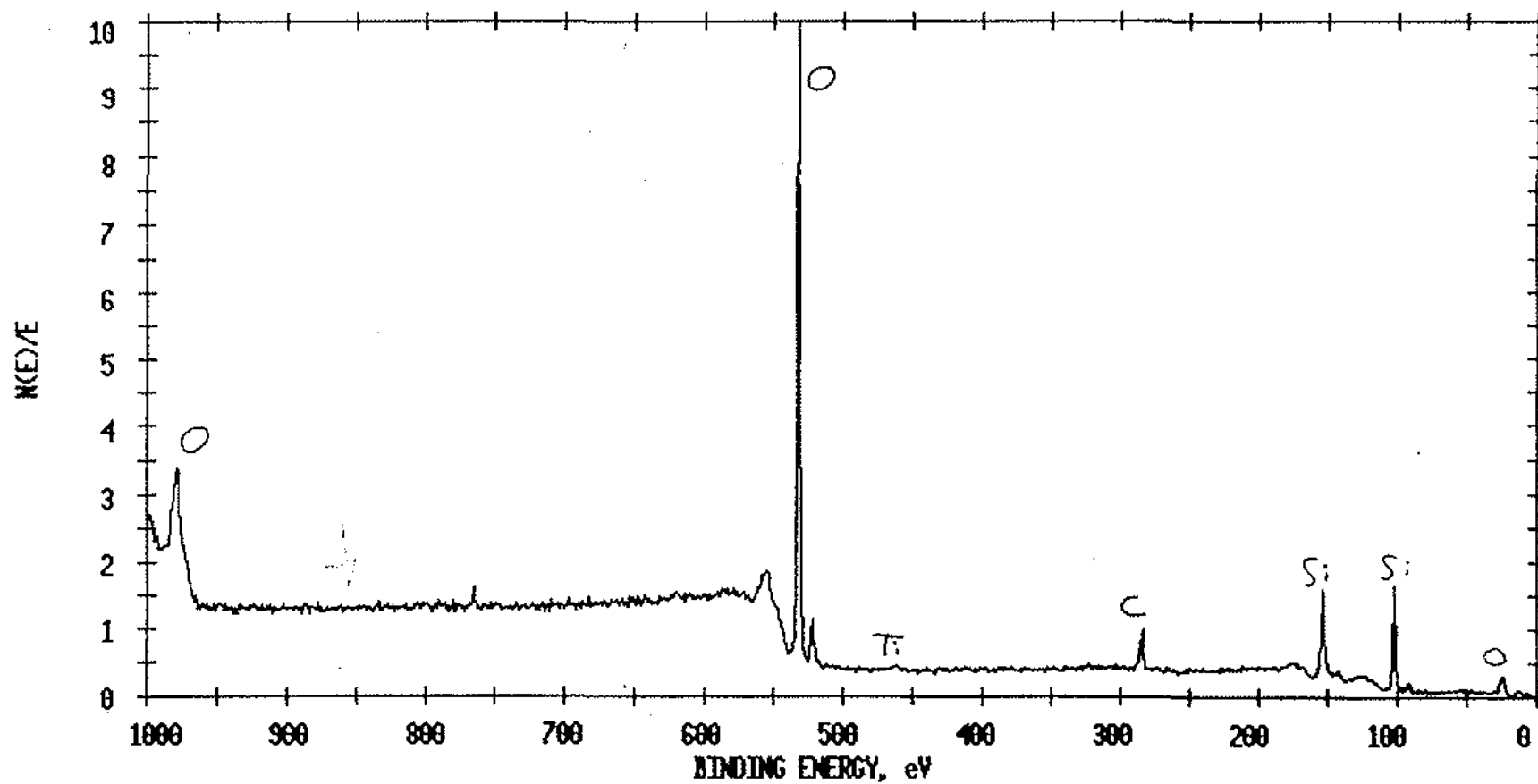


©

ESCA SURVEY 11/8/87 ANGLE= 45 deg ACQ TIME=3.75 min

FILE: nfh1 Milled IOTA quartz

SCALE FACTOR= 5.882 k c/s, OFFSET= 0.329 k c/s PASS ENERGY= 44.750 eV Al 300 W

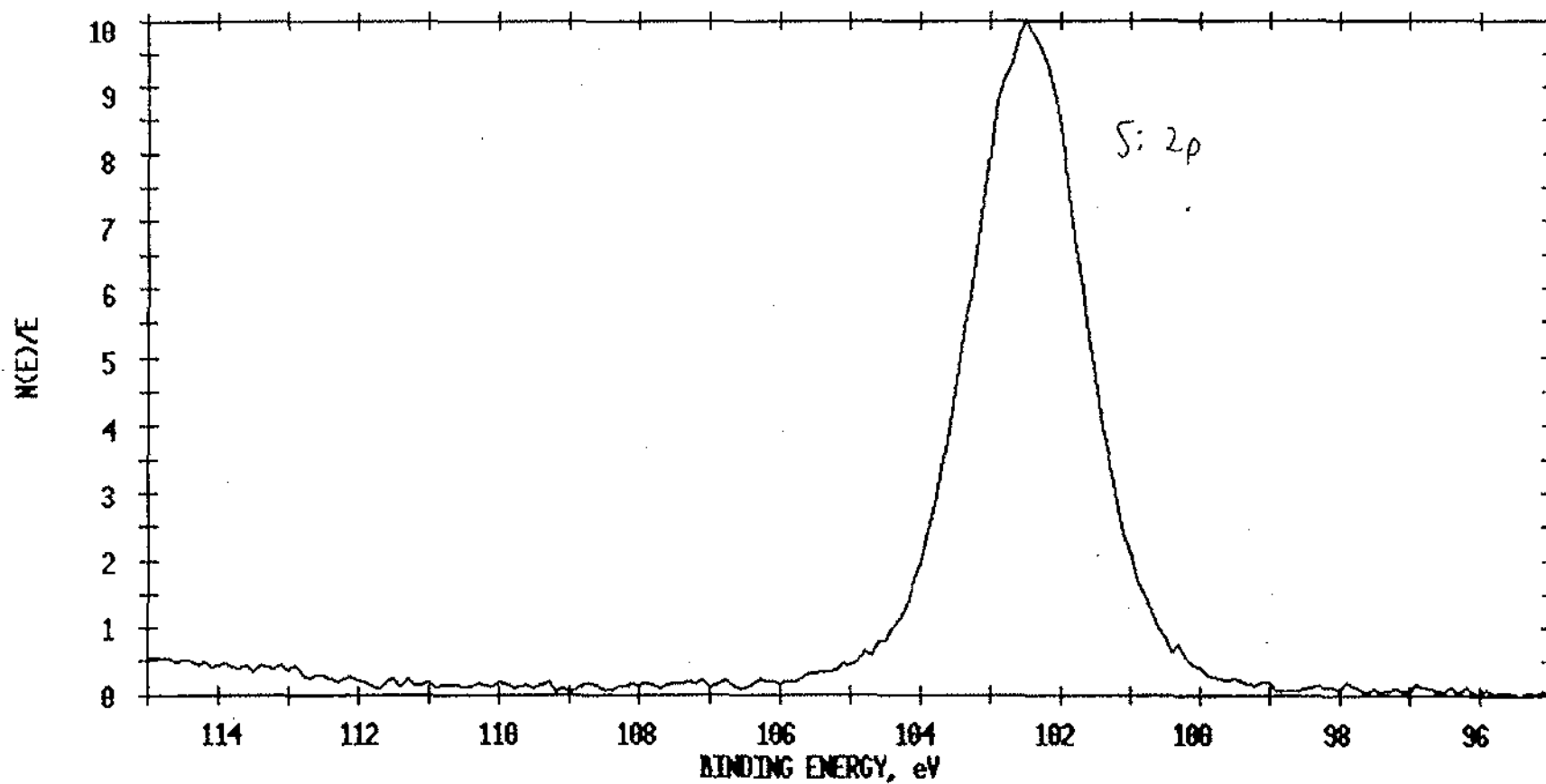


(5)

ESCA MULTIPLEX 11/8/87 EL=Si1 REG 1 ANGLE= 45 deg ACO TIME=6.53 min

FILE: afh5 Milled IOTA quartz

SCALE FACTOR= 0.331 k c/s, OFFSET= 0.278 k c/s PASS ENERGY= 17.900 eV Al 300 W

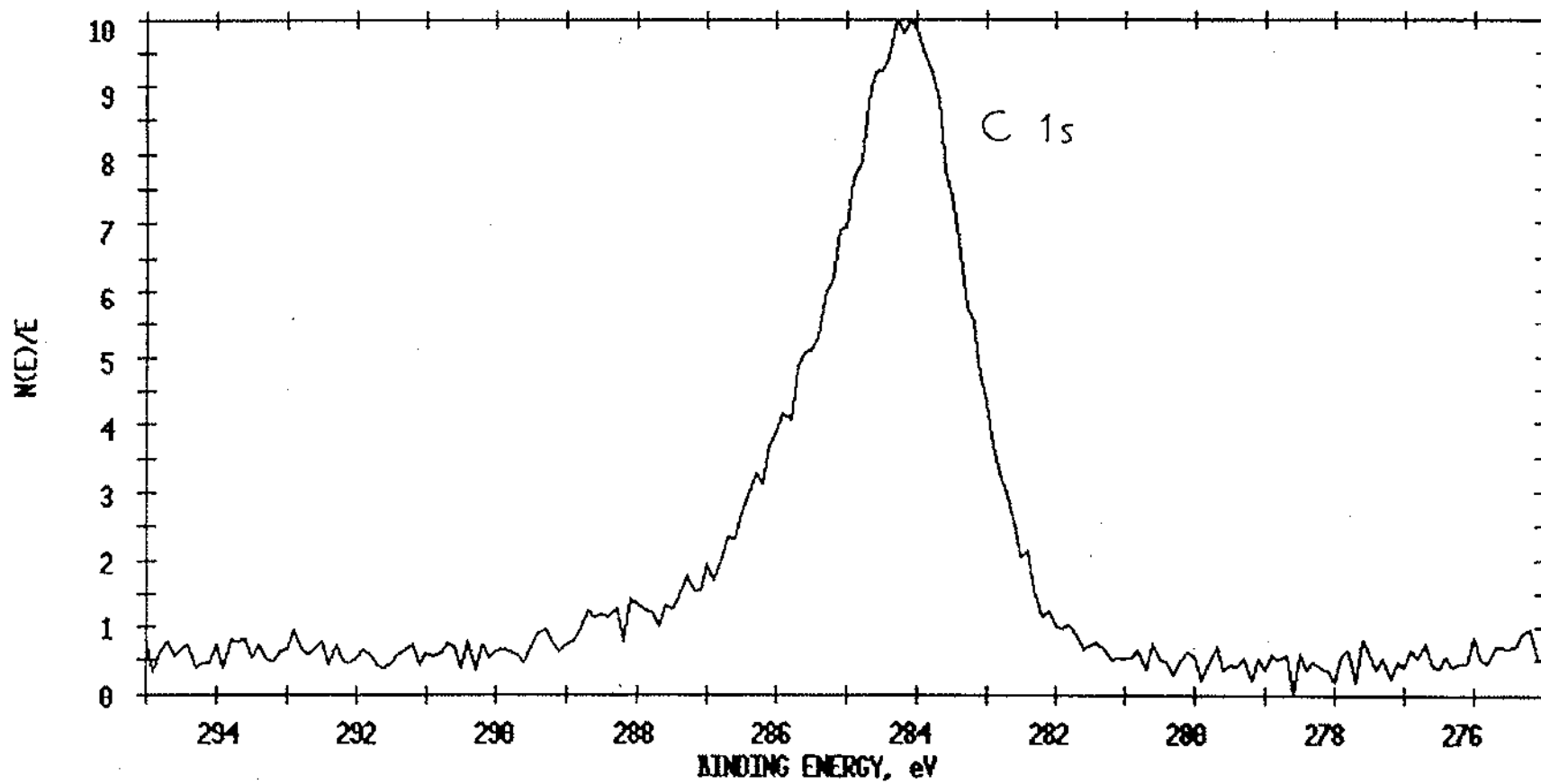


(4)

ESCA MULTIPLEX 11/8/87 EL=C1 REG 2 ANGLE= 45 deg ACO TIME=6.37 min

FILE: afh5 Milled IOTA quartz

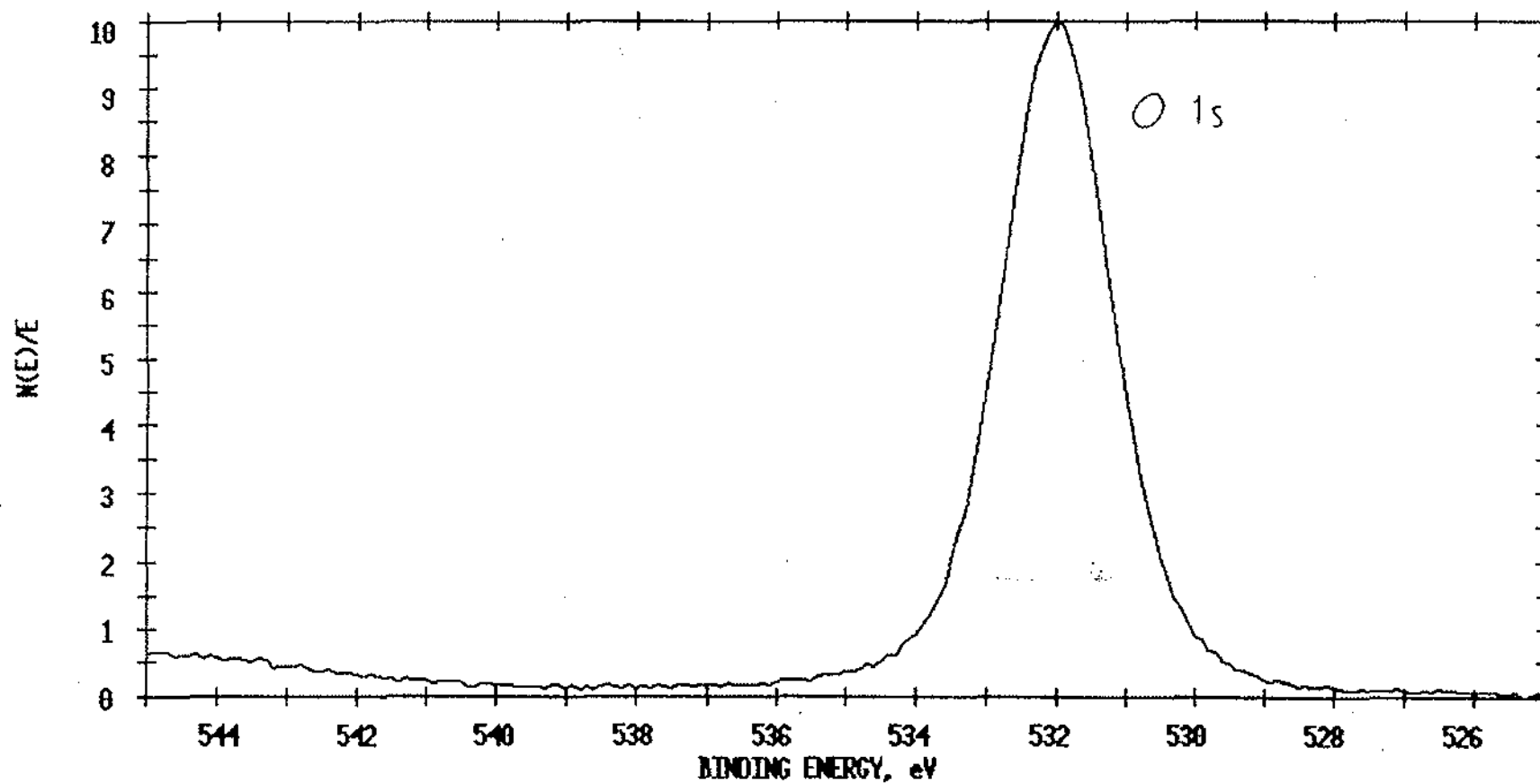
SCALE FACTOR= 0.132 k c/s, OFFSET= 0.739 k c/s PASS ENERGY= 17.900 eV Al 300 W



ESCA MULTIPLEX 11/8/87 EL=01 REG 3 ANGLE= 45 deg ACQ TIME=3.18 min

FILE: wfh5 Milled IOTA quartz

SCALE FACTOR= 1.735 k c/s, OFFSET= 0.960 k c/s PASS ENERGY= 17.900 eV Al 300 W



(9)

The Pathogenic Properties of the Disturbed Surface on Quartz

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Draft of Final Report

to the

Sorptive Minerals Institute

Abstract—Quartz dissolution studies in incubated batch reactors using several natural sand samples and one crushed quartz sample showed an initial fast silica dissolution rate that closely matches that of amorphous silica. This phenomenon is generally explained to be the result of a disturbed surface. The disturbed surface on the sand grains consists of only a small amount of silica and might be explained by reactive siloxane bridge sites and/or amorphous silica produced by abrasion of the dry grains. The disturbed surface on the crushed powder contains much more silica and may be the result of local melting caused by particle impacts during crushing.

This study found no evidence that the disturbed surfaces on any of these grains has any relationship to the pathogenic properties of quartz. This lends additional credence to the idea that the pathogenic features are free radicals and/or sharp edges that are present on freshly broken grains. Because these features are rather labile they would be rapidly destroyed on quartz grains in the natural environment making this quartz less likely to cause disease. This is consistent with the rarity of quartz related diseases in the general

population even though exposure to natural quartz dust is common and unavoidable, especially in arid regions.

Notation

A	surface area (m^2)
A_{as}	surface area of amorphous silica exposed to solution
A_{as}^0	initial surface area of amorphous silica exposed to solution
A_{as}	molar area of amorphous silica ($= 7.56 \times 10^4 \text{ m}^2$)
a_i	activity of species i
A_{qz}	surface area of quartz exposed to solution
A_{sp}	specific surface area (m^2/g)
b	geometric constant ($= 4.836$ for spheres)
B	geometric constant ($= 16.8$ for spheres)
c_i	concentration (ppm)
ΔG	free energy (kJ/mol)
K	equilibrium constant
$\hat{K}$	equilibrium constant for small particles
$k(i)_+$	dissolution rate constant for mineral i ($\text{mol}/\text{m}^2 \text{ sec}$)
M	mass of water (kg)
m_i	concentration (mol/kg)
n	number of moles (mol)
r	radius (m)
R	gas constant ($8.314 \text{ J}/\text{mol K}$)
$r(i)_+$	rate of dissolution of i (mol/sec)
σ	surface free energy ($120 \times 10^{-7} \text{ J}/\text{m}^2$ for quartz)
t	time (sec)
T	temperature (K)
t_l	lifetime of a particle or sharp edge (sec)
V	volume (cm^3)
$V_m(i)$	molar volume of species i (cm^3/mol)
W_m	molecular weight (g/mol)
x	thickness of layer (m)

Introduction

It has long been known that exposure to mineral dusts produced by mining operations can lead to disease. Georgius Agricola pointed out in his 1556 treatise *De Re Metallica* (Agricola, 1556) that "...some mines are so dry that they are entirely devoid of water, and this dryness causes the workmen even greater harm, for the dust which is stirred and beaten up by digging penetrates into the windpipe and lungs, and produces difficulty in breathing and the disease which the Greeks call $\alpha\sigma\theta\mu\alpha$ ". Of course, today we recognize that several different diseases are caused by several different kinds of mineral dusts (see, for example, Guthrie Jr. and Mossman, 1993). Of all these minerals that are known to cause lung diseases quartz is perhaps the most important. Quartz is known to cause several different diseases. Acute silicosis is caused by exposure to extremely high levels of quartz dust and is characterized by the filling of the air sacs in the lung with lipid-rich protein released by the damaged tissues. This is a highly fatal disease with death occurring within a few months after the exposure event. Accelerated silicosis is caused by exposure to high levels of quartz dust over a period of 5 to 10 years. It is characterized by irregular fibrosis in the upper chest with numerous nodules visible on X-rays. This is a progressive disease with continuing decrease in lung function even if there is no further exposure to quartz dust. Death, from cardiopulmonary failure, usually occurs within 10 years of the onset of symptoms. Chronic silicosis is caused by 10 or more years of exposure to lower concentrations of quartz dust. It is characterized by patches of fibrotic scarring in the lung tissue that show up as nodules on chest X-rays. This scarring eventually leads to heart or respiratory failure. Silicotuberculosis is associated with all kinds of silicosis. It is thought that the damage to the lung tissue caused by the quartz reduces the effectiveness of the immune system in dealing with the *M. tuberculosis* pathogen. Antibiotics have greatly reduced this problem in developed countries but in the past this was a major cause of death among those afflicted with silicosis. Finally, the International Agency for Research on Cancer has classified crystalline silica (quartz and cristobalite) as probable human carcinogens so that there is now widespread concern that exposure to quartz dust can cause lung cancer. See Goldsmith (1994), Ross et al. (1993), and Saffiotti et al. (1993) for extensive descriptions of these diseases. Thus, there is widespread evidence that inhalation of quartz dust can be quite dangerous.

Given the great abundance of quartz in the natural environment, it is surprising that these diseases seem to be limited to victims exposed to quartz dusts in the workplace. Oxygen and silicon are the two most abundant elements in the earth's crust so it is not surprising that silica minerals are quite common. Figure 1 shows that, after the feldspars, quartz is the next most abundant mineral in the crust and although the other silica minerals do not have such a great abundance they dominate certain geochemical facies. For example, essentially all of the silica that precipitates in shallow hydrothermal systems is amorphous silica (Rimstidt and Cole, 1983). Over time, this amorphous silica dissolves and reprecipitates as quartz because quartz is the silica phase with the lowest solubility (Figure 2). This dissolution/reprecipitation process is responsible for the eventual transformation of all other silica phases to quartz in the geologic environment. Furthermore, many of the other silicate minerals are chemically unstable under earth-

surface conditions so they are destroyed or converted to clay minerals by chemical weathering. This causes a relative increase in the abundance of quartz in surficial deposits. Much of this quartz is sand-sized and thus is too large to be respired but a significant amount is small enough to become airborne dust. A summary of 22 studies of the mineral composition of airborne dusts (Pye, 1987) shows that in every case quartz was a major component. About 500 million metric tons of aeolian dust is transported through the atmosphere annually, mostly derived from arid regions. This suggests that there is a significant amount of human exposure to quartz dust outside of the workplace, especially in arid areas.

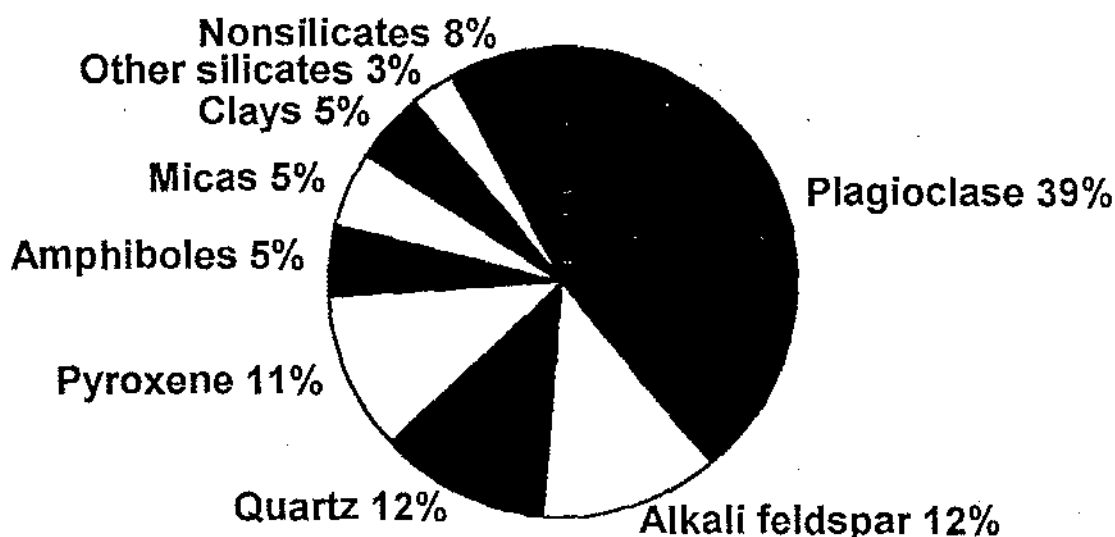


Figure 1. Relative abundances of minerals in the earth's crust. Note that quartz makes up 12% of the rocks in the crust so human exposure to quartz dust is unavoidable.

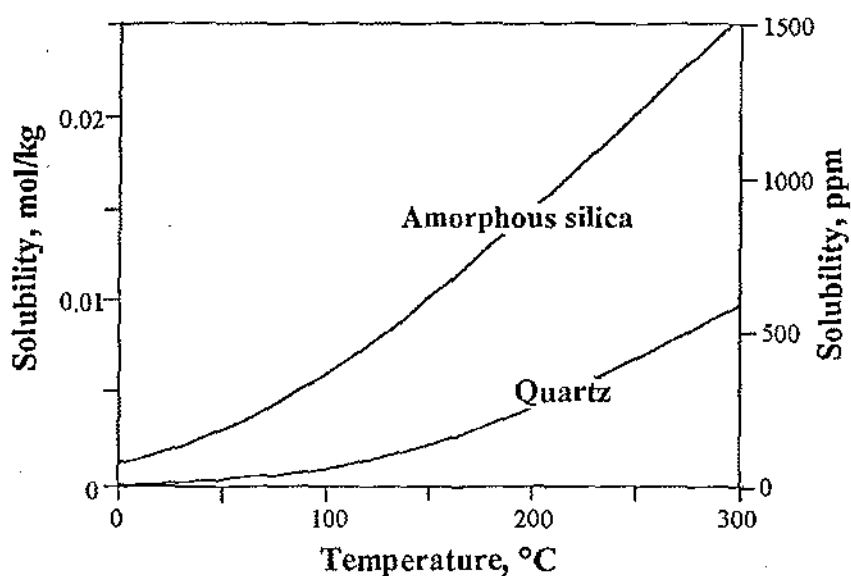


Figure 2. Solubility of amorphous silica and quartz as a function of temperature. When amorphous silica is placed in contact with aqueous solutions it dissolves and the aqueous silica reprecipitates as less-soluble quartz.

There is a longstanding literature that suggests that the chemical reactivity (i.e. rate of silica release) of mineral dusts is somehow related to their pathogenic effects (e.g. Briscoe et al., 1937). Much of this literature probably arose from early studies of the disturbed surface on quartz. This disturbed surface was first identified in quartz dissolution experiments that were carried out more than 50 years ago. There is no doubt that most quartz grains show an anomalously high dissolution rate at the beginning of dissolution experiments (e.g. Clelland et al., 1952; Dempster and Ritchie, 1952; Henderson et al., 1970; Holt and King, 1955; Liberti and Devitofrancesco, 1963; van Lier et al., 1960; Rimstidt and Barnes, 1980) but it is not yet clear just what causes this phenomenon or whether it has any relationship to the pathogenic properties of quartz. Several authors have suggested that quartz surfaces are coated with a layer of amorphous silica which they call the disturbed layer. Because, as this report will show, there is little evidence that this rapid dissolution phenomenon is related to a continuous layer, I believe the term disturbed surface is more appropriate. It is important to realize that at this time the disturbed surface on quartz is an operationally defined feature and that attempts to characterize this highly soluble surface feature by x-ray and electron diffraction produced conflicting and equivocal results (Bergman et al., 1963; Gibb et al., 1953; Talbot et al., 1963; Talbot and Kempis, 1960).

The purpose of this paper is to try to determine the nature of the disturbed surface on quartz, to distinguish between the disturbed surface on freshly broken and aged natural grains, and to evaluate how the disturbed surface on quartz might be related to respiratory diseases.

Experimental methods

Several different commercial sand samples (Table 1) were used in this study. All the samples had been sieved to create a rather narrow range of grain sizes. The Ione and Feldspar Corporation samples contained a visible amount, estimated to be less than 0.1 wt %, of hydrophobic clay material. However, all of the peaks visible on the x-ray diffraction (XRD) patterns (Appendix I) were from quartz. The Iota sample had been treated with hydrochloric and then hydrofluoric acid and those treatments removed all clays, titanium and iron oxides, and almost all zircon. In addition, it appears to have etched the quartz grains along certain crystallographic directions so the grains became oriented when they were mounted for XRD analysis; this caused a reduction in the intensity of some of the peaks. The milled Iota sample had been crushed to produce a powder with a particle size of less than about 1 μm . All of these samples were used without further treatment. The Corning sample is the same material used by Rimstidt and Barnes (1980) to determine the dissolution rates of quartz. The specific surface area of each sample was determined by N_2 BET analysis using a Nova 1000 surface area analyzer.

Table 1. Run materials and run compositions used in the silica dissolution experiments.

Sample	Source	A_{sp} m^2/g	H_2O	SiO_2 g	A/M m^2/kg

			<i>g</i>		
Corning	Dune sand, northern IN	0.092	10.0	10.0	92
Feldspar Corporation	Residue from kaolinite recovery. Location ?	0.156	10.0	10.0	156
Ione	Residue from kaolinite recovery. Ione CA	0.072	10.0	10.0	72
Iota	Chemically treated pegmatite quartz. Spruce Pine NC	0.075	5.0	5.0	75
Milled Iota	Chemically treated and crushed pegmatite quartz. Spruce Pine NC	2.52	20.0	0.20	25.2
Ottawa	Glacial outwash sand. Location ?	0.039	10.0	10.0	39

Dissolution experiments were carried out in a series of incubated batch reactors. For each experiment, a series of identical containers, in this case 30 mL Teflon™ bottles, were filled with identical amounts of solid and solution (see Table 1) and deposited in a constant temperature bath set at 65°C. Each container was weighed before and after incubation to insure that it had not leaked. The containers were mixed by inversion once every 24 hr. After a container had incubated the required amount of time, it was removed and some of the solution was sampled by decanting into a clean 30 mL polyethylene bottle. The solution was separated from the milled Iota powder by filtering through a 0.45 μ m membrane. After all of the samples were collected, the silica concentration in each sample was determined using the molybdate blue colorimetric method (Govett, 1961).

Results

The results of these experiments are tabulated in Appendix II and shown in Figures 3 through 8. All of the experiments that used sand grains show an initial period of rapid increase in silica concentration followed by a period with a slower rate of increase. The dissolution rates of the grains for these two periods were determined from the slopes of the two straight line segments shown on the graphs (fit by linear regression). A dissolution rate constant (Table 2) was calculated for each line segment using the A/M values listed in Table 2. Because these dissolution rate constants closely match the values for amorphous silica and quartz determined by Rimstidt and Barnes (1980), they were labeled as k_{as} and k_{qz} . The dissolution rate of the milled Iota sample remained high over more than 300 hr so no value of k_{qz} was calculated for that sample.

A reasonable interpretation of the dissolution pattern shown by the sand grains is that the disturbed surface consists of a continuous layer of amorphous silica that dissolves away and then the dissolution rate is controlled by the underlying quartz. If this silica were distributed on the grains' surface as a continuous layer, we can estimate of the thickness of

this disturbed layer using the silica concentration where the two line segments intersect (c) on Figures 3, 4, 5, 6, and 8, and the following equation

$$x = \left(\frac{cV_m}{W_m(A/M)} \right) (10^{-9}) \quad (1)$$

The values of x estimated using this equation are listed in Table 2. In addition, this equation was used to estimate that the thickness of the disturbed layer on the Corning sand using the data from experiment 2F in Rimstidt and Barnes (1980) (see Fig. 3 in that paper) was 0.45 Angstroms. This calculation shows that the silica that contributes to the initial rapid rate of dissolution cannot exist as a continuous layer on the surface because its thickness is less than the dimensions of a SiO_2 molecule. Therefore, it must be heterogeneously distributed on the grains' surfaces. The A_{ss} column in Table 2 is the fraction of the quartz surface that would be covered by the disturbed surface if it exists as a monolayer of amorphous silica, 3.56 Å thick, on the surface. Note that the calculated thickness of disturbed surface, using the amount of silica in solution at 300 hr, on the milled Iota grains is equivalent to two monolayers of coverage. This is 2.5 to 10 times more than was found for the sand grains.

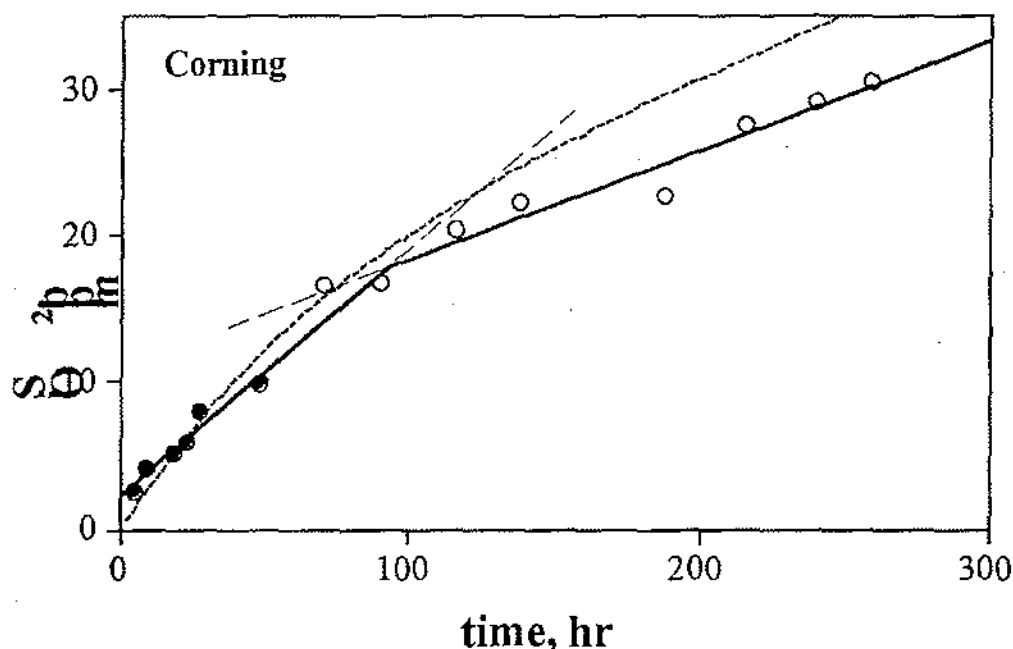


Figure 3. Concentration of dissolved silica versus time data for experiments using Corning sand. These data are tabulated in Appendix II. The two straight lines were fit by linear regression and the dashed line was calculated using the equation derived in Appendix III using the data given in Table 2.

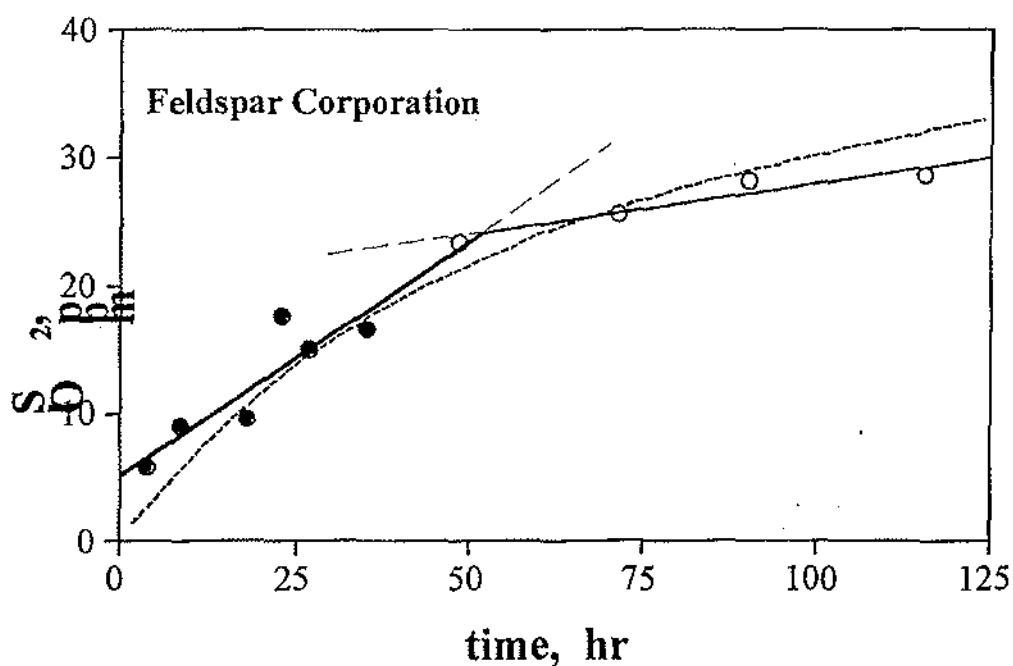


Figure 4. Concentration of dissolved silica versus time data for experiments using Feldspar Corporation sand. These data are tabulated in Appendix II. The two straight lines were fit by linear regression and the dashed line was calculated using the equation derived in Appendix III using the data given in Table 2.

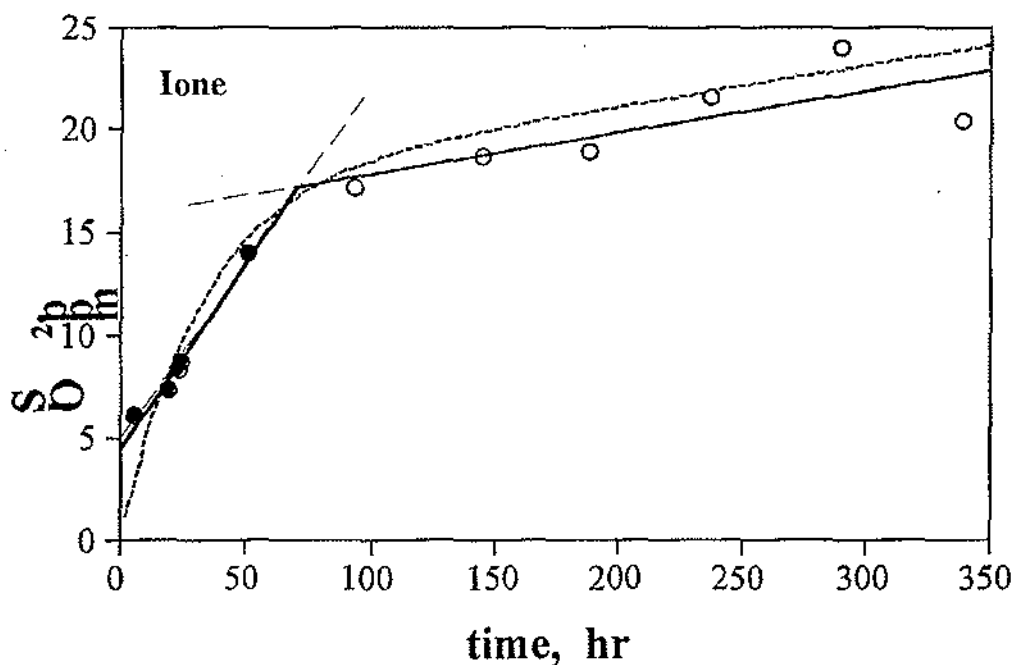


Figure 5. Concentration of dissolved silica versus time data for experiments using Feldspar Corporation sand. These data are tabulated in Appendix II. The two straight lines were fit

by linear regression and the dashed line was calculated using the equation derived in Appendix III using the data given in Table 2.

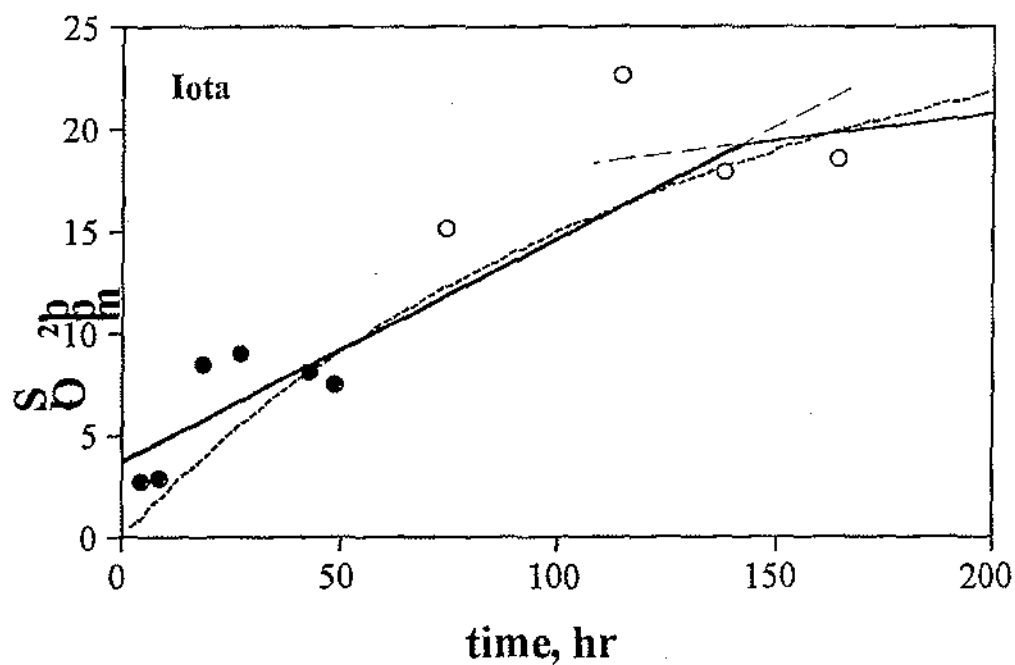


Figure 6. Concentration of dissolved silica versus time data for experiments using Iota sand. These data are tabulated in Appendix II. The two straight lines were fit by linear regression and the dashed line was calculated using the equation derived in Appendix III using the data given in Table 2.

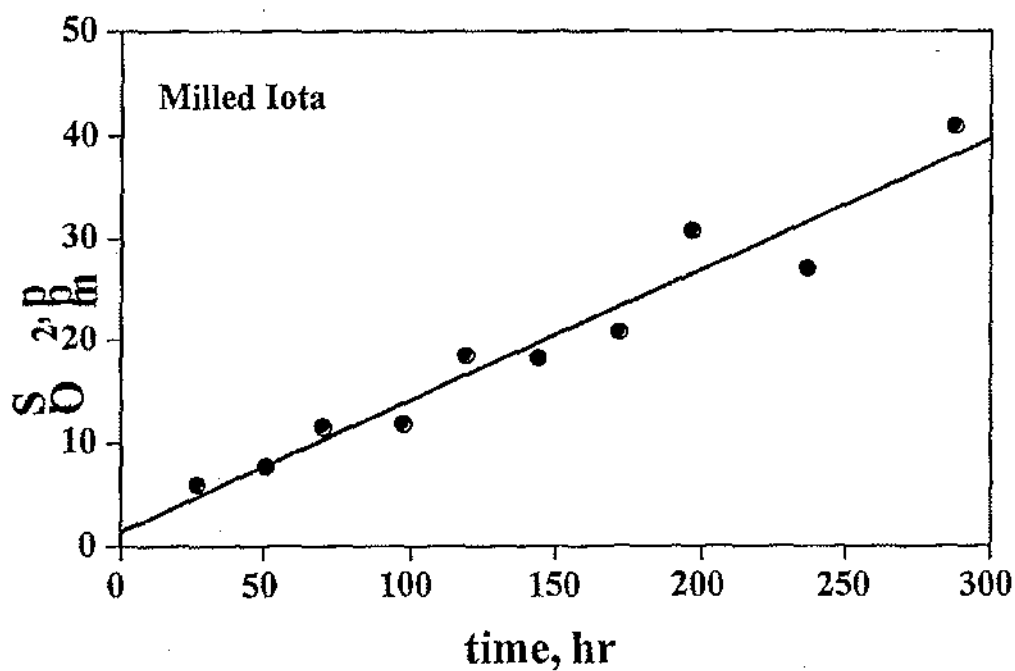


Figure 7. Concentration of dissolved silica versus time data for experiments using milled Iota sand. These data are tabulated in Appendix II. The straight lines was fit by linear regression.

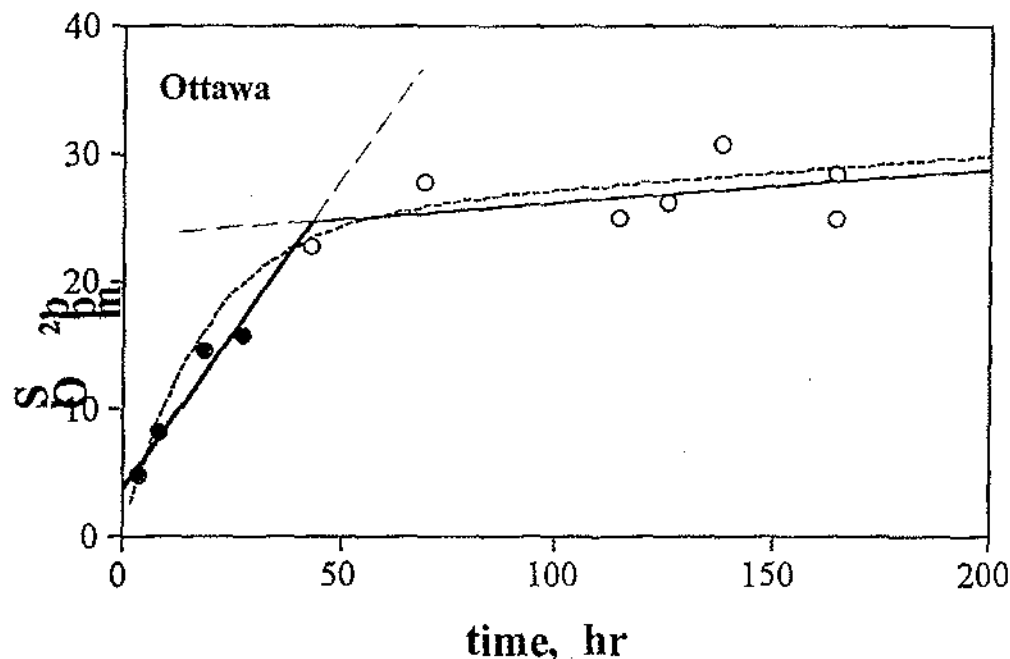


Figure 8. Concentration of dissolved silica versus time data for experiments using Ottawa sand. These data are tabulated in Appendix II. The two straight lines were fit by linear regression and the dashed line was calculated using the equation derived in Appendix III using the data given in Table 2.

Table 2. Results of the silica dissolution experiments.

Sample	k_{as} mol/m ² sec	k_{qz} mol/m ² sec	m_x ppm	x Å	A_{as}	k_{as}^* mol/m ² sec
Corning	8.39×10^{-12}	3.77×10^{-12}	18	0.888	0.249	5.0×10^{-11}
Feld. Corp.	1.08×10^{-11}	2.40×10^{-12}	24	0.698	0.196	1.0×10^{-10}
Ione	1.16×10^{-11}	1.28×10^{-12}	17.1	1.08	0.303	1.2×10^{-10}
Iota	6.78×10^{-12}	1.73×10^{-12}	18.9	1.14	0.321	4.0×10^{-11}
Milled Iota	2.33×10^{-11}	ND	40	7.20	2.02	ND
Ottawa	5.79×10^{-11}	3.08×10^{-12}	24.7	2.87	0.807	2.0×10^{-10}

ND = no data

Another way to analyze these data is based on the model derived in Appendix III. This model is based on the assumption that the surfaces of the quartz grains are partially covered by a monolayer of amorphous silica that rapidly dissolves away and uncovers the

underlying quartz which dissolves more slowly. This model predicts that the concentration of dissolved silica at any time is given by the equation

$$m = \left(\frac{A}{M} \right) \left(\left(\frac{A_{as}^0}{A_{as}} \right) \left(\frac{k_{(as)+} - k_{(qz)+}}{k_{(as)+}} \right) (1 - \exp(-A_{as} k_{(as)+} t)) + k_{(qz)+} t \right) \quad (2)$$

The dashed lines in Figures 3, 4, 5, 6, and 8 were calculated using this equation and the values of (A/M) , A_{as} , and k_{qz} listed in Tables 1 and 2; however, the values of k_{as} were increased by approximately 10 times, to those listed in the k_{as} column of Table 2, in order to make the slope of the initial line segment steep enough to match the experimental results. Attempts to fit the experimental data to this equation using nonlinear regression failed because of the high degree of correlation between the terms of this expression.

These experiments strongly support the results of Rimstidt and Barnes (1980), who found that the initial silica dissolution rate of quartz grains from several different studies closely matched the dissolution rate of amorphous silica. The average of the k_{as} values in Table 2 is 1.98×10^{-11} mol/m² sec which is very close to the value of 1.71×10^{-11} mol/m² sec calculated for 65°C using the temperature function for the amorphous silica dissolution rate constant given in Rimstidt and Barnes (1980). In addition, the average of the values of k_{qz} given in Table 2 is 2.45×10^{-12} mol/m² sec is only slightly larger than the value of 1.54×10^{-12} mol/m² sec for the dissolution rate constant at 65°C from Rimstidt and Barnes (1980).

Discussion

Nature of the disturbed surface

Four reasonable hypotheses have been proposed to explain the disturbed surface on quartz. They are briefly described here in the order presented in Talbot et al. (1963).

1. The *strained lattice/etch pit hypothesis* suggests that the faster dissolution rate is caused by strain energy in the crystal caused by grinding or abrasion and the crystal defects, which harbor this energy, dissolve faster than unstrained quartz. Brantley et al., (1986) and Brantley et al. (1986) have performed a modern quantitative study of this phenomenon. This hypothesis, however, was never a very strong contender to explain the dissolution behavior of disturbed quartz surfaces because in order for the etch pits to produce dissolution rates comparable to that of amorphous silica they would have to completely cover the grain surfaces and the strained area would have to have a free energy of formation comparable to amorphous silica. This is the same as saying that the surface of the grains are coated with amorphous silica (hypothesis number 4). Blum et al. (1990) showed that, in fact, the dissolution rate of quartz is not significantly affected by dissolution at defect sites that leads to the formation of etch pits. Thus, this hypothesis is not viable.

2. The *siloxane/silanol surface hypothesis* is based on the fact that the surfaces of freshly broken quartz are hydrophobic because they consist of mostly siloxane bridges (Figure 9). Over time, these bridging oxygen atoms react with moisture from the air or with liquid water to form silanol groups and this causes the surface to become more hydrophilic. This conversion from hydrophobic to hydrophilic surface is illustrated in the

study of Janczuk et al. (1986), which shows how the contact angle of water droplets on quartz surfaces changes over time. Many details of this reaction are discussed in Iler (1979). The original hypothesis suggested that the silanol covered surface of the grains dissolved faster than the siloxane surface but perhaps the reverse is true.

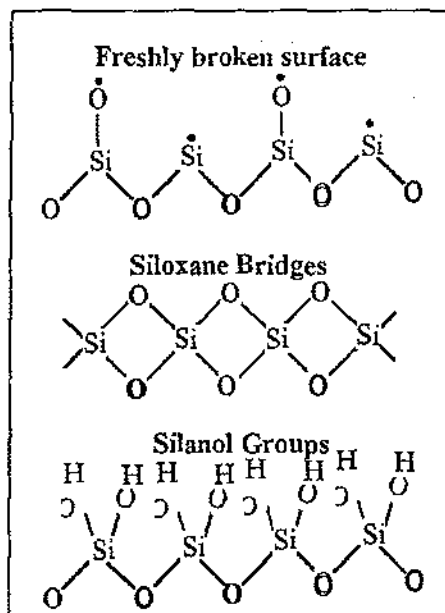
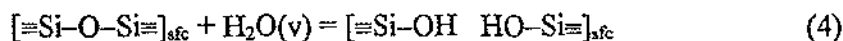


Figure 9. Schematic diagram showing unsatisfied bonds on freshly broken quartz (top). These unsatisfied bonds are Si• and O• free radicals that rapidly react with each other to form siloxane bridges, Si-O-Si (center). The siloxane bridges are highly strained and they eventually react with water, either from humidity in the air or from solution, to form silanol groups (bottom).

This hypothesis might explain the observed fast dissolution behavior of these quartz grains. The predominance of silanol groups on the surface of quartz in contact with normal air is well established (Iler, 1979) but this does not rule out the possibility that some of the surface might be covered by siloxane bridges that are thermodynamically more unstable. I assume that these sites would dissolve at a faster rate than the silanol sites; however, there is no proof of this. The amount of coverage would likely be determined by an equilibrium reaction



and the equilibrium constant would be defined as

$$K = \frac{a_{[=\text{SiOHHSi}=]}}{a_{[=\text{SiOSi}=]} a_{\text{H}_2\text{O}}} \quad (5)$$

where $a_{\text{H}_2\text{O}}$ would be determined by the relative humidity of water in the air. If the sand samples had been exposed to approximately the same relative humidity ($a_{\text{H}_2\text{O}}$) prior to the dissolution experiments then the surface coverage of more reactive siloxane bridge sites on the surface would be comparable so the initial rate of dissolution would be comparable. However, there is no particular reason why that rate should match the dissolution rate of

amorphous silica so closely. Thus, although this is a possible explanation of the experimental results, it is not the best one.

3. The *surface free energy hypothesis* is based on the fact that when a mineral is broken it takes work (expressed as surface free energy, $\sigma = \Delta G_{sf}/A$) to create the new mineral surface. Thus, the total free energy of formation of a quartz grain consists of the free energy of formation of the bulk crystal plus the surface free energy

$$\Delta G_{total} = \Delta G_f^\circ + A\sigma \quad (1)$$

Rimstidt and Cole (1983) estimated that for quartz, $\sigma \approx 120 \times 10^{-7} \text{ J/cm}^2$. This relationship leads to an equation that gives the solubility of a quartz particle or edge as a function of its equilibrium solubility and its radius of curvature (Dove and Rimstidt, 1994)

$$\log(c) = \log(c_{eq}) + \frac{(2/3)\sigma BV}{rRT} \quad (2)$$

Figure 10 shows that this effect is really not significant until the radius of curvature of the grains is less than about $0.1 \mu\text{m}$ but it becomes very important below this value so when the radius of curvature is less than $0.012 \mu\text{m}$ the solubility of the quartz grain is greater than the solubility of bulk amorphous silica.

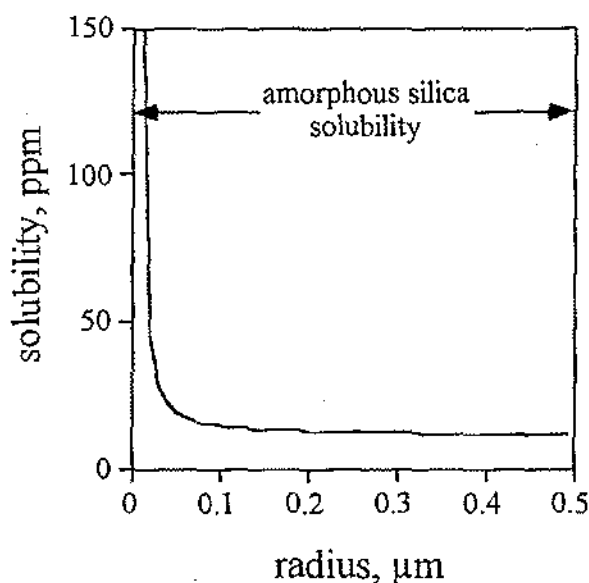


Figure 10. The solubility of quartz at 25°C as a function of the radius of curvature of the grain or edge. Note that quartz solubility exceeds amorphous silica solubility when the radius of curvature is less than $0.012 \mu\text{m}$.

The sand grains used in this experiment are fairly well rounded with no sharp edges. Such features would have been removed in the geologic environment by dissolution and/or abrasion. Figure 11 based on the equation derived in Appendix IV shows that sharp

edges and very fine grains should dissolve away rather quickly in the aqueous environment. Furthermore, the sand grains used in these experiments are all on the order of 100 to 300 μm in diameter so their radii of curvature are far too large for them to exhibit enhanced solubility from surface free energy effects. More importantly, Figure 10 shows that there is only one radius of curvature that produces a solubility that exactly matches that of amorphous silica. All others have either higher or lower solubility. If surface free energy effects were the cause of the disturbed surface phenomenon, the edges with the smallest radii of curvature should dissolve away first at a rate that is much greater than that of amorphous silica and then the rate should slow in a monotonic fashion as the edges with larger radii of curvature dissolve away until all the edges have dissolved and the dissolution rate matches bulk quartz. This is in direct contrast to the two distinct periods of dissolution behavior shown by the sand grains.

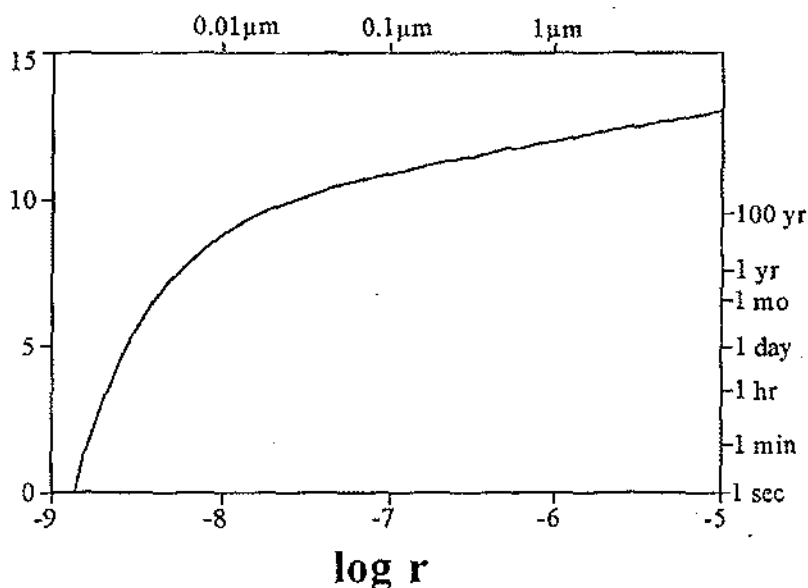


Figure 11. Estimated lifetime of sharp edges and/or small particles of quartz with radii of curvature of r based on the equation derived in Appendix IV.

4. The *amorphous surface hypothesis* postulates that abrasion and/or surface deformation related to wetting and drying, which causes the siloxane/silanol transformation, produces a layer of amorphous silica on the grain surface that is similar to the Beilbe layer on polished metals. This has been the most persistent explanation for the disturbed layer on quartz but it has also been widely criticized; see for example, Talbot and Kempis (1960).

Based on the results presented here this is still the most plausible explanation of the observed dissolution behavior, but it too has problems. The close match between the dissolution rate of the disturbed layer on the sand grains and the dissolution rate of amorphous silica is difficult to explain without postulating the existence of amorphous silica on the sand surfaces. However, if amorphous silica does exist on these surfaces, very little is present and it cannot exist as a continuous coating. Furthermore, the model developed in Appendix III to account for the changing coverage of the surface by

amorphous silica suggests that the material in the disturbed layer may have a solubility that is as much as 10 times higher than amorphous silica. How could such highly reactive material form?

It seems clear that this disturbed surface is a characteristic only of dry quartz grains. Because amorphous silica is about ten times more soluble than quartz (Figure 2), any amorphous silica associated with quartz in the geologic environment would dissolve and the silica would reprecipitate as quartz. The results presented here show that for the sands studied in these experiments the disturbed surface would dissolve away in only a few days. This is consistent with the observation that groundwaters in sandstone aquifers contain dissolved silica concentrations in equilibrium with quartz, not amorphous silica (Rimstidt, 1997). This means that the disturbed surface on sand grains likely formed after these materials were mined. They either could have been produced by abrasion of the dry surface during handling and transport or by the drying process itself.

Finally, there is an important difference between the dissolution behavior of the sand grains and the milled Iota quartz. The milled Iota quartz clearly has much more amorphous silica associated with it. This amorphous silica probably has a different origin than that on the sand grains. A likely explanation is that it was produced by the grinding process. Figure 12, based data taken from Figure 1 of Lin and Somasundaran (1972), shows that when quartz is ground in air the resulting powder has a lower density than quartz. This amorphous silica may have been produced melting of small parts of the quartz surface caused local heating from violent impacts during grinding. The quartz ground under water shows only a small decrease in density because the liquid water cools the grains. Any amorphous silica produced by grinding would not dissolve away during grinding in water as suggested by Lin and Somasundaran (1972) because the dissolution rate is too low; Figure 7 shows that the amorphous silica on the milled Iota sample has not dissolved away after 300 hr in a 65°C solution. This diagram suggests that quartz powders produced by dry grinding should have surface properties that are quite different from those produced by wet grinding.

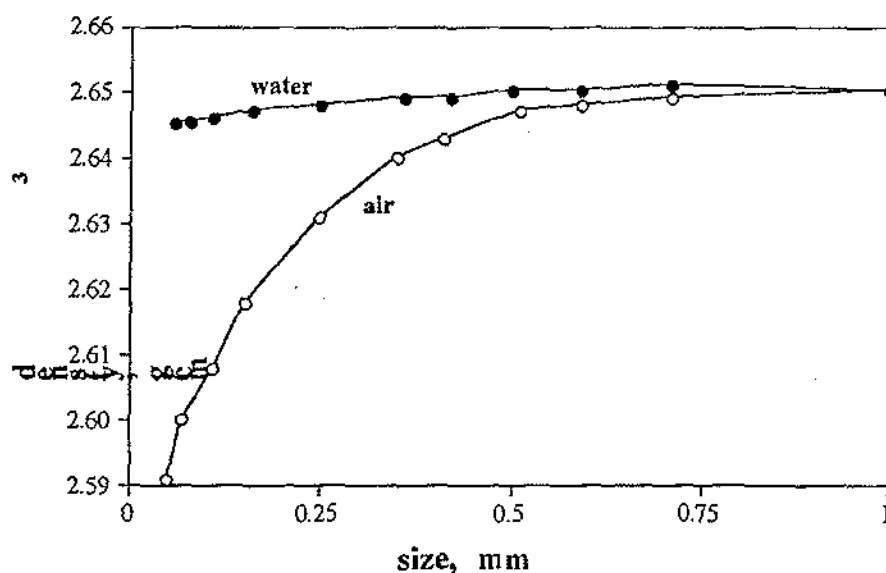


Figure 12. Density of quartz particles ground in air and in water. The density of amorphous silica is 2.20 g/cm^3 . This diagram is based data taken from Figure 1 of Lin and Somasundaran (1972); the authors of this paper explain the lower density of the smaller particles produced by grinding in air as the result of amorphous silica produced by the grinding process.

Pathogenic effects

Many of the early studies of the disturbed surface on quartz surfaces were carried out to identify a link between the disturbed surface and pathogenic effects of respired quartz dust. This results presented in this paper offer very little support for such a link. There are at least three credible explanation of how respired quartz causes lung diseases.

1. One of the early ideas was that cells near quartz grains were damaged or killed by dissolved silicic acid released from the rapidly dissolving disturbed surface (e.g. Gibb et al., 1953); however, studies of silicate mineral dissolution rates in our laboratory at Virginia Tech show that nonpathogenic minerals like olivine, wollastonite, and talc dissolve and release silicic acid to solution much faster than it is released from the disturbed layer on quartz. Furthermore, the rate of dissolution of amorphous silica at body temperature (37°C) is 8.5 times slower than the rate at the temperature of this experiment (65°C) so at body temperature the disturbed layer would dissolve away in slightly more than 18 days. This rate is not affected by pH, even at the pH 4 conditions found in macrophages (Jaurand et al., 1984), because the rate of quartz (and amorphous silica) dissolution is independent of pH below pH 8, as shown by the study of Knauss and Wolery (1988). However, we do know that the catalytic effect of sodium ion (Dove, 1994) increases silica dissolution rates so the lifetime of the disturbed surface in the lung environment might be only a few days. It is not clear how such an ephemeral feature could produce the pathogenic properties of quartz grains. In addition, if the disturbed surface on quartz is nothing more than amorphous silica then it should have pathogenic properties that are identical to silica glass. There is little evidence for pathogenic effects from amorphous silica. This leads to a fairly clear conclusion that the disturbed surface on quartz is not related to lung diseases.
2. Another explanation for the pathogenic properties of quartz dusts involves physical damage to cells produced by sharp edges and points on the grains. However, such features are unlikely to persist for long on natural quartz grains because of their high solubility (Figure 10) so the lifetime of sharp edges in the water-rich geologic environment would be very short (Figure 11). On the other hand, such features would be common on freshly broken quartz dusts such as those produced in mining and sand blasting operations. This might explain the perceived difference in pathogenic potential between aged, natural quartz and freshly broken quartz dust that is common in workplace exposure.
3. A more recent explanation of the pathogenic properties of quartz dusts relates the diseases to cellular and DNA damage caused by free radicals that are present at freshly broken quartz surfaces (Castranova et al., 1996; Saffiotti et al., 1993)(see Figure 9). There is a growing number of papers that show that quartz surfaces, and especially the

surfaces of freshly broken grains, produce a variety of highly reactive oxygen and peroxide species (e.g., Costa et al., 1991; Giamello et al., 1990) that can produce cell damage and alter DNA. It is worth noting that these reactive species have been found in association with freshly broken quartz surfaces that have a high concentration of siloxane bridging oxygens but not on crystal growth faces that are covered with silanol groups. The concentration of these free radicals decreases with time as shown in Figure 13 so their concentration on natural, unbroken quartz grains is likely to be quite small. It is important to note that both sharp edges and free radicals are not only viable explanations for the pathogenic nature of quartz dusts but they are both features associated with freshly broken quartz surfaces but not with aged, natural grains.

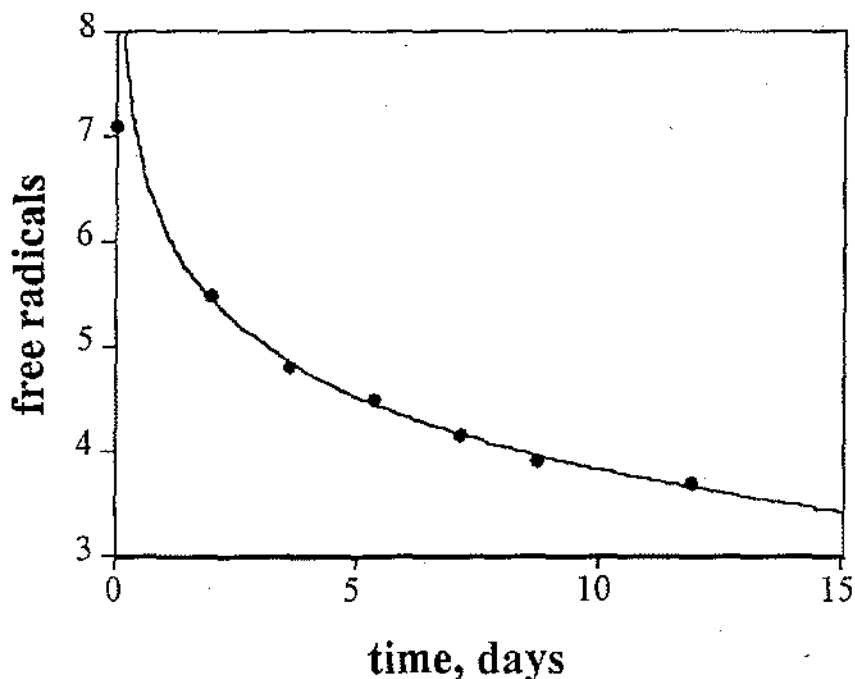


Figure 13. Graph of the relative amount of free radicals on the surface of ground quartz as a function of time of exposure to air. It seems that the amount of free radicals on quartz surfaces decreases as a logarithmic function of time (radicals = $-2.31\log(t) + 6.12$). This figure is based on data from Figure 4 in Castranova et al. (1996).

Conclusions

This study showed that quartz sands from several sources have a disturbed surfaces that dissolve at rates comparable to amorphous silica. This finding is consistent with other studies including the one by Rimstidt and Barnes (1980) that found that the initial rate of dissolution of several quartz samples was the same as that of amorphous silica. However, the disturbed surfaces on these sand grains studied here contained only a very small amount of silica so that if it evenly distributed over the grains' surfaces it would amount to less than a monolayer of SiO_2 and it would dissolve away in lung tissue in only a few days. This disturbed layer might be related to siloxane bridges on the quartz surface

or it might be caused by abrasion of the grain. Furthermore, there is no evidence that this disturbed surface on natural and aged sand grains is related to the pathogenic properties of quartz. On the other hand, the disturbed surface on the milled Iota grains involves much more amorphous silica. This is consistent with a study by Lin and Somasundaran (1972) that showed that grinding quartz in air produces a powder with a density that is lower than that of quartz; this observation is explained by the formation of amorphous silica during grinding, perhaps as a result of localized fusion of the quartz caused by particle impacts. When such powders are immersed in water silica is released at a rate comparable to amorphous silica until most of the amorphous silica has dissolved away. Thus, although the disturbed surface on both natural quartz sand and on crushed quartz grains seems to consist of amorphous silica the amounts and mechanisms of formation of the disturbed surfaces appears to be quite different.

These results strengthen the hypotheses that suggest that sharp edges and/or free radicals on freshly broken quartz surfaces are responsible for its reported pathogenic tendencies. These hypotheses are particularly compelling in light of the lack of quartz-related diseases in the the general population even though they are subject to widespread exposure to natural quartz dust. Figure 10 shows that sharp edges, which have a very small radius of curvature, are very soluble and would quickly dissolve in the natural environment (Figure 11) . Furthermore, Figure 13 shows that the concentration of free radicals on freshly broken quartz also declines rather quickly. Together, these observations imply that natural quartz grains are likely to have few if any of these potentially dangerous features. This draws a sharp distinction between pathogenic, freshly-broken quartz particles, which are produced in mining or sand blasting operations, and more benign, aged quartz particles, which are present in almost all surficial geological materials.

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Appendix I. X-ray diffraction patterns for the samples used in this study.

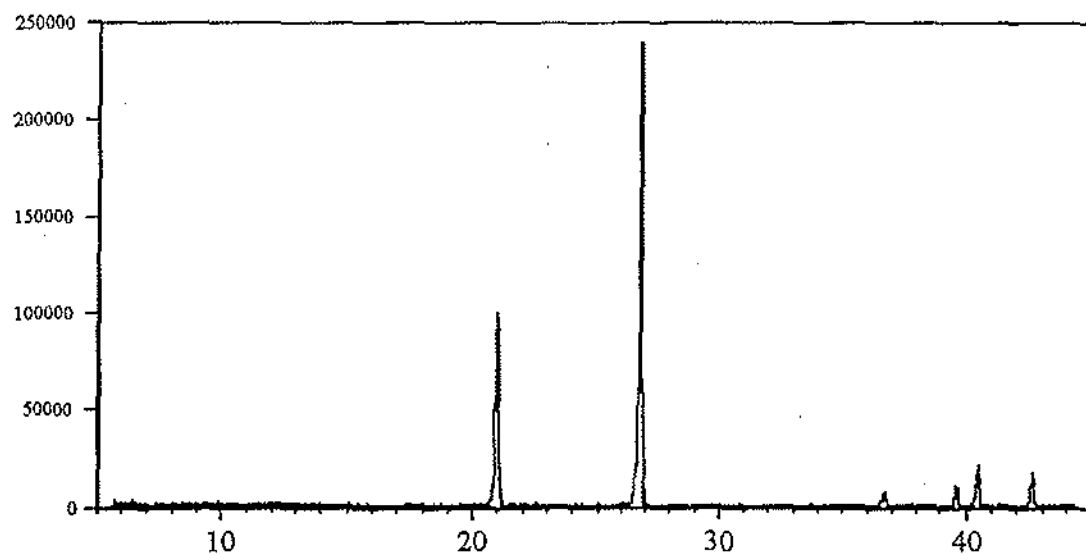


Figure I.1. X-ray diffraction pattern for Corning sand.

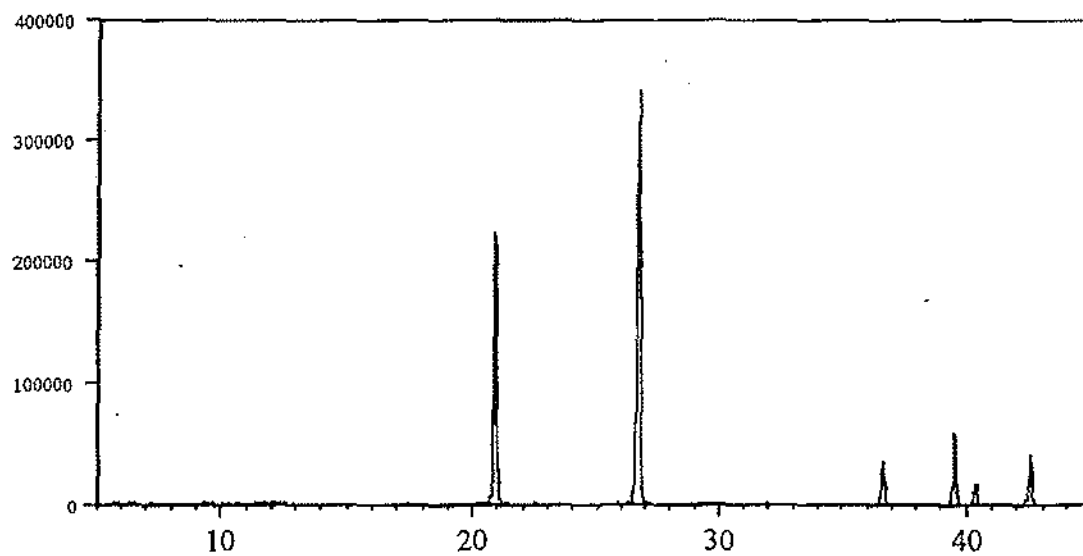


Figure I.2. X-ray diffraction pattern for Feldspar Corporation sand.

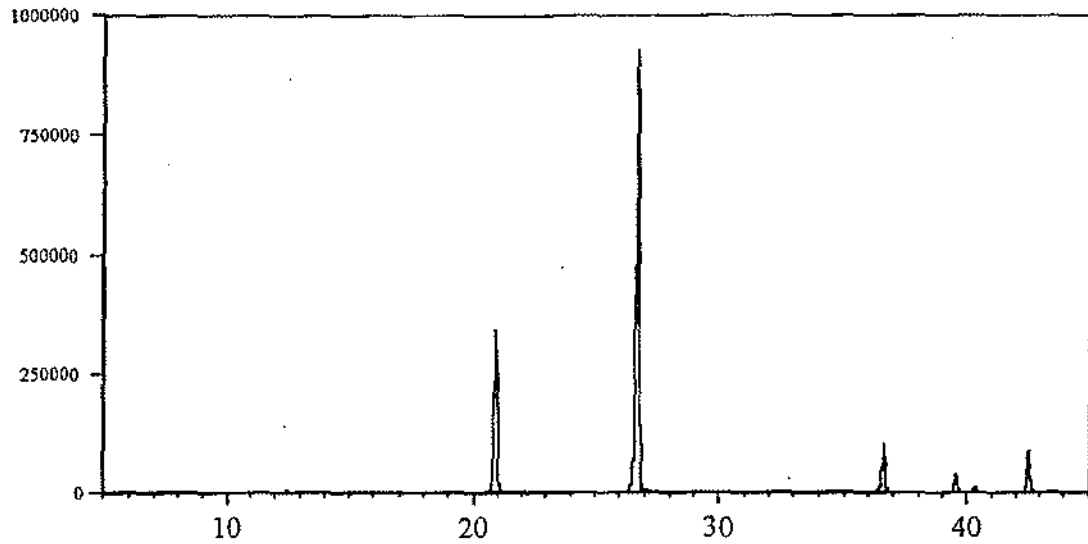


Figure I.3. X-ray diffraction pattern for Ione sand.

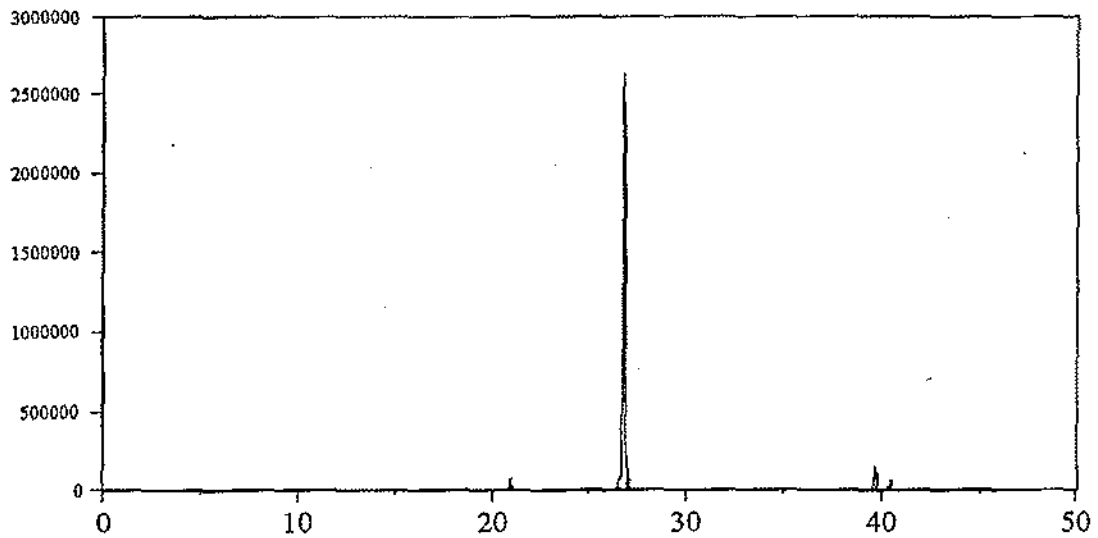


Figure I.4. X-ray diffraction pattern for Iota sand. Note that some peaks are missing suggestion that the sample is oriented. This might be due to crystallographically controlled leaching by HF during production.

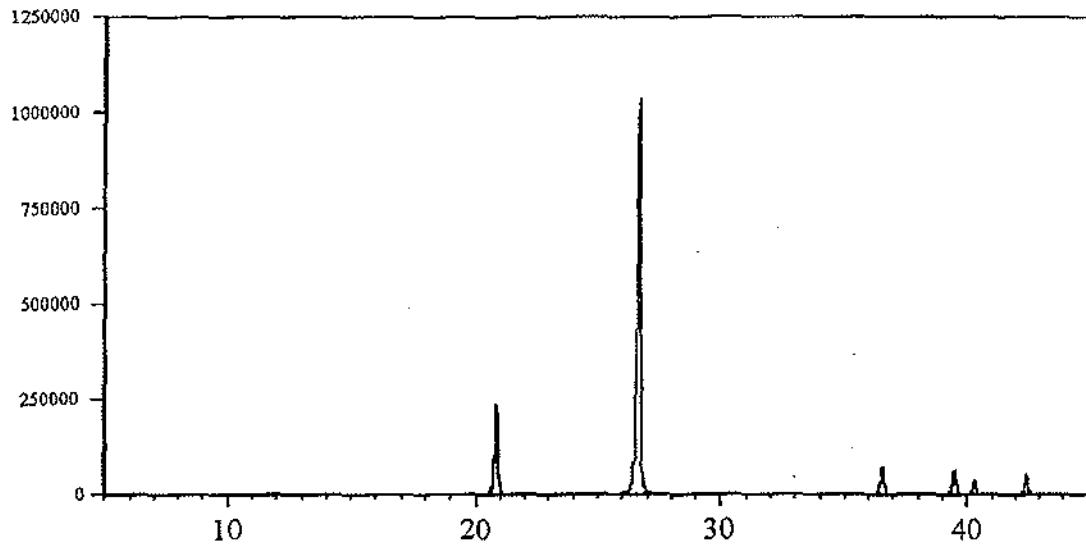


Figure I.5. X-ray diffraction pattern for milled Iota sand.

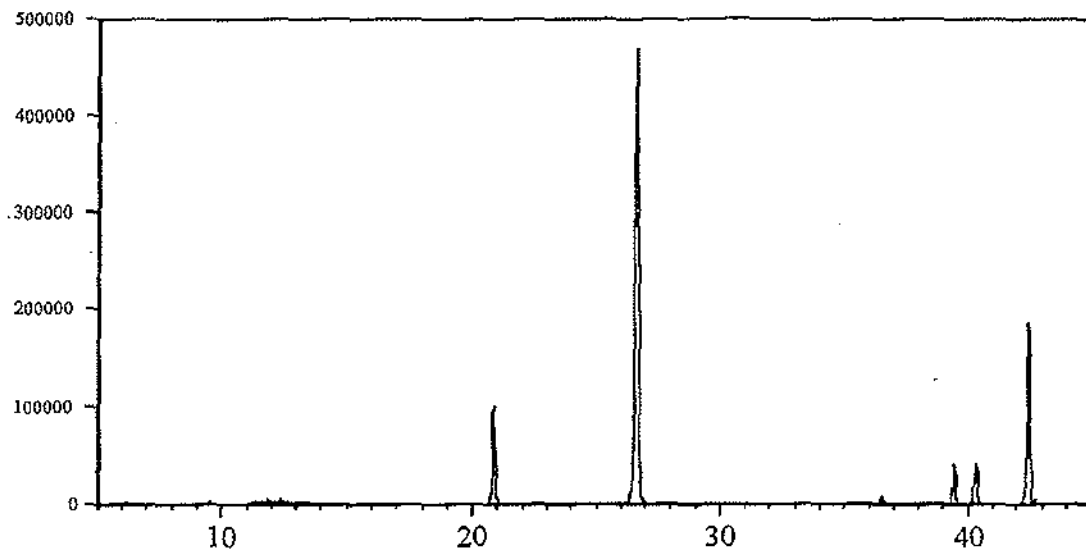


Figure I.6. X-ray diffraction pattern for Ottawa sand.

Appendix II. Concentration versus time data from the dissolution experiments.

Corning		Ione		Milled Iota	
time, hr	SiO ₂ ppm	time, hr	SiO ₂ ppm	time, hr	SiO ₂ ppm
4.3	2.54	5.1	6.14	26.6	5.89
8.9	4.15	18.8	7.34	50.8	7.63
18.2	5.12	22.5	8.34	70.2	11.3
22.9	5.84	23.9	8.77	98.0	11.6
27.5	7.94	50.8	14.0	118.4	18.3
48.2	9.95	93.7	17.1	143.9	18.2
71.2	16.5	144.8	18.7	171.6	20.7
90.0	16.8	187.9	18.8	196.2	30.7
115.5	20.4	236.2	21.5	236.6	27.0
138.0	22.3	289.3	24.0	288.2	40.8
187.8	22.6	338.9	20.3		
215.0	27.5				
239.8	29.2				
258.2	30.6				
Feldspar Corporation		Iota		Ottawa	
time, hr	SiO ₂ ppm	time, hr	SiO ₂ ppm	time, hr	SiO ₂ ppm
3.8	5.77	4.0	2.71	3.9	4.71
8.4	8.91	8.7	2.92	8.5	8.27
18.2	9.55	18.2	8.52	18.2	14.7
22.9	17.7	27.2	9.06	27.1	15.7
27.0	15.0	42.9	8.08	42.9	22.7
35.2	16.6	49.3	7.53	69.0	27.9
48.2	23.4	74.3	15.1	114.5	25.0
71.2	25.6	114.5	22.6	126.1	26.3
90.0	28.2	138.2	17.9	138.2	30.8
115.5	28.6	164.3	18.5	164.3	25.1
				164.3	28.4

Appendix III. Derivation of monolayer model to describe the rate of dissolution of the “disturbed” surface on quartz.

When aged quartz grains are immersed in water, the concentration of dissolved silica increases rapidly, at a rate comparable to the dissolution rate of amorphous silica, and then abruptly slows to a rate that is comparable to quartz dissolution. At the time when the abrupt change in rate occurs, the amount of dissolved silica that has been released to solution is less than that needed to form a complete monolayer of SiO_2 on the quartz surface. The purpose of this section is to derive a model that is based on reasonable assumptions about the nature of the grains’ surfaces that gives a concentration versus time graph that matches that found in the experiments. This model is based on the following assumptions:

- The solution is so undersaturated with respect to quartz solubility that the precipitation rate is insignificant. Thus, the rate of silica release to solution is expressed by the simple dissolution rate laws:

$$r_{(as)+} = \frac{dn}{dt} = A_{as}k_{(as)+} \quad (1)$$

and

$$r_{(qz)+} = \frac{dn}{dt} = A_{qz}k_{(qz)+} \quad (2)$$

- We will consider exactly 1 m^2 of total surface area and this total area remains constant. However, it is the sum of the surface area of amorphous silica and of quartz. Although the relative amount of these areas change with time they always sum to 1 m^2 :

$$A_{as} + A_{qz} = 1 \quad (3)$$

- The layer of amorphous silica is exactly one monolayer thick ($3.56 \text{ \AA}$)(based on a molar volume of $27.27 \times 10^{-6} \text{ m}^3/\text{mol}$ from Robie and Hemmingway, 1997). This means that each SiO_2 molecule occupies $1.27 \times 10^{-19} \text{ m}^2$ of surface area so 1 m^2 of amorphous silica monolayer contains 1.31×10^{-5} moles of SiO_2 ; this makes the molar area of amorphous silica

$$\Lambda_{as} = \left(\frac{A_{as}}{n_{as}} \right) = 7.56 \times 10^4 \text{ m}^2/\text{mol} \quad (4)$$

Equation (4) can be used to convert the number of moles of amorphous silica on the surface to the amount of area that is occupied by that silica. This information can be combined with (1) to find the rate of decreases of this area caused by the dissolution of the amorphous silica

$$\left(\frac{dA_{as}}{dt} \right) = -A_{as}A_{as}k_{(as)+} \quad (5)$$

and (5) can be rearranged and integrated from the initial amount of surface area covered by amorphous silica to find the amount of amorphous silica-covered surface at time, t

$$\int_{A_{as}^0}^{A_{as}} \frac{dA_{as}}{A_{as}} = -A_{as} k_{(as)+} \int_0^t dt \quad (6)$$

These integrals are evaluated to give

$$\ln\left(\frac{A_{as}}{A_{as}^0}\right) = -A_{as} k_{(as)+} t \quad (7)$$

and this equation can be recast to give the amount of surface covered by amorphous silica at any time

$$A_{as} = A_{as}^0 \exp(-A_{as} k_{(as)+} t) \quad (8)$$

The rate of release of SiO_2 from the grains' surfaces is the sum of the release rates from the amorphous silica surface and the quartz surface

$$\frac{dn}{dt} = A_{as} k_{(as)+} + A_{qz} k_{(qz)+} \quad (9)$$

but according to equation (3), $A_{qz} = 1 - A_{as}$ so

$$\frac{dn}{dt} = A_{as} k_{(as)+} + (1 - A_{as}) k_{(qz)+} \quad (10)$$

which can be rearranged to

$$\frac{dn}{dt} = A_{as} (k_{(as)+} - k_{(qz)+}) + k_{(qz)+} \quad (11)$$

Then equation (8) is substituted into (11) to give

$$\frac{dn}{dt} = \left(A_{as}^0 \exp(-A_{as} k_{(as)+} t)\right) (k_{(as)+} - k_{(qz)+}) + k_{(qz)+} \quad (12)$$

which can be rearranged and integrated

$$\int_0^n dn = A_{as}^0 (k_{(as)+} - k_{(qz)+}) \int_0^t (\exp(-A_{as} k_{(as)+} t)) dt + k_{(qz)+} \int_0^t dt \quad (13)$$

$$n = -\left(\frac{A_{as}^0}{A_{as}}\right) \left(\frac{k_{(as)+} - k_{(qz)+}}{k_{(as)+}}\right) (\exp(-A_{as} k_{(as)+} t)) + \left(\frac{A_{as}^0}{A_{as}}\right) \left(\frac{k_{(as)+} - k_{(qz)+}}{k_{(as)+}}\right) + k_{(qz)+} t \quad (14)$$

or

$$n = \left(\frac{A_{as}^0}{A_{as}} \right) \left(\frac{k_{(as)_+} - k_{(qs)_+}}{k_{(as)_+}} \right) (1 - \exp(-A_{as} k_{(as)_+} t)) + k_{(qs)_+} t \quad (15)$$

Equation (15) gives the number of moles of silica released to solution from 1 m² of surface area at a given time. This can be converted to the concentration of silica in an aqueous solution in contact with an arbitrary amount of silica surface by multiplying both sides by the (A/M) value for the system.

$$m = \left(\frac{A}{M} \right) \left(\left(\frac{A_{as}^0}{A_{as}} \right) \left(\frac{k_{(as)_+} - k_{(qs)_+}}{k_{(as)_+}} \right) (1 - \exp(-A_{as} k_{(as)_+} t)) + k_{(qs)_+} t \right) \quad (16)$$

Appendix IV. Derivation of a model for the lifetime of particles and edges of radius, r , taking the effect of surface free energy into consideration.

The purpose of this model is to estimate the lifetime of a small particle or a small sharp edge on a larger particle. The model is derived for spherical geometry, and because spheres have the smallest surface area to volume ratio of any solid, it estimates the longest possible lifetime for the particles. The first part of the derivation simply accounts for the reduction in the dissolution rate caused by the decreasing surface area of the shrinking particle. The second part of the derivation accounts for the increased dissolution rate that results from the higher solubility of very small particles caused by surface free energy effects. When these two effects are combined they show that for quartz the surface free energy effects become important when the radius of curvature is less than $\approx 0.1 \mu\text{m}$ and that when the radius of curvature is less than $0.01 \mu\text{m}$ the lifetime of the feature is less than a few months. This means that these features are not common in geological quartz samples.

The first step in developing this model is to find the lifetime of a shrinking particle or arbitrary size that accounts for the reduced rate of dissolution as the surface area of the particle decreases. This begins with the general relationship between the surface area of a sphere and its volume

$$A = bV^{2/3} \quad (1)$$

Substituting the equations that relate the radius of a sphere to its surface area and volume into (1) allows us to determine that

$$b = \frac{4\pi^{2/3}}{(4/3)^{2/3}} \quad (2)$$

This relationship can be recast in terms of the number of moles of substance in this particle using the relationship

$$V = nV_m \quad (3)$$

so

$$A = bn^{2/3}V_m^{2/3} \quad (4)$$

and this expression can be combined with the rate law for quartz dissolution

$$r_+ = \frac{dn}{dt} = -Ak_+ \quad (5)$$

to give

$$\frac{dn}{dt} = -bn^{2/3}V_m^{2/3}k_+ \quad (6)$$

and the principle of detailed balancing (Rimstidt and Barnes, 1980) requires that $k_+ = k_-K$ so

$$\hat{r}_+ = k_-K \quad (15)$$

However, the the solubility of sharp edges and small particles is larger than for bulk material so K must be adjusted to account for this. This higher value of K is related to the radius of curvature of the surface by the Kelvin equation

$$\log \hat{K} = \log K + \frac{(2/3)\sigma BV_m}{rRT} \quad (16)$$

which can be rearranged to the form

$$\hat{K} = K \exp\left(\frac{(2/3)\sigma BV_m}{rRT}\right) \quad (17)$$

Equation (17) can be combined with (15) to give

$$\hat{r}_+ = k_-K \exp\left(\frac{(2/3)\sigma BV_m}{rRT}\right) \quad (18)$$

Furthermore, this expression can be combined with equation (12) to find the lifetime of a particle where surface free energy increases the solubility and therefore the rate of dissolution of small particles.

$$t_i = \left(r / V_m k_-K \exp\left(\frac{(2/3)\sigma BV_m}{rRT}\right) \right) \quad (19)$$

but this equation can be simplified by replacing k_-K with k_+ to give

$$t_i = \left(r / V_m k_+ \exp\left(\frac{(2/3)\sigma BV_m}{rRT}\right) \right) \quad (20)$$

and it can be put into a more convenient form by taking the logarithm of both sides to give

$$\log t_i = \log r - \log(V_m k_+) + \left(\frac{(2/3)\sigma BV_m}{rRT} \right) \quad (21)$$

Figure IV.1 shows that the lifetimes of particles of radius greater than 0.1 μm are not significantly affected by surface free energy effects while the additional solubility produced by surface free energy causes particles with radii smaller than 0.1 μm to dissolve fairly quickly. This means that sharp edges and microfine particles of quartz, with radii of

curvature less than $\approx 0.01 \mu\text{m}$, will not persist in an aqueous environment for more than a few so they are unlikely to be important features in quartz dusts.

APPENDIX II

Nature and Distribution of Crystalline Silica in Pet Litters

II-1.0 General Approach

The study, carried out by Dr. Robert D. Hamilton of the Johns Manville Technical Center, Littleton, Colorado, encompassed several parts. These included --

- analysis of the bulk samples
- water separation of the bulk sample into grit fractions (plus and minus 44 micrometers)
- heavy liquid separation of the grit fractions into greater than and less than specific gravity of 2.52
- capture of respirable equivalent $\leq 10\mu\text{m}$ fractions in customer exposure estimates
- air separation of the respirable fraction using a tubular aerosol dust chamber
- examination of fractions by x-ray diffraction, scanning electron microscopy, optical microscopy

Hamilton's general analytical scheme appears in Table II-1. A description of his findings appears in this report Characterization of Quartz in Selected Cat Litter Products, December 1997, and ancillary tables in this Appendix.

II-2.0 Aerosol dust generation

The procedure and equipment used are familiar to sedimentary petrologists, X-ray technologists, and electron microscopists. However, one particular problem was obtaining sufficient $\leq 10\mu\text{m}$ dust for analysis. The air monitor samples do not have sufficient dust for detailed chemical treatments, separations, and analysis.

II-2.1 General information

Davis⁵ has described a device for preparing X-ray diffraction mounts that could be adaptable to collecting $\leq 10\mu\text{m}$ fraction. This device is the tubular aerosol dust chamber, or "TADC". Use of this device is not identical to pouring and tends to overestimate the amount of $\leq 10\mu\text{m}$ dust, but does give an approximation. The charge to the TADC is -100 mesh material sieved from the bulk sample. Glass beads were needed to help fluidize the bed during the test, even though they abraded the granules, somewhat.

II-2.2 Preparation of sample for aerosol dust generation

Sieve sufficient bulk material in 250g batches through a 100 mesh screen on a RoTap™ to collect at least 1 g of -100 mesh material.

II-2.3 Collection chamber design

The tubular aerosol dust collector (TADC) was constructed by mounting a Millipore Pyrex™ glass filter assembly for 47 mm diameter filters to the open end of a 500mL glass burette. The filter funnel and the burette have identical diameters and the resulting column is roughly 15 inches long and three inches in diameter with a stopcock at one end and a 47 mm filter assembly at the other. Near the center of the tube, a ¼ inch diameter side arm with a stopcock valve was attached to allow additional control of airflow into the chamber. The hole in the plastic stopcock at the bottom of the gas burette tube is restricted by the insertion of two small diameter plastic tubes. The tubes allow adequate airflow through the stopcock while preventing the glass beads from the fluidized bed from dropping into the stopcock assembly. The chamber was mounted vertically with the stopcock down. A ball type airflow monitor was connected to the open end of the stopcock and the inverted base of the filter assembly at the top was connected to a vacuum line.

II-2.3 Chamber operation

With the bottom stopcock closed the chamber is loaded from the top with a small number of 2 mm. glass beads. Beads are added until they fill the straight portion of the upper side of the stopcock. The desired quantity of test material, 100 mg, is then added to the glass beads. A weighed 47 mm diameter cellulose acetate Millipore™ filter with a pore size of 1.2 µm is then mounted on the filter base using the vacuum to hold the filter and support screen in place. The filter base is then inverted and attached to the column with a mounting clamp.

Sample collection is initiated by opening the stopcock at the base until the desired airflow through the chamber is reached. The airflow should result in a steady boiling movement of the glass beads and the deposition of particles with a diameter of 10 µm or less on the filter. The correct airflow was determined by collecting samples at a number of flow rates and determining the maximum diameter of the particles collected by microscopy.

Each sample was run for a minimum of 3 minutes with a gentle tapping on the chamber sides if dust begins to accumulate during the collection process. When collection is complete, the bottom stopcock is closed, the vacuum line is closed, and air is bled into the chamber from the side vent. The filter is then carefully removed and weighed to determine the amount of dust collected. In general, the maximum amount of dust on a single filter should not exceed 1 to 20 mg. Repeat the procedure until collecting approximately 50 mg of dust, usually 2 to 3 filters.

II-2.4 Dust analysis

X-ray and microscopic examination follow the procedures described in II-4-0.

II-3.0 Experimental Details - Sample Processing.

II-3.1 100 mesh dry screening

The full 1 kg sample is divided in 300 gram lots. Each lot was dry screened with an 8-inch sieve in a Rotap™ for 5 minutes. The resulting pan and screen fractions from each run were combined into a single batch of each size fraction.

II-3.2 325 mesh wet screening

A 20-25 gram sample of the +100 mesh material is placed in a laboratory Waring™ blender with about 250-300 mL of water and 0.1 grams of TSPP. The sample is run for 1 minute and the resulting slurry poured through a 325 mesh screen. The fines are collected and the +325 mesh retained fraction is washed back into the blender jar and processed again with the same volume of water and TSPP. The dispersion and washing process is repeated once more and the fines from all three stages are combined and dried for later analysis. The +325 mesh fraction retained on the screen is thoroughly washed with water, air-dried, and retained for analysis.

II-3.3 Heavy mineral separation

About 0.5 grams of +325 mesh grit is placed in a 100mL-centrifuge tube with 50mL of bromoform which has been diluted with DMF to a specific gravity of 2.52. The mixture is shaken and allowed to separate for about 1 hour. The light top fraction is stirred gently and allowed to separate again for an hour. The stirring process is repeated one more time and then the tubes are allowed to stand over night. The lower ¼ inch of the centrifuge tube is immersed briefly in liquid nitrogen to freeze the bromoform in the lowest portion of the tube. The unfrozen bromoform with the light fraction is poured off, filtered and washed to recover the light fraction. The frozen bromoform with the heavy fraction is allowed to thaw and then poured off, filtered and washed.

II-3.4 NIOSH Method 7601 digestion

The standard procedure is followed up to the point at which the insoluble residue is collected on a filter after the phosphoric acid digestion. At this point the filter and residue are dried and ashed in a crucible at 600°C for four hours. The ash normally contains some residual amorphous silica and is suitable for XRD analysis and not suitable for SEM analysis.

II-3.5 Modified NIOSH Method 7601 digestion

Prior to filtering the residue from the phosphoric acid digestion, 25 mL of 1N KOH is added to the digestion flask, heated to boiling, swirled around the inside of the flask and then added to the filter with the residue. The addition of the fluoroboric and the rinse with hot KOH removes the

residual silica that obscures particles during SEM analysis. The effect on the quantitative determination of quartz and cristobalite has not been evaluated.

II-4.0 Analytical Methods and Instrumentation

II-4.1 Bulk powder X-ray diffraction

Used for qualitative mineralogical analysis of bulk samples, -325 mesh water washed fines, grits and residues from NIOSH Method 7601 as noted.

Instrumentation: Siemens D-500 x-ray diffractometer in standard focus Cu tube operating at 40 kV and 30 ma. The goniometer is equipped with a curved graphite monochromator and operated with 1° slits and a 0.06° scatter slit. Samples are loaded and run with a 40-position sample chamber, with 30-rpm sample rotation. Unless otherwise specified all samples are scanned from 4 to 76 degrees 2θ with a step size of 0.05 degrees 2θ and counting time of 1 second per step. The system is regularly calibrated for peak position using NIST reference quartz as a standard.

Sample mounting for analysis: Bulk samples with a mass of greater than about 0.25 grams are hand ground gently to -325 mesh and packed into an aluminum sample holder with an aperture of 1 inch. Samples with weights less than 0.25 grams are ground, dispersed in propanol and sedimented on a zero background quartz plate supplied by D. K. Smith at Penn State University. The zero background plate is prepared from a single crystal of quartz that is cut with an orientation that produces no diffraction peaks over the interested range.

Data processing and analysis: Raw count data are processed with a five point smoothing and diffraction peaks with magnitudes of greater than 1 percent relative are extracted using the Autoxray program supplied by Omni International. Mineral phases are identified qualitatively by comparison with the ASTM / JCPDS Diffraction DataBase.

II-4.2 Determination of Murata ratios by X-ray diffraction

Samples are mounted in a bulk mount, on a zero background plate on a silver membrane filter depending on the amount of sample present and the mode of preparation. Samples are scanned with the D-500 Siemens x-ray diffractometer from 66°2θ with a step size of 0.02°2θ and a counting time of 5 seconds per step. The resulting data are smoothed with a five-point algorithm and plotted with an xy plotter on 8 ½ by 11 inch paper. The peak heights are measured from the plots following the procedure by Murata and Norman and the Murata ratio or “crystallinity index” of the quartz is calculated following the authors’ procedure.

II-4.3 Quantitative determination of quartz by NIOSH method 7500

Bulk samples on air filter membranes containing the material for analysis are ashed thermally, at 600°C and analyzed following the procedures outlined in the published method. The system is calibrated with well-characterized reference standards containing from 25 to 2000µg of NIST

reference respirable silica. All analyses are performed with the Siemens D-599 diffractometer as described above.

II-4.4 Scanning Electron Microscope (SEM) analysis

Instrumentation: All samples are examined with one of two scanning electron microscopes as described below. All EDS analyses and most particle imaging are performed on the JEOL-840 SEM. High magnification imaging of the aerosol dust and cross-section of the grit is performed on the Hitachi S-4200 SEM.

SEM-1: JEOL-840 SEM with a lanthanum hexaboride electron source. Equipped with an EDAX DX-4 energy dispersive x-ray analyzer and two, dual crystal wavelength x-ray spectrometers. For normal imaging and EDS analysis the instrument is operated at 15 kV, 10-8 A beam current and a working distance of 15 mm.

SEM-2: Hitachi S-4200 field emission source SEM. For high resolution and surface imaging, the instrument is usually operated at 2 kV, 10-8 A emission current and a working distance of 10 mm.

SEM sample preparation: Loose powder samples with particle diameters greater than 5 μ m are mounted by sprinkling on a stub coated with conductive carbon tape. Finer samples are dispersed in water and deposited on 0.2 μ m pore diameter Nuclepore™ filter. The filter is mounted while still damp on a SEM sample stub with double sided tape. All samples that are prepared for imaging only are coated with Au-Pd using a Hummer VI sputter coater. Samples prepared for both imaging and EDS analysis are coated with vacuum evaporated carbon in a Kinney KVC-503 vacuum evaporator.

II-4.5 SEM-EDS analysis

All SEM-EDS analysis is conducted with carbon coated samples in the JEOL-840 SEM as described above. The EDS system is equipped with an ultra thin window detector that permits detection of elements with atomic number 5 and up. Elemental identification and standardless quantitative analysis is done with software provided by EDAX.

II-5.0 Materials

II-5.1 Set One

These are samples of pet litter purchased from store shelves by the Executive Director of the Sorptive Mineral Institute in January 1997, and sent to Dr. Robert Hamilton at the Johns Manville Technical Center for analysis. They include products made from the Monterey formation, the Oregon diatomite, the Porters Creek formation, the Twiggs fullers earth, the Meigs fullers earth, and Wyoming bentonite.

II-5.2 Set Two

These are samples of pet litter purchased from store shelves of retail grocery stores, pet supply stores and mass merchandisers by TRC Environmental Corporation in August 1997. They were sent to both Dr. Robert Hamilton at the Johns Manville Technical Center for analysis and to Dr. Karen Vetrano at TRC Environmental Corporation for generation of dust by pouring.

A complete description of the samples tested in Sets One and Two is included in Figure II-1.

II-6.0 Results

II-6.1 Mineralogy

The results appear in Table II-2

II-6.2 Dust Analysis

The results appear in Table II-3

II-6.3 Size Fraction and Distribution Analysis

The results appear in Tables II-4 through II-13.

Table II-1. - Processing Scheme

Table II-2. - Mineralogy of Pet Litters.

<u>Name</u>	<u>Sample Date</u>	<u>Bulk Mineralogy*</u>
Twiggs	January, 1997	major: smectite, opaline silica minor: quartz, kaolinite
Meigs	January, 1997	major: attapulgite, opaline silica minor: quartz
Wyoming Bentonite	August, 1997	major: smectite minor: quartz, mica, feldspar, calcite, gypsum, opaline silica
Porters Creek	August, 1997	major: smectite, opaline silica minor: quartz, mica, kaolinite
Monterey	August, 1997	major: opaline silica minor: quartz, feldspar, mica, dolomite
Oregon Diatomite	January, 1997	major: opaline silica (diatomite) minor: feldspar

* = estimated from major diffraction peak intensities.
major = >10%.
minor = <10%.

**Table II-3. - Dust Analysis
Sets One and Two.**

<u>Sample ID</u>	<u>Sample Date</u>	<u>% - 100 mesh in bulk</u>	<u>% -10 micron in bulk</u>	<u>% quartz in 10 micron dust</u>	<u>% -10 micron quartz. in bulk</u>	<u>C.I. ratio of dust</u>
Bentonite						
JMTC dust	August, 1997	0.19	0.01	6.70	0.0006	10
JMTC dust	January, 1997	0.29	0.04	6.90	0.0027	<1
TRC dust				nr		nr
Monterey						
JMTC dust	August, 1997	0.24	0.07	7.10	0.0050	9.3
JMTC dust	January, 1997	0.08	0.03	7.50	0.0017	5.6
TRC dust	August, 1997			1.71		7.3
Porters Creek						
JMTC dust	August, 1997	0.35	0.05	9.80	0.0053	9.7
JMTC dust	January, 1997	0.11	0.02	5.30	0.0013	8.1
TRC dust	August, 1997			2.44		5.7

JMTC = Johns Manville Technical Center

TRC = TRC Environmental Corporation

JMTC dust = Generated with tubular aerosol dust chamber (TADC).

TRC dust = Generated by pouring at TRC facility.

C.I. = Crystallinity Index

nr = Insufficient sample for analysis

**Table II-4. - Size Fraction Analysis
Sets One and Two**

Sample	Sample Date	SizeFraction			
		+325 mesh	-325 mesh	-100 mesh	-10 micron
Monterey	August, 1997	28.5	71.5	0.24	0.0710
	January, 1997			0.08	0.0335
Bentonite	August, 1997	5.3	94.7	0.19	0.0094
	January, 1997			0.29	0.0389
Porters Creek	August, 1997	4.4	95.6	0.35	0.0542
	January, 1997			0.11	0.0187
Twiggs	January, 1997	30.2	69.8	0.10	0.0280
Meigs	January, 1997	29.0	71.0	0.05	0.0075
Oregon Diatomite	January, 1997	15.5	84.5	0.45	0.2095

+325 mesh from wet sieve analysis of bulk material.

-325 mesh from wet sieve analysis of bulk material.

-100 mesh from dry sieve analysis of bulk material

-10 micron mesh (-10µm) dust in bulk as recovered from the -100 mesh fraction in the tubular aerosol chamber (TADC).

**Table II-5. - Distribution of Quartz
Sets One and Two**

Sample ID	Sample Date	Size Fraction			
		Bulk	Grit	Fines	Dust
Wyoming Bentonite	August, 1997	2.7	8.6	2.3	6.7
	January, 1997				6.9
Monterey	August, 1997	7.2	9.2	4.8	7.1
	January, 1997				7.5
Porters Creek	August, 1997	9.5	34.9	8.5	9.8
	January, 1997				5.3
Twiggs	January, 1997	5.3	13.7	5.1	7.5
Meigs	January, 1997	8.6	10.2	6.4	9.8
Oregon Diatomite	January, 1997	0	0	0	0

Bulk = Pet litter as received.
 Grit = +325 mesh fraction from wet sieving.
 Fines = -325 mesh fraction from wet sieving.
 Dust = -10 μ m dust from tubular aerosol dust chamber (TADC).

**Table II-6. - Crystallinity Index
Sets One and Two.**

Sample	Sample Date	Size Fraction			
		Bulk	Grit	Fines	Dust
Wyoming Bentonite	August, 1997	8.0	8.7	3.5	10.0
TRC dust, only	August, 1997				n.d.(1)
JMTC dust, only	January, 1997				<1
Monterey	August, 1997	9.0	9.8	5.8	9.3
TRC dust, only	August, 1997				7.3
JMTC dust, only	January, 1997				5.6
Porters Creek	August, 1997	9.0	7.4	7.7	9.7
TRC dust, only	August, 1997				5.7
JMTC dust, only	January, 1997				8.1
Twiggs	January, 1997	7.5	8.9	5.4	7.3
Meigs	January, 1997	6.7	7.2	7.6	7.3
Oregon Diatomite (2)	January, 1997	n.d.	n.d.	n.d.	n.d.

TRC = TRC Environmental Corporation
 JMTC = Johns Manville Technical Center
 Bulk = Pet litter as received.
 Grit = +325 mesh fraction from wet sieving.
 Fines = -325 mesh fraction from wet sieving.
 Dust = -10 μ m dust from tubular aerosol dust chamber (TADC).
 (1) = no sample available

(2) = no quartz detected

Table II-7. - Grit Analysis - Monterey Formation.

Grit content:	wt % +325 mesh	=	28.5
	vol % S.G. >2.52	=	5
	vol % S.G. <2.52	=	95

Table II-8. - Grit Analysis - Oregon Diatomite.

Table II-9. - Grit Analysis - Porters Creek Formation.

Table II-10. - Grit Analysis - Twiggs Fullers Earth.

Table II-11. - Grit Analysis - Meigs Fullers Earth.

Table II- 12. - Grit Analysis - Wyoming Bentonite (Sct One).

Table II-13. - Grit Analysis - Wyoming Bentonite (Set Two).

Sorptive Minerals Institute

Attachment 2

Physicochemical and Mineralogical Characterization of Test Materials used in 28-Day and 90-Day Intratracheal Instillation Toxicology Studies in Rats

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Two recent intratracheal instillation toxicology studies in rats clearly show that a naturally occurring quartz, with occluded crystal surfaces (quartz isolate), produced significantly less inflammatory response than a crushed reference quartz (DQ12). Respirable-size quartz isolate was isolated from bentonite parent rock, without crushing or the use of chemicals, to ensure that the surface properties of the quartz particles were unaltered. The isolation technique utilized gentle mechanical dispersion followed by sedimentation in an aqueous medium. Extensive mineralogical and chemical characterizations were undertaken to define the physicochemical properties of the test materials. The characterizations showed significant, measurable physicochemical differences between the two quartz types. These differences may help to explain the difference in toxicological response associated with these materials. The evaluation methods and resulting data that characterized the chemical and physical properties of the instillation test materials are discussed. The data presented show that such characterizations are essential if meaningful correlations are to be made between test materials and their toxicological profiles.

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In recent years, investigators have determined that the human toxicity of quartz varies depending on the circumstances of exposure (Castranova et al., 1996b; International Agency for Research on Cancer, 1997). Mechanistic studies by Donaldson and Borm (1998) emphasized that the biological toxicity of quartz is related to its surface activity and that this toxicity may vary dramatically depending on the origin of the quartz. Fubini (1998) also found that the biological toxicity of quartz is reduced by substances affecting its surface activity. Vallyathan et al. (1991) noted that while the toxicity of crushed quartz declines with time after crushing, crushed quartz that has been kept in a clean, dry environment can retain significant measurable toxicity for years. Vallyathan et al. (1995) showed that inhalation of respirable-size, “freshly fractured” (crushed within days) quartz leads to enhanced lung injury which may be the result of free radicals on the freshly fractured surfaces. Hamilton (1999) suggested a function that could quantify the

toxicity related to surface activity of crystalline silica. This function, expressed in surface activity units, takes into account mass, surface area, silica polytype, origin, and time-dependent activity decay coefficients. Most naturally occurring quartz found in the environment has been in contact with a variety of contaminants, such as aluminosilicates, carbon compounds, and metal ions, for long periods of time. These environmental contaminants can occlude the surface of the quartz particles with a thin layer of material that can alter the particle surface characteristics. Quartz particles with these occluded surfaces can have a level of biological activity that is very different from that of quartz particles with unoccluded surfaces, or the surfaces of particles of freshly crushed quartz.

The most direct way to compare the surface differences of crushed quartz and quartz with occluded surfaces, and to relate these differences to their toxicological properties, is to dose laboratory animals with the different quartz species and compare the toxicological effects. A major challenge to this comparison is the ability to obtain a sufficient quantity of respirable-size, occluded quartz particles without changing the particles' surfaces. A method was developed that produced sufficient quantities of material for characterization and toxicological testing.

The Fraunhofer Institute of Technology and Experimental Medicine conducted two rat intratracheal instillation toxicology studies to compare the biological effects of respirable-size, crushed quartz and a naturally occurring quartz with occluded surfaces. The results of these studies are reported in Creutzenberg et al. (2003, 2008). In the first study, conducted in 2001, the animals were evaluated for 28 days post dosing. In the second study, conducted in 2004, the post-dosing observation period was 90 days. Both studies reported significant, persistent inflammation with the crushed quartz, while the natural quartz with occluded surfaces produced a much lower degree of inflammation that was nonpersistent. The physicochemical properties of the instillation test materials used in both studies were characterized in order to better understand the nature of their toxicity.

MATERIALS AND METHODS

Test Materials

Any toxicological study must be based on a thorough understanding of the chemical and physical properties of the test material. Any characterization of the test material should also include an understanding of the history of the material and how that may have affected its properties. The materials used in these studies were crushed quartz, quartz isolated from sodium bentonite, montmorillonite clay isolated from sodium bentonite, and titanium dioxide. All test materials were composed of respirable-size particles.

Reference Quartz

DQ12 quartz obtained from Bergbauforschung, Essen, Germany, was used as the reference quartz. DQ12 quartz has well-documented biological activity, and has served as a fre-

quent positive control in toxicological studies. This material is a purified sand obtained from the Dörentrop kaolin deposit in Germany (Robok, 1973; Clouter et al., 2001). The sand was crushed approximately 30 years ago and then air centrifuged in 1985 to separate the bulk material into fine, midsize, and coarse fractions. The fine fraction, used in the 28-day study, was designated as "Reference Quartz 2001." The mid-size fraction, used in the 90-day study, was designated as "Reference Quartz 2004."

Quartz Isolate

"Quartz Isolate" as used in this article refers to the naturally occurring, respirable-size, occluded quartz separated from sodium bentonite, without the use of crushing or chemical additives. Obtaining a sufficient quantity of natural, respirable-size quartz with occluded surfaces, without altering the surface characteristics of the particles, requires great care. Many possible sources for these particles were examined. Of these, crude sodium bentonite was found to be the only material from which the quartz particles could be readily isolated without the need to dry, crush, or chemically treat the material. Crude sodium bentonite, from the 110- to 112-million-year-old Newcastle formation in Wyoming, was used to obtain the occluded quartz particles used in these studies.

Sodium bentonite is composed principally of the clay mineral montmorillonite, which occurs in water-dispersible particles that are typically $<0.5 \mu\text{m}$ in size. A low-shear, aqueous dispersion technique was developed that, over time, allows the quartz particles in the bentonite to become separated from the clay particles. This technique makes it unnecessary to dry, crush, or chemically disperse the crude clay to liberate the quartz particles from the clay matrix, and thereby minimizes the potential for altering the natural occluded crystal surfaces of the quartz grains. The naturally occurring, respirable-size quartz particles in the bentonite were separated from the aqueous clay dispersion and concentrated by centrifugation. (A more detailed description of the separation procedure is available from the authors upon request. Initial separation and concentration of the Quartz Isolate material was done by Southern Clay Products, Gonzales, TX.). The resulting quartz concentrates were purified by repeated suspension in deionized water and sedimentation, using Stokes's Law to calculate sedimentation time. Following this process, the $<0.5 \mu\text{m}$ montmorillonite particles were first removed, followed by the $>5.0 \mu\text{m}$ particles. This process allowed the final particle size distribution of the quartz concentrate to be brought into the 1 to $5 \mu\text{m}$ range. Each resulting quartz concentrate was maintained as an aqueous slurry to eliminate possible effects that drying might have on the surface properties of the particles. The quartz concentrates used in the 28-day and 90-day studies were designated as "Quartz Isolate 2001" and "Quartz Isolate 2004," respectively.

This isolation technique may be applicable to isolating quartz particles from other sedimentary source rocks as well.

Titanium Dioxide

The titanium dioxide used was Bayertitan T, lot 85/13928, from Bayer AG, Krefeld, Germany. This material was produced by flame hydrolysis of TiCl_4 and is the rutile polymorph.

Positive Control

Both toxicological studies were designed to dose the animals, in the control and test groups, with the same mass of test material and the same dose of quartz. The quartz content in the two reference quartzes was greater than that in the quartz isolates. In order to achieve the proper dosing parameters, titanium dioxide was added to dilute the reference quartzes, to produce positive control mixtures that contained the same quartz content as their respective quartz isolate. The resulting positive control mixtures were designated Positive Control 2001, for the 28-day study, and Positive Control 2004, for the 90-day study.

Clay Isolate

The montmorillonite clay separated from the sodium bentonite used to obtain Quartz Isolate 2001 was designated as the clay isolate. This material, which contained less than 0.04% quartz, was obtained using the aqueous dispersion and centrifugation techniques described for the quartz isolates.

Chemical Composition

The chemical composition of the test materials was determined using the standard lithium metaborate/tetraborate fusion method for inductively coupled plasma (ICP) determination of the major and trace element concentrations (Johnson et al., 1981). (Testing was conducted by Activation Laboratories Ltd., Ancaster, Ontario, Canada.) The lithium metaborate/tetraborate method ensures that the entire sample is dissolved, enabling major oxides and high field strength elements to be put into solution. The results of chemical analysis of the quartz isolates were used to confirm their mineralogy as determined by x-ray diffraction analysis.

Mineralogical Composition

An understanding of the mineralogy of the reference quartz and quartz isolate samples is important for determining the purity and crystallinity of the quartz. In the case of the quartz isolates it was also important to determine the presence of other mineral components that might affect the surface properties and toxicity of these materials. X-ray diffraction (XRD) analysis was used for determination and quantification of the mineralogical composition of test materials. The quantification of quartz employed the standard NIOSH method 7500, adapted for bulk samples (National Institutes of Occupational Safety and Health, 2003). The latest quartz standard, NIST Standard Reference Material (SRM) 1878a, which is currently used for α -quartz determinations, was used as the calibration standard (National Institute

of Standards and Technology, 2005). Quantification of the trace minerals analcime and calcite used standard XRD procedures (Klug et al., 1974). Quantification of the swelling clay mineral, montmorillonite, poses special problems due to its variable composition. Following standard practice, the chemical contributions of the nonmontmorillonite mineral phases were deducted from the total chemical analysis. The remaining chemical values were used to generate a chemical formula matching that of the montmorillonite unit cell structure, following the method of Ross and Hendricks (1945). The amount of montmorillonite could then be estimated from the resulting chemical formula. Any excess SiO_2 was identified as undefined silica.

XRD analysis also allows determination of the domain size, which is an important characteristic of quartz. A domain is defined as a volume within a crystalline material that has a coherent diffracting alignment. A single crystal quartz particle can have a multitude of domains. Domain size is determined by measuring the diffraction line broadening in an XRD pattern, and applying the Warren-Averbach equation (Warren & Averbach, 1953). Murata and Norman (1976) described a semi-empirical treatment of line broadening they termed the "crystallinity index." The crystallinity index appears to be largely a function of domain size (up to about 1 μm diameter) but may also be affected by crystal lattice distortions induced by mechanical stress.

Knowledge of the domain size or its surrogate, crystallinity index, is critical in the quantification of crystalline silica by x-ray diffraction. Using x-ray diffraction, the intensity of a quartz sample is compared with that of a standard reference material to determine the quartz content of the sample. Quartz that has a significantly smaller domain size than that of the standard reference material, as a result of either the natural crystallization process that formed it (e.g., quartz isolates) or postformation stress-induced trauma, such as that produced by intense grinding (e.g., reference quartz), can yield a diffraction intensity per unit mass that may be as little as 50% of that produced by large domain, untraumatized quartz. As a result of this effect, the quartz content of materials containing quartz with a low crystallinity index may be significantly underreported when using a high-crystallinity-index standard reference material, such as NIST SRM 1878a. When this happens most analysts attribute the missing silica to an amorphous phase. In these circumstances, there is no distinct, physically separable, amorphous phase, and reporting amorphous silica as an identified phase is incorrect. Rather, the missing material should be included in the quartz content or simply listed as undefined and not assigned to a specific phase. The mineralogical composition of the quartz materials used in this study, and reported in Table 2, was obtained using SRM 1878a as the calibration standard. When compared with the chemical analysis (Table 1), the quartz values do not account for all of the silica present in these materials. Because no physically separable, identifiable, amorphous silica phases were found, the excess silica has been identified here as "undefined silica."

TABLE 1
Chemistry of instillation materials used in the 2001 and 2004 Fraunhofer rat studies

Element ^a	2001 Rat study			2004 Rat study	
	Quartz Isolate 2001	Reference Quartz 2001	Clay Isolate	Quartz Isolate 2004	Reference Quartz 2004
SiO ₂	87.61	99.39	65.00	87.88	99.30
Al ₂ O ₃	7.79	0.10	22.53	7.58	0.00
Fe ₂ O ₃	1.33	0.04	4.67	1.39	0.04
MnO	0.01	0.00	0.01	0.00	0.00
Na ₂ O	1.05	0.16	4.00	0.78	0.21
K ₂ O	0.23	0.05	0.00	0.75	0.05
MgO	0.76	0.04	2.51	0.79	0.00
CaO	0.88	0.14	0.44	0.19	0.00
TiO ₂	0.08	0.05	0.11	0.08	0.02
P ₂ O ₅	0.05	0.02	0.02	0.04	0.02
F	0.07	0.00	0.14	0.07	0.02
S	0.01	0.01	0.36	0.01	0.01
Total C	0.12	0.00	0.11	0.39	0.33

Note. Chemical composition of the instillation test materials was determined using standard lithium metaborate/tetraborate fusion methods for inductively coupled plasma (ICP) analysis. Trace element chemistry data is available from the authors.

^aIn weight percent. Expressed as oxides.

TABLE 2
Physical properties of instillation materials for the 2001 and 2004 Fraunhofer rat studies

Property	2001 Rat study			2004 Rat study	
	Quartz Isolate 2001	Reference Quartz 2001	Clay Isolate	Quartz Isolate 2004	Reference Quartz 2004
Mineralogy ^a :					
Quartz	62	87	0.04	60	87
Undefined silica	13	13	0	8	13
Montmorillonite	23	0	99.9	30	0
Analcime	2	0	0	2	0
Calcite	< 1	0	0	0	0
Crystallinity:					
Quartz crystallinity index	1.6	6		1.6	5
Quartz XRD domain size (μm)	0.085	0.16		0.076	0.19
Scanning electron microscopy:					
Mass mean geometric diameter, (μm)	3.69	1.31		3.58	3.01
Surface area:					
EGME (m^2/g)	173.7	16.01	479.5	213.0	5.6
Nitrogen BET (m^2/g)	3.2	1.51	6.4	21.2	3.7

^aIn weight percent. Quartz, analcime and calcite concentrations were determined by x-ray diffraction analysis. NIST SRM 1878a was used as the standard reference for quartz. Montmorillonite concentrations were estimated by subtracting the chemical contributions of the non-montmorillonite mineral phases from the total chemical analysis and then fitting a chemical formula matching that of the montmorillonite unit cell to the remaining chemical values following the method of Ross and Hendricks (1945). Excess SiO₂ is identified here as undefined silica.

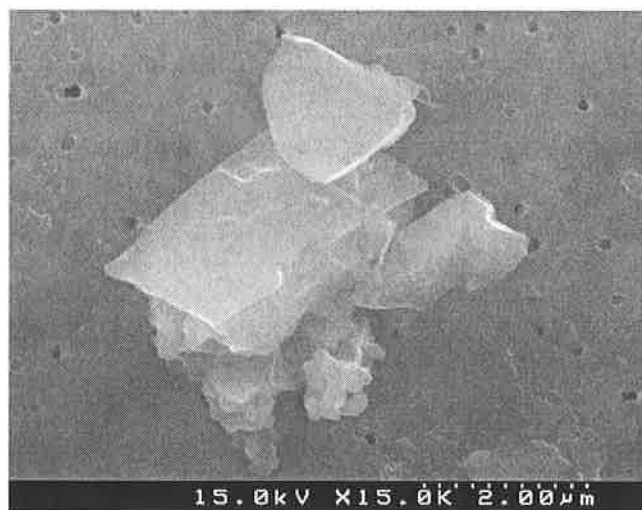


FIG. 1. SEM photomicrograph of Reference Quartz 2004 (15,000 $\times$).

Thermal Analysis

Thermal measurements, such as differential scanning calorimetry (DSC), can be used to obtain information about certain mineral characteristics that cannot be obtained by other methods. DSC measures thermal reactions, either endothermic or exothermic, during heating of the sample from room to high temperatures. DSC testing was conducted using a Netzsch simultaneous thermal analyzer (STA) 409 CD equipped with platinum crucibles for differential scanning calorimetry. Additionally, high-temperature XRD analysis was conducted using a Rigaku DMAX2200 in theta–theta configuration with HT Di-

rect Heating Pt/Rh carriers and an HT 1600C furnace attachment (thermal XRD analyzer).

Particle Size Distribution and Surface Characteristics

Scanning electron microscope (SEM) examination of mineral particles allows direct determination of the shape of particles in a sample, as well as the statistical particle size distribution of the sample. Samples of test materials were carbon coated using a Denton Vacuum DV-502A vacuum evaporator and then viewed in a Hitachi S-4200 field emission scanning electron microscope (SEM), operating at 15 kV, with a 7-mm working distance. The long and short particle dimensions were measured from digital images for 1000 particles of each test material using ImagePro 4.5 image analysis software. The diameter and mass distributions for each material were then determined. The mass distribution data was then averaged for each test material to obtain the mass mean geometric diameter (MMGD) of that material.

Surface Area

Surface-area measurements become problematic when dealing with samples containing expandable clays. The conventional Brunauer–Emmett–Teller (BET) technique, which is typically used to determine surface area for most minerals, involves gas adsorption after heating the sample in vacuo. However, minerals such as montmorillonite will expand in water and reveal a much larger surface area. Because tissues of living organisms are aqueous systems, the BET method does not provide the proper environment in which to measure the surface area of expandable minerals used for in vivo toxicological testing. The ethylene glycol monoethyl ether (EGME) technique has been shown to be useful where expandable minerals are involved (Bower et al., 1959; Carter et al., 1965). This method assumes that EGME is absorbed as a single monolayer on all exposed surfaces, both internal and external. For swelling clays, like montmorillonite, this results in much larger surface area measurements than those obtained with the BET method.

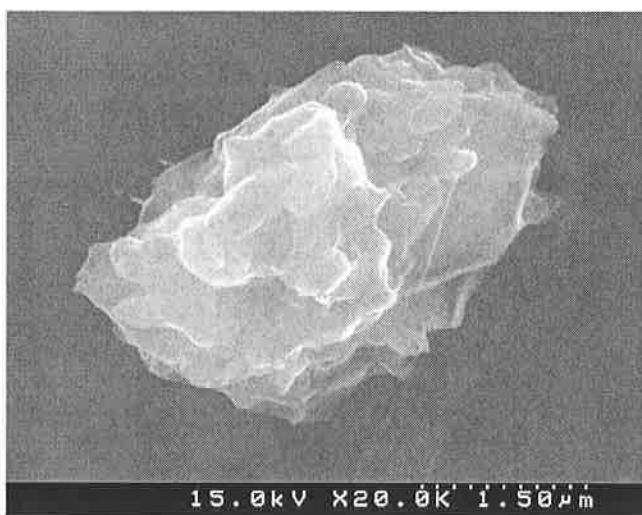


FIG. 2. SEM photomicrograph of Quartz Isolate 2004 (20,000 $\times$).

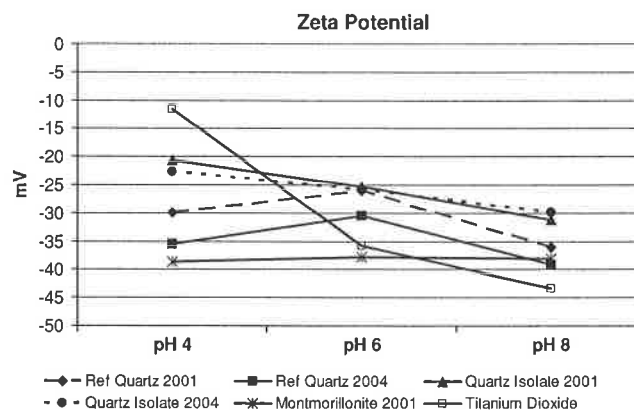


FIG. 3. Zeta potential of instillation test materials at pH 4, 6, and 8.

Electron Spin Resonance

The ability of quartz to generate free radicals appears to be an important aspect of its potential biological toxicity (Castranova et al., 1996a; Chen et al., 1998). Electron spin resonance spectroscopy (ESR) enables identification and quantification of unpaired electrons in paramagnetic compounds that may act as free radicals. Because most ESR work on quartz has been conducted on pure quartz, this study sought to determine whether ESR would also be predictive of biological activity in mixed-mineral systems, such as the quartz isolates. ESR measurements were obtained using a Bruker EMX spectrometer at a frequency of 9.80 GHz; power 63.5 mW; receiver gain 50,000; time constant 0.04 s; modulation amplitude 1.0 gauss; scan time 41 s; and magnetic field 3487 ± 100 G. The Quartz Isolate 2004, Reference Quartz 2004, and an additional quartz sample, Min-U-Sil 5 (a very fine, crushed, single crystal quartz), were first analyzed in aqueous slurry form using the DMPO/hydrogen peroxide spin adduct method described by Castranova et al. (1996b) to compare their abilities to generate relatively long-lived $\text{OH}^\bullet$ free radicals. These analyses were conducted both with and without deferoxamine, an iron chelator that has been shown to eliminate $\text{OH}^\bullet$ radicals generated by quartz (Castranova et al., 1996b; Chen et al., 1998). The samples were then also analyzed as dry powders to determine their silicon source free radicals. Each dry sample was then dry crushed in a Fritsch IEC ball mill for 10 min and then immediately reanalyzed.

Zeta Potential Measurements

Zeta potential measures the electrical potential of colloidal particles in an aqueous suspension and can provide useful information about the boundary between colloidal particles and the medium in which they are suspended. Zeta potential values are affected by the pH of the solution and so are typically measured at several pH levels. Three 0.1% suspensions of each test material were prepared by adding the material to deionized water and processing for 3 min in a Waring blender at 14,500 rpm. The pH of the three suspensions for each material was then adjusted to 4, 6, and 8, respectively, using HCl or NaOH. Each suspension was then injected into a test cell and tested with a Malvern Zetasizer Nano ZS using the standard ZTS program.

Examination of Recovered Particles

Techniques that were developed to study the fate of inorganic and organic fibers in the lung have also been found to be useful in detecting changes to quartz particles after residence in the lung (Hesterburg et al., 1996a, 1996b). Plasma ashing is considered the best method for isolating particles from lung tissue without introducing artifacts. In this technique, samples are not heated above 40°C so delicate inorganic structures remain unaltered. The residue from plasma ashing is typically composed of mineral phases and a mixture of anhydrous oxides and salts from the inorganic constituents of the tissue.

A preliminary validation study was conducted with samples of the quartz isolate and reference quartz, mixed with lung tissues harvested from untreated rats. This study shows no effects of the plasma ashing procedures on the morphological properties of the particles.

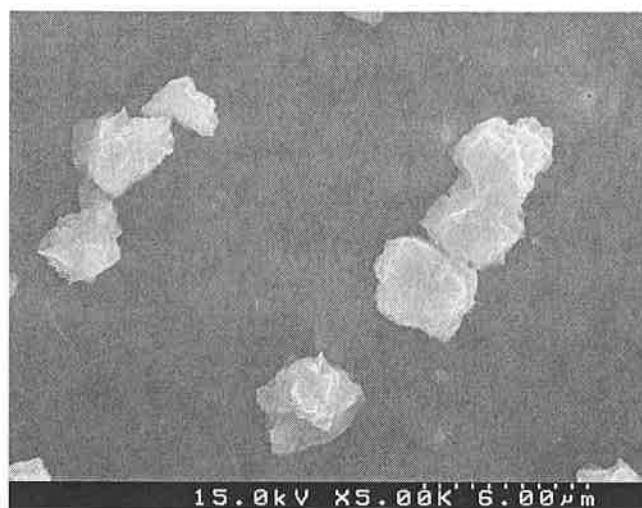
At the conclusion of the life portion of the 90-day study whole lungs were harvested from the test animals and cartilaginous airway tissue was then removed by dissection. The lungs were then freeze-dried. Standard preservation methods, using formalin and phosphate buffers, were avoided so as not to alter the particle surfaces. Lung samples included lungs from untreated animals (air control) and saline-instilled control animals, Quartz Isolate 2004-instilled samples, and Positive Control 2004-instilled samples. In each case, a 100-mg lung sample was ashed in an oxygen plasma using an LTA-504 low-temperature oxygen plasma asher (LFE Plasma Systems, Clinton, MA) for 16 h at a chamber pressure of 1 mm mercury. The resulting ash was dispersed in 100 ml hot water. An aliquot of the suspended ash was then filtered onto a $0.2\text{-}\mu\text{m}$ pore diameter Nuclepore polycarbonate membrane filter. The filtered ash was then mounted on an SEM stub, carbon coated in a vacuum evaporator, and placed in an SEM for imaging. SEM photomicrographs were examined to determine whether there were any differences between pre- and post-instillation quartz particle surface morphology and particle size. Semi-quantitative analysis of 10 particles from each lung of each of six animals was carried out for seven major oxides, using the energy-dispersive spectroscopy (EDS) function of the microscope. The same EDS analyses were also performed on 10 particles from each of the stock samples of Quartz Isolate 2001 and Quartz Isolate 2004.

RESULTS AND DISCUSSION

The 28-day and 90-day studies show significant toxicological differences between the reference quartz, composed of crushed DQ12, and quartz isolates, with occluded surfaces. These differences are paralleled by striking differences in the physicochemical nature of the respective quartz particles.

Table 1 summarizes the chemical analysis of the test materials used in the 28-day and 90-day studies. The chemistry of each quartz isolate was used to confirm its mineralogy as determined by x-ray diffraction analysis. These data show that the chemical compositions of the two quartz isolates are very similar, as are the chemical compositions of the two reference quartz samples. The data also shows that the chemical compositions of the quartz isolates differ significantly from those of the reference quartz samples. Some of these differences are due to the presence of residual montmorillonite and other trace minerals associated with the quartz isolates.

The physical properties of the test materials used in these studies are summarized in Table 2. SEM analysis shows the MMGD of the Quartz Isolate 2001 to be $3.69\text{ }\mu\text{m}$, which is nearly 3 times larger than the $1.31\text{ }\mu\text{m}$ MMGD of the reference quartz used in the 28-day study. Particle size is known to play a significant role in the toxicity of crystalline silica. Due to the



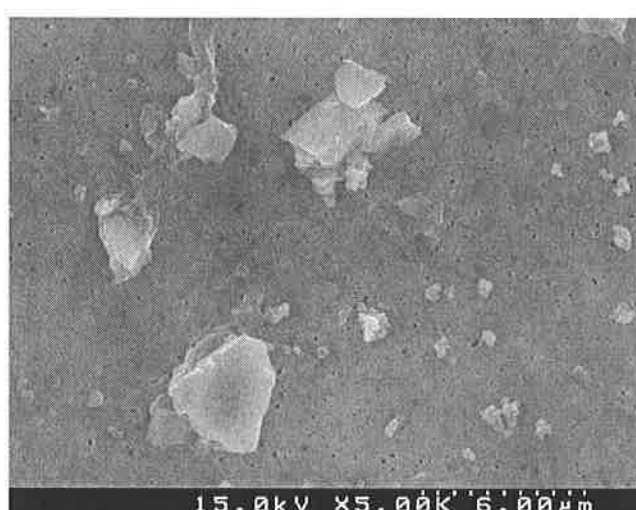
(a)



(a)



(b)



(b)

FIG. 4. (a) SEM photomicrograph of quartz isolate before instillation in rat lung (5000 $\times$). (b) SEM photomicrograph of quartz isolate recovered from rat lung after 90 days by low-temperature plasma ashing (5000 $\times$).

FIG. 5. (a) SEM photomicrograph of reference quartz before instillation in rat lung (5000 $\times$). (b) SEM photomicrograph of reference quartz recovered from rat lung after 90 days by low-temperature plasma ashing (5000 $\times$).

large difference in particle size between the test materials used in the 28-day study, it was not possible to determine whether the results of the study were due to differences in the toxicity of the quartz species, or related to their particle size. To eliminate this problem, the quartz test materials used in the 90-day study were selected to have similar particle sizes. The MMGD of Quartz Isolate 2004 was 3.58 μm , which compared well with the MMGD of 3.01 μm for the reference quartz. The Bayertitan T titanium dioxide, used to create the positive control material, had an MMGD of 0.81 μm .

Recent research has shown that sample surface area, sample particle size, and sample particle number, rather than sample

weight (mass), are the proper metrics in toxicological studies of particulate materials (Oberdörster et al., 2005; Stoeger et al., 2006; Wittmaack, 2007). Although surface area may provide a useful metric when dealing with pure test substances, the presence of materials having very large surface areas, such as montmorillonite in the quartz isolates, can overwhelm the contribution of other materials, making data interpretation problematic. Further, use of the standard BET surface area measurement does not reflect the actual surface area of water-expandable minerals, such as montmorillonite, when they are used for in vivo toxicological studies. As previously

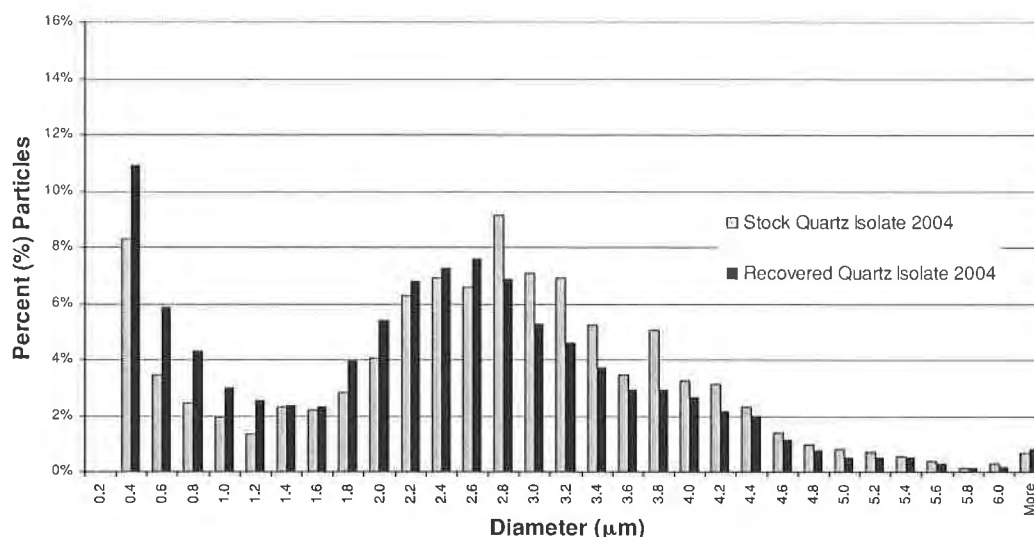


FIG. 6. Recovered Quartz Isolate 2004 particle diameter distribution vs. stock material. Median diameter: stock particles, 2.6 μm ; recovered particles, 2.3 μm .

discussed, the surface area values from the EGME method are considered to be a more accurate measure of surface area when dealing with expandable-mineral-containing materials, such as the quartz isolates used in these studies. The surface area data, presented in Table 2, show the dramatic difference between the results obtained using these two methods. These data also show the significant difference in surface area between the reference quartz and quartz isolate test materials.

XRD analysis of Reference Quartz 2001 and Reference Quartz 2004 showed these materials to be mineralogically identical, with both materials containing 87% quartz. Neither kaolin nor rubber contamination, from the grinding process, was detected in either material as previously reported (Robok, 1973). Though not absolutely identical, Quartz Isolate 2001 and Quartz Isolate 2004 have very similar mineralogical compositions containing 62% and 60% quartz, respectively.

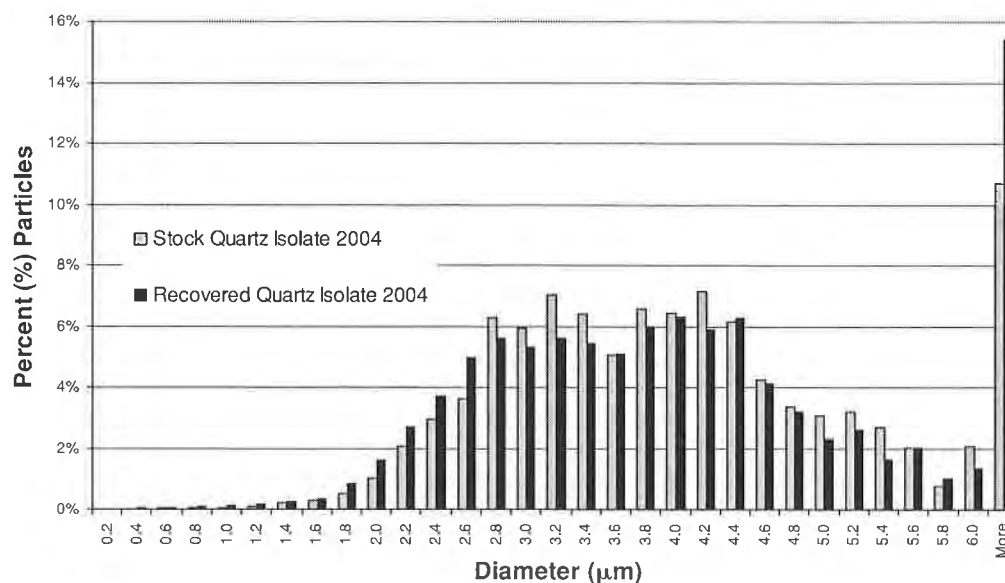


FIG. 7. Recovered Quartz Isolate 2004 particle mass distribution vs. stock material. Median diameter: stock particles, 3.8 μm ; recovered particles, 3.8 μm .

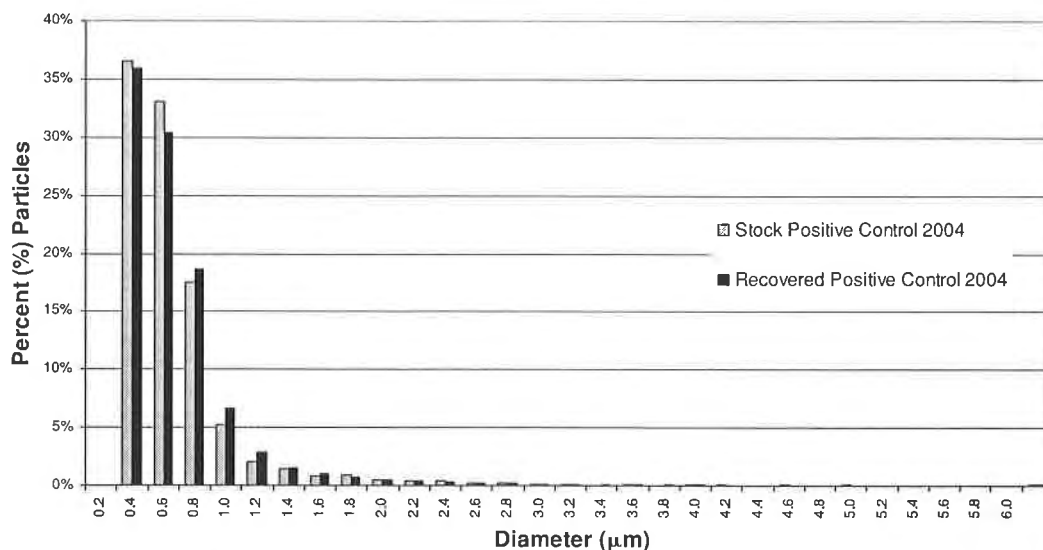


FIG. 8. Recovered Positive Control 2004 particle diameter distribution vs. stock material. Median diameter: stock particles, 0.5 μm ; recovered particles, 0.5 μm .

Chemical analysis of the two reference quartzes showed that they were both composed of 99.3% silica. The lack of correlation of these results with the XRD mineralogical results indicates either that the crystallinity of both reference quartz samples is significantly less than that of the NIST SRM 1878a reference standard or that they may contain 13% undefined silica. In consideration of the method used to prepare these samples, the authors feel it is likely that the undefined silica, quantified by XRD, may actually be composed of quartz particles whose crystalline structure has been altered by the crushing process. These particles yield XRD patterns that differ in crucial details from the pattern of the NIST SRM 1878a standard quartz. In either case, this indicates that NIST SRM 1878a, although commonly used as a calibration standard for toxicological studies, may not be the appropriate standard to use for all quartz studies.

XRD analysis also revealed that the domain size and crystallinity index values of Quartz Isolate 2001 and 2004 are 0.085 $\mu\text{m}/1.6$ and 0.076 $\mu\text{m}/1.6$, respectively. The domain size and crystallinity index values of Reference Quartz 2001 and 2004 are 0.16 $\mu\text{m}/6.0$ and 0.19 $\mu\text{m}/5.0$, respectively. These results show a significant, fundamental difference in the characteristics of the crystal structure between the quartz isolate and reference quartz materials.

A unique characteristic of quartz is that it undergoes a reversible, crystallographic phase transition from α quartz to β quartz at 573°C. This transition can be detected using DSC methods. A DSC scan of Reference Quartz 2004 clearly showed an α quartz to β quartz transition at 573°C. This transition was confirmed by high temperature XRD analysis. In contrast, DSC analysis of Quartz Isolate 2004 did not reveal any indication of an α quartz to β quartz phase transition even when the sample was heated to 775°C. However, when 13% Reference Quartz

2004 was mixed with 87% Quartz Isolate 2004 the resulting DSC plot did show the reversible $\alpha \leftrightarrow \beta$ transition at 573°C. As a result, a high-temperature XRD study was conducted to investigate this unusual thermal behavior of the quartz isolates. When a sample of Quartz Isolate 2004 was heated to well above the $\alpha \leftrightarrow \beta$ phase transition temperature, the resulting XRD pattern showed that the quartz was in the β form. This confirmed that the phase transition did indeed take place, even though it was undetected in the DSC study. This same behavior has previously been observed in polycrystalline quartz from soils (Drees et al., 1989). The lack of an $\alpha \leftrightarrow \beta$ transition endotherm in this type of quartz may be attributed either to a very high degree of crystal lattice distortion, resulting from impurities, or to the presence of many very small domains, all with slightly different transition temperatures.

These data reveal that the nature of the internal crystal organization of the reference quartz is significantly different from that of the quartz isolates. The domain size of the quartz isolates is quite small, showing that these crystals have numerous disruptions that affect the crystalline continuity within a single particle. In contrast, the reference quartz has a much larger domain size indicating that there are significantly fewer disruptions within the crystal structure of each particle. The thermal data also show that, in contrast to the particles of reference quartz, the quartz isolate particles are not acting as single crystals but, rather, as assemblages of numerous smaller crystals.

SEM photomicrographs show that the reference quartz is composed of highly angular, crushed fragments of larger quartz crystals (Figure 1). In contrast, SEM photomicrographs of the quartz isolate show it to be composed of agglomerates of unbroken quartz intergrown with montmorillonite and undefined silica. Additionally, the quartz isolate particles appear to have

filmy edges that may be an expression of thin layers of montmorillonite that coat the particles (Figure 2).

The results of ESR testing showed that Quartz Isolate 2004, Reference Quartz 2004, and Min-U-Sil 5 all produced ESR spectra that indicate they possess the ability to generate Si (Si• and SiO•) and long-lived OH• free radicals. In wet preparations, the OH• radical peak heights in these ESR spectra show that the relative ability to generate free radicals was in the order of Reference Quartz 2004 = Min-U-Sil 5 > Quartz Isolate 2004. The Si radicals peak heights show an order of Quartz Isolate 2004 > Reference Quartz 2004 = Min-U-Sil 5 (Table 3). The addition of deferoxamine greatly reduced or eliminated the induced free radical signal from all the samples tested. Many earlier studies relate the intensity of the ESR signals for OH•, Si•, and SiO• directly to biological activity (Fubini et al., 1990; Castranova et al., 1996a). The results of this study indicate that this relationship appears to be valid only when dealing with pure quartz, however. While the quartz isolate in the present study generates a significant ESR signal, the results of the toxicological studies show that it is significantly less biologically active than the reference quartz samples (Creutzenberg, 2008). The explanation for this apparent discrepancy may lie in the presence of Fe²⁺ and Fe³⁺ ions in the residual montmorillonite associated with the quartz isolates, which may create radicals through a Fenton reaction. Apparently, these radicals are not as biologically active as the radicals generated on the fractured surfaces of the reference quartz.

Castranova (1997), Fubini (1998), and others have shown that there is a relative decline of the ESR signal and biological toxicity of crushed quartz with time. After the initial dry ESR analyses, the Quartz Isolate 2004, Reference Quartz 2004, and Min-U-Sil 5 samples were each crushed for 10 min in a Fritsch IEC centrifugal ball mill. The resulting crushed samples were immediately reexamined by ESR. The ESR peak height for all three quartz samples increased slightly. This experiment shows that the strength of the ESR signal may not be indicative of relative biological toxicity. Further, when dealing with materials in which quartz particles are intimately associated with other minerals, such as the quartz isolates, the ESR data could be misleading and may not be a predictor of biological activity.

Some researchers feel that the electrical potential of the surface of respirable-size particles, as represented by their zeta potential, may be an indicator of their toxicological potential. The results of zeta potential measurements of the test materials in the present studies are presented in Figure 3. It is apparent that the patterns of the zeta potential plots of the quartz isolate samples are different from those of the other test materials. Depending on pH, the zeta potential of Reference Quartz 2001 and 2004 is from 3 to 57% higher than that of Quartz Isolates 2001 and 2004 respectively. This indicates that the reference quartzes have a much more electrically reactive surface than do the quartz isolates.

SEM photomicrographs of quartz isolate and reference quartz before instillation (stock material) and after recovery from rat

lungs from the 90-day study are shown in Figure 4, a and b, and Figure 5, a and b, respectively. These figures show that the surface morphology of both quartz materials was unchanged, either by 90 days of residence in the rat lungs or by the plasma ashing technique used to recover the particles.

SEM images of the Quartz Isolate 2004 and Positive Control 2004 samples were also evaluated to determine particle size distribution, prior to instillation and following recovery from rat lungs. Particles recovered from the Quartz Isolate 2004-exposed lungs appear to show an increase in the number of fine particles compared to the stock material (Figure 6). The median diameter of the recovered Quartz Isolate 2004, based on particle number, is 2.3 μm , compared to 2.6 μm for the stock material. The difference between these two materials is most pronounced in particles with diameters between 3.8 and 2.8 μm , and less than 1.4 μm . Increased lung inflammation is known to contribute to an increase in the amount of inorganic fine particulate residue present after low-temperature plasma ashing. The presence of this residue, associated with lung injury, may account for at least a portion of the difference in particle size distribution between the stock and recovered Quartz Isolate 2004. There was no difference in the mass median diameter between the recovered Quartz Isolate 2004 and the stock Quartz Isolate 2004 (Figure 7). The mass of the finer particles in the recovered material was balanced by the occurrence of a few larger more massive particles.

The particle size distribution of both the positive control stock material and the recovered positive control material, based on particle number, is dominated by the particles of titanium dioxide used as the diluent in their preparation (Figure 8). The median diameter of both positive control materials, and the titanium dioxide, is 0.5 μm . The mass median diameters of the positive control stock and recovered materials were 3.1 and 3.4 μm , respectively. The smaller mass median diameter of the stock material may reflect the affects of the ultrasonic processing, which was necessary to break up aggregates of titanium dioxide in that material, prior to measurement, rather than being indicative of any changes occurring in the lung. It does not appear that there was any meaningful change in the particle size distribution of the positive control material after 90 days in rat lungs.

The results of the EDS analysis of the Quartz Isolate 2004, recovered from rat lungs, and from the Quartz Isolate 2004 stock are summarized in Table 4. Also included in this table are wet chemical analysis results of the stock Quartz Isolate 2004. The analyses are normalized to 100% for the seven oxides reported. The EDS results for the stock Quartz Isolate 2004 compares well with the wet chemical analysis data. There is no significant difference in the chemistry of the recovered quartz isolate particles and the stock material. The lack of change in the aluminum oxide and magnesium oxide values is evidence that no clay is being lost from the particles during their 90-day residence time in the animal lung.

The toxicological studies showed a noticeable initial inflammation with the reference quartz, quartz isolate, and clay isolate test materials. In the 28-day study the initial inflammatory

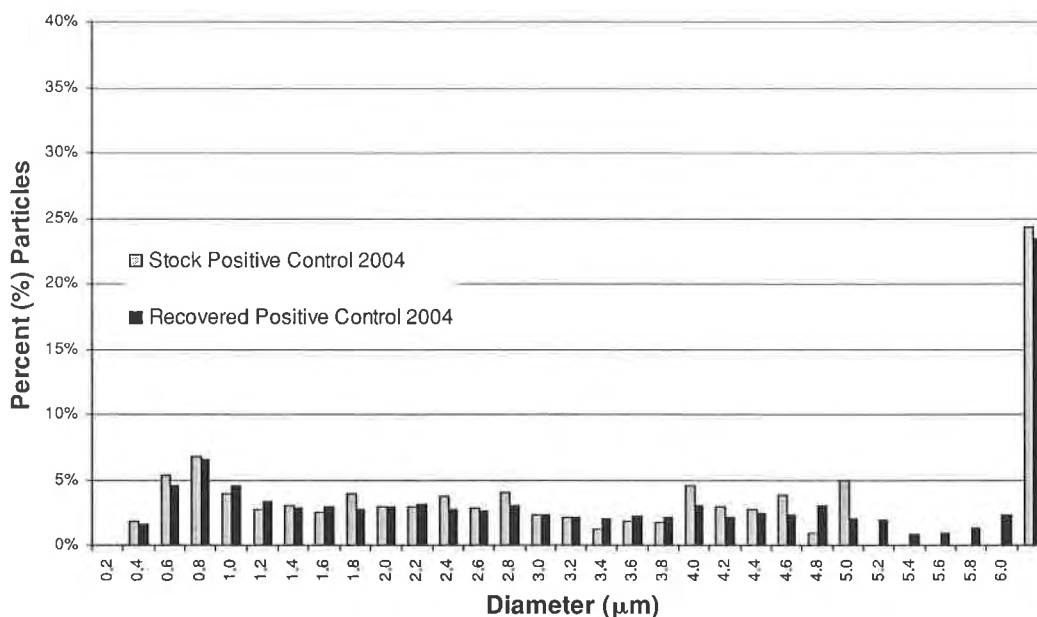


FIG. 9. Recovered Positive Control 2004 particle mass distribution vs. stock material. Median diameter: stock particles, 3.1 μm ; recovered particles, 3.4 μm .

TABLE 3
Electron spin resonance of Quartz Isolate 2004 and Reference Quartz 2004

Test material	Wet ^a		Dry ^b unground	Dry ^c ground
	Si radicals peak height	OH• radical quartet peak height	Si radicals peak height	Si radicals peak height
Fenton reaction: FeSO ₄ + H ₂ O ₂ control	None	Very strong ^d	—	—
Min-U-Sil 5 crushed quartz	None	65	21	32
Reference Quartz 2004	None	65	31	44
Quartz Isolate 2004 before filtration	Very low	50	90	131
Quartz Isolate 2004 after filtration ^e	None	50	—	—

^aTests conducted using 10 mg samples in aqueous suspension. DMPO added for ESR signal generation.

^bTests conducted using 40 mg dry powder samples.

^cTests conducted using 40 mg dry powder samples that had been crushed in a Fritsch IEC centrifugal ball mill for 10 min immediately prior to testing.

^dNot specifically defined but on the order of 100× greater than the values for the test materials.

^eSamples filtered through 0.45- μm nitrocellulose filter to remove quartz particulate material.

TABLE 4
EDS chemical analysis of stock Quartz Isolate 2004 and Quartz Isolate 2004 recovered from rat lungs after 90 days by plasma ashing

Sample	Na ₂ O	MgO	Al ₂ O ₃	SiO ₂	K ₂ O	CaO	Fe ₂ O ₃
EDS analysis results							
Stock Quartz Isolate 2004	0.81	1.28	5.94	89.71	0.21	0.61	1.46
Recovered Quartz Isolate 2004 (Average of 6 animals)	0.88	1.3	6.17	89.41	0.51	0.32	1.42
Wet chemical analysis results							
Stock Quartz Isolate 2004	0.79	0.78	7.63	88.44	0.19	0.75	1.4

response to the clay isolate was equal to that of the reference quartz but decreased significantly by day 3 postdosing. In both the 28-day and 90-day studies the inflammation from the reference quartz samples persisted and increased over time. In contrast, the inflammation from the quartz isolates declined significantly over the course of the studies, though not as rapidly or to the degree found with the clay isolate. The presence of montmorillonite in the quartz isolates and the similarity of the inflammatory response profile between the quartz isolates and the clay isolate suggest that montmorillonite plays a role in the quartz isolate's inflammatory response. The amount of inflammation, as well as the progression of the inflammation over time, attributable to either the montmorillonite or quartz in the quartz isolate has yet to be determined.

SUMMARY AND CONCLUSIONS

Physicochemical and mineralogical characterization tests conducted on the quartz samples used in the 28-day and 90-day rat intratracheal instillation toxicology studies show that there are significant differences between the chemical, physical, and mineralogical properties of the crushed reference quartz and the quartz isolates with occluded surfaces. These differences are particularly striking when viewed in the context of the results of the toxicological studies, which clearly showed that the quartz isolates produced a significantly lower degree of inflammatory response than the reference quartz at the termination of the studies. The physicochemical and mineralogical differences of the test materials used in these studies provide useful information that helps to elucidate their toxicological differences.

In the published literature on quartz toxicology the quartz materials that have been used have not always been properly or thoroughly characterized. The results presented here clearly show that there can be significant differences between quartz species. The importance of a comprehensive characterization of any test substance, especially those that contain quartz, cannot be overstated. Without proper and thorough physicochemical and mineralogical characterization of mineral test materials used in toxicology studies, it may be impossible to draw meaningful conclusions from resulting toxicological data.

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Attachment 3

Toxicity of a Quartz with Occluded Surfaces in a 90-Day Intratracheal Instillation Study in Rats

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This 90-day study was aimed at characterizing the differences in biological activity between a crystalline ground reference quartz (DQ12) and a quartz with occluded surfaces (quartz isolate) obtained from a clay deposit formed 110 to 112 million years ago. In different test groups, rats were dosed with the same total mass and quartz level by intratracheal instillation, with a total high dose of 15.2 mg/kg (body weight, bw) or approximately 4.7 mg/rat of each quartz species in a saline suspension. The reference quartz was mixed with titanium dioxide to achieve a positive control mixture, which contained the same quartz content as in the quartz isolate. At 3 days post dosing, both quartz groups showed a significant inflammatory response based on total and differential cell counts from bronchoalveolar lavageate (BAL) analysis. At 28 and 90 days, the quartz isolate values were no longer statistically different from vehicle control group values; however, the positive control group values were approximately 12 and 65 times greater than those of the control group, respectively. After 28 days, histopathological evaluation showed moderate effects in the quartz isolate group compared to the saline control animals. These effects did not progress in severity at 90 days. In contrast, the positive control group exhibited more severe effects than the quartz isolate group and these effects showed a progression to a persistent and self-perpetuating inflammatory state. The toxicological properties of quartz particles can vary significantly dependent on their surface characteristics. Toxicity can range from a high-dose-induced, modest, transient inflammation from quartz with occluded surfaces, to a severe and persistent inflammatory state caused by ground quartz with fractured surfaces.

In its 1997 monograph, the International Agency for Research on Cancer (IARC) assessed the carcinogenic potential of respirable crystalline silica using data from human epidemiological and chronic animal inhalation studies. Their general conclusion, that respirable crystalline silica is a human carcinogen, was supplemented by the restrictive statement “that carcinogenicity in humans was not detected in all industrial circumstances studied. Carcinogenicity may be dependent on inherent characteristics of

the crystalline silica or external factors affecting its biological activity or distribution of its polymorphs.”

Research conducted by Hemenway (1986, 1990) has emphasized the crucial role of specific particle properties, such as surface area, protein binding ability, and solubility for initiation of an inflammatory response in lungs. In these rat studies, different lung responses were obtained from α -cristobalite and various types of α -quartz. An important outcome of these studies was that some quartz types show a low and transient toxicological response after uptake in the lungs, as defined by inflammatory cells in the bronchoalveolar lavage (BAL) fluid. Mechanistic studies conducted by Donaldson and Borm (1998) have emphasized that the toxic impact of quartz is related to surface reactivity and may vary dramatically depending on the origin of the quartz sample. These studies also showed that the mechanism of quartz toxicity includes the generation of free radicals and reactive oxygen species. Additionally, Fubini (1998) has found

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that the biological response to quartz can be reduced by substances affecting the quartz surface. These studies point out the need to better understand the relationship between quartz types and toxicological responses.

The current study was undertaken to investigate the intrinsic lung toxicity of a respirable-size quartz, with naturally occluded surfaces (quartz isolate), that had been isolated from a sodium bentonite clay deposit which formed 110 to 112 million years ago. The isolation techniques used to obtain the quartz isolate enriched the quartz content while avoiding the use of chemicals or mechanical grinding that might artificially affect the occluded surfaces of the quartz particles. Lung effects were characterized using BAL and histopathological parameters at 3, 28, and 90 days after intratracheal instillation of test materials into rats. The effects were compared to those of a reference quartz (DQ12) with a well-characterized biological activity.

This study was preceded by a 28-day intratracheal rat instillation study that investigated the inflammatory and genotoxic effects of similar quartz isolate and reference quartz (DQ12 fine size) samples (Creutzenberg et al., 2003). In the 28-day study, the intrinsic toxicity of the quartz isolate sample (geometric mean: 3.70) was compared to that of the reference quartz (geometric mean: 1.31). The inflammatory endpoints, bronchoalveolar lavage (BAL) and histopathology of lungs, revealed a moderate effect induced by the quartz isolate, whereas the reference quartz showed a strong effect, e.g., 36% PMNs in BAL fluid on day 28 after instillation versus 65%, respectively.

Since it is known that particle diameter can play a significant role in the toxicity of crystalline silica, it could not be determined whether the results from the 28-day study were attributable to particle size differences or to other factors. In the current study, a quartz isolate and a reference quartz with similar particle size distributions, geometric means of 3.58 and 3.01 μm , respectively, were used. A 3-mo postdosing observation period was also used, which gave a broader base for evaluating the effects on the lungs. This study also included lung retention measurements of the test materials, which provided some information on the toxicokinetics and effects on the morphological properties of the quartz species after 90 days in the lungs.

MATERIALS AND METHODS

Test Materials

Quartz Isolate

A quartz isolate sample was extracted from crude, undried and unprocessed bentonite clay obtained from the 100- to 112-million-year-old, lower Cretaceous age, northeastern Wyoming Newcastle formation. As used here, the term "bentonite" is restricted to a geologic deposit derived from the alteration of silica-rich, glassy volcanic ash that fell into a shallow sea, dissolved rapidly, and re-precipitated primarily as the aluminosilicate mineral montmorillonite. Excess silica resulting from this re-precipitation formed quartz and the amorphous silica hydrate mineral opal. Quartz formed in this manner bears the name "au-

thigenic" to differentiate it from quartz resulting from crystallization from the molten state. Authigenic quartz, in bentonite, exhibits a low degree of internal crystal organization as compared to quartz formed at high temperature, which exhibits a much higher degree of internal crystal organization. The surfaces of quartz particles formed in this manner are also intimately associated with, and occluded by, coatings of the clay and amorphous silica with which they were formed, as well as water and metal ions such as Al^{3+} and $\text{Fe}^{2+/3+}$.

The quartz isolate was gently extracted from an aqueous suspension of crude, unground bentonite clay, without the addition of any chemicals, using a centrifugal method designed to avoid alteration of the quartz particle surfaces. The physicochemical properties of the quartz isolate obtained in this manner are presented in Table 1. The quartz isolate particles consist of a sedimentary, chert-like, microcrystalline form of quartz formed at low temperature in the presence of liquid water, intergrown with the amorphous hydrous mineral opal and the clay mineral montmorillonite. Figure 1 shows a scanning electron photomicrograph of a representative sample of quartz isolate. The convoluted nature of the quartz particles and their occluding coating can clearly be seen in this figure. The quartz content of the quartz isolate was 60%. Prior to use, the quartz isolate was stored at 3°C as a 34.8% suspension in deionized water.

Reference Quartz

The quartz sample DQ12 middle size was obtained from Bergbauforschung, Essen, Germany. This material, which was produced by mechanical grinding, was a mid-size fraction of a classified Dörentrop quartz sample, and was used as the reference quartz. It is thought to consist entirely of broken fragments of particles of single crystal α -quartz. α -Quartz is a polymorph of quartz crystallized from an igneous melt. The physicochemical properties of the reference quartz are presented in Table 1. Figure 2 shows a scanning electron photomicrograph of a representative sample of the reference quartz material. The clean, flat surfaces and highly angular nature of the particles of this material can clearly be seen here.

Titanium Dioxide

The titanium dioxide sample Bayertitan T (TiO_2) was obtained from Bayer AG, Krefeld, Germany. Based upon information obtained from the supplier, the chemical composition is 98.174% TiO_2 , the annealing weight loss is 0.22%, and the mineralogical composition is 99.5% rutile polymorph. The density of this material is 4.26 g/cm^3 . Additional physicochemical properties of the titanium dioxide are also presented in Table 1. Figure 3 shows a scanning electron photomicrograph of a representative sample of the titanium dioxide.

Animals and General Procedures

Male Wistar rats (strain CrI:WU), approximately 6 wk of age at delivery, were obtained from Charles River Deutschland, Sulzfeld, Germany. The age of the animals at the start

TABLE 1
Physicochemical properties of test materials used in 90-day study

Characteristic	Material		
	Quartz isolate	Reference quartz	TiO ₂
Surface area			
By BET method ^a (m ² /g)	21.2	3.7	1.9
By EGME method ^b (m ² /g)	213	5.6	21.7
XRD average domain size ^c (μm)	0.076	0.19	—
XRD mineralogy			
Quartz (%) (per NIST 1878A standard reference)	60	87	0
Montmorillonite (%)	30	0	0
Undefined amorphous silica (%)	8	13	0
Analcime (%)	2	0	0
Rutile (%)	0	0	99.5
Major element composition ^d (normalized)			
SiO ₂ (%)	87.88	99.30	—
Al ₂ O ₃ (%)	7.58	0.00	—
Fe ₂ O ₃ (%)	1.39	0.04	—
MnO (%)	0.00	0.00	—
Na ₂ O (%)	0.79	0.21	—
K ₂ O (%)	0.19	0.05	—
MgO (%)	0.78	0.00	—
CaO (%)	0.75	0.00	—
TiO ₂ (%)	0.08	0.02	98.17
P ₂ O ₅ (%)	0.04	0.02	—
F (%)	0.07	0.02	—
S (%)	0.01	0.01	—
Total C (%)	0.39	0.33	—
Organic carbon (%)	0.05	0.00	—
Particle size by scanning electron microscopy			
Mass mean geometric diameter (μm)	3.58	3.01	1.80
Geometric Standard deviation	1.30	1.53	1.90
Differential scanning calorimetry (DSC)			
α Quartz → β quartz transition temperature (°C)	Not detected	573	—
β Quartz → α quartz transition	None	Yes	—
Thermal XRD Analysis			
α Quartz → β quartz transition temperature (°C)	by 675	573	—
Zeta potential ^e			
At pH 4 (mV)	−20.49	−35.42	−11.47
At pH 6 (mV)	−25.32	−30.49	−35.08
At pH 8 (mV)	−31.14	−39.09	−43.03
Electron spin resonance (ESR) ^f			
Hydroxyl radical (•OH) relative peak height	54	65	—
Endotoxin content			
ng/g (EU/g)	100 (1000)	1.78 (17.8)	<0.562 (<5.62)

^aBrunauer–Emmet–Teller method (Brunauer et al., 1938).

^bEthylene glycol monoethyl ether method (Carter et al., 1965; Bower and Goertzen, 1959).

^cUsing the Warren–Averbach procedure (Warren and Averbach, 1953).

^dUsing standard lithium metaborate/tetraborate fusion methods with inductively coupled plasma (ICP) analysis.

^eA measurement of the magnitude of surface charge.

^fA measurement of the presence of free radicals.

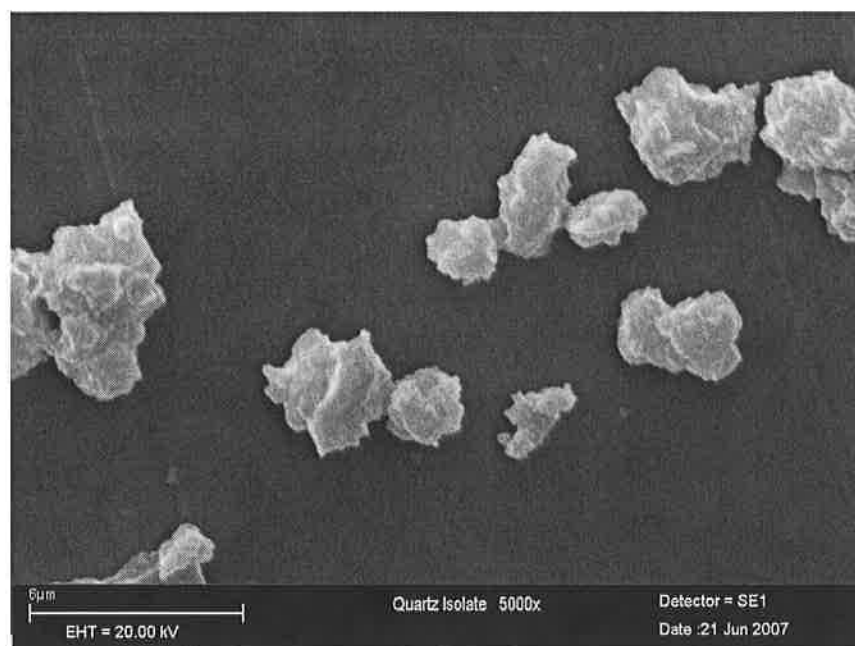


FIG. 1. Quartz isolate (5000×).

of treatment was approximately 11 wk and their weight was approximately 300 g. Rats were dosed with the test materials by intratracheal instillation. Males were preferred to females because literature data indicate a higher sensitivity in the fibrotic response induced by crystalline silica (Honnon & Porcher, 2000).

Study Design and Dosing Scheme

The test materials were administered on a body weight basis (Table 2). The quartz isolate and positive control were dosed at 15.2 mg/kg body weight. Aliquots of 3.8 mg/kg body weight, suspended in approximately 0.3 ml saline, were administered intratracheally in 4 consecutive daily applications. To minimize

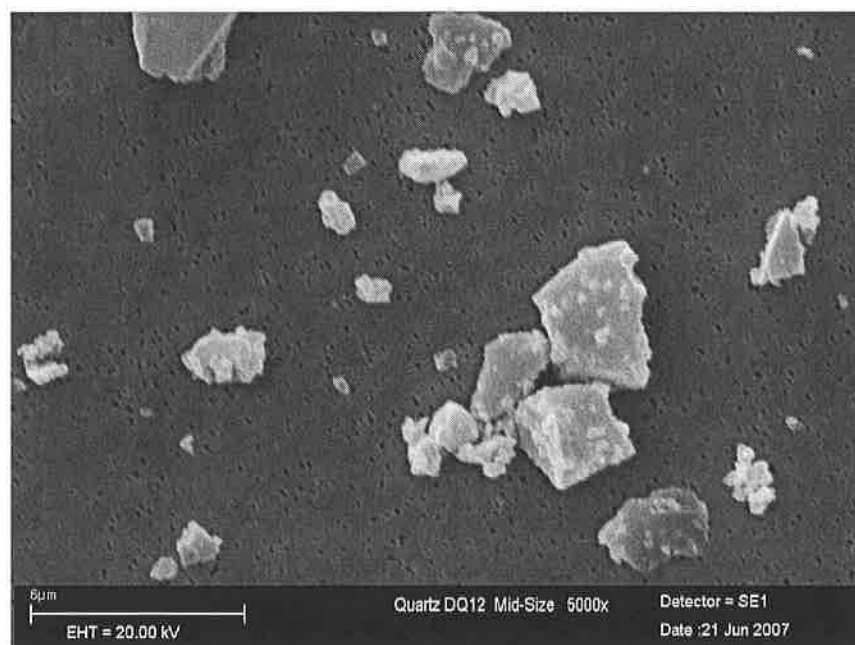
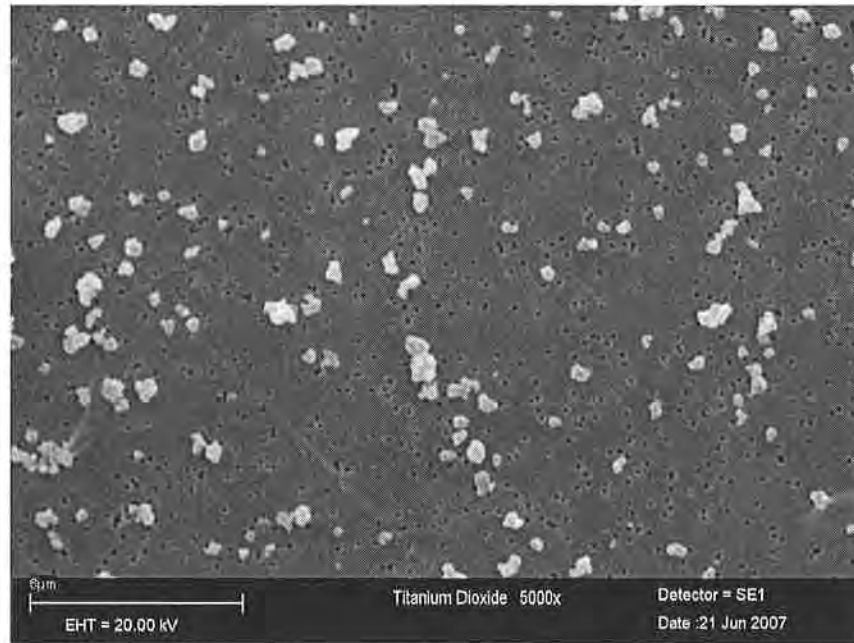


FIG. 2. Reference quartz (5000×).

FIG. 3. Titanium dioxide Bayertitan T (5000 $\times$).

particle agglomeration, the suspensions were shaken moderately prior to instillation but did not undergo an ultrasonic treatment. The study was designed to dose the animals, in the different test groups, with the same total mass and the same quartz level. The quartz content of the quartz isolate was 60% while the quartz content of the reference quartz was 87%. Thus, in order to deliver the same mass dose as well as the same dose of quartz, the reference quartz had to be supplemented with additional particulate material to produce a positive control mixture that contained 60% quartz. Fine titanium dioxide particles were selected because they represent particles that have been used and recognized for decades, in inhalation toxicology, as a negative control particle (Ferin et al., 1965; Ferin, 1994). In the previously conducted 28-day study (Creutzenberg et al., 2003), separate

groups of rats were dosed with reference quartz (quartz DQ12 fine size) or a mixture of 71.25% reference quartz and 28.75% titanium dioxide. In both cases the total amount of reference quartz was identical. The results of the 28-day study clearly showed that, at all time periods, the effects caused by reference quartz alone were essentially identical to those seen in rats dosed with the mixture of reference quartz and titanium dioxide. Based on these results, it was concluded that the presence of titanium dioxide would not alter the toxicological effects of the reference quartz in the present study. Therefore, in the present study, the effects seen in the animals dosed with the positive control mixture are assumed to be attributable to the reference quartz.

The quartz isolate was supplied as a 38.8% aqueous suspension in deionized water. Sodium chloride was added to reach a

TABLE 2
Study design

Group	Test material	Cumulative dose (mg/kg) ^a	Cumulative dose: quartz (mg/kg) ^a	Cumulative dose: other particles (mg/kg) ^a	Number of animals per group
1	Saline	Vehicle only	Vehicle only	Vehicle only	30
2	Quartz isolate	15.2	9.1	6.1	30
3	Positive control = 69.1% reference quartz + 30.9% titanium dioxide	10.5 Reference quartz + 4.7 titanium dioxide	9.1	1.4 + 4.7	30

^aAll groups were dosed, over 4 days, with 4 aliquots of the test materials at a volume of 0.3 ml.



FIG. 4. Quartz isolate before instillation (5000 $\times$).

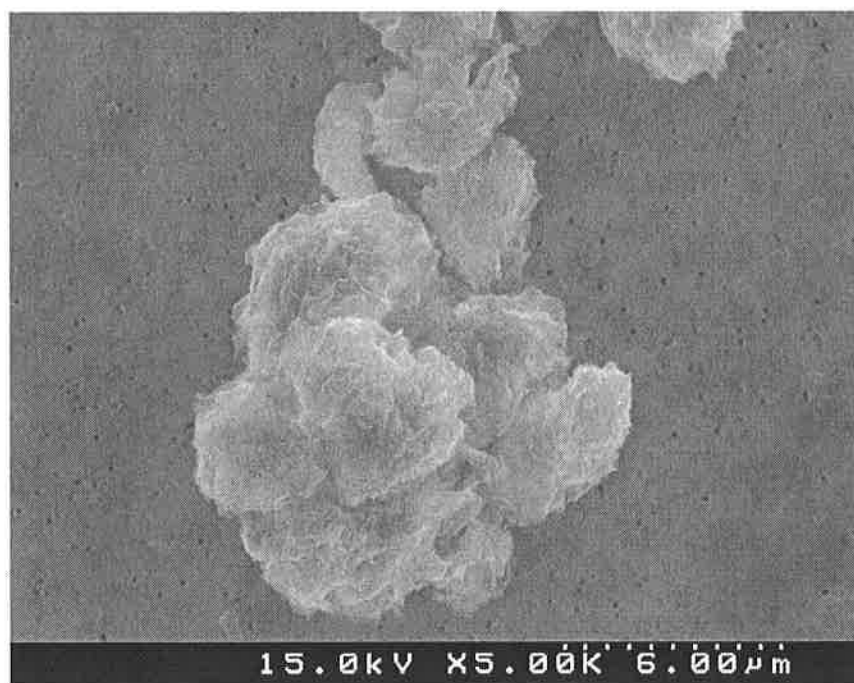


FIG. 5. Quartz isolate recovered from rat lung after 90 days by low-temperature plasma ashing (5000 $\times$).

0.9% isotonic saline concentration. The suspension was further diluted with sterile and pyrogen-free saline to reach the target concentration for dosing. Only saline was added to the positive control mixture to prepare the positive control dosing suspension. Concurrent control animals were treated with the saline vehicle only. Prior to dosing, the rats were slightly anesthetized with CO₂.

Sacrifice of Rats

Rats scheduled for bronchoalveolar lavage (BAL) were anesthetized by an intraperitoneal application of sodium pentobarbital (Narcoren at 0.1 ml per 100 g body weight) and exsanguinated by cutting the vena cava caudalis. Rats scheduled for histopathological examination were sacrificed by an overdose of carbon dioxide and subsequent bleeding via cutting the vena cava caudalis.

Endpoints

Bronchoalveolar Lavage (BAL)

On days 3, 28, and 90 posttreatment, a bronchoalveolar lavage (BAL) was performed and the lavage fluid collected for differential cell counts and analysis of biochemical parameters (see Tables 3 and 4). With minor alterations, the procedure followed the method described by Henderson et al. (1987). Following preparation, the lungs were lavaged with saline using 5 lavages of 5 ml each. The lavage fluid was collected separately for lavages 1 and 2 (first series, without massage of lungs) and for lavages 3 to 5 (second series, using gentle massage of lungs). After centrifugation of the first series, biochemical indicators relevant for diagnosis of lung damage were determined in the supernatant (lactic dehydrogenase [LDH], β -glucuronidase, total protein). The cell pellet of the first lavage series was resuspended in the residual supernatant, combined with the second lavage series, and the resulting cell pool of five lavages was used for cytology. The leukocyte concentration of the lavageate was determined using a counting chamber and two cytospin slides were prepared with a cytocentrifuge (Shandon Co., Frankfurt, Germany) for differential cell count (macrophages, neutrophils, eosinophils, lymphocytes, epithelial cells).

Histopathology

On days 28 and 90 posttreatment, histopathology of all 5 lung lobes, including bronchi and the lung-associated lymph nodes (LALN), was conducted on 5 animals per group. Lungs were inflated under a pressure of approximately 20 cm water, with 10% neutral-buffered formalin, and fixed by immersion. They were embedded in paraffin, sectioned, and stained with hematoxylin and eosin (H&E). A special stain (Masson's trichrome) was applied for diagnosis of fibrotic changes.

Morphology of Test Materials in Lungs

Lungs were harvested from dosed and undosed (reserve) rats and lyophilized 90 days after treatment of the animals.

The lungs were then ashed using a low-temperature plasma technique and the inorganic content of the ash was analyzed by scanning electron microscopy to determine general particle morphology, particle size distribution, and particle chemistry.

Statistics

Differences between groups were considered to be statistically significant at the level of $p < .05$. Data were analyzed using analysis of variance. If the group means differed significantly by the analysis of variance the means of the treated groups were

TABLE 3
Means of parameters measured in BAL fluid 3, 28, and 90 days after the last of 4 instillations

Group	Parameter	Lactic dehydrogenase (U/L)		
		Day 3	Day 28	Day 90
Saline control	Mean	52	30	38
	SD	21	7	6
	<i>n</i>	6	6	7
Quartz isolate	Mean	166	107	128
	SD	39	25	18
	<i>n</i>	6	6	7
Positive control	Mean	284***	361**	734***
	SD	132	258 [#]	132###
	<i>n</i>	6	6	7
		β -Glucuronidase (U/L)		
		Day 3	Day 28	Day 90
Saline control	Mean	0.3	0.2	0.3
	SD	0.1	0.1	0.1
	<i>n</i>	6	6	7
Quartz isolate	Mean	1.1***	0.8	1.1
	SD	0.2	0.1	0.3
	<i>n</i>	6	6	7
Positive control	Mean	1.6***	2.7***	12.6***
	SD	0.5 [#]	1.0 [#]	4.6###
	<i>n</i>	6	6	7
		Total protein (mg/L)		
		Day 3	Day 28	Day 90
Saline control	Mean	73	101	83
	SD	14	27	8
	<i>n</i>	6	6	7
Quartz isolate	Mean	204***	185	209
	SD	17	26	23
	<i>n</i>	6	6	7
Positive control	Mean	346***	512***	875***
	SD	60###	129###	182###
	<i>n</i>	6	6	7

Note. Statistics: ANOVA + Tukey test, * $P \leq 5\%$, ** $P \leq 1\%$, and *** $P \leq 0.1\%$ (vs. control); [#] $P \leq 5\%$, ^{##} $P \leq 1\%$, and ^{###} $P \leq 0.1\%$ (positive control vs. QI).

TABLE 4
Differential cell count 3, 28, and 90 days after intratracheal instillation (means)

		3 Days						
Group	Dose (mg/kg)		Leukocyte number (cells)	Macrophages (%)	Eosinophil PMNs (%)	Neutrophil PMNs (%)	Lymphocytes (%)	Cell viability (%)
Saline control	0	Mean	4.73×10^6	99.7	0.0	0.0	0.3	99
		SD	0.71×10^6	0.5	0.0	0.0	0.3	1
Quartz isolate	15.2	Mean	$12.42 \times 10^6^*$	79.9***	0.0	18.1***	2.0**	100
		SD	2.17×10^6	2.7	0.1	2.6	0.8	1
Positive control	10.5 + 4.7	Mean	$19.95 \times 10^6^{***}$	58.0***	0.0	39.0***	2.9***	100
		SD	$6.88 \times 10^6^{\#}$	7.3###	0.1	7.3###	1.2	1
28 Days								
Saline control	0	Mean	3.44×10^6	99.4	0.0	0.3	0.3	99
		SD	1.04×10^6	0.5	0.0	0.3	0.4	1
Quartz isolate	15.2	Mean	10.88×10^6	72.7***	0.3*	24.9***	2.3	99
		SD	5.16×10^6	7.3	0.2	7.1	1.1	1
Positive control	10.5 + 4.7	Mean	$40.82 \times 10^6^{***}$	49.5***	0.0	44.8***	5.8***	100
		SD	$7.55 \times 10^6^{\#}$	4.9###	0.0#	6.2###	2.0##	0
90 Days								
Saline control	0	Mean	3.34×10^6	99.3	0.0	0.1	0.6	100
		SD	0.36×10^6	0.7	0.0	0.2	0.7	0
Quartz isolate	15.2	Mean	6.81×10^6	64.5***	0.0	31.6***	4.0***	99
		SD	2.07×10^6	11.6	0.0	11.3	0.7	1
Positive control	10.5 + 4.7	Mean	$218.21 \times 10^6^{***}$	51.2***	0.0	45.8***	3.0***	100
		SD	$79.36 \times 10^6^{\#}$	6.2#	0.0	5.5##	1.2	0

Note. SD, standard deviation; $n = 6-7$ = number of animals. Statistics: ANOVA + Tukey test, * $P \leq 5\%$, ** $P \leq 1\%$, and *** $P \leq 0.1\%$ (vs. control); # $P \leq 5\%$, ## $P \leq 1\%$, and ### $P \leq 0.1\%$ (positive control vs. QI).

compared with the means of the control groups. Tukey's test was used. Incidences of histopathological microscopic findings were compared using the two-tailed Fisher test at a level of $p < .05$.

RESULTS

Survival of Animals and Clinical Observations

No deaths occurred during the course of the study. Body weights were in the normal range and treatment-related effects were not observed.

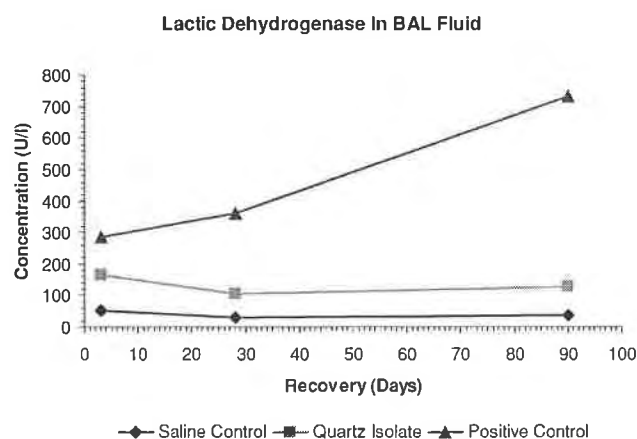


FIG. 6. Lactic dehydrogenase levels in bronchoalveolar lavage fluid measured on days 3, 28, and day 90 after end of treatment.

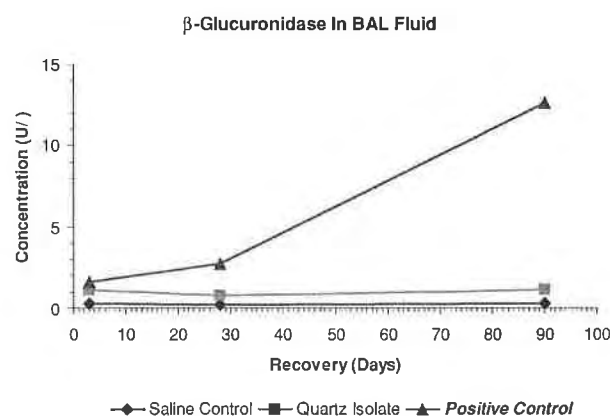


FIG. 7. β-Glucuronidase levels measured in bronchoalveolar lavage fluid on days 3, 28, and day 90 after end of treatment.

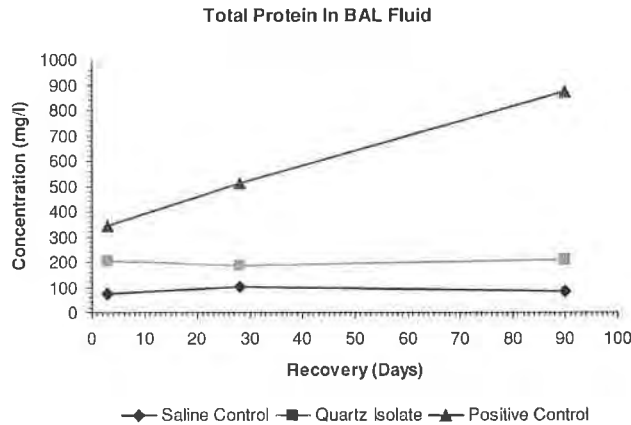


FIG. 8. Total protein levels in bronchoalveolar lavage fluid measured on days 3, 28, and day 90 after end of treatment.

BAL Enzyme and Total Protein Levels

The results of BAL fluid analysis are summarized in Tables 3 and 4. Plots of lactic dehydrogenase, β -glucuronidase and total protein are presented in Figures 6 through 8. The saline control group values were at historical levels.

At day 3, the quartz isolate group's β -glucuronidase and total protein values were statistically greater than those of the saline

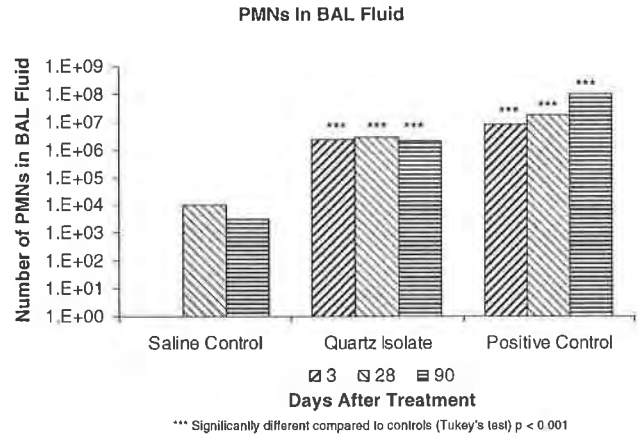


FIG. 9. Absolute numbers of polymorphonuclear neutrophils (PMNs) in bronchoalveolar lavage fluid (note log scale).

TABLE 5
Terminal body and lung wet weights 3, 28, and 90 days after the last of 4 instillations—BAL rats

Group	Dose (mg/kg)		Body weight (g)	Lung weight (g)	Lung weight/body weight × 1000
Day 3					
Saline control	0	Mean	316.3	1.1007	3.479
		Std	12.7	0.0662	0.129
Quartz isolate	15.2	Mean	317.4	*1.3298	4.191
		Std	15.4	0.0759	0.136
Positive control	10.5 + 4.7	Mean	317.4	***1.5630	***4.950
		Std	17.7	#0.2025	#0.808
Day 28					
Saline control	0	Mean	360.2	1.2076	3.350
		Std	34.4	0.1707	0.307
Quartz isolate	15.2	Mean	363.3	1.3729	*3.787
		Std	34.6	0.1335	0.286
Positive control	10.5 + 4.7	Mean	372.8	***1.8735	***5.032
		Std	16.4	###0.0771	###0.254
Day 90					
Saline control	0	Mean	422.0	1.2215	2.892
		Std	14.8	0.1094	0.194
Quartz isolate	15.2	Mean	442.9	1.4174	3.208
		Std	29.9	0.0881	0.233
Positive control	10.5 + 4.7	Mean	415.8	***2.4602	***5.908
		Std	31.0	###0.2948	###0.420

Note. Statistics: ANOVA + Tukey test, * $P \leq 5\%$, ** $P \leq 1\%$, and *** $P \leq 0.1\%$ (vs. control); # $P \leq 5\%$, ## $P \leq 1\%$, and ### $P \leq 0.1\%$ (positive control vs. QI).

TABLE 6
Histopathological findings on days 28 and 90 posttreatment

Finding	Grade	Day 28		Day 90	
		Quartz isolate	Reference quartz/TiO ₂ positive control	Quartz isolate	Reference quartz/TiO ₂ positive control
Lungs					
Multifocal alveolar accumulations of particle-laden macrophages	slight	3		5	
	moderate	2	5		4
	severe				1
Interstitial accumulations of particle-laden macrophages	very slight	1		1	
	slight	4	5	4	5
Multifocal interstitial fibro-histiocytic granulomata	slight		3		5
	moderate		1		
Multifocal alveolar lipoproteinosis ^a	slight		2		5
	moderate		3		
Multifocal interstitial (predominantly perivascular) mononuclear cell infiltrations	very slight	4		2	1
	slight	1	2	1	2
	moderate		3		2
Alveolar granulocytic infiltration	very slight	3		4	
	slight	2	1	1	2
	moderate		4		3
Multifocal bronchiolo-alveolar hyperplasia (bronchiolar type)	very slight	5	2	3	
	slight		3	1	5
Multifocal mucus (goblet) cell hyperplasia of the bronchi/bronchioles	very slight	2		1	2
	slight	1	5		3
Multifocal very slight (minimal) interstitial fibrosis ^b	very slight	5	2	4	
	slight		3		5
LALN					
Accumulation of particle-laden macrophages	(very) slight	5		3	
	moderate		4	2	
	severe		1		5
Reactive lymphoid hyperplasia	slight	1	1	3	
	moderate	1	4	2	5

^aThe pulmonary macrophages of the quartz isolate-treated group showed a marked foamy appearance, but without significant signs of degeneration and necrosis. In contrast, in the positive control group, the majority of alveolar macrophages were degenerating or necrotic, as reflected by alveolar lipoproteinosis. Lipoproteinaceous intra-alveolar material appeared to be mainly derived from decaying macrophages.

^bInterstitial fibrosis developed in close association with accumulation of (particle-laden) macrophages and inflammatory cells (PMN, mononuclear cells) and was characterized by localized discrete thickening of intra-alveolar septa due to increased deposition of collagen. In the positive control group, the interstitial fibro-histiocytic granulomas, when present, were characterized by central foci of foamy macrophages which at the periphery were rimmed by minimal amounts of collagenous (fibrotic) tissue that was continuous with the interstitium.

Total and Differential Cell Counts

The results of differential cell counts are presented in Table 4. Differential cell counts for the saline control group resulted in polymorphonuclear neutrophil (PMN) levels of 0.0% to 0.3% at 3, 28, and 90 days after the last intratracheal instillation. These values are comparable to historical values.

After 3 days, the PMN value for the quartz isolate group reached 18%, while the value for the positive control group

was 39%. The PMN values for the quartz isolate and positive control groups after 28 days were 25% and 45%, respectively. After 90 days they were 32% and 46%, respectively. These data indicate a moderate inflammatory response for the quartz isolate group and a strong response for the positive control group. When considering the large increase in the total cell number by quartz (Table 4, Fig. 9), the number of PMNs for the positive quartz control and quartz isolate (in parenthesis) show even greater

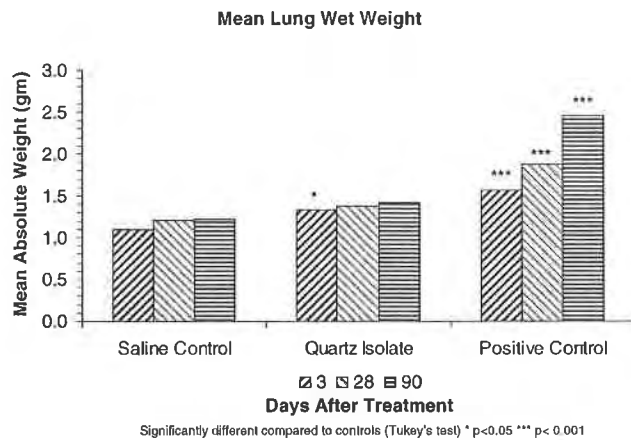


FIG. 10. Mean absolute lung wet weights, BAL rats.

differences: 7.78×10^6 (2.25×10^6) on day 3; 18.29×10^6 (2.71×10^6) on day 28; and 99.94×10^6 (2.15×10^6) on day 90. This means, that the elicited PMNs in the quartz exposed rats were manyfold higher than those of quartz isolate exposed rats: 3.5 fold on day 3; 6.8 fold on day 28; and 46 fold on day 90. These data indicate the strongest and persistent inflammatory response in the quartz positive control group, and this response was evidently considerably smaller in the quartz isolate group.

Terminal Body and Lung Wet Weights (BAL Rats)

Absolute and relative wet lung weights of BAL animals were statistically significantly elevated, in both treatment groups, after 3 and 28 days (Table 5; Figure 10). After 90 days, lung weights in the quartz isolate group were not statistically different from

those in the saline control group. By contrast, the positive control group continuously increased lung weights throughout the postexposure period and showed a high degree of statistical difference when compared to the quartz isolate and saline control groups.

Histopathology

Histopathologically, the quartz isolate group showed a very slight to slight inflammatory response compared to the saline control group (Table 6). At 28 days and 90 days, there was no progression of the effects. The positive control group, after 28 days and 90 days, exhibited a significantly stronger reaction than the quartz isolate group and the effects showed a continued progression in the intensity.

Lungs

Very slight interstitial fibrosis of the lungs was observed in the quartz isolate group in all 5 rats after 28 days. At 90 days, the incidence was 4 of 5 rats (Table 6). In contrast, all animals of the positive control group, at 28 and 90 days, showed a higher grade of interstitial fibrosis. Alveolar lipoproteinosis, which is a measure of alveolar macrophage degeneration or necrosis, was statistically significantly increased, after 28 and 90 days, in the positive control group. Alveolar lipoproteinosis was not observed in the quartz isolate or saline control groups (Table 6).

Lymph Nodes

Accumulation of particle-laden macrophages was most pronounced in the positive control group (moderate: 4/5; severe: 1/5). Particle-laden macrophages were present only to a minimal degree in rats in the quartz isolate group (very slight: 5/5).

TABLE 7
Summary of the toxicological effects of the quartz isolate and positive control

Parameter	Quartz isolate			Positive control		
	3 days post treatment	28 days post treatment	90 days post treatment	3 days post treatment	28 days post treatment	90 days post treatment
BAL of lungs						
Total protein (BAL) (ratio treatment/control)	***2.8	-1.8	-2.5	***4.7	***5.1	***10.5
Total cell number (BAL) (ratio treatment/control)	*2.6	-3.1	-2.0	***4.2	***11.9	***65.3
PMN (BAL) (% in differential cell count)	***18.1	***24.9	***31.6	***39.0	***44.8	***45.8
Lung wet weights (absolute)	*	—	—	***	***	***
Lung wet weights (relative)	—	*	—	***	***	***
Histopathology						
Inflammation	(+) to +	(+) to +		+ to ++		+ to ++
Interstitial fibrosis	(+)	(+)		+		+
Alveolar lipoproteinosis	—	—		++		+

Note. Significant effects as compared to positive control group: *5% **1% *** 0.1% (numerical values: see Tables 7–13). Histopathology: (+) very slight effect, + slight effect, ++, moderate effect, +++ high effect, — not significant.

Associated with this finding was reactive lymphoid hyperplasia, which occurred at slight to moderate degrees in 2/5 (slight: 1/5; moderate: 1/5) animals in the quartz isolate group and 5/5 (slight: 1/5; moderate: 4/5) animals in the positive control group.

DISCUSSION AND CONCLUSION

The focus of this study was to characterize differences in biological activity between different quartz species of similar particle size. A vast literature exists suggesting that (1) geologically old mixtures, (2) aging of freshly cleaved quartz (not for all varieties observed), and (3) experimental surface modification have an attenuating effect on the intrinsic toxicity of quartz-containing particles. For example, contaminants such as coal dust or metals (aluminum, iron) can diminish the toxic potential of quartz in quartz-containing materials (Fubini, 1998), and crushed quartz and aged quartz behave differently in terms of toxicological potency (Vallyathan et al., 1995; Castranova et al., 1997). The fracturing of quartz generates silicon-based free radicals that are able to produce reactive oxygen species (ROS) (Donaldson & Borm, 1998). In terms of toxicological potency, freshly ground quartz-containing materials are significantly more capable of causing lung injury than corresponding aged materials. The surfaces of freshly ground quartz particles become less active as they age due to the reaction of free radicals on the fractured surfaces with other ions or molecules. Although an aging effect may not be observed for every quartz modification, it is, nevertheless, an important parameter that must be considered in evaluating the toxic potential of quartz-containing materials from different sources.

Ross et al. (1962) exposed rats to a mixture of quartz dust and very low quartz coal dust (ground together at a ratio of 5% and 95%). Although the quartz/coal dust mixture was inhaled 16 h/day at 60 mg/m³ for 1 yr, the high level of inhaled quartz induced only little fibrosis, demonstrating the reduction of toxicity due to the accompanying coal dust. In a complementary rat experiment, Le Bouffant et al. (1982) compared a quartz-containing coal dust to an artificial quartz/coal dust mixture (not ground together) of the same quartz content. Following a 12-mo inhalation, lung collagen analysis revealed that the artificial mixture had induced 3 times more collagen as compared to the quartz occurring naturally in association with the coal.

Other examples include studies showing that the strong hydrogen bonding agent polyvinylpyrrolidone *N*-oxide (PVPNO), after surface treatment, can powerfully attenuate the hemolytic activity of quartz (Nolan et al., 1981). Another study of Ernst et al. (2002), investigating the quartz surface-modifying effect of PVPNO, confirmed strong reduction of the inflammogenic effect of quartz DQ12 in terms of BAL endpoints or histopathological effects.

Still other studies have demonstrated that aluminum ions can lower quartz-induced toxicity. Brown et al. (1990) found that aluminum-coated quartz was substantially less inflammogenic to the rat lung than native quartz as evaluated by a differential cell count. Thus, as this quenching effect of aluminum lactate

is quartz specific it can be used to modify the surface of quartz-containing particles and to distinguish between a quartz-specific induction of inflammation and a general particle effect.

More recently, Fubini et al. (2004) characterized four different quartz samples and could attribute physicochemical properties to the varying lung toxicity potential observed by Seiler et al. (2004) in parallel rat studies. Two samples induced inflammation *in vivo*, whereas the other two were mostly inert. With respect to physicochemical parameters, the two less inflammogenic quartzes were more resistant to solubility in HF, irreversibly coordinated H₂O molecules, and exhibited strong adsorption sites. In addition, they appeared to be covered by aluminum ions known to quench quartz-specific toxicity.

The quartz isolate investigated in this study was derived from a sodium bentonite deposit formed 110 to 112 million years ago. It is composed of quartz particles that were formed by precipitation from aqueous solution, at the time the clay was formed, and has been in contact with the clay since that time. In contrast, the reference quartz in the positive control is relatively recently crushed quartz that has been accepted as a biologically active standard in other toxicological studies (Robock, 1973; Clouter et al., 2001). The physicochemical properties of the quartz isolate and the reference quartz sample reveal that these materials are substantially different (Table 1). In particular, the crystal domain size of the reference quartz is 2.1 times that of the quartz isolate, indicating that the reference quartz has a much coarser crystal structure than does the quartz isolate; the zeta potential of the reference quartz is 20 to 75% higher than that of the quartz isolate, depending on pH, indicating that the reference quartz has a much more electrically reactive surface than does the quartz isolate; and the mineralogical composition, as determined by XRD analysis, and the chemical analysis show the reference quartz to be nearly pure silicon dioxide while the quartz isolate contains 30% montmorillonite clay as an inseparable coating on the surface of the quartz particles. Additionally, the morphological characteristics of particles of these materials are also quite different, as illustrated in Figures 1 and 2. These, and other differences, provide some physicochemical basis for the differences observed in the overall toxicological results. Based on the greater specific surface area of the quartz isolate compared to the reference quartz (Table 1), one would expect that the toxicity of quartz isolate should be significantly greater than for quartz if surface reactivity were the same for both. The opposite was found, i.e. quartz isolate was significantly less toxic than quartz, indicating that surface reactivity of quartz isolate is considerably less reactive than that of the reference quartz.

Samples of the quartz isolate and positive control from low-temperature plasma-ashed lungs of treated animals were evaluated at the end of the study. No significant difference in particle size distribution, morphology, or chemistry between the recovered and original materials was found. Figures 4 and 5 show the appearance of the quartz isolate before and after 90-day retention in rat lungs. The only significant change noted was a decrease in the reference quartz/TiO₂ ratio in the recovered

positive control, possibly indicating an overproportional clearance of the reference quartz. This effect can be explained by the high cytotoxicity of the reference quartz allowing the permeation of free reference quartz particles through the alveoli walls into the interstitium/lymph nodes. This translocation of quartz particles retained in lungs to lung-associated lymph nodes has been described by Porter et al. (2004). The result of the histopathological examination of the lung associated lymph nodes, presented in Table 6, provides evidence that there was significant movement of the reference quartz particles, from the lungs, into the lymph nodes. In addition, there was a marked difference in the degree of accumulation of particle-laden macrophages, between the quartz isolate and the positive control groups. The effect was significantly more progressive in the positive control group, from the 28-day to the 90-day sacrifice. This indicates a greater degree of tissue damage in the positive control group relative to the quartz isolate group. A similar pattern of particle accumulation was also seen in the previously conducted 28-day study (Creutzenberg et al., 2003).

Geh et al. (2006) found that respirable bentonite particles, with up to 6% quartz, did not induce micronuclei (MN) in human lung fibroblasts, whereas the positive control material (quartz DQ12) did. In the 28-day study preceding this 90-day study, micronuclei (MN) in alveolar macrophages were evaluated as an endpoint to determine genotoxic potential. This work revealed a significant increase in MN in the alveolar macrophages from animals treated with the 28-day study reference quartz only. In contrast, no abnormal MN increase was noted in alveolar macrophages from animals treated with the quartz isolate from the 28-day study, the quartz isolate from the 90-day study or the positive control from the 90-day study.

The results of this study consistently reveal that the lung inflammatory response for the quartz isolate was significantly lower than that of the positive control. The biochemical, differential cell count, and histopathology data, summarized in Table 7, show a persistent and progressive inflammatory response in the positive control animals. In contrast, the data show a moderate response for the quartz isolate group with an initial inflammatory effect that, for all but the PMN values, remained constant or declined after the first few days.

- In conclusion, this study showed that:
- The vehicle control did not induce lung inflammation.
- The positive quartz control produced significant and progressive lung inflammation.
- The quartz isolate induced a significantly weaker inflammatory response than the positive control, and this response was not progressive.

These results show that there are significant differences in the characteristics and the severity of the biological responses from quartz with occluded surfaces that had been isolated from sodium bentonite and those of crushed quartz known to cause substantial toxicological effects. These differences between quartz types must be considered by researchers and by

those considering the development of health and safety regulations associated with exposure to quartz.

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Attachment 4

Genotoxic Potential of Respirable Bentonite Particles with Different Quartz Contents and Chemical Modifications in Human Lung Fibroblasts

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Crystalline silica has been classified as a human carcinogen, but there is still considerable controversy regarding its fibrogenic and carcinogenic potential. In the present study, we investigated the genotoxic potential of bentonite particles (diameter $<10\ \mu\text{m}$) with an α -quartz content of up to 6% and different chemical modifications (alkaline, acidic, organic). Human lung fibroblasts (IMR90) were incubated for 36 h, 48 h, or 72 h with bentonite particles in concentrations ranging from 1 to $15\ \mu\text{g}/\text{cm}^2$. Genotoxicity was assessed using the micronucleus (MN) assay and kinetochore analysis. The generation of reactive oxygen species (ROS) caused by bentonite particles via Fenton-like mechanisms was measured acellularly using electron spin resonance (ESR) technique and intracellularly by applying an iron chelator. Our results show that bentonite-induced genotoxic effects in human lung fibroblasts are weak. The formation of micronuclei was only slightly increased after exposure of IMR90 cells to an acidic sample of bentonite dust with a quartz content of 4–5% for 36 h ($15\ \mu\text{g}/\text{cm}^2$), 48 h ($5\ \mu\text{g}/\text{cm}^2$), and 72 h ($1\ \mu\text{g}/\text{cm}^2$), to an alkaline sample with a quartz content of 5% for 48 h and 72 h ($15\ \mu\text{g}/\text{cm}^2$), and to an acidic bentonite sample with 1% quartz for 72 h ($1\ \mu\text{g}/\text{cm}^2$). Native (untreated) and organic activated bentonite particles did not show genotoxic effects in most of the experiments. Also, bentonite particles with a quartz content $<1\%$ were negative in the micronucleus assay. Generation of ROS measured by ESR was dependent on the content of transition metals in the sample but not on the quartz content or the chemical modification. Reduction of MN after addition of the iron chelator 2,2'-dipyridyl showed that ROS formation also occurs intracellularly. Altogether, we conclude that the genotoxic potential of bentonite particles is generally low but can be altered by the content of quartz and available transition metals.

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Crystalline silica were classified as human carcinogen by the U.S. Department of Health and Human Services in the National Toxicology Program (NTP), the IARC, and the German Research Foundation (DFG) (U.S. Department of Health and Human Services, 2001; IARC, 1997; DFG, 1999). Since then, many studies have shown that the biological activity of quartz is highly variable (Donaldson & Borm, 1998; Fubini et al., 2001; Schins et al., 2002). Occupational exposure to mineral dust, including quartz and quartz-containing particles, has been studied extensively. Fibrosis and pneumoconiosis have been documented in workers involved in mining and processing of different silicates, bentonite, fuller's earth, montmorillonite, and kaolin (Adamis et al., 1986; Bassermann, 1966; Gibbs and Pooley, 1994; Mikhailova-Docheva et al., 1986; Phibbs et al., 1971; Rombola and Guardascione, 1955; Vigliani 1954; Elmore & Cosmetic Ingredient Review Expert Panel, 2003).

Several studies regarding genotoxic effects of quartz particles showed that chromosomal aberrations, sister chromatid exchanges, and micronucleus formation occur in lung fibroblasts and lung epithelial cells (for review see IARC, 1997). Additional to these findings, Liu et al. (1996) have shown that surfactant-coated silica did not induce micronucleus (MN) formation in V79 cells. The authors concluded that silica can cause chromosomal aberrations and/or aneuploidy in V79 cells; however, pretreatment of particles with surfactant reduces or delays their genotoxicity. In line with these observations, surface modification of silica particles with aluminium salt or polyvinylpyridine *N*-oxide (PVNO) inhibits its ability to elicit DNA single-strand breakage, as well as inhibiting formation of the oxidative DNA lesion 8-hydroxydeoxyguanosine (Schins et al., 2002).

Cyto- and genotoxic effects of silicates are poorly investigated. Gao et al. (2000) described decreased genotoxic effects in rat alveolar macrophages after exposure to kaolin compared to quartz particles. In the present study we investigated the genotoxic potential of respirable bentonite particles with different

quartz contents (0.5–6%) and chemical modifications (alkaline, acidic or organic treatment) in human lung fibroblasts (IMR90) in vitro. In earlier work from our laboratory (Geh et al., 2006) we showed that cytotoxic effects of bentonite particles are enhanced after chemical treatment of the particles. Activated bentonite particles were better taken up by IMR90 cells than were untreated (native) bentonite particles. The cytotoxic potential of the different bentonite samples was mainly characterized by a lysis of the cell membrane.

In the present experiments we were interested in investigating if a correlation exists between genotoxic effects of bentonite particles in human lung cells, their quartz content, and the chemical treatment (acidic, alkaline, organic) of the bentonite samples. IMR90 cells (human lung fibroblasts) were chosen for the investigations because of their untransformed primary properties.

MATERIALS AND METHODS

Cell Culture

Human lung fibroblasts (IMR90) were purchased from ATCC, USA (No. CCL-136). Cells were cultivated in MEM with 10% fetal calf serum (FCS), 1% L-Glutamine, 1% sodium Pyrovate, 0.1% nonessential amino acids (NAA), and antibiotics (100 U/ml penicillin, 100 µg/ml streptomycin) and were maintained at 37°C and 5% CO₂. All supplements were obtained from CC-Pro, Germany.

Particles

The bentonite samples were provided by the Süd-Chemie AG Moosburg, Germany. Particles were industrially treated with Na₂CO₃ (bentonite sample 1403: 3–5% Na₂CO₃; bentonite sample 1400: 5% Na₂CO₃; bentonite sample 1401: 3% Na₂CO₃), with HCl (samples 1402 and 1405), and with distearyl dimethylammonium chloride (organic treatment, sample 0015) (Table 1). The chemical modification of the native bentonite particles causes an intercrystalline exchange of ions like

TABLE 1
Applied bentonites with different activation, and contents of quartz and transition metals

Mineral	Activation	Quartz content (%)	Content of all transition metals (%)	Iron content (%)	Other transition metals ^a (%)	pH
0015	Organic: distearyl dimethyl ammonium chloride	1–2 (Cristobalite)	1.75	1.70	0.05	6.9
1403	Alkaline: Na ₂ CO ₃ (3–5%)	0.5	2.39	2.20	0.19	10.4
1400	Alkaline: Na ₂ CO ₃ (5%)	5–6	3.90	3.70	0.20	10.1
1401	Alkaline: Na ₂ CO ₃ (3%)	5–6	3.79	3.60	0.19	9.1
1405	Acidic: HCl	1	0.79	0.64	0.15	7.9
1402	Acidic: HCl	4–5	2.06	1.80	0.26	4.6
1404	Native (untreated)	1	1.22	1.10	0.12	8.3
1408	Native (untreated)	5–6	4.01	3.80	0.21	8.4
Quartz (DQ-12)	—	95				7.4
Gypsum	—	0				7.5

^aCr, Ni, Cu, Mn, Co, V, La, Sc.

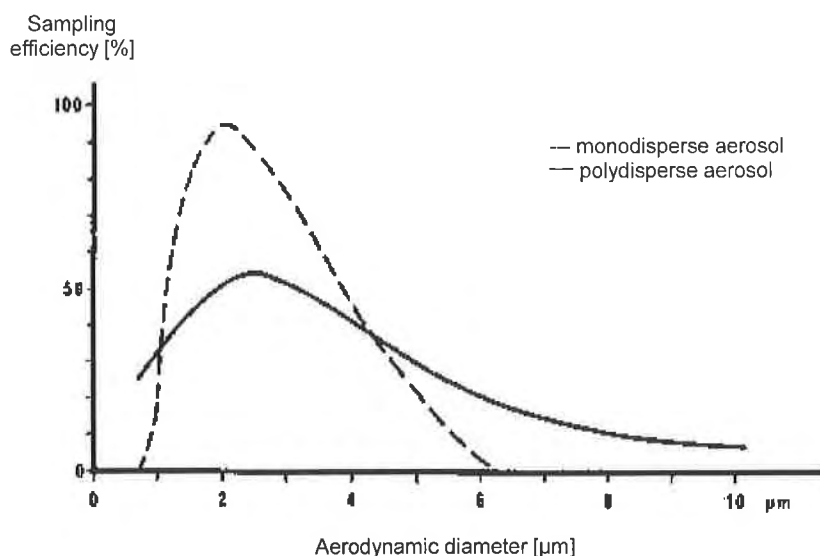


FIG. 1. Sampling efficiency (%) of the used classifier (TBF 50) for the alveolar fraction determined with mono- and polydisperse aerosols.

Na⁺ or Ca²⁺. More than 90% of the particles had a diameter <10 μm, and about 50% were smaller than 2.5 μm (Figure 1). The quartz content of the particles varied from 0.5% to 6%. Gypsum (CaSO₄) and quartz particles (DQ12) were used as negative and positive control particles, respectively. All mineral dust samples were suspended in isotonic NaCl solution, homogenized and sterilized by ultrasonification (Brandson Sonifier 250-D, USA).

The number of particles taken up by IMR90 cells at a concentration of 10 μg/cm² after 24 h of exposure ranged from 1.3×10^{-3} to 4.6×10^{-3} particles/μm². A maximum of particle uptake can be found at a diameter of 0.4 to 1.6 μm. Particles were solely located in the cytoplasm (Geh et al., 2006).

Electron Spin Resonance

Hydroxyl radical formation by bentonite, quartz and gypsum particles was measured by electron spin resonance (ESR) with spin trapping in the presence and absence of H₂O₂; 100 μl of the particle suspension (80 mg/ml) was mixed with 200 μl of the spin trap 5,5-dimethyl-1-pyrroline *N*-oxide (DMPO, 0.05 M in double-distilled water) and 100 μl H₂O₂ (0.5 M in phosphate-buffered saline). For measurement of the ESR spectrum without addition of H₂O₂, 125 μl of the particle suspension (200 mg/ml) was mixed with 200 μl DMPO (0.2 M) in double-distilled water (ddH₂O). ddH₂O was used as negative control. The suspension was incubated for 15 min (3 h without H₂O₂) at 37°C in a water bath and filtered through a 0.2-μm filter (acrodisc 25-mm syringe filter, Pall Gelman Laboratory, USA). The filtrate was immediately transferred to a capillary and measured with an ESR spectrometer (Miniscope MS 100, Magnettech, Germany). The ESR spectra were recorded at room temperature using the fol-

lowing instrumental conditions: magnetic field 3360 G, sweep width 100 G, scan time 30 s, number of scans 3, modulation amplitude 1975 G, receiver gain 1000. Quantification was done by accumulation of three different spectra each averaging three different scans. All four peaks were the amplitude. Outcomes were expressed as the total amplitude in arbitrary units (ESR units) (Shi et al., 2003).

Micronucleus Assay and Kinetochore Analysis

IMR90-cells were seeded in a density of 2×10^4 cells/cm² on each well of Quadriperm dishes (Greiner, Germany) and cultured up to a density of 80% confluence. The cells were exposed to the particles for 36 h, 48 h, and 72 h at various concentrations ranging from 1 μg/cm² to 15 μg/cm². After the exposure time, cytochalasin B was added to the culture medium in a concentration of 3 μg/ml for an additional 24 h. After the treatment, cells were fixed in cold methanol (−20°C) for 30 min. The nuclei were stained with bisbenzimidazole (concentration 5 μg/ml; Hoechst 33258, Sigma, Germany) for 1 min. The slides were mounted for fluorescence microscopy (DAKO antifade mounting media, USA) and examined for the presence of micronuclei. One thousand binucleated cells were evaluated for the presence of micronuclei in each case. The experiments were repeated three times. The micronucleus assay without cytochalasin B treatment was used to analyse the influence of the iron chelator 2,2'-dipyridyl (DPD, Fluka, Germany) on the particle-induced genomic effects. DPD was dissolved in ddH₂O and added to the culture medium (final concentration 100 μM). The treatment of cells with DPD was done simultaneously with the particle treatment (particle concentration 1 μg/cm² and 15 μg/cm², exposure time 72 h).

For further analysis of the particle-induced micronuclei, kinetochores were stained by incubating the fixed cell preparations with CREST serum (Antibodies Incorporated, Davis, CA) for 1 h in a humidified chamber at 37°C. After rinsing the slides with PBS/CMF (calcium/magnesium-free phosphate-buffered saline) with 5% Tween 20 (AppliChem, Germany), the cells were incubated with fluorescein isothiocyanate (FITC)-conjugated goat immunoglobulin (Ig) G anti-human antibodies (Sigma, Germany). Subsequently, the DNA was stained with bis-benzimide as described earlier. At least 200 micronuclei were examined for the presence of kinetochores in each case. The experiments were repeated three times.

Statistical Analysis

Student's *t*-test (two-tailed) was used for comparison of micronucleus and kinetochore results with the untreated control in each set of experiments.

RESULTS

Induction of Micronuclei

The particle concentrations used in the experiments were previously tested for cytotoxicity and were found to be non-cytotoxic (Geh et al., 2006). The formation of micronuclei was only slightly increased after exposure of IMR90-cells to the bentonite samples 1402 for 36 h ($15 \mu\text{g}/\text{cm}^2$) (Figure 2), 48 h ($5 \mu\text{g}/\text{cm}^2$), and 72 h ($1 \mu\text{g}/\text{cm}^2$) (Table 2), 1400 for 48 h and 72 h ($15 \mu\text{g}/\text{cm}^2$) (Figure 2), and 1405 for 72 h ($1 \mu\text{g}/\text{cm}^2$) (Table 2). The maximum rate of 17 MN/1000 cells was found

after exposure of IMR90-cells to the sample 1400 (concentration $15 \mu\text{g}/\text{cm}^2$, 72 h) (Figure 2) and 1402 (concentration $5 \mu\text{g}/\text{cm}^2$, 48 h) (Table 2) compared to the untreated control with 10.3 ± 2.9 MN/1000 cells (1.7-fold increase). These effects are weak but statistically significant ($p < 0.05$).

DQ12-quartz induced the highest number of micronuclei in IMR90 cells (22 ± 2.8 MN/1000 cells) at a concentration of $10 \mu\text{g}/\text{cm}^2$ and an exposure time of 48 h compared to the untreated and the negative (gypsum) control (Table 2). Native (untreated) and organic activated bentonite particles did not influence micronucleus formation. Also, bentonite particles with a quartz content $< 1\%$ were negative in the micronucleus assay (Figure 2 and Table 2).

The kinetochore analysis revealed that the number of kinetochore-positive micronuclei is statistically not significantly reduced in IMR90 cells exposed to the bentonite samples 1400 and 1402, which were positive in the MN assay (Table 3). The number of kinetochore-positive MN in IMR90 cells exposed to 1403, 1404, 1405, and 1408 were at the control level (data not shown). DQ12 quartz induced clastogenic effects (increased proportion of kinetochore-negative MN), which were statistically significant compared to the negative control (Table 3).

Generation of Hydroxyl Radicals Measured by ESR

Both chemically treated and untreated bentonites induced the formation of hydroxyl radicals in the presence and absence of hydrogen peroxide and the spin trap DMPO (Figures 3 and 4, respectively). Apart from sample 1408, the highest generation

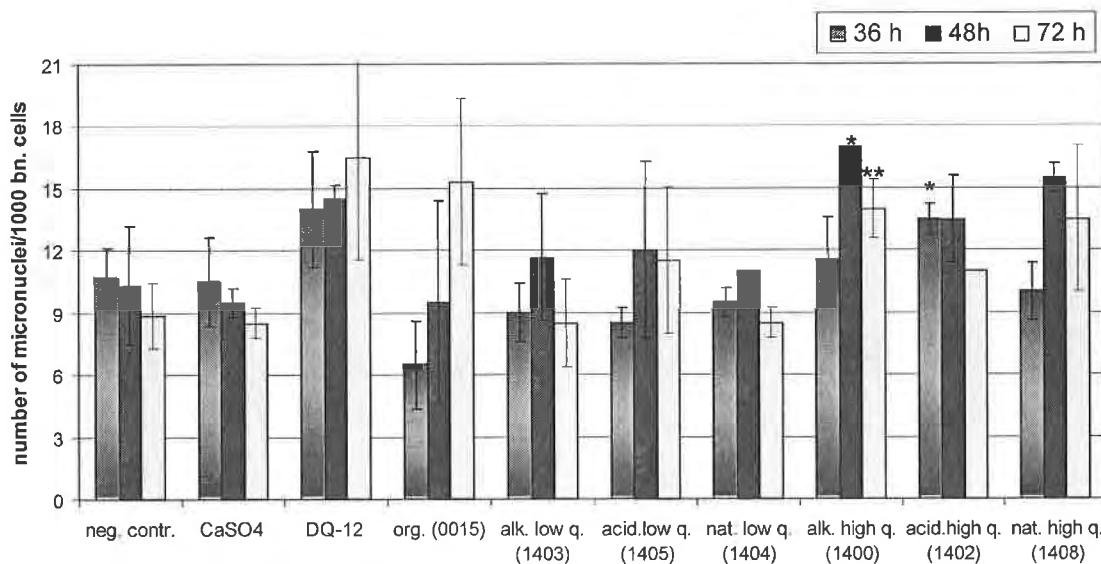


FIG. 2. Induction of micronuclei in human lung fibroblasts exposed to different modified bentonites with various contents of quartz at a concentration of $15 \mu\text{g}/\text{cm}^2$. The exposure times vary between 36 h and 72 h. Statistical significance: * $p < 0.05$; ** $p < 0.01$.

TABLE 2

Induction of micronuclei in 1000 binucleated IMR90 cells after bentonite exposure at different concentrations and exposure times

Sample	1 $\mu\text{g}/\text{cm}^2$	5 $\mu\text{g}/\text{cm}^2$	10 $\mu\text{g}/\text{cm}^2$
Micronuclei $\pm$ SD, 36 h			
Neg. contr.	10.7 $\pm$ 1.5	10.7 $\pm$ 1.5	10.7 $\pm$ 1.5
CaSO ₄	12.5 $\pm$ 0.7	8.5 $\pm$ 0.7	11.5 $\pm$ 3.5
DQ-12	12.0 $\pm$ 2.8	14.0 $\pm$ 0.0 ^a	13.5 $\pm$ 2.1
0015 (q: 1%–2%)	9.5 $\pm$ 4.9	13.5 $\pm$ 2.1	5.5 $\pm$ 0.7
1403 (q: 0.5%)	12.0 $\pm$ 1.4	12.0 $\pm$ 1.0	10.0 $\pm$ 2.8
1405 (q: 1%)	12.5 $\pm$ 0.7	11.5 $\pm$ 2.1	9.0 $\pm$ 0.0
1404 (q: 1%)	10.0 $\pm$ 1.4	10.5 $\pm$ 2.1	9.5 $\pm$ 2.1
1400 (q: 5%–6%)	11.0 $\pm$ 1.4	9.7 $\pm$ 1.2	11.5 $\pm$ 2.1
1402 (q: 4%–5%)	11.0 $\pm$ 1.4	10.0 $\pm$ 1.7	9.5 $\pm$ 0.7
1408 (q: 5%–6%)	10.5 $\pm$ 0.7	10.0 $\pm$ 1.4	12.0 $\pm$ 1.4
Micronuclei $\pm$ SD, 48 h			
Neg. contr.	10.3 $\pm$ 2.9	10.3 $\pm$ 2.9	10.3 $\pm$ 2.9
CaSO ₄	7.0 $\pm$ 3.5	9.0 $\pm$ 2.8	12.5 $\pm$ 3.5
DQ-12	16.0 $\pm$ 0.0 ^a	15.5 $\pm$ 3.5	22.0 $\pm$ 2.8 ^b
0015 (q: 1%–2%)	10.0 $\pm$ 0.0	9.5 $\pm$ 2.1	12.0 $\pm$ 1.4
1403 (q: 0.5%)	12.3 $\pm$ 2.5	10.0 $\pm$ 3.0	10.7 $\pm$ 2.3
1405 (q: 1%)	8.0 $\pm$ 1.4	8.0 $\pm$ 1.4	9.0 $\pm$ 1.4
1404 (q: 1%)	13.5 $\pm$ 2.1	10.0 $\pm$ 1.4	11.0 $\pm$ 1.4
1400 (q: 5%–6%)	9.5 $\pm$ 0.7	15.0 $\pm$ 4.2	15.0 $\pm$ 4.2
1402 (q: 4%–5%)	14.5 $\pm$ 2.1	17.0 $\pm$ 0.0 ^a	14.5 $\pm$ 2.1
1408 (q: 5%–6%)	9.0 $\pm$ 1.7	13.5 $\pm$ 0.7	14.5 $\pm$ 3.5
Micronuclei $\pm$ SD, 72 h			
Neg. contr.	8.8 $\pm$ 1.6	8.8 $\pm$ 1.6	8.8 $\pm$ 1.6
CaSO ₄	9.0 $\pm$ 1.4	8.5 $\pm$ 0.7	10.5 $\pm$ 0.7
DQ-12	13.5 $\pm$ 3.5	15.0 $\pm$ 0.0 ^b	13.0 $\pm$ 5.7
0015 (q: 1%–2%)	8.5 $\pm$ 0.7	14.0 $\pm$ 4.2	9.7 $\pm$ 3.5
1403 (q: 0.5%)	9.0 $\pm$ 2.8	6.5 $\pm$ 2.1	7.5 $\pm$ 0.7
1405 (q: 1%)	14.0 $\pm$ 0.0 ^b	11.0 $\pm$ 2.8	7.5 $\pm$ 0.7
1404 (q: 1%)	12.0 $\pm$ 3.5	8.0 $\pm$ 2.8	10.5 $\pm$ 0.7
1400 (q: 5%–6%)	12.0 $\pm$ 4.2	11.0 $\pm$ 0.0	9.0 $\pm$ 1.4
1402 (q: 4%–5%)	13.5 $\pm$ 0.7 ^b	8.5 $\pm$ 2.1	8.5 $\pm$ 3.5
1408 (q: 5%–6%)	11.0 $\pm$ 4.0	7.0 $\pm$ 1.4	9.7 $\pm$ 3.8

^aSignificant at $p < 0.05$.

^bSignificant at $p < 0.01$.

of DMPO-OH was yielded by bentonites with a high content of transition metals mainly represented by iron (Table 1). The maximum increase in radical generation was caused by 1400 (and 1401) and was in the presence of hydrogen peroxide 10-fold higher compared to the control (Figure 3) and in the absence of hydrogen peroxide 5.5-fold higher than the control (Figure 4). In most bentonite samples, a high content of transition metals was aligned with a high content of quartz (exception: 1403). The comparison of all ESR data including those of quartz showed that the crucial factor for the generation of bentonite-

TABLE 3

Kinetochores analysis after CREST-staining of bentonite-exposed IMR90 cells (concentration 15 $\mu\text{g}/\text{cm}^2$, exposure time 72 h)

Sample	Number of scored MN	K ⁺ [%] $\pm$ SD	p Value
Neg. contr.	500	59.2 $\pm$ 5.2	—
DQ-12	300	49.0 $\pm$ 4.6	<.05
1400	300	56.0 $\pm$ 2.6	n.s.
1402	400	56.8 $\pm$ 2.6	n.s.

Note. At least 300 micronuclei were evaluated for the presence of kinetochore-positive (K⁺) signals.
n.s., Not significant.

induced OH[•] radicals is probably the content of transition metals.

Suppression of MN Formation by Application of an Iron Chelator

A significantly decreased number of MN could be observed just after exposure of IMR90 cells (concentration 15 $\mu\text{g}/\text{cm}^2$, 72 h) to the bentonite sample 1408 ($p < 0.05$), which was co-treated with the iron chelator 2,2'-DPD (final concentration 100 μM , Figure 5).

DISCUSSION

The purpose of this study was to investigate the genotoxic potential of respirable bentonite particles in relation to their quartz contents and chemical modifications in human lung cells. Our results show that bentonite particles induce just weak or no genotoxic effects in IMR90 cells. The formation of micronuclei was only slightly increased after exposure of IMR90 cells to 3 samples of chemically treated bentonite dust; 2 samples of these had a quartz content of 4–5%. Native and organic activated bentonite particles did not show any genotoxic effects. The maximum number of induced MN in bentonite-exposed IMR90 cells was just 1.7-fold increased compared to the untreated control. Even the maximum increase in MN formation (2.2-fold) after exposure of cells to DQ12-dust, which was used as positive control, was relatively low.

These results are in agreement with those of Gao et al. (2000), who reported a lower genotoxicity of kaolin, another clay mineral similar to bentonite, in comparison to quartz in alveolar rat macrophages.

The relatively low genotoxic properties (MN formation, generation of free radicals) of DQ12 quartz in the own experiments might be explained by the age of the sample. Fubini et al. (1995) have shown that “fresh” quartz samples are more reactive than “aged” material. Also, Cakmak et al. (2004) observed large differences in toxic properties of different quartz samples. Other authors were able to induce a four- to sixfold

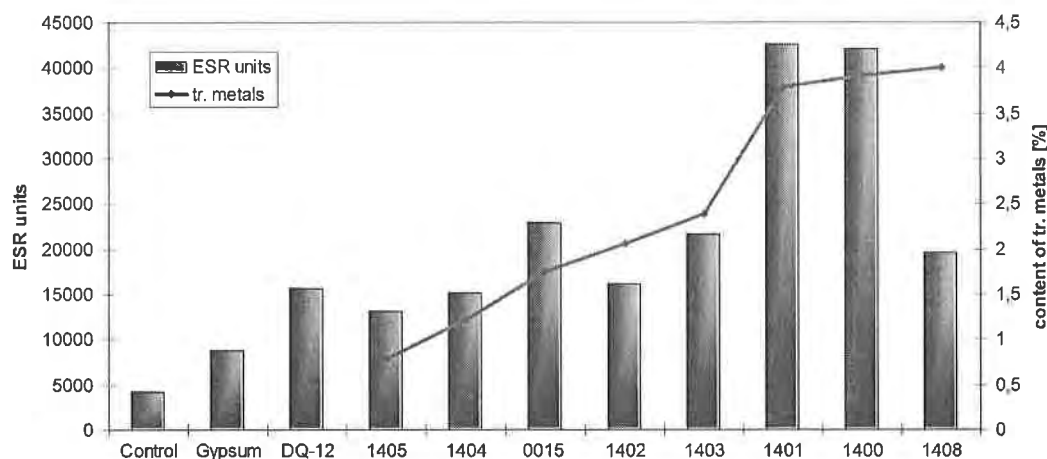


FIG. 3. DMPO-OH generation by bentonite particles in the presence of hydrogen peroxide in relation to other mineral particles (SD below 8%). The dependency of the DMPO-OH signal, represented by ESR units, upon the content of transition metals is shown by the curve.

increase of micronucleated cells after exposure of human embryonic lung cells (HEL299) to $320 \mu\text{g}/\text{cm}^2$ Min-U-Sil quartz for 24 h (Nagalakshmi et al., 1995) or hamster lung fibroblasts (V79) to $160 \mu\text{g}/\text{cm}^2$ Min-U-Sil quartz for 24 h (Liu et al., 1996). However, the quartz concentrations used by Nagalakshmi et al. (1995) and Liu et al. (1996) are 10 to 20 times higher than the concentrations used in our own experiments. The dust concentrations for the genotoxicity tests in our own study were chosen according to the results of the cytotoxicity tests in the former study of Geh et al. (2006). These used non-cytotoxic dust concentrations are even >10-fold higher compared to real exposure conditions, according to the allowed maximum work place concentration of $4 \text{ mg}/\text{m}^3$ (MAK value for inhalable dust; DFG, 2005).

Geh et al. (2006) have also shown that chemically treated bentonites (acidic or alkaline) are better taken up by IMR90 cells than untreated bentonite particles. This effect might ex-

plain the slightly increased genotoxic properties of chemically modified bentonite particles with a higher quartz and/or transition metal content compared to natural bentonites with a low quartz/transition metal content. Although quartz particles were not detected inside the IMR90 cells (Geh et al., 2005), they are able to generate extracellularly free radicals like hydrogen peroxide, hydroxy radicals, superoxide, and singlet oxygen, which can "act at a distance" and cause oxidative DNA damage (Schins et al., 2002; Knaapen et al., 2004). Bioavailable transition metals are also of importance for oxidative stress mediated free radical generation via Fenton or Haber-Weiss reactions (Shi et al., 2003). The data of our ESR study show clearly that the content of transition metals for generated acellular ESR units is even more important than the quartz content of the dust sample. We found a positive but statistically not significant correlation of $R^2 = 0.45$ between transition metal content of the dust sample and detected ESR units, whereas the correlation

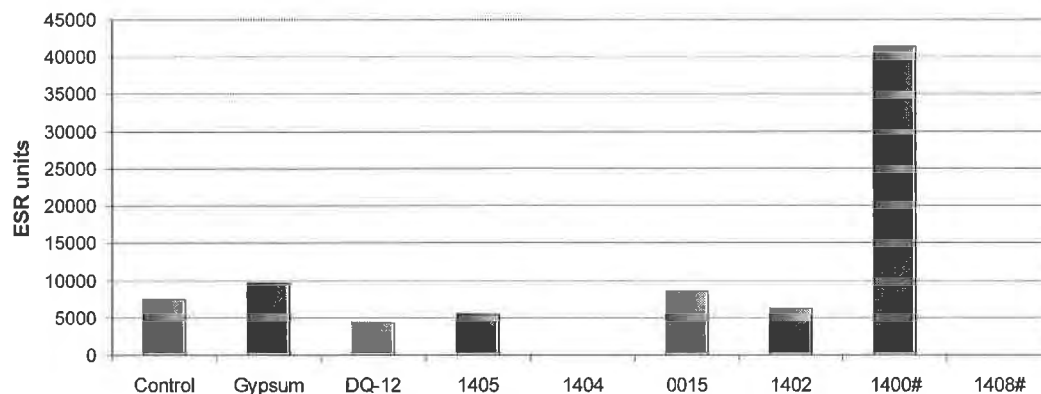


FIG. 4. DMPO-OH generation by bentonite particles in the absence of hydrogen peroxide in relation to other mineral particles (SD below 5%). Values for 1405 and 1408 are zero.

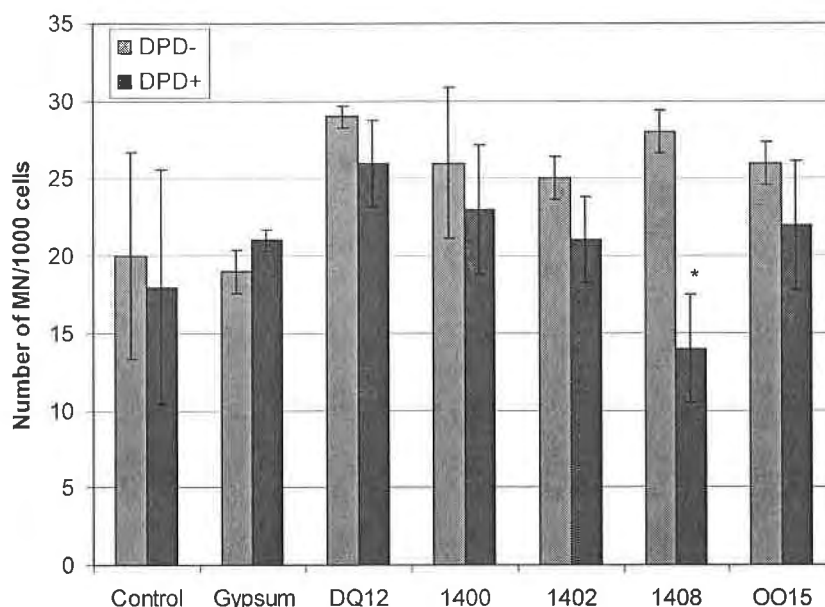


FIG. 5. Inhibition of micronucleus induction in bentonite, quartz (DQ12), and gypsum exposed IMR90 cells after addition of the iron chelator 2,2'-dipyridyl (final concentration 100 μ M). The treatment of cells with 2,2'-DPD was done simultaneously with the particle treatment (particle concentration 15 μ g/cm², exposure time 72 h).

between quartz content and ESR units was comparably lower ($R^2 = 0.31$). The calculated correlation between number of induced MN and quartz content of the bentonite samples was just $R^2 = 0.26$.

In the case of sample 1408 the content of iron was relatively high (3.8%) but the effects measured by ESR were comparatively low. It might be that the iron of this sample was less bioavailable due to the native composition of the bentonite sample. The aluminum content of sample 1408 was elevated (9.8%) compared to the other investigated bentonite samples. It might be possible that the aluminium content of the sample reduces its toxicity. In agreement with this, cytotoxicity and DNA breakage effects of DQ12 were found to be inhibited in vitro as well as in vivo upon its surface coating with aluminum lactate (Schins et al., 2002; Knaapen et al., 2002). Earlier, Duffin et al. (2001) showed that aluminum lactate-treated quartz particles showed a reduced reactivity both in terms of hydroxyl radical generation and in terms of the induction of molecular signaling events leading to pulmonary inflammation. In the current experiments, micronucleus formation after exposure of IMR90 cells to sample 1408 was reduced after addition of the iron chelator DPD. An explanation for this effect could be that aluminum catalyzes the DPD-induced complexation of iron ions.

Altogether, we conclude from our in vitro experiments that the genotoxic potential of bentonite particles is generally low but can be altered by the content of quartz and available transition metals. There are indications that the chemical modification of the dust could enhance the genotoxic properties. Further in vivo

experiments are necessary to investigate the genotoxic effects of bentonite dust on lung cells at low doses that are comparable to real exposure conditions.

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Sorptive Minerals Institute

Attachment 5

FINAL REPORT

**28-Day Study: Inflammatory and Genotoxic Effects
of Native or Treated Quartz Samples
Following Intratracheal Instillation in the Rat**

Fraunhofer ITEM Study No. 02G01030

Submitted to
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August 1, 2003

This final report consists of 123 pages.

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CERTIFICATIONStatement of Principal Scientists

Fraunhofer ITEM Study No.: 02G01030

Study Title: 28-Day Study: Inflammatory and Genotoxic Effects
of Native or Treated Quartz Samples Following
Intratracheal Instillation in the Rat

Test Items: Clay fraction, Non-Clay fraction

Reference Items: Quartz DQ 12 (positive control)
Titanium Dioxide (negative control)

We, the undersigned, hereby declare that the work in this study was performed by us or under our supervision according to the procedures herein described and that this report provides a correct and faithful record of the results obtained.

Fraunhofer Institute of Toxicology and Experimental Medicine

Signature/Date

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Statement of Study Director

Fraunhofer ITEM Study No.: 02G01030
Study Title: 28-Day Study: Inflammatory and Genotoxic Effects of
Native or Treated Quartz Samples Following Intratracheal
Instillation in the Rat
Test Items: Clay fraction, non-clay fraction
Reference Items: Quartz DQ 12 (positive control)
Titanium Dioxide (negative control)

The study described in this report was conducted in compliance with Principles of Good Laboratory Practice (German Chemicals Law ' 19a Appendix 1 pp. 2119-2129, June 28, 2002) and with regulations of the German Animal Protection Law (Tierschutzgesetz of May 25, 1998).

I, the undersigned, hereby declare that this report provides a correct and faithful record of the results obtained. I accept the responsibility for the validity of the study.

The endotoxin assay (see section 2.2) has been outsourced to the School of Veterinary Medicine, Hannover and was not performed in compliance with GLP.

The micronucleus test on alveolar macrophages (see supplement at the end of the report) was done as a supplementary investigation on genotoxicity and was not performed in compliance with GLP.

Date

Signature

Fraunhofer Institute of Toxicology and Experimental Medicine
(Fraunhofer ITEM)

Study Director: Dr. O. Creutzenberg

Quality Assurance Statement

Fraunhofer ITEM Study No.: 02G01030
Study Title: 28-Day Study: Inflammatory and Genotoxic Effects of
Native or Treated Quartz Samples Following Intratracheal
Instillation in the Rat

Test Items: Clay fraction, non-clay fraction
Reference Items: Quartz DQ 12 (positive control)
Titanium Dioxide (negative control)

The Quality Assurance Unit (QAU) hereby declares that the study was inspected at intervals adequate to ensure the integrity of the study and the findings reported to the Study Director and the management of Fraunhofer ITEM. The exact dates of inspections and reports are given below. The report describes the methods and procedures used in the study and the documented results accurately reflect the raw data of the study.

Date of Inspection	Type of Inspection	Date of Report to Study Director/management
June 22 – October 19, 2001	Study plan	October 19, 2001
October 22-26, 2001	Intratracheal instillation	October 30, 2001
October 29-31, 2001	Necropsy, clinical chemistry, BAL	October 31, 2001
November 7-9, 2001	Differential cell count	November 9, 2001
November 22-26, 2001	Necropsy, organ weight, clinical chemistry, BAL	November 26, 2001
Nov. 22 – Dec. 4, 2001	Isolation of type II cells	December 4, 2001
Dec. 20, 2001 – Jan. 15, 2002	Alkaline phosphatase staining	January 15, 2002
March 15, 2002 – July 30, 2003	Draft reports, raw data	July 30, 2003

Date

Signature

Fraunhofer Institute of Toxicology and Experimental Medicine
(Fraunhofer ITEM)
Head, Quality Assurance Unit: Dr. M. Ketkar

Copy of the GLP Certificate



Niedersächsisches
Landesamt für Ökologie

Gute Laborpraxis/Good Laboratory Practice

GLP-Bescheinigung/Statement of GLP Compliance

(gemäß/according to § 19 b Abs. 1 Chemikaliengesetz)

Eine GLP-Inspektion zur Überwachung der Einhaltung
der GLP-Grundsätze gemäß Chemikaliengesetz bzw.
Richtlinie 86/320/EEG wurde durchgeführt in:

Assessment of conformity with GLP according to
Chemikaliengesetz and Directive 86/320/EEC at:

☒ Prüfeinrichtung / Test facility ☐ Prüfstandort / Test site

Fraunhofer-Institut für Toxikologie und Experimentelle Medizin
in D-30625 Hannover, Nikolai-Fuchs-Straße 1

(Unverwechselbare Bezeichnung und Adresse/Unequivocal name and address)

Prüfungen nach Kategorien/Areas of Expertise (gemäß/according ChemVwV-GLP Nr. 5.3/ OECD guidance)

- 1 - Prüfungen zur Bestimmung der physikalisch-chemischen Eigenschaften und Gehaltsbestimmungen
- 2 - Prüfungen zur Bestimmung der toxikologischen Eigenschaften
- 3 - Prüfungen zur Bestimmung der erbgutverändernden Eigenschaften (in vitro und in vivo)
- 5 - Prüfungen zum Verhalten im Boden, im Wasser und in der Luft, Prüfungen zur Bioakkumulation und zur Metabolisierung
- 8 - Analytische Prüfungen an biologischen Materialien
- 9 - Sonstige Prüfungen:
 - Sicherheitspharmakologie, Teilbereich Lung
 - Molekultoxikologische und molekularbiologische Prüfungen zu Chemikalien, Pflanzenschutzmittel-Wirkstoffen, Bioziden und pharmakologischen Wirkstoffen

Datum der Inspektion / Date of inspection
(Tag, Monat, Jahr / day, month, year)

25. - 27. November 2002

Die/Der genannte Prüfeinrichtung/Prüfstandort befindet
sich im nationalen GLP-Überwachungsverfahren und
wird regelmäßig auf Einhaltung der GLP-Grundsätze
überwacht.

The above mentioned test facility / test site is included in
the national GLP Compliance Programme and is
inspected on a regular basis.

Auf der Grundlage des Inspektionsberichtes wird hiermit
bestätigt, dass in dieser Prüfeinrichtung/diesem
Prüfstandort die oben genannten Prüfungen unter
Einhaltung der GLP-Grundsätze durchgeführt werden
können.

Based on the inspection report it can be confirmed, that
this test facility / test site is able to conduct the
forementioned studies in compliance with the Principles
of GLP.

Niedersächsisches Landesamt für Ökologie/Lower Saxony State Agency for Ecology

Hildesheim, 05.02.03

Im Auftrage

Teufelberg
(Dauamtmann)



INFORMATION ON THE STUDY

Fraunhofer ITEM Study No :	02G01030
Test Facility:	Fraunhofer ITEM Animal Room T1.030A Instillation T1.029 Necropsy Room T1.07
Test Items:	Clay material (SMI R-01-353A) Non-clay fraction (SMI R-01-353B)
Reference Items	Titanium Dioxide (negative control) Quartz DQ 12 (positive control) Quartz DQ 12 + Titanium Dioxide
Fraunhofer ITEM Study Director:	Dr. O. Creutzenberg
Sponsor's Study Monitor:	Prof. Dr. G. Oberdörster
Study Initiation Date:	October 19, 2001
Experimental Start Date: (Start of animal exposure = first date the test substance was applied to the test system)	October 22, 2001
Experimental Termination Date: (Last sacrifice = last date on which data were collected directly from the study)	December 6, 2001
Study Completion Date:	August 1, 2003

Conduct of Study

Good Laboratory Practice (GLP)

The study procedures and data comply with current Standard Operating Procedures and the Principles of Good Laboratory Practice (German Chemicals Law, Paragraph 19a, Appendix 1, pp. 2119-2129, June 28, 2002).

The endotoxin assay (see section 2.2) has been outsourced to the School of Veterinary Medicine, Hannover and was not performed in compliance with GLP.

The micronucleus test on alveolar macrophages (see supplement at the end of the report) was done as a supplementary investigation on genotoxicity and was not performed in compliance with GLP.

Deviations from the study plan

No deviations from the study plan were necessary.

Amendments to the study plan

Three amendments to the study plan were made (for details see Appendix XIV).

Study Staff

Study Director	Dr. rer. nat. O. Creutzenberg
Laboratory Animal Veterinarians	Prof. Dr. med. vet. C. Dasenbrock - Dr. med. vet. Th. Tillmann
Biochemist/BAL	Dr. rer. nat. O. Creutzenberg
Clinical Chemist	Prof. Dr. rer. nat. W. Bartsch
Genotoxicologist	Dr. med. vet. B.-D. Görlitz
Immunotoxicologist	Dr. rer. nat. M. Müller
Histopathologist	Dr. med. vet. H. Ernst
Biostatistician	Prof. Dr. rer. nat. H. Hecker
Quality Assurance	Dr. M. Ketkar

EXECUTIVE SUMMARY

- This study was performed to investigate the intrinsic toxicity of quartz in its native state in clays. Lung effects of a clay fraction (< 0.035% quartz) and a non-clay fraction (about 67% quartz) both isolated from bentonite were followed after intratracheal instillation in the rat. The total dose administered was 15.2 mg/kg (equal to 5 mg per 330 g body weight). To compare and rank the observed effects a positive control (quartz DQ 12) and a negative control (titanium dioxide) were analyzed in parallel.
- All animals survived during the study.
- Effects indicating systemic toxicity were not observed.
- Results in bronchoalveolar lavagate (BAL) showed a high acute inflammatory effect in the clay group three days after end of treatment. After 28 days these effects mostly recovered. The quartz-containing non-clay group induced moderate effects after 3 days, which did not change significantly by day 28. Compared to the positive control group DQ 12 the response of the non-clay group was weaker.
- Histopathologically pulmonary inflammation in lungs was most pronounced in the DQ12 and DQ12 + TiO₂ groups, followed by the non-clay and clay groups. The negative control TiO₂ showed no significantly increased inflammatory lesions. In the clay group, the type of inflammation differed from the quartz groups by the absence of lesions, which are typical for a persistent and self-perpetuating inflammation. It was classified as chronic stage of a severe acute damage.

Interstitial fibrosis in all treatment groups, when present, developed in close association with accumulation of (particle-laden) macrophages and inflammatory cells (PMN, mononuclear cells) and was characterized by localized discrete thickening of intraalveolar septa due to increased deposition of collagen. In the non-clay group, both amount and extension of fibrotically thickened septa were dose-dependent and much less than in the quartz DQ 12 positive control group.

As the HPRT gene mutation assay was negative for all groups, a genotoxic assessment could not be done in this study. However, the evaluation of micronuclei in alveolar macrophages revealed a significant increase in the quartz DQ12 group whereas the non-clay group did not.

The effects are summarized and graded in the table below.

Summary of main effects after intratracheal instillation

	Effects after instillation (day 3) and after end of the recovery period (day 28)									
Test substance	TiO ₂		Clay slurry		Non-clay slurry		Quartz DQ 12		Quartz DQ 12 + TiO ₂	
Post-Treatment Period (days)	3	28	3	28	3	28	3	28	3	28
Parameter										
Total Protein (BAL)	-	-	+++	(+)	(+)	+	+++	+++	+++	+++
Total Cell Number (BAL)	-	-	+++	(+)	++	(+)	+++	+++	+++	+++
PMN (BAL)	-	-	+++	+	+	++	+++	+++	+++	+++
Lung Wet Weights	-	-	+++	+	+	-	+++	++	+++	++
Inflammation (histopathology; day 28)	(+)		(+)		+		++		++	
Interstitial Fibrosis (histopathology; day 28)	-		+		(+)		+		+	
Alveolar Lipoproteinosis (histopathology; day 28)	-		-		-		++		++	

Significant effects, also graded by numerical values:

(+) very slight effect
 + slight effect
 ++ moderate effect
 +++ high effect

- not significant

ABSTRACT

The scientific approach in this study on various quartz species was to characterize differences in biological activity between various quartz species. It is known that freshly ground and geological old quartz-containing materials behave differently in terms of toxicological potency.

An intratracheal instillation study with 28 days post-observation was conducted in male Wistar WU rats to investigate the treatment-related effects of two particle suspensions isolated from bentonite. The aim was to characterize the toxicological behavior of a nearly quartz-free fraction consisting of > 99.9% montmorillonite (called clay fraction) and a second fraction containing about 67% quartz and accompanying aluminosilicates, e.g. montmorillonite (called non-clay fraction). To compare and to rank the effects, a negative control (titanium dioxide; rutile modification) and a positive control (quartz DQ 12) were analyzed in parallel.

For titanium dioxide and the test items a total dose of 15.2 mg/animal was administered to rats by four consecutive daily applications of aliquots, 3.8 mg/kg each suspended in 0.3 ml saline. Controls received the vehicle only. For quartz DQ 12 the total dose was 11.7 mg/kg to use the same quartz dose as given with the non-clay sample. To consider the lower total dose, an additional DQ 12 group was investigated where the missing mass of 3.5 mg/kg was substituted by titanium dioxide.

As toxicological endpoints bronchoalveolar lavages (BAL) were performed 3 and 28 days after treatment, the HPRT test after 28 days and organ weights (lungs) and histopathological examination of inflammation and fibrosis in the respiratory tract after 28 days.

The animal clinical health status was within normal limits; no unscheduled deaths occurred. Body weight measurements did not show significant changes with the exception of lower body weights, observed in the group treated to the clay fraction one and two weeks after treatment. This seemed to be due to an initial impairment of the general health status of rats after treatment with the clay fraction.

Bronchoalveolar lavage (BAL) analysis after 3 days resulted in percentages of polymorphonuclear neutrophils (PMNs) of 61% for clay, 15% for non-clay, 67% for quartz DQ 12 and 3% for titanium dioxide. The values after 28 days were 14%, 36%, 65% and 3.5%,

respectively. Vehicle control levels were $\leq 0.5\%$. These data indicate a high acute response in the clay group recovering evidently within 4 weeks. In the non-clay group the response was

initially moderate and did not change significantly in the 4-week observation period. Titanium dioxide and DQ 12 worked well as negative and positive controls. In the DQ 12 group supplemented by titanium dioxide the response was slightly weaker than in the pure DQ 12 group. Principally, for the biochemical parameters lactic dehydrogenase and total protein and for the lung wet weights the same trends of effects were observed.

Histopathological examination revealed, based on mononuclear cell and granulocyte infiltration, that pulmonary inflammation was most pronounced in the DQ12 and DQ12 + TiO₂ groups, followed by the non-clay and clay groups. In the TiO₂ group, no significant inflammatory lesions could be observed. In the clay group, the type of inflammation differed from the quartz groups by the absence of lesions, which are typical for a persistent and self-perpetuating inflammation, i.e. macrophage degeneration, lipoproteinosis and granulocyte infiltration. Instead, the type of inflammation in the clay group is considered to represent the chronic stage of a severe acute damage as evidenced by the presence of interstitial granulomata and prominent interstitial fibrosis.

Interstitial fibrosis in all treatment groups, when present, developed in close association with accumulation of (particle-laden) macrophages and inflammatory cells (PMN, mononuclear cells) and was characterized by localized discrete thickening of intraalveolar septa due to increased deposition of collagen. In the non-clay group, both amount and extension of fibrotically thickened septa were dose-dependent and much less than in the quartz DQ 12 positive control group. Only in the latter group, concentric areas of fibrosis as a component of the cholesterol granulomas were observed in addition to thickened intraalveolar septa. The cholesterol granuloma-associated fibrosis was more pronounced than the fibrotic thickening of intraalveolar septa and tended to fill also alveolar spaces.

The HPRT gene mutation assay was negative for all groups and could not be used for a genotoxic assessment in this study. However, an alternative assay, i.e. the evaluation of micronuclei in alveolar macrophages, revealed a significant increase in the quartz DQ12 group. In contrary, the data in the non-clay group were not significantly increased as compared to concurrent controls.

1. INTRODUCTION

1.1 General Outline

This 28-day study with intratracheal instillation was a pilot study to assess the toxicological potential of geologically aged, unaltered quartz of respirable size in clay. The experimental design of this study was similar to that described by Johnston and coworkers (2000).

1.2 Objectives of the Study

The objectives of this study were:

- To investigate the intrinsic toxicity of geologically aged, unaltered quartz of respirable size in clays.
- To characterize lung effects by using bronchoalveolar lavage (BAL), histopathological and genotoxic parameters.
- To rank the observed effects compared to well-characterized reference items (quartz DQ 12 - positive control and TiO₂ - negative control).

2. MATERIALS AND METHODS

2.1 Preparation and Characterization of Test and Reference Items

The sponsor supplied all the information in this section with the exception of the endotoxin data.

The samples investigated in the study are listed in Table 1.

Clay Fraction

The sample contains more than 99% montmorillonite, a smectite-type clay mineral that has a surface area of 480 m²/g as measured by the ethylene glycol monoethyl ether (EGME) technique. The fraction used in this study has the code no. SMI R-01-353A.

SEM photographs of the clay fraction appear in Appendix XIII. The minute, platy clay particles form coalescing sheets. The seemingly elongated particles in the photographs are merely folds in these sheets.

Non-Clay Fraction

The sample contains 62 to 71 % quartz, according to the weight % NIOSH 7500 calibration technique used in the X-ray diffraction analysis. This study used the value of 67%. This quartz has an X-ray domain scattering size of 0.085 μm . The remaining non-quartz material is predominately the smectite clay mineral montmorillonite. The fraction used in this study has the code no. R-01-353B.

SEM photographs of the non-clay fraction appear in Appendix XIII. The material is in the form of aggregates of intergrown quartz and clay.

Both the clay fraction and the non-clay fraction were gently isolated using a wet sedimentation and centrifugal process, which was used to avoid mechanical and chemical alteration of the surface of these geologically old materials. The concentrations of these two aqueous suspensions were determined by lyophilization of aliquots (see Appendix I).

Silica, SiO_2 , 'DQ 12' (positive control)

This quartz sample was purchased from Bergbauforschung, Essen, Germany. This material is a fine fraction of a classified Dörentrup quartz sample and is the positive control. The full name is DQ12<5 μm . More information is given in a paper of Robock (1973).

The test sample contains 87% quartz as determined by NIOSH 7500. The remaining 13% was not defined by X-ray diffraction. The sample contains no detectable kaolinite. The chemical analysis shows 99.39% SiO_2 and very minor amounts of other elements. The X-ray scattering domain size is 0.16 μm , which is significantly larger than that in the non-clay fraction.

Titanium Dioxide, TiO_2 , Type 'Bayerlitan T' (negative control)

This titanium dioxide was purchased from Bayer AG, Krefeld, Germany. Chemical analysis shows 98.174% TiO_2 ; annealing weight loss 0.22%; 95% of the TiO_2 material is the rutile

polymorph. Its density is 4.26 g/cm³. The lot number was 85/13928. This material is still available at Fraunhofer ITEM.

An overview of the characteristics appears in Table 1.

Table 1 Test and Reference Items

Sample #	Test and Reference Items (data supplied by sponsor except endotoxin data)
1	Saline
2	Titanium dioxide, type Bayertitan T (negative control) Particle size, μm (Malvern laser particle size): Mean 1.72 Particle size, μm (electron microscope): Mean 0.91, STD 0.46 Geometric mean 0.81, geom. STD 1.62 Min. 0.22, max. 3.95, median 0.81 Mineral composition, %: rutile 99.5 Chemical composition, %: SiO_2 0.10, Al_2O_3 0.06, Fe_2O_3 0.01, MnO 0.00, MgO 0.03, CaO 0.00, Na_2O 0.13, K_2O 0.07, TiO_2 98.174, P_2O_5 0.00 Surface area, m^2/g: BET 1.9, EGME 21.7 Endotoxin concentration: 56.2 ng/g = 562 endotoxin units per gram (EU/g)
3	Clay Fraction SMI R-01-353A isolated from bentonite Particle size, μm (centrifugal measurement): < 1 Mineral composition, %: montmorillonite > 99.9, quartz 0.035 Chemical composition, %: SiO_2 59.97, Al_2O_3 20.76, Fe_2O_3 4.30, MnO 0.006, MgO 2.31, CaO 0.44, Na_2O 3.69, K_2O 0.00, TiO_2 0.104, P_2O_5 0.02, LOI 8.59 Surface area, m^2/g: BET 6.4, EGME 480 Endotoxin concentration: 1000 ng/g = 10,000 EU/g
4	Non-clay Fraction SMI R-01-353B isolated from bentonite Particle size, μm (centrifugal measurement): 1-10 Particle size, μm (Malvern laser particle size): Mean 4.8 Particle size, μm (electron microscope): Mean 4.7, STD 3.25 Geometric mean 3.70, geom. STD 2.07 Min. 0.15, max. 28.95, median 4.00 X-ray scattering domain size, μm: 0.085 Mineral composition, %: quartz 62-71, opal 3-13, montmorillonite 23-33, analcime 2, calcite < 1 Chemical composition, %: SiO_2 84.81, Al_2O_3 7.54, Fe_2O_3 1.29, MnO 0.008, MgO 0.74, CaO 0.85, Na_2O 1.02, K_2O 0.22, TiO_2 0.079, P_2O_5 0.05, LOI 3.35 Surface area, m^2/g: BET 3.2, EGME 174 Endotoxin concentration: 316 ng/g = 3160 EU/g
5	Quartz, type DQ 12<5μm – Dörentrup (positive control) Particle size, μm (Malvern laser particle size): Mean 1.29 Particle size, μm (electron microscope): Mean 1.02, STD 0.68 Geometric mean 0.86, geom. STD 1.78 Min. 0.18, max. 5.81, median 0.82 X-ray scattering domain size, μm: 0.16 Mineral composition, %: quartz 87, undetermined: 13 Chemical composition, %: SiO_2 99.39, Al_2O_3 0.10, Fe_2O_3 0.04, MnO 0.00, MgO 0.00, CaO 0.14, Na_2O 0.16, K_2O 0.05, TiO_2 0.049, P_2O_5 0.02 Surface area, m^2/g: BET 1.51, EGME 16.0 Endotoxin concentration: 56.2 ng/g = 562 EU/g
6	Quartz, type DQ 12<5μm + Titanium dioxide, type Bayertitan T (TiO_2 added to reach same total mass dose as in sample # 4)

STD: standard deviation

BET: Brunauer, Emmett, and Teller method

EGME: ethylene glycol monoethyl ether method

Safety protection, handling, storage, disposal

Biological waste material, including food, bedding and other disposable materials generated in the animal facility was collected and disposed of in compliance with local, state and federal regulations.

2.2 Analysis on Contamination with Endotoxins

Prior to study start, all test items have been tested for endotoxin contamination using the limulus assay. A detailed description of the analysis is given in Appendix II. This test has been done at the School of Veterinary Medicine, Dept. of Animal Nutrition in Hannover resulting in the values given below (Table 2).

This assay was not performed in compliance with GLP.

Table 2 Endotoxin Data

Test Item	Lipopolysaccharides (LPS) (ng/g)
Clay SMI R-01-353A	1000
Non-clay SMI R-01-353B	316
Quarz DQ 12	56.2
Titanium Dioxide	56.2
Embedding Material (softwood granulate from spruce)	3160

Among the test items, DQ 12 and titanium dioxide showed very low values. The others were in the range up to 1000 ng/g.

For comparison: background levels for animal feed are 10,000 to 15,000 ng/g.

Note: The often used endotoxin units (EU) correspond to nanograms by: 10 EU = 1 ng endotoxin.

From experimental work (Elder et al., 2000) it is known that 80 EU = 8 ng endotoxin administered to rat lungs by intratracheal instillation induced PMN levels of 8% in lung lavage fluid after 24 hours. At a given concentration of 1000 ng/g (e.g. SMI R-01-353A) 8 ng are

contained in a total dose of 8 mg. In this study the total doses were 5 mg/lung. Therefore, the endotoxin contents of the test items were acceptable.

2.3 Test System

Animal model

Male Wistar WU rats (strain Crl:(WI)WU) were purchased from Charles River Deutschland, Sulzfeld, Germany. The age of the animals at the start of exposure was about 12 weeks and the weight approx. 330 grams. Rats were exposed to the test/reference items by intratracheal instillation. Males were preferred to females because literature data indicated a higher sensitivity with regard to silicotic response (Honnons & Porcher, 2000).

Wistar and Wistar WU rats are commonly used in subchronic and chronic inhalation toxicity studies. They fulfill the criteria stated by a U.S. EPA Workshop (Vu et al., 1996, U.S. EPA, 1988) such as (i) a low background rate of neoplasia (ii) a low background rate of pulmonary disease (iii) longevity and (iv) a history of laboratory use. In this study the specified Wistar WU strain is preferred to the Fischer strain because young Fischer rats available in Germany sporadically show a slight latent inflammation of lungs which might interfere with the scheduled investigations.

A total of 132 rats were used for this study. Thirty further rats were ordered with the same delivery to provide homogeneous test groups at study start (see randomization). After randomization, the remaining 30 rats were taken:

- for use in trials to check the tolerance of test items and administration mode
- as sentinel animals for health control (bacteriology, virology) or for substitution of losses during the instillation procedure.

Acclimatization

Upon arrival on September 4, 2001 all animals were thoroughly examined by a veterinarian or his representative. This examination included but was not limited to the skin and hair coat,

eyes, ears, nose, body openings and the respiratory tract. Prior to the start of the treatment period, the following procedures were completed for all rats.

1. Approximately seven weeks were allowed for the animals to adjust and become

acclimatized to the Fraunhofer ITEM environment.

2. Clinical observations were done every day. Body weight was measured three times during the acclimatization period.

Rats were accepted into the study of the completion of items 1 and 2 and absence of disease as demonstrated by general physical condition during the acclimatization period.

Randomization

The animals were allocated to groups on a body weight basis. At that time, the animals were weighed, randomized and grouped by the DATATOX system (management of toxicology laboratory data, Instem Computer System Ltd., Walton Industrial Estate, Stone, Staffs, ST 15 0LT, Great Britain). This process assured that all the animals in groups 1-6 had body weights within 20% of the mean weight across those groups, at that time.

Identification

To each animal in the study a unique six digit identification number was assigned, which was a combination of the group number and the consecutive animal number. These six digits are necessary for data storage in the computer system. The number assigned is GG00NN where GG denotes the two digit (01-99) group number, 00 is a common separator and NN are the two digits (01-99) consecutive animal number. Each animal was permanently identified by a numbered metal plate on the cage corresponding to that identification number. All data collected from an animal were filed under that number.

In addition, the right ear of the animals was tattooed with the group number and the left ear with a two digit animal number.

Housing and Maintenance

Two animals were housed per Makrolon™ cage type III (800 cm²). Cages and softwood ('altromin 3/4') bedding material were changed twice a week or more often when necessary. Tap water was offered fresh weekly or more often when necessary in 300 ml Makrolon® bottles fitted with a stainless steel nipple top with a hole approximately 0.5 mm in diameter.

As diet a commercial chow in pellet form was used, identified as altromin "1324 N spec. prepared", purchased from Altromin International, Lage, Germany. Diet was stored in room

T1.015 with a temperature range of $18 \pm 5^\circ \text{C}$ and relative humidity of $40 \pm 15\%$. No batch of diet was used after 4 months have elapsed from manufacturing date. Diet was offered fresh weekly or more often when necessary.

Environmental Conditions

The temperature and relative humidity of the animal room were recorded on a continuous basis, and the records are maintained with the raw data.

The limits were set at $22^\circ \text{C} \pm 2^\circ \text{C}$ for temperature and $55\% \pm 15\%$ for relative humidity. The air change rate was approximately 15 times per hour. Animal room lighting was on a 12-hour light/dark cycle controlled by an automatic timing device.

2.4 Study Design and Dosing Scheme

The experimental set-up is shown in Table 3. In Table 4 the investigations to be performed are listed. Detailed information on the parameters is given in sections 2.9 (bronchoalveolar lavage), 2.10 (histopathology) and 2.11 (HPRT assay).

For dosing the non-clay fraction served as the basis with 15.2 mg test item per kg body weight (equal to 5 mg per 330 g body weight). For dosing purposes this fraction was assumed to consist of 67% of quartz. To reach the same quartz dose in the positive control, DQ 12 quartz was dosed with 11.7 mg/kg (instead of 10.2 mg/kg) to adjust for the purity of this quartz sample being 87%.

On four consecutive days the total doses were administered in four instillative administrations each aliquot suspended in 0.3 ml saline (or 0.9 ml/kg). This nominal volume refers to a 'standard body weight' of the rats at the initiation of the study of 330 g. As test items were administered on a body weight basis the actual body weights of animals on the first of the 4-day treatment series were taken to calculate the animal-specific dose. Initially a dosing of two aliquots was scheduled. However, results of a pilot experiment revealed that rats would not have tolerated this scheme in the clay group, so the dose was divided into four daily aliquots.

Intratracheal instillations were followed by a 28-day observation period.

Animals were sacrificed at 3 and 28 days post-treatment for BAL investigations. After 28 days, subgroups of the rats were sacrificed for histopathological and genotoxic investigations.

Table 3 Study Design

Group	Treatment	Cumulative Dose (mg/kg)	Number of animals per group
1	Saline	0.9 ml/kg	22
2	TiO ₂ - Bayertitan T (negative control)	15.2	22
3	Clay Material R-01-353A	15.2	22
4	Non-Clay Fraction R-01-353B	15.2	22
5	DQ 12 Quartz (positive control)	11.7	22
6	DQ 12 Quartz + TiO ₂	11.7 + 3.5	22
Total			132

Table 4 Investigations

Investigation	Days of post-exposure at which investigation has to be performed	Number of animals per group/ time-point	Group No. (see Table 3)	Consecutive Animal No.
Clinical observation	daily	22	1-6	1-22
Body weight	weekly	22	1-6	1-22
Gross pathology observations	upon sacrifice	22	1-6	1-22
Bronchoalveolar lavage (BAL) Total cell count, differential cell count (macrophages, neutrophils, lymphocytes, eosinophils, epithelial cells), biochemical parameters (lactic dehydrogenase (LDH), β -glucuronidase, total protein), cell viability - Lung wet weights	3 28	6 6	1-6 1-6	1-5, 21 6-10, 22
Histopathology (scoring of inflammation and fibrosis)	28	5	1-6	11-15
HPRT assay	28	5	1-6	16-20

2.5 Intratracheal Instillation of Test and Reference Items

Test items were suspended in saline. The total dose to be administered was intratracheally instilled in four applications of a nominal volume of 0.9 ml/kg body weight. Concurrent controls were treated with the vehicle only. For the procedure of intratracheal instillation the rats were slightly anesthetized with CO₂.

To prepare the test material, sterile and pyrogene-free saline was added under a laminar flow bench. As the test items clay and non-clay were delivered as aqueous suspension, the water amount of the used suspension volume was calculated and dry sodium chloride added as much as needed to reach 0.9% isotonic saline concentrations. For details see Appendix I.

2.6 Daily and Weekly Observations

All animals were observed in their cages daily. Animals were also removed from the cages once a week, and carefully examined for abnormalities.

2.7 Body Weights

Individual body weights were recorded to the nearest 0.1 g once a week throughout the study for all animals. All body weight data were collected using electronic balances, interfaced with a computer and programmed for direct on line data acquisition.

2.8 Gross Pathology/Necropsy - Organ Weights

All animals were subjected to a complete necropsy, which included careful examination of the external surface of the body, all orifices, and the cranial, thoracic and abdominal cavities and their contents. The rats were anesthetized with an overdose pentobarbital sodium (Narcoren™) and killed by cutting the vena cava caudalis (BAL rats). Rats for histopathological investigations were sacrificed by using an overdose of carbon dioxide. The abdominal cavity was opened and the diaphragm was cut carefully allowing the lungs to collapse. From the animals used for histopathology the following organs were sampled and wet weights were recorded: liver, kidneys, adrenals, testes, epididymides, thymus, spleen, brain, and heart.

2.9 Bronchoalveolar Lavage (BAL)

Bronchoalveolar lavages were performed in 6 rats per group, per time point, i.e. 3 and 28 days after intratracheal instillation. The method of Henderson et al. (1987) was used with minor modifications.

Following preparation, the lungs were lavaged with saline using two lavages of 5 ml each. Leukocyte concentration of the lavagate was determined using a counting chamber and two cytoslides were prepared for differential cell count (macrophages, PMNs, lymphocytes). Epithelial cells were counted per 200 total cells on the same cytoslides. A special stain technique to label epithelial cells was not applied but these were identified by their ciliae.

Another aliquot was used for a viability assay of lavaged cells (trypan blue exclusion test after 3 minutes of incubation).

After centrifugation of the lavage fluid at 160 g, in the supernatant three biochemical analytes relevant for diagnosis of lung damage were determined (lactic dehydrogenase - LDH, β -glucuronidase, total protein) by the kinetic HCC method (Lorentz et al., Eur J Clin Chem Biochem 31, 897-899 1993), an enzymatic color reaction (Stehl PD, Fishman WH in: Bergmeyer HU (ed). Methods of Enzymatic Analysis, Verlag Chemie, Weinheim, Vol IV, pp. 246-256, 1984) and by the Lowry method (Bergmeier HU, Methoden der enzymatischen Analyse, Verlag Chemie, Weinheim, p. 140, 1974), respectively. The justification of the parameters is given below:

Cytological parameters

- total cell count (recruitment of lung leukocytes)
- viability test (giving percentage of alive leukocytes among the total number of cells)
- differential cell count (inflammatory (neutrophils = PMNs) or immunological (lymphocytes) reactions)

Biochemical parameters

- lactic dehydrogenase (LDH = cytosolic marker enzyme; increased permeability of membranes, cell damage and lysis)
- β -glucuronidase (measure of phagocytic activity of macrophages; lysis of macrophages)
- total protein (marker of transsudation; damage of epithelial cells).

2.10 Histopathology

At 28 days post-treatment histopathology of all 5 lung lobes, including bronchi and the lung-associated lymph nodes (LALN) was done in 5 animals per group. Three sections per lung were prepared: section no. 1: lobe 5, section no. 2: lobe 3, section no. 3: lobes 1, 2 and 4. Lungs were inflated under a pressure of about 20 cm water with neutral-buffered formalin (10%) and fixed by immersion for a minimum of 2 hours. They were embedded in paraffin, sectioned, and stained with hematoxylin and eosin (H & E). A special stain (Masson trichrome) was applied for diagnosis of fibrotic changes.

2.11 HPRT Gene Mutation Assay

At 28 days post-treatment freshly isolated epithelial lung cells (predominantly alveolar type II cells) were cultured and then fed with medium containing 6-thioguanine (6-TG) to select for mutation in the HPRT gene. After 3-4 weeks in culture the cells were fixed and immunostained to count 6-TG resistant colonies.

Type II Cell Isolation

The isolation of the lung epithelial cells followed the procedure published by the group of Driscoll and Carter (Driscoll et al., 1997; Johnston et al., 2000) and was done as documented in Fraunhofer ITEM SOP 180439.

For that, the animals were sacrificed by an intraperitoneal injection of 0.8 ml Narcoren™. The abdomen was opened, the diaphragm cut and the vena cava caudalis severed. The skin was removed from the neck and through an incision in the trachea a 16 gauge i.v. catheter was placed into the trachea. The lungs were perfused through the left atrium.

After that, the lungs were removed from the animal and placed in a 100mm sterile petri dish. The lungs were lavaged with 50 ml solution B (125 mM NaCl, 5 mM KCl, 2.5 mM Na₂HPO₄, 17 mM Hepes, 1.2 mM MgSO₄, 0.1% Glucose, 1% Antibiotic/Antimycotic, 1% Nystatin) and 20 ml solution A (125 mM NaCl, 5mM KCl, 2.5 mM Na₂HPO₄, 2.5 mM CaCl₂, 17 mM Hepes, 1%Antibiotic/Antimycotic, 0.1 % Glucose, 1% Nystatin). Lungs were then placed in a beaker of sterile PBS that had been warmed to 37°C. 10 ml of sterile 0.25% Pronase in solution A were instilled into the lungs at 5 minute intervals for a total volume of 30 ml and 15 minutes.

After removal from trachea and connective tissue, the lung lobes were placed in a 100 ml - 200 ml beaker containing 1 mg DNase/4 ml solution A and 5 ml fetal bovine serum on ice and minced into tiny pieces. The pieces were strained through 20 µm and 100 µm nylon filters rinsing with solution A for a final volume of 40 ml. The cells were washed two times at 500g at 4°C for 10 minutes. The cells were resuspended in 3 ml Ham's F12 + 2% FBS and layered on the previously prepared Nycodenz gradient. The gradient was spun for 20 minutes at 15°C

at 1500g. To collect the epithelial cells, the second layer was removed using a 5 ml syringe.

The cells were washed two times and counted using a CASY cell counter in accordance to Fraunhofer ITEM SOP 180422. The cells were resuspended in the appropriate volume in BRFF-RLuE medium containing 1% Antibiotic/Antimycotic solution.

To determine the number of epithelial cells in the isolated cell fraction, the cells were stained using the alkaline phosphatase kit from Sigma. Additionally, after 48 hours of culture the percentages of adherent cells with lamellar bodies were determined on cytoslides. Results of these two methods are presented in Appendix XII.

HPRT Protocol

To perform the HPRT mutation selection, 40 M 6-thioguanine was added to the culture medium. 1×10^6 cells were placed into 6 T25 flasks per sample to achieve 5×10^6 cells per sample. After 24 hours culture to allow adherence of the cells, the plating efficiency was determined by trypsinization and counting of the attached cells. The remaining flasks were cultured for 6 weeks in HPRT selection medium containing 6-thioguanine. The cells were fed two times per week with fresh medium. After that period the number of colonies was determined and the mutation frequency determined as follows ($\#$ colonies stained / % plating efficiency) / $\#$ cells of initial plate.

2.12 Data Collection and Documentation

All data to be collected were recorded either by direct printout following electronic data processing or in laboratory journals/data sheets.

Recording and analysis of body weight and biochemical BAL data was performed using the DATATOX system (Instem Computer Systems - release C.10).

The original of this final report will be submitted to the sponsor. A duplicate will be kept in the archives of Fraunhofer ITEM.

2.13 Statistical Methods

Differences between groups were considered statistically significant at $P < 0.05$. Data were

analyzed using analysis of variance. If the group means differed significantly by the analysis of variance the means of the treated groups were compared with the means of the control groups based on the LSD or Tukey test. The statistical evaluation of the histopathological findings was done with the two-tailed Fisher test by the PLACES system.

3. RESULTS

3.1 Investigations of Health Status Regarding Background Values of PMNs

The Wistar WU rats received from the breeder Charles River Deutschland, Germany were checked for 'lung health' status. In the pretreatment phase of the study a bronchoalveolar lavage (BAL) was performed. A completely accurate status of a group of 6 rats could be demonstrated analyzing the polymorphonuclear neutrophils (PMN) level. No evidence of background inflammation in the lungs was detected.

For details of this investigation see Appendix III.

3.2 Survival of Animals and Clinical Observations

No deaths occurred during the anesthesia and intratracheal instillation procedure. No test item-related deaths were observed in the observation period till sacrifice.

After treatment the rats were carefully inspected. During the anesthesia/instillation procedure single animals (1-2%) had to be ventilated directly after administration of test items. In the clay group animals exhibited moderate to marked dyspnoea for 1-3 hours after instillation but recovered within one day. Several rats exhibited slight dyspnoea for up to one week following the last intratracheal instillation. Thereafter the general health of the animals was within normal limits. The macroscopical observations made upon sacrifice of animals are summarized in Table 5 (lungs and lung-associated lymph nodes).

3.3 Body Weights

Body weights were recorded weekly during the acclimatization period and following intratracheal instillation. Day 6 is the first measurement after animal treatment. A significant difference compared to controls was observed only in the clay group. A statistically significant lower body weight was measured on day 6 and day 13 corresponding to the acute inflammatory lung response seen in parallel (see results of bronchoalveolar lavage (BAL) of day 3 in section 3.4). Following day 13 a fast recovery of body weight development was

Means of body weights are plotted in Figure 1 and 2. The individual data with means are presented as tables in Appendix IV.

Note: All necropsy findings, other than those presented in Table 5, were incidental findings commonly seen in rats of this strain and age. None were considered related to test substances.

	Saline	Negative Control TiO ₂	Clay	Non-Clay	Positive Control DQ 12	DQ12 + TiO ₂
Day	3 28	3 28	3 28	3 28	3 28	3 28
Lungs						
Puffy consistency	1		6 3			
Grey/white area	1	4	1 2	4 4	3 2	3 2
White + red areas			1	2 2	2	2
White + grey/glassy areas				1	1 1	
White + grey/lardaceous areas					8	8
Red/lardaceous areas			4			
Red/glassy areas			4			
Lardaceous/glassy areas			2			
Red + white/lardaceous areas						
Patchy grey/white discolored			9			
Lung-Associated						

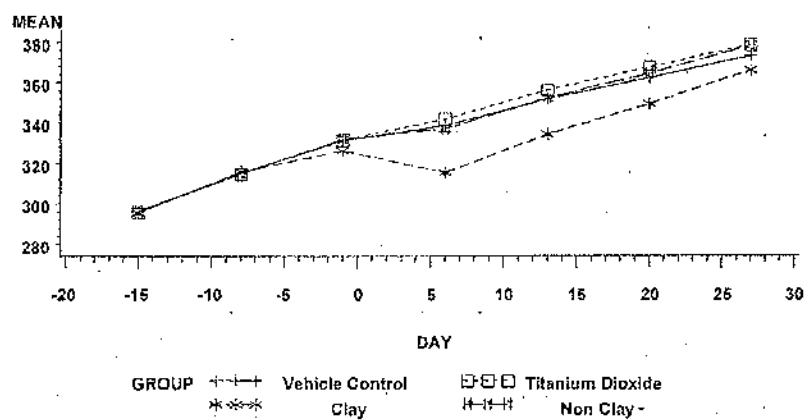
Lymph Nodes (LALN)	1						
Enlargement			1	3	1	11	10

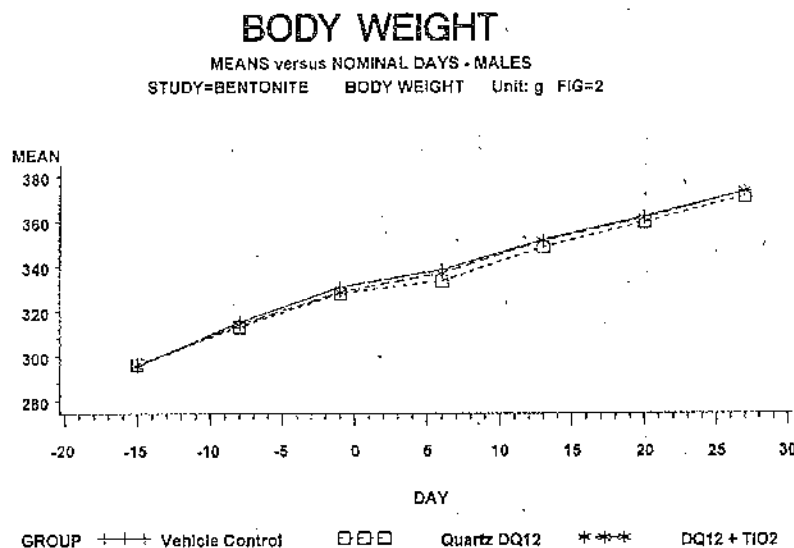
number of animals per group = 6 (Day 3) n = 11 (Day 28)

BODY WEIGHT

MEANS versus NOMINAL DAYS - MALES

STUDY=BENTONITE BODY WEIGHT Unit: g FIG=1





Figures 1 and 2 Body weight development

3.4 Bronchoalveolar Lavage (BAL)

3.4.1 Enzyme and Total Protein Levels (Table 6)

The results of bronchoalveolar lavage fluid (BALF) analysis are summarized in Table 6. Plots of lactic dehydrogenase, β -glucuronidase and total protein are presented in Figures 3-5.

The individual data is given in Appendix V.

The vehicle control shows data being at historical baseline levels. The titanium dioxide group (negative control) is in all parameters very close to data of the vehicle control (Fraunhofer ITEM internal database).

In the clay group, an inflammatory response was observed at day 3. After 28 days post-observation, however, the enzyme and total protein levels were no longer statistically different from controls. On day 3 the enzyme and protein levels of the non-clay group were not statistically different from controls. After 28 days the lavage protein showed a statistically

significant increase in the non-clay group (Table 6).

Quartz DQ 12 as the positive control showed an acute response which persisted over the 28-day period. There were no significant differences between DQ 12 and the DQ 12 plus TiO₂ groups.

3.4.2 Differential cell count (Tables 7 and 8)

Results of differential cell count are presented in Table 7 and 8. The individual data is given in Appendix VI.

In controls, differential cell count resulted in polymorphonuclear neutrophil (PMN) levels of 0.2% - 0.5% three days and 28 days after the last intratracheal instillation. These values are close to historical data and confirm the result of the 'lung health' check (see Appendix III) that rat lungs were without any background inflammation.

After 3 days, percentages of PMNs reached 61% for clay, 15% for non-clay, 67% for quartz DQ 12 and 3% for titanium dioxide. The values after 28 days were 14%, 36%, 65% and 3.5%, respectively. These data indicate an acute response in the clay group recovering partly within 4 weeks. In the non-clay group the response was initially moderate but increased in the 4-week observation period. Titanium dioxide and DQ 12 worked well as negative and positive controls.

Table 6 Means of parameters measured in BAL fluid 3 and 28 days after last of 4 instillations

28-Day Study: Inflammatory and Genotoxic Effects of Native or Treated Quartz Samples

Following Intratracheal Instillation

Study : BENTONITE-01030

Species: Rat

Sex : Male

Group Means

M A L E S		Day 3	Day 3	Day 3	Day 28	Day 28	Day 28
		LDH	β-GLU	Total Protein	LDH	β-GLU	Total Protein
		U/l	U/l	mg/l	U/l	U/l	mg/l
Control	Mean	48	0.3	80	31	0.2	88

	SD	7	0.1	9	6	0.1	9
	n	6	6	6	6	6	6
TiO ₂	Mean	63	0.2	94	37	0.2	92
	SD	19	0.1	15	6	0.1	11
	n	6	6	6	6	6	6
Clay	Mean	392***	2.2***	557***	52	0.2	131
	SD	190	0.8	129	9	0.1	8
	n	6	6	6	6	6	6
Non-Clay	Mean	121	0.8	175	117	1.0	201**
	SD	22	0.2	34	20	0.2	27
	n	6	6	6	6	6	6
Quartz DQ12	Mean	413***	3.3***	552***	428***	7.1***	877***
	SD	99###	0.4###	65###	98###	3.0###	55###
	n	6	6	6	6	6	6
DQ 12 + TiO ₂	Mean	405***	3.3***	505***	498***	8.9***	902***
	SD	73###	0.8###	62###	101###	2.8###	107###
	n	6	6	6	6	6	6

Statistics: Anova + Tukey's tests. * P<=5%; ** P<=1%; *** p<=0.1%; (vs. Control)
 ### p<=0.1%; (DQ12 and DQ12/TiO₂ vs. Non-Clay)

see Appendix VII for Anova + Tukey-test tables

LDH: Lactic Dehydrogenase

β-GLU: beta-Glucuronidase

LACTIC DEHYDROGENASE IN BALF

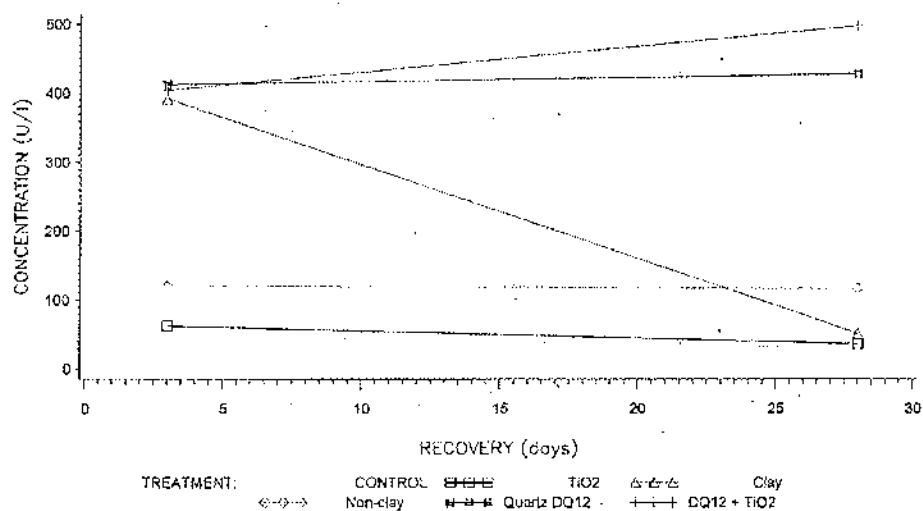
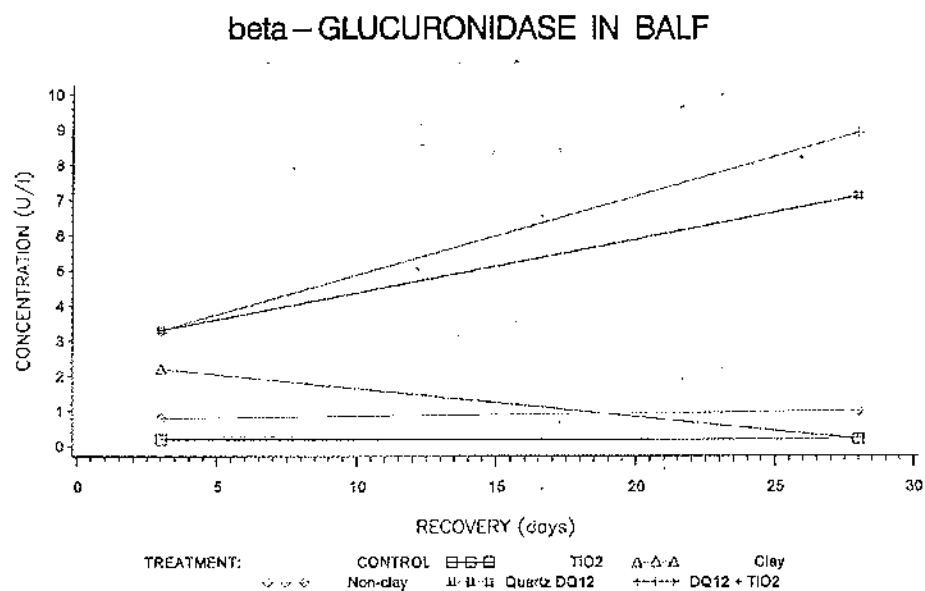


Figure 3 LDH levels measured on day 3 and day 28 after end of treatment

Figure 4 β -Glucuronidase levels measured on day 3 and day 28 after end of treatment

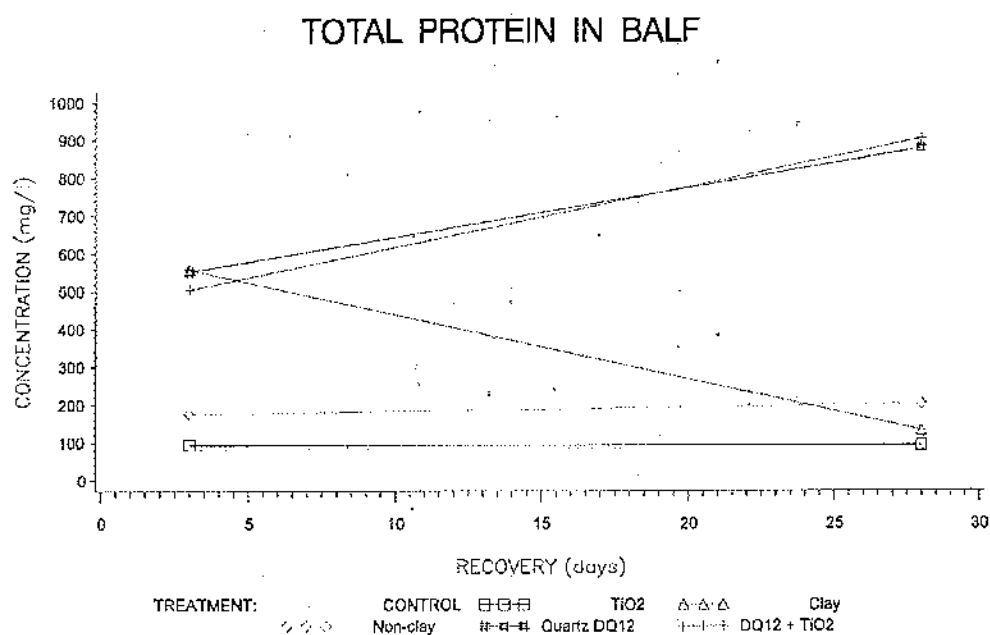


Figure 5 Total protein levels measured on day 3 and day 28 after end of treatment

Table 7 Differential cell count 3 days after intratracheal instillation (means)

BENTONITE - 02G01030

TABLE OF DIFFERENTIAL CELL COUNT

Wistar WU, males			RECOVERY PERIOD AFTER LAST INSTILLATION (days)					
			3					
			LEUKOCYTE NUMBER [cells]	MACRO- PHAGES [%]	EOSINO- PHILS [%]	NEUTRO- PHILS [%]	LYMPHO- CYTES [%]	Cell bili- ty(%)
Group	Dose [mg]							
Vehicle Control	0	MEAN	884531	99.3	0.0	0.2	0.5	96
		SD	224815	0.6	0.0	0.2	0.6	2
		n	6	6	6	6	6	6
TiO2	5	MEAN	1181042	94.9	0.0	2.9	2.2	98
		SD	275842	2.4	0.1	1.7	1.3	1
		n	6	6	6	6	6	6
Clay	5	MEAN	*** 7133333	*** 36.0	0.0	*** 60.9	* 3.1	* 99
		SD	1132456	5.9	0.1	6.7	1.6	1
		n	6	6	6	6	6	6
Non-Clay	5	MEAN	** 3986458	*** 81.5	0.1	*** 15.1	** 3.4	98
		SD	924182	4.5	0.2	3.6	1.3	2
		n	6	6	6	6	6	6
DQ 12	3.9	MEAN	***10145000	*** 30.8	0.0	*** 67.3	1.9	98
		SD	### 2270890	### 6.6	0.1	### 6.3	0.7	1
		n	6	6	6	6	6	6
DQ 12 + TiO2	3.9 + 1.1	MEAN	*** 7259583	*** 35.3	0.1	*** 62.4	2.2	99
		SD	### 1923279	### 4.7	0.1	### 6.1	1.5	1
		n	6	6	6	6	6	6

significantly different compared to vehicle controls

* p < 0.05 (Tukey's test)

** p < 0.01

*** p < 0.001

p < 0.001 (DQ12 and DQ12/TiO2 vs. Non-clay)

SD = standard deviation

n = number of animals

Table 8 Differential cell count 28 days after intratracheal instillation (means)

BENTONITE - 02G01030

TABLE OF DIFFERENTIAL CELL COUNT

Wistar WU, males			RECOVERY PERIOD AFTER LAST INSTILLATION					
			28					
			LEUKOCYTE NUMBER [cells]	MACRO- PHAGES [%]	EOSINO- PHILS [%]	NEUTRO- PHILS [%]	LYMPHO- CYTES [%]	Cell Viabi- lity [%]
Group	Dose [mg]							
Vehicle Control	0	MEAN	506823	99.3	0.0	0.5	0.3	99
		SD	206162	1.0	0.0	0.7	0.4	1
		n	6	6	6	6	6	6
TiO2	5	MEAN	480260	95.9	0.0	3.5	0.6	99
		SD	82606	1.4	0.0	1.3	0.3	1
		n	6	6	6	6	6	6
Clay	5	MEAN	1214271	** 85.2	0.0	** 14.3	0.5	* 96
		SD	362936	6.4	0.0	6.2	0.2	4
		n	6	6	6	6	6	6
Non-Clay	5	MEAN	2107292	*** 62.5	0.1	*** 35.5	** 2.0	99
		SD	545483	6.6	0.2	6.2	1.1	1
		n	6	6	6	6	6	6
DQ 12	3.9	MEAN	***22462500	*** 33.7	0.0	*** 64.8	1.4	99
		SD	### 8098650	### 9.3	0.1	### 9.7	1.1	1
		n	6	6	6	6	6	6
DQ 12 + TiO2	3.9 + 1.1	MEAN	***28617708	*** 36.8	0.0	*** 62.1	1.1	100
		SD	### 8967198	### 3.2	0.0	### 3.2	0.4	1
		n	6	6	6	6	6	6

significantly different compared to vehicle controls

* p < 0.05 (Tukey's test)

** p < 0.01

*** p < 0.001

p < 0.001 (DQ12 and DQ12/TiO2 vs. Non-clay)

SD = standard deviation

n = number of animals

In Figure 6 the absolute numbers of PMNs are shown.

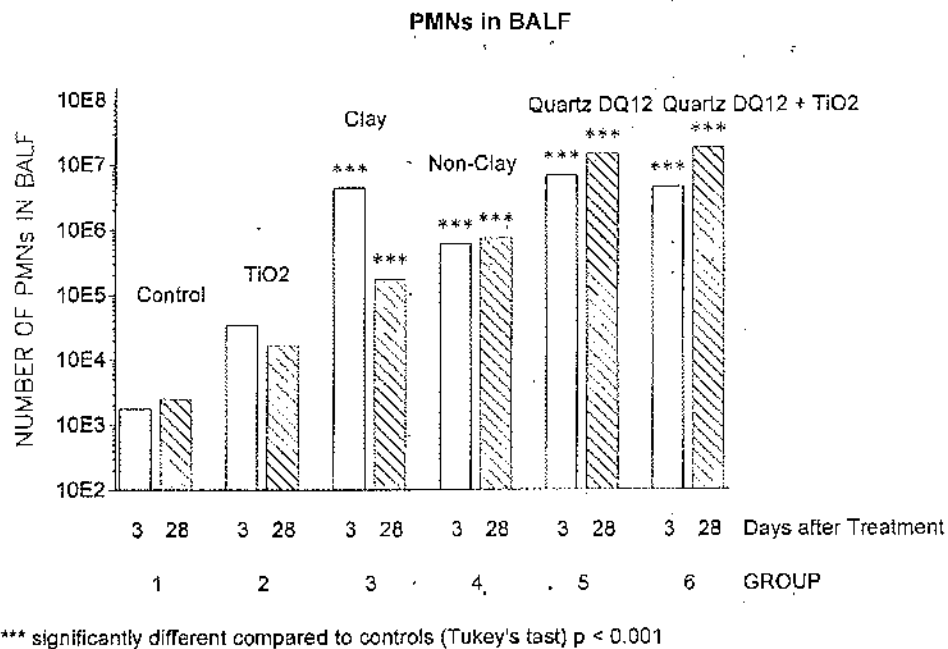


Figure 6 Absolute numbers of PMNs

3.4.3 Viability Test/Epithelial Cells

The trypan blue exclusion assay for cell viability did not show treatment-related effects (Tables 7 and 8, last column).

For epithelial cells significant differences between groups were not observed (see Appendix VIII).

3.4.4 Terminal Body and Lung Wet Weights

Absolute and relative wet lung weights of BAL animals were significantly elevated in all

treatment groups with the exception of the negative control (TiO₂) after 3 days (Tables 9 and 10; Figure 7). The clay group had the largest values. The clay group also had the lowest body weight values at 3 and 28 days.

The individual data is given in Appendix IX.

Table 9 Terminal body and lung wet weights 3 days after last of 4 instillations

Study : BENTONITE-01030
Species: Rat Sex: Male

Group Means				
M A L E S				
		Day 3	Day 3	Day 3
		TermBW	Rat lung	rel. Lung
		g	g	g/kg
Control	Mean	343.7	1.197	3.483
	SD	8.9	0.085	0.214
	n	6	6	6
TiO ₂	Mean	339.2	1.244	3.674
	SD	15.2	0.030	0.200
	n	6	6	6
Clay	Mean	310.6**	2.764***	8.896***
	SD	12.5	0.226	0.517
	n	6	6	6
Non-Clay	Mean	335.2	1.502**	4.482***
	SD	15.0	0.081	0.193
	n	6	6	6
Quartz DQ 12	Mean	338.0	1.853***	5.472***
	SD	17.2	0.174##	0.290###
	n	6	6	6
DQ 12 + TiO ₂	Mean	335.2	1.790***	5.340***
	SD	19.0	0.117#	0.215###
	n	6	6	6

significantly different compared to vehicle controls

* p < 0.05 (Tukey's test)

** p < 0.01

*** p < 0.001

p < 0.05

p < 0.01

p < 0.001 (DQ12 and DQ12/TiO₂ vs. Non-clay)

SD - standard deviation

n = number of animals

Table 10 Terminal body and lung wet weights 28 days after last of 4 instillations

Study : BENTONITE-01030

Species: Rat Sex: Male

 Group Means

M A L E S		Day 28	Day 28	Day 28
		TermBW g	Rat lung g	rel.Lung g/kg
Control	Mean	366.5	1.345	3.673
	SD	18.1	0.067	0.184
	n	6	6	6
TiO ₂	Mean	373.0	1.400	3.755
	SD	15.6	0.047	0.095
	n	6	6	6
Clay	Mean	357.8	2.050***	5.728***
	SD	13.9	0.149	0.344
	n	6	6	6
Non-Clay	Mean	374.3	1.482	3.962
	SD	9.8	0.116	0.319
	n	6	6	6
Quartz DQ 12	Mean	370.5	2.366***	6.388***
	SD	25.5	0.227###	0.457###
	n	6	6	6
DQ 12 + TiO ₂	Mean	370.2	2.395***	6.472***
	SD	19.2	0.204###	0.488###
	n	6	6	6

 significantly different compared to vehicle controls

* p < 0.05 (Tukey's test)

** p < 0.01

*** p < 0.001

p < 0.001 (DQ12 and DQ12/TiO₂ vs. Non-clay)

SD = standard deviation

n = number of animals

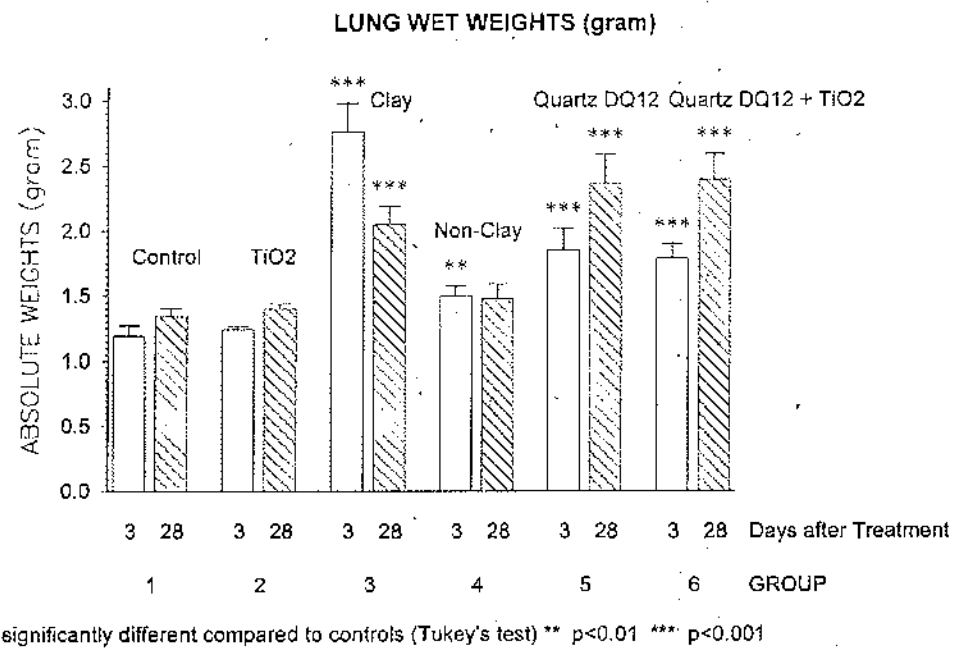


Figure 7 Absolute lung wet weights

3.5 Histopathology

3.5.1 Organ Weights

At terminal sacrifice 28 days after end of treatment organ weights were determined. In Table 11 the absolute and in Table 12 the relative weights are given of those animals taken for histopathological examination. Significant increases compared to vehicle controls were observed for the absolute and relative lung weights, in all treatment groups but titanium dioxide, correlating with the results of BAL animals. The significant decrease of liver weights in the DQ12/TiO₂ group is considered to be an incidental finding and may not be biologically relevant.

Table 11 Absolute terminal body and organ weights

Study : BENTONITE-01030
Species: Rat
Sex : Male

Group		Means							
Nominal days in study		28							
M A L E S		TermBW	Liver	L Kidney	R Kidney	L Adrenl	R Adrenl	L Testcl	R Testcl
		g	g	g	g	g	g	g	g
Control	Mean	385.9	16.370	1.259	1.281	0.019	0.018	1.772	1.784
	SD	13.5	1.343	0.102	0.110	0.001	0.001	0.066	0.091
	n	5	5	5	5	5	5	5	5
TiO ₂	Mean	394.8	16.733	1.364	1.344	0.018	0.018	1.761	1.774
	SD	24.5	0.966	0.100	0.104	0.003	0.002	0.037	0.067
	n	5	5	5	5	5	5	5	5
Clay	Mean	382.5	15.250	1.300	1.318	0.020	0.017	1.635	1.606
	SD	11.4	1.091	0.166	0.101	0.002	0.003	0.232	0.259
	n	5	5	5	5	5	5	5	5
Non-Clay	Mean	388.1	16.560	1.282	1.245	0.019	0.018	1.737	1.719
	SD	12.9	0.538	0.059	0.047	0.003	0.001	0.087	0.141
	n	5	5	5	5	5	5	5	5
Quartz	Mean	382.0	15.783	1.329	1.338	0.021	0.019	1.762	1.735
	SD	10.7	0.697	0.061	0.046	0.003	0.003	0.060	0.093
	n	10	5	5	5	5	5	5	5
DQ 12	Mean	379.2	14.056**	1.280	1.319	0.018	0.017	1.680	1.698
	SD	18.4	0.799	0.057	0.063	0.002	0.002	0.071	0.060
	n	10	5	5	5	5	5	5	5

significantly different compared to controls * P<=5%; ** P<=1%; *** P<=0.1%;
(Tukey's test)

P<=5%; ## P<=1%; ### P<=0.1%; (DQ12 and DQ12/TiO2 vs. Non-clay)
SD = standard deviation n = number of animals

Table 11 Absolute terminal body and organ weights – cont'd

M A L E S		R Epid g	L Epid g	Thymus g	Spleen g	Brain g	Heart g	Rat lung g
Control	Mean	0.617	0.606	0.453	0.710	1.927	1.281	1.365
	SD	0.036	0.036	0.076	0.099	0.050	0.065	0.075
	n	5	5	5	5	5	5	5
TiO ₂	Mean	0.679	0.622	0.569*	0.741	1.952	1.308	1.419
	SD	0.045	0.042	0.082	0.073	0.033	0.143	0.060
	n	5	5	5	5	5	5	5
Clay	Mean	0.629	0.609	0.364	0.724	1.927	1.324	2.419***
	SD	0.043	0.060	0.046	0.053	0.068	0.073	0.080
	n	5	5	5	5	5	5	5
Non-Clay	Mean	0.642	0.633	0.435	0.704	1.941	1.227	1.607
	SD	0.043	0.024	0.135	0.065	0.111	0.057	0.080
	n	5	5	5	5	5	5	5
Quartz DQ 12	Mean	0.607	0.628	0.440	0.688	1.829	1.294	2.626***
	SD	0.038	0.051	0.079	0.052	0.135	0.049	0.303
	n	5	5	5	5	5	5	5
DQ 12 + TiO ₂	Mean	0.645	0.610	0.415	0.698	1.929	1.296	2.400***
	SD	0.016	0.054	0.047	0.107	0.109	0.099	0.185
	n	5	5	5	5	5	5	5

significantly different compared to controls * P<=5%; ** P<=1%; *** P<=0.1%;
(Tukey's test)

P<=5%; ## P<=1%; ### P<=0.1%; (DQ12 and DQ12/TiO₂ vs. Non-clay)

SD = standard deviation n = number of animals

Lung weights were determined from rats used for histopathology; therefore these data differ from those of rats used for BAL (see section 3.4.4).

Table 12 Relative organ weights

Study : BENTONITE-01030

Species: Rat Sex: Male

Group		Means						
Nominal days in study		28						
M A L E S		r.Liver g/kg	r.L Kid g/kg	r.R Kid g/kg	r.L Adr g/kg	r.R Adr g/kg	r.L Tes g/kg	r.R Tes g/kg
Control	Mean	42.47	3.267	3.326	0.0498	0.0471	4.59	4.62
	SD	3.88	0.296	0.352	0.0027	0.0042	0.15	0.12
	n	5	5	5	5	5	5	5
TiO ₂	Mean	42.40	3.454	3.410	0.0463	0.0455	4.48	4.50
	SD	1.05	0.110	0.135	0.0071	0.0062	0.30	0.23
	n	5	5	5	5	5	5	5
Clay	Mean	39.83	3.392	3.442	0.0517	0.0443	4.27	4.19
	SD	1.72	0.343	0.167	0.0052	0.0079	0.59	0.65
	n	5	5	5	5	5	5	5
Non-Clay	Mean	42.69	3.303	3.211	0.0479	0.0472	4.48	4.44
	SD	1.51	0.087	0.134	0.0069	0.0045	0.32	0.42
	n	5	5	5	5	5	5	5
Quartz DQ 12	Mean	41.34	3.483	3.507	0.0555	0.0504	4.62	4.55
	SD	2.31	0.226	0.199	0.0092	0.0079	0.24	0.34
	n	5	5	5	5	5	5	5
DQ 12 + TiO ₂	Mean	37.06**	3.378	3.483	0.0485	0.0444	4.43	4.48
	SD	0.72	0.170	0.193	0.0039	0.0047	0.14	0.18
	n	5	5	5	5	5	5	5
M A L E S		r.R Epi g/kg	r.L Epi g/kg	r.Thymus g/kg	r.Spleen g/kg	r.Brain g/kg	r.Heart g/kg	rel.Lung g/kg
Control	Mean	1.602	1.572	1.175	1.838	5.00	3.32	3.538
	SD	0.125	0.130	0.198	0.237	0.13	0.17	0.152
	n	5	5	5	5	5	5	5
TiO ₂	Mean	1.726	1.579	1.442	1.875	4.96	3.31	3.610
	SD	0.171	0.105	0.184	0.089	0.24	0.18	0.337
	n	5	5	5	5	5	5	5
Clay	Mean	1.644	1.590	0.950	1.895	5.04	3.46	6.325***
	SD	0.098	0.136	0.105	0.153	0.08	0.16	0.204
	n	5	5	5	5	5	5	5
Non-Clay	Mean	1.655	1.633	1.116	1.816	5.01	3.16	4.146
	SD	0.129	0.081	0.332	0.192	0.42	0.15	0.269
	n	5	5	5	5	5	5	5
Quartz DQ 12	Mean	1.590	1.646	1.156	1.802	4.79	3.39	6.878***
	SD	0.119	0.169	0.236	0.145	0.33	0.16	0.822###
	n	5	5	5	5	5	5	5
DQ 12 + TiO ₂	Mean	1.703	1.609	1.097	1.835	5.09	3.42	6.337***
	SD	0.096	0.106	0.136	0.204	0.22	0.29	0.516###
	n	5	5	5	5	5	5	5

significantly different compared to controls * P<=5%; ** P<=1%; *** P<=0.1%;
(Tukey's test)

$P \leq 5\%$; ## $P \leq 1\%$; ### $P \leq 0.1\%$; (DQ12 and DQ12/TiO₂ vs. Non-clay)
SD = standard deviation n = number of animals
Lung weights were determined from rats used for histopathology; therefore these data differ from those of rats used for BAL (see section 3.4.4).

3.5.2 Histopathological Findings

The results of the histopathological examination of the lungs (all lobes) and the lung-associated lymph nodes (LALN) are summarized in Table 13.

The individual animal data are shown in Appendix X. Photographs of the characteristic histopathological findings are presented for all groups in Appendix XI.

Lungs:

No lesions were observed in the saline control group.

All rats of the particle instillation treatment groups (5/5, each group) had multifocal slight to moderate alveolar accumulations of particle-laden macrophages. A slight degree of alveolar macrophage accumulation was observed in 5/5 animals of the TiO₂ group, while all rats (5/5 each) of the clay group, the DQ12 group and the DQ12/TiO₂ group showed moderate alveolar accumulations of particle-laden macrophages. The non-clay group had both slight (4/5) and moderate (1/5) degrees of alveolar macrophage accumulation. The particle-laden macrophages were preferentially located in the alveolar duct region. Interstitial accumulations of particle-laden macrophages were observed at increasing degrees of severity in the TiO₂ group (very slight: 5/5), the non-clay group (very slight: 3/5; slight: 2/5), the DQ12 group (slight: 5/5) and the DQ12/TiO₂ group (slight: 4/5; moderate: 1/5). In the clay group, the interstitial accumulations of particle-laden macrophages were most pronounced, but had a more granulomatous appearance with a peripheral zone of fibrosis (= collagen deposition) and therefore were termed 'interstitial fibro-histiocytic granulomata' (slight: 1/5; moderate: 4/5). This change was also seen in single rats of the DQ12 and DQ12/TiO₂ groups, respectively. The pulmonary macrophages of the rats of the clay group showed a marked foamy appearance, but without significant signs of degeneration and necrosis. This was also true for the TiO₂ group, in which, however, the pigment obscured cellular details. In contrast, in the DQ12 and DQ12/TiO₂ groups, the majority of the alveolar macrophages were degenerating or necrotic, as reflected by alveolar lipoproteinosis. In the non-clay group, some macrophage degeneration was observed but to a much lesser extent than in the DQ12 and DQ12/TiO₂ groups. Multifocal alveolar lipoproteinosis was diagnosed in all rats of the DQ12

(moderate: 4/5; severe: 1/5) and DQ12/TiO₂ (slight: 1/5; moderate: 4/5) groups, respectively, and in 1/5 animals (slight, focal) of the non-clay group. The lipoproteinaceous intra-alveolar material appeared to be mainly derived from decaying macrophages.

Table 13: Summary of histopathological findings - with score expansion

		INCIDENCE OF LESIONS (PERCENT)					
		Males					
LESIONS	TREATMENT	Saline	5 mg TiO2	5 mg Clay	5 mg Non- Clay	3.9 mg DQ 12	3.9 + 1.1 mg DQ 12+ TiO2
LUNGS		(5)	(5)	(5)	(5)	(5)	(5)
No abnormality detected		5 (100%)	0** (0%)	0** (0%)	0** (0%)	0** (0%)	0** (0%)
Alveolar accumulation of particle-laden macrophages	slight	0 (0%)	5** (100%)	0 (0%)	4* (80%)	0 (0%)	0 (0%)
	moderate	0 (0%)	0 (0%)	5** (100%)	1 (20%)	5** (100%)	5** (100%)
Interstitial accumulation of particle-laden macrophages	very slight	0 (0%)	5** (100%)	0 (0%)	3 (60%)	0 (0%)	0 (0%)
	slight	0 (0%)	0 (0%)	0 (0%)	2 (40%)	5** (100%)	4* (80%)
	moderate	0 (0%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)	1 (20%)
Interstitial mononuclear cell infiltration	very slight	0 (0%)	4* (80%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)
	slight	0 (0%)	0 (0%)	5** (100%)	5** (100%)	0 (0%)	0 (0%)
	moderate	0 (0%)	0 (0%)	0 (0%)	0 (0%)	5** (100%)	3 (60%)
	severe	0 (0%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)	2 (40%)
Alveolar granulocytic infiltration	very slight	0 (0%)	0 (0%)	5** (100%)	2 (40%)	0 (0%)	0 (0%)
	slight	0 (0%)	0 (0%)	0 (0%)	3 (60%)	2 (40%)	0 (0%)
	moderate	0 (0%)	0 (0%)	0 (0%)	0 (0%)	3 (60%)	5** (100%)
Interstitial fibro-histiocytic granulomata	slight	0 (0%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)	1 (20%)
	moderate	0 (0%)	0 (0%)	5** (100%)	0 (0%)	1 (20%)	0 (0%)

Significance of difference in a pairwise Fisher's test between control and treatment groups: *P<0.05, **P<0.01, ***P<0.001
Figures in parentheses represent the number of animals from which this

tissue was examined microscopically

*** Continued

Table 13: Summary of histopathological findings - with score expansion (cont'd)

LESIONS	TREATMENT	INCIDENCE OF LESIONS (PERCENT)					
		Males					
		Saline	5 mg TiO ₂	5 mg Clay	5 mg Non- Clay	3.9 mg DQ 12	3.9 + 1.1 mg DQ 12+ TiO ₂
LUNGS		(5)	(5)	(5)	(5)	(5)	(5)
Bronchiolo-alveolar hyperplasia							
	very slight	0	1	3	2	0	0
		(0%)	(20%)	(60%)	(40%)	(0%)	(0%)
	slight	0	0	2	3	5**	5**
		(0%)	(0%)	(40%)	(60%)	(100%)	(100%)
Goblet cell hyperplasia, bronchi/bronchioles	slight	0	0	1	0	1	1
		(0%)	(0%)	(20%)	(0%)	(20%)	(20%)
Interstitial fibrosis *							
	very slight	0	0	0	5**	2	3
		(0%)	(0%)	(0%)	(100%)	(40%)	(60%)
	slight	0	0	5**	0	3	2
		(0%)	(0%)	(100%)	(0%)	(60%)	(40%)
Alveolar lipoproteinosis							
	slight	0	0	0	1	0	1
		(0%)	(0%)	(0%)	(20%)	(0%)	(20%)
	moderate	0	0	0	0	4*	4*
		(0%)	(0%)	(0%)	(0%)	(80%)	(80%)
	severe	0	0	0	0	1	0
		(0%)	(0%)	(0%)	(0%)	(20%)	(0%)
Foreign-body granuloma	slight	0	1	0	0	0	1
		(0%)	(20%)	(0%)	(0%)	(0%)	(20%)
Osseous metaplasia	slight	0	2	2	0	0	0
		(0%)	(40%)	(40%)	(0%)	(0%)	(0%)
Haemorrhage	slight	0	0	3	0	0	1
		(0%)	(0%)	(60%)	(0%)	(0%)	(20%)

Interstitial fibrosis *

Note: For qualitative comparison of this finding in the clay and non-clay group see text on page 47

Significance of difference in a pairwise Fisher's test between control and treatment groups: *P<0.05, **P<0.01, ***P<0.001

Figures in parentheses represent the number of animals from which this tissue was examined microscopically

*** Continued

Table 13: Summary of histopathological findings -with score expansion (cont'd)

LESIONS	TREATMENT	INCIDENCE OF LESIONS (PERCENT)					
		Males					
		Saline	5 mg TiO ₂	5 mg Clay	5 mg Non- Clay	3.9 mg DQ 12	3.9 + 1.1 mg DQ 12+ TiO ₂
LUNG ASSOCIATED LYMPH NODES		(5)	(5)	(5)	(5)	(5)	(5)
No abnormality detected		4 (80%)	0* (0%)	0* (0%)	0* (0%)	0* (0%)	0* (0%)
Accumulation of particle-laden macrophages	very slight	0 (0%)	1 (20%)	0 (0%)	1 (20%)	0 (0%)	0 (0%)
	slight	0 (0%)	4* (80%)	2 (40%)	4* (80%)	0 (0%)	0 (0%)
	moderate	0 (0%)	0 (0%)	3 (60%)	0 (0%)	4* (80%)	4* (80%)
	severe	0 (0%)	0 (0%)	0 (0%)	0 (0%)	1 (20%)	1 (20%)
Lymphoid hyperplasia	slight	0 (0%)	1 (20%)	2 (40%)	2 (40%)	0 (0%)	0 (0%)
	moderate	0 (0%)	0 (0%)	1 (20%)	0 (0%)	4* (80%)	3 (60%)
	severe	0 (0%)	0 (0%)	0 (0%)	0 (0%)	1 (20%)	2 (40%)
Mast-cell infiltration	moderate	1 (20%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)

Significance of difference in a pairwise Fisher's test between control and treatment groups: *P<0.05, **P<0.01, ***P<0.001

Figures in parentheses represent the number of animals from which this tissue was examined microscopically *** Listing Complete ***

Nearly all rats of the particle treatment groups showed focal or multifocal interstitial (predominantly perivascular) mononuclear cell infiltrations (TiO₂ group: 4 /5 very slight; clay and non-clay groups: 5/5 slight, each; DQ12 group: 5/5 moderate; DQ12/TiO₂ group: 3/5 moderate, 2/5 severe). A further hallmark of the inflammatory activity in the lungs was the degree of granulocytic infiltration of alveoli (and less obvious also of the interstitium). This change was not observed in the TiO₂ group but in all rats of the other particle treatment groups (5/5, each). The degree of granulocytic infiltration was very slight (5/5) in the clay group, very slight (2/5) to slight (3/5) in the non-clay group, slight (2/5) to moderate (3/5) in

the DQ12 group and moderate (5/5) in the DQ12/TiO₂ group.

Bronchiolo-alveolar hyperplasia, mainly of the bronchiolar type (alveolar bronchiolization) was another change which occurred at very slight (TiO₂ group: 1/5; clay group: 3/5; non-clay group: 2/5) to slight (clay group: 2/5; non-clay group: 3/5; DQ12, and DQ12/TiO₂ groups: 5/5, each) degrees in the particle-treatment groups. The hyperplastic epithelium often consisted of only a few cells and usually developed in association with inflammatory and fibrotic foci. In addition, single animals (1/5 per group) of the clay-, DQ12- and, DQ12/TiO₂ groups, respectively, showed multifocal slight mucus (goblet) cell hyperplasia of the bronchi/bronchioles.

Multifocal slight interstitial fibrosis, usually surrounding the interstitial fibro-histiocytic granulomata, was diagnosed in 5/5 rats of the clay group. In the non-clay group, all rats (5/5) had very slight (minimal) interstitial fibrosis, while in the DQ12 and DQ12/TiO₂ groups, 2/5 and 3/5 animals had very slight and 3/5 and 2/5 rats, respectively, showed slight interstitial fibrosis (see photographs in Appendix XII). Interstitial fibrosis could not be detected in any animal of the control or TiO₂ group. Interstitial fibrosis in all treatment groups, when present, developed in close association with accumulation of (particle-laden) macrophages and inflammatory cells (PMN, mononuclear cells) and was characterized by localized discrete thickening of intraalveolar septa due to increased deposition of collagen. In the non-clay group, both amount and extension of fibrotically thickened septa were dose-dependent and much less than in the quartz DQ 12 positive control group. Only in the latter group, concentric areas of fibrosis as a component of the cholesterol granulomas were observed in addition to thickened intraalveolar septa. The cholesterol granuloma-associated fibrosis was more pronounced than the fibrotic thickening of intraalveolar septa and tended to fill also alveolar spaces.

In the clay group, the interstitial fibro-histiocytic granulomas were characterized by central foci of foamy, but non-degenerating macrophages, which at the periphery were rimmed by collagenous (fibrotic) tissue that was continuous with the interstitium.

Other findings in the lungs such as foreign body granuloma, osseous metaplasia and haemorrhage occurred incidentally in some animals of different groups.

Lung-associated lymph nodes (LALN)

In all rats of the particle treatment groups, very slight to severe accumulation of particle-laden macrophages could be detected. This change was most pronounced in animals instilled with DQ12 and DQ12 + TiO₂ (4/5 moderate, 1/5 severe, each group), followed by the clay group (2/5 slight, 3/5 moderate) and the TiO₂ and non-clay groups (1/5 very slight, 4/5 slight, each group). Associated with the accumulations of particle-laden macrophages were slight to severe reactive lymphoid hyperplasias, also most pronounced in the DQ12 and DQ12 + TiO₂ groups.

The histopathological findings listed in Table 13 were individually scored attributing the following points to the grade of lesion:

no finding detected	zero points
very slight	one point
slight	two points
moderate	three points
severe	four points.

This scoring allows a statistical evaluation of histopathological findings using the Tukey test (see Table 14).

Table 14 Table of histopathological findings (numerical scoring)

Wistar WU, males	B1	B2	B3	B4	B5	B6	B7	B8	B9	B10	B11	B12	B13	B14
Group														
Vehicle	MEAN	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Control	SD	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
	n	5	5	5	5	5	5	5	5	5	5	5	4	4
TiO2	MEAN	* 2.0	* 1.0	0.0	0.0	0.0	0.2	0.0	0.0	0.0	0.4	0.6	0.0	* 1.8
	SD	0.0	0.0	0.4	0.0	0.0	0.4	0.0	0.0	0.0	0.9	1.1	0.0	0.4
	n	5	5	5	5	5	5	5	5	5	5	5	4	4
Clay	MEAN	* 3.0	0.0	* 2.0	* 1.0	* 3.0	* 1.4	0.4	* 2.0	0.0	0.0	0.8	* 1.2	* 2.6
	SD	0.0	0.0	0.0	0.0	0.0	0.5	0.9	0.0	0.0	0.0	1.1	1.1	0.5
	n	5	5	5	5	5	5	5	5	5	5	5	4	4
Non-Clay	MEAN	* 2.2	* 1.4	* 2.0	* 1.6	0.0	* 1.6	0.0	* 1.0	0.4	0.0	0.0	0.0	* 1.8
	SD	0.4	0.5	0.0	0.5	0.0	0.5	0.0	0.0	0.9	0.0	0.0	0.0	0.4
	n	5	5	5	5	5	5	5	5	5	5	5	4	4
DQ 12	MEAN	* 3.0	* 2.0	* 3.0	* 2.6	0.6	* 2.0	0.4	* 1.6	* 3.2	0.0	0.0	0.0	* 3.2
	SD	0.0	0.0	0.0	0.5	1.3	0.0	0.9	0.5	0.4	0.0	0.0	0.0	0.4
	n	5	5	5	5	5	5	5	5	5	5	5	4	4
DQ 12 + TiO2	MEAN	* 3.0	* 2.2	* 3.4	* 3.0	0.4	* 2.0	0.4	* 0.8	* 2.2	0.4	0.0	0.4	* 3.2
	SD	0.0	0.4	0.5	0.0	0.9	0.0	0.9	0.8	0.8	0.9	0.0	0.9	0.4
	n	5	5	5	5	5	5	5	5	5	5	5	4	4

LUNGS

B1	Alveolar accumulation of particle-laden macrophages
B2	Interstitial accumulation of particle-laden macrophages
B3	Interstitial mononuclear cell infiltration
B4	Alveolar granulocytic infiltration
B5	Interstitial fibro-histiocytic granulomata
B6	Bronchiole-alveolar hyperplasia
B7	Goblet cell hyperplasia, bronchi/bronchioles
B8	Interstitial fibrosis
B9	Alveolar lipoproteinosis
B10	Foreign-body granuloma
B11	Osseous metaplasia
B12	Hemorrhage

LALN

B13	Accumulation of particle-laden macrophages
B14	Lymphoid hyperplasia

* significantly different compared to vehicle controls p < 0.05 (Tukey's test)

significantly different (clay and non-clay vs. DQ 12) (Tukey's test)

SD = standard deviation

n = number of animals

3.6 HPRT Gene Mutation Assay

One single HPRT mutation, consisting of more than 50 cells was observed in this study. This occurred in one rat of the clay group (group 3, animal # 20). A differentiation of effects was not possible among the different groups. In the positive control DQ12 no mutants were detected.

Supplement (non-GLP)

As alternative genotoxic assay, micronuclei in alveolar macrophages have been evaluated using cytoslides from bronchoalveolar lavage. This additional investigation was done not in compliance with GLP.

Results are presented in the supplement at the end of the report. In summary, the result was that DQ12 quartz induced a significantly increased number of micronuclei in macrophages after 3 and 28 days. In contrary, for the non-clay fraction isolated from bentonite this effect was not observed.

4. DISCUSSION AND CONCLUSION

The scientific approach in this study was to characterize differences in biological activity between various quartz species. It is known that freshly ground and geological old quartz-containing materials behave differently in terms of toxicological potency (Valliyathan et al., 1995; Castranova et al., 1997).

The composition of the natural bentonite material is about 85% clay and about 15% non-clay grit. Bentonite is an alteration product of volcanic ash, and is principally smectite minerals, which are hydrous aluminium silicates. Montmorillonite is the predominate smectite mineral encountered in this study.

The clay fraction used in this study showed a strong acute effect (three days after termination of treatment) in inflammatory BAL parameters which was similar to that of the positive control quartz DQ 12. This result may be related to the very small particle size ($< 1 \mu\text{m}$), the high dose and correspondingly large surface area of this test material ($480 \text{ m}^2/\text{g}$; EGME method; see Table 1). Similar effects of a very fine particle size has been observed with other

particulate matter, such as TiO_2 and carbon black (Oberdörster et al., 1994; Driscoll et al., 1996), when they have been administered in the ultrafine or fine particle mode. The non-clay particles were in the size range 1-10 μm . The positive controls of DQ 12 and DQ12 plus TiO_2 with particle sizes of 1-5 μm show a similar high acute response. In contrast to the clay particles, the non-clay particles with a size range of 1-10 μm induced reactions much weaker than the positive control. After 28 days, however, the clay group showed a significant recovery.

Based on the histopathological findings, especially with respect to mononuclear cell and granulocyte infiltration, pulmonary inflammation was most pronounced in the DQ12 and DQ12 + TiO_2 groups, followed by the non-clay and clay groups. In the TiO_2 group, no significant inflammatory lesions could be observed.

A comparison of the characteristics of lesions between non-clay and quartz DQ12 results in the following interpretation. Pulmonary lesions in the non-clay group at this early timepoint after instillation (i.e. 28 days) are much different to those seen in the DQ12-control groups, mainly quantitatively but also qualitatively, as demonstrated by the finding of prominent alveolar lipoproteinosis in the quartz group only for example. Due to this qualitative difference, it is very unlikely, that in the non-clay groups a progression of the lesions towards permanent lung damage would occur over time as is typical for quartz DQ12. The lesions observed in the quartz groups were the same as seen in other toxicological studies with DQ12 at this timepoint, whereas the lesions in the non-clay groups were similar to the spectrum of lesions which can be observed in the rat lung also after instillation of a large variety of other particles with less cytotoxicity than DQ12.

In the clay group, the type of inflammation differed from the quartz groups by the absence of lesions which are typical for a persistent and self-perpetuating inflammation, i.e. macrophage degeneration, lipoproteinosis and granulocyte infiltration. Instead, the type of inflammation in the clay group is considered to represent the chronic stage of a severe acute damage as evidenced by the presence of interstitial granulomata and prominent interstitial fibrosis.

The morphological changes observed in the lungs of the clay group of this study resemble very much the lesions seen for example after instillation of (soluble) amorphous (colloidal)

silica. With this material, which has a half-life of about 10 days in the lungs, there was observed a significant reduction in the amount of fibrosis over time, although thickened (fibrotic) interalveolar septa were still recognizable 2 years after instillation. Other lung lesions e.g. such as potentially preneoplastic hyperplasias, however, could not be detected in the amorphous silica-treated rats. This means that the fibrotic lesions seen in the clay group might resolve with time.

If rats had been treated with similar doses of TiO_2 or carbon black instead of clay, these would have caused fibrotic lesions of much less severity at this early timepoint. However, due to the long persistence of these particles in the lung, a gradual increase of interstitial (focal) fibrosis and emergence of other lesions, e.g. such as cholesterol granulomas and hyperplastic changes, over time is typical.

With respect to the HPRT assay, the evaluation of mutants delivered only one colony at all (for animal # 030020 – clay group) and no background mutations were detected. Therefore, the results of the assay cannot be used for a mutagenic assessment in this study. A positive outcome of this assay has been demonstrated in previous Fraunhofer ITEM work (background mutations in controls; mutants induced by Ethylmethane-sulfonate - EMS). Therefore, a reason for the non-occurrence of mutants, particularly in the DQ12 quartz group, is not known. EMS or N-Ethyl-N-nitroso-urea (ENU) as a second positive agent was not run in parallel in this study.

Supplement (non-GLP)

As an alternative, the evaluation of micronuclei in alveolar macrophages was found to be a suitable tool for detection of genotoxic effects. Results of this parameter allowed a differentiation between quartz DQ12 (significantly increased response) and the non-clay fraction (no significant increase). Etoposide was identified as a well-working positive control.

From the results of the study the following conclusions can be derived:

- The vehicle control did not induce any lung background inflammation. With the titanium dioxide (Bayertitan T) and quartz DQ 12 two appropriate representatives for a negative and a positive control were chosen.

The clay fraction induced a high acute inflammatory response. After 28 days effects in this group recovered mostly. Histopathological examination characterized the type of inflammation as not typical for a persistent and self-perpetuating inflammation; it was specified as a chronic stage of a severe acute damage.

- The non-clay fraction induced effects that were evidently weaker than those of quartz DQ 12 and DQ 12 + TiO₂.

Overall, the above-mentioned biological effects of the test items can be ranked in the following order considering single parameters:

A. Lavage data: acute (3-day result):

DQ 12 ≈ DQ 12/TiO₂ = Clay >> Non-Clay > TiO₂ > Vehicle Control

B. Lavage data: subchronic (28-day result):

DQ 12 ≈ DQ 12/TiO₂ >> Non-Clay >> Clay > TiO₂ > Vehicle Control

C. Histopathology: inflammation:

DQ 12 ≈ DQ 12/TiO₂ > Clay > Non-Clay >> TiO₂ = Vehicle Control

D. Histopathology: fibrosis:

DQ 12 ≈ DQ 12/TiO₂ > Non-Clay > Clay >> TiO₂ > Vehicle Control

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Reg Tox Pharm 24, 202-212 (1996)

Appendix I Concentration of Solid Material in SMI Clay/Non-Clay Slurries

Small aliquots of the samples were lyophilized to determine the concentrations (n=2):

Sample Identification No.	Concentration (mg/ml)	Density (g/cm ³)
SMI R-01-353A	34.6	1.0044
SMI R-01-353B	429.8	1.2386
(SMI R-01-312A	40.2	0.9841)
(SMI R-01-312C	mostly solid precipitant; aqueous moiety 56.4%)	

Test items in parentheses were not used in the main study.

Conclusion

The concentrations of all suspensions/slurries are high enough to adjust the administration concentrations by adding of water/sodium chloride to final values of 1.25 mg per 0.3 ml. The latter is the instillation volume per daily administration in the rat. This means that the dusts can remain in suspension form till administration to the animals (no evaporation of water necessary).

Appendix II Endotoxin Analysis

The lipopolysaccharide (LPS) concentration of test and reference items was determined according to the following sequence:

- the material to be tested was weighed in a 25 ml Erlenmeyer flask and the 99-fold amount of sterile water added
-
- the sample was digested in an oven at 80° C for 1 hour followed by a centrifugation at 2000 x g for 10 minutes
-
- a dilution series was prepared from the supernatant mixing 64.8 µl sterile water with 30 µl sample solution; using this initial dilution step of 3.16 a series up to a dilution of 1 : 316000 was prepared
-
- from each dilution step a volume of 30 µl was pipetted on a test plate (LAL Test, Labortechnik Peter Schulz) together with sterile water (negative control) and an endotoxin standard (1 EU/ml); positive control).
-
- the test plate covered with Parafilm was incubated for one hour
-
- after incubation to each test cavity 20 µl of a staining solution was pipetted; the covering liquid was sucked off by a Pasteur pipet connected to a water stream pump making visible the gel formation
-
- formation of a purple gel in the cavity indicates a positive reaction the dilution step of the last positive reaction was recorded; this was multiplied with the sensitivity of the plate (1 EU = 0.1 ng) and the dilution factor deriving from the sample preparation (1 g sample in 100 g solution). The unit of the result is ng LPS/g sample.

Appendix III Background Values of PMNs in Rat Lungs

Before starting the intratracheal instillation study, on October 2, 2001 a check of lung leukocytes was performed with 5 rats to control the animal breeder/delivery. The results of this bronchoalveolar lavage (BAL) investigation are shown in the table below.

Investigation done on October 2, 2001

Rat #	Age (weeks)	Body Weight (g)	Cell Concentration (1/ml)	Macrophages (%)	PMNs (%)	Lymphocytes (%)
5 rats from same colony, 9 weeks aged; sacrifice 28 days after receipt at Fraunhofer ITEM						
157	9	256.7	69,375	99.75	0.25	0
158	9	247.7	113,750	98.25	1	0.75
159	9	269.7	81,875	99.75	0	0.25
160	9	253.7	124,375	99.75	0.25	0
161	9	243.7	73,750	96.65	2.85	0.5
Means			92,625	98.83	0.87	0.30
SD			24,833	1.38	1.17	0.33
N			5	5	5	5

SD Standard Deviation

N Number of rats

Conclusion

The quality of the BAL cell status was satisfactory in all rats but rat # 161. This animal showed a moderately increased PMN level. This may be an incidental finding. Overall, the delivery of Wistar WU rats for study no. 02G01030 had lung PMN levels, which were in the normal range observed for control animals. As no evidence for a background inflammation in lungs was found the animals can be used for the study without any reservation.

Appendix IV Body Weights - Means

Study : BENTONITE-01030

Species: Rat

Sex : Male

B o d y W e i g h t (g)

M A L E S		Day -15	Day -8	Day -1	Day 6	Day 13	Day 20
Control	Mean	295.9	315.4	331.0	338.6	351.9	362.3
	SD	11.0	10.6	10.8	12.8	13.2	14.5
	n	22	22	22	16	16	16
TiO2	Mean	295.8	314.8	331.5	341.9	356.3	367.6
	SD	10.4	13.4	14.3	15.4	16.3	18.3
	n	22	22	22	16	16	16
Clay	Mean	295.5	315.9	326.2	315.4***	334.3**	349.4
	SD	10.9	12.7	14.1	20.9	15.3	16.8
	n	22	22	22	16	16	16
Non-Clay	Mean	296.2	314.7	331.9	337.0	352.4	364.5
	SD	10.8	11.7	12.3	14.3	12.0	14.1
	n	22	22	22	16	16	16
Quartz DQ 12	Mean	296.7	313.3	328.2	333.6	348.6	359.9
	SD	12.0	13.3	16.0	16.4	16.5	17.7
	n	22	22	22	16	16	16
DQ 12 + TiO2	Mean	295.8	314.3	328.8	337.2	351.5	361.5
	SD	11.7	12.7	14.0	16.9	17.0	17.3
	n	22	22	22	16	16	16
		Day 27	Day 34	Day 41			
Control	Mean	373.3	387.9	397.6			
	SD	17.2	10.9	0.0			
	n	16	3	1			
TiO2	Mean	378.9	389.6	395.9			
	SD	19.3	6.3	0.0			
	n	16	3	1			
Clay	Mean	366.1	386.8	404.3			
	SD	17.5	1.4	0.0			
	n	16	3	1			
Non-Clay	Mean	378.4	395.7	381.2			
	SD	15.4	24.0	0.0			
	n	16	3	1			
Quartz DQ 12	Mean	371.2	378.7	392.7			
	SD	17.4	2.7	0.0			
	n	16	3	1			
DQ 12 + TiO2	Mean	373.6	399.1	419.6			
	SD	16.9	8.0	0.0			
	n	16	3	1			

Statistics: Anova + L.S.D. tests. (Two-Sided) * $P \leq 5\%$; ** $P \leq 1\%$; *** $P \leq 0.1\%$;
SD = standard deviation n = number of animals

Appendix IV Body Weights - Individual Data with Means - cont'd

Study : BENTONITE-01030

Species: Rat

Sex : Male

Body Weight (g)

M A L E S		Day -15	Day -8	Day -1	Day 6	Day 13	Day 20
Animal No.							
Control	010001	293.8	317.9	335.4			
	010002	284.9	308.9	324.3			
	010003	319.2	339.9	352.6			
	010004	307.5	320.6	342.7			
	010005	289.8	314.7	334.4			
	010006	287.2	313.5	327.7	333.0	343.9	361.0
	010007	309.4	325.7	339.4	353.8	370.4	376.0
	010008	292.4	308.9	326.3	340.0	347.6	361.3
	010009	278.7	297.2	312.1	317.1	332.8	339.4
	010010	285.0	302.9	319.0	322.6	340.5	353.4
	010011	306.8	319.3	329.7	335.2	351.0	360.3
	010012	301.0	319.0	330.6	342.4	358.4	364.6
	010013	292.2	313.9	330.8	345.9	362.5	372.5
	010014	302.1	329.7	344.8	359.3	375.6	392.1
	010015	298.1	320.3	331.2	341.7	354.0	366.7
	010016	289.3	310.5	321.7	339.5	355.1	365.4
	010017	303.6	321.6	336.2	340.7	353.5	363.4
	010018	294.0	309.3	325.3	332.8	340.2	351.0
	010019	309.2	327.7	345.6	350.2	360.0	372.9
	010020	292.9	312.0	335.6	350.1	359.1	366.5
	010021	298.7	311.9	330.4			
	010022	272.9	292.7	306.2	313.3	325.7	329.7
Control	Mean	295.9	315.4	331.0	338.6	351.9	362.3
	SD	11.0	10.6	10.8	12.8	13.2	14.5
	n	22	22	22	16	16	16

M A L E S		Day -15	Day -8	Day -1	Day 6	Day 13	Day 20
Animal No.							
TiO2	020001	297.1	315.7	333.3			
	020002	282.7	301.4	319.2			
	020003	313.9	337.4	355.4			
	020004	305.4	322.2	339.4			
	020005	289.6	302.4	317.4			
	020006	290.4	307.9	327.2	334.8	352.5	363.9
	020007	302.2	325.7	345.6	353.2	374.7	382.4
	020008	290.4	304.7	317.4	329.4	343.0	349.4
	020009	282.6	294.8	310.7	315.3	331.6	344.0
	020010	283.0	298.4	314.3	323.6	339.3	347.6
	020011	307.8	336.9	351.4	362.4	384.1	400.2
	020012	295.5	315.0	330.6	340.2	352.6	364.0
	020013	293.2	308.3	322.6	332.6	343.4	353.7
	020014	314.4	339.2	358.4	367.9	383.8	401.7
	020015	305.0	319.7	334.7	352.3	367.1	381.2
	020016	282.0	300.3	317.4	327.9	343.1	353.6
	020017	305.9	327.6	351.9	357.6	373.6	388.6
	020018	296.1	317.1	336.3	347.2	355.4	367.7
	020019	301.7	326.8	340.0	357.0	362.7	369.0
	020020	291.4	312.3	324.7	340.0	351.8	355.7
	020021	297.7	312.9	326.4			
	020022	279.2	299.0	318.3	328.3	342.2	358.4
TiO2	Mean	295.8	314.8	331.5	341.9	356.3	367.6
	SD	10.4	13.4	14.3	15.4	16.3	18.3
	n	22	22	22	16	16	16

Statistics: Anova + L.S.D. tests. (Two-Sided) * P<=5%; ** P<=1%; *** P<=0.1%;

SD = standard deviation

n = number of animals

Appendix IV Body Weights - Individual Data with Means - cont'd

Study : BENTONITE-01030

Species: Rat

Sex : Male

Body Weight (g)

M A L E S		Day -15	Day -8	Day -1	Day 6	Day 13	Day 20
Animal No.							
Clay	030001	299.2	325.8	339.9			
	030002	284.0	302.2	317.6			
	030003	308.4	329.0	345.5			
	030004	310.4	326.4	346.8			
	030005	288.9	303.2	317.4			
	030006	282.9	304.9	322.2	299.2	325.1	343.1
	030007	307.2	324.1	339.1	324.7	350.8	367.9
	030008	289.7	308.5	324.0	302.4	322.2	334.7
	030009	278.5	294.6	308.7	288.4	313.7	326.7
	030010	278.5	294.0	311.6	287.1	314.9	325.0
	030011	310.3	334.3	326.0	326.8	354.8	375.3
	030012	299.3	323.7	317.5	318.0	338.6	354.4
	030013	297.4	317.7	318.9	314.6	337.3	354.1
	030014	306.4	328.2	322.9	325.1	352.6	366.9
	030015	303.7	325.2	321.2	328.3	356.5	374.6
	030016	288.3	303.0	294.6	294.8	320.1	335.5
	030017	299.7	326.2	342.4	316.9	342.1	361.1
	030018	296.3	317.5	332.1	328.4	329.3	352.2
	030019	303.9	326.4	339.4	351.8	335.3	349.8
	030020	296.3	318.6	339.4	352.7	345.0	340.3
	030021	297.8	321.2	341.0			
	030022	274.9	295.0	308.5	287.4	311.1	328.9
Clay	Mean	295.5	315.9	326.2	315.4***	334.3**	349.4
	SD	10.9	12.7	14.1	20.9	15.3	16.8
	n	22	22	22	16	16	16
Non-Clay	040001	301.7	326.2	349.5			
	040002	280.9	298.8	313.6			
	040003	316.8	339.1	352.5			
	040004	305.0	319.7	339.4			
	040005	291.1	309.3	329.6			
	040006	287.1	302.4	320.9	321.4	339.5	347.1
	040007	305.3	323.6	337.0	347.5	361.9	371.6
	040008	293.9	310.3	329.4	331.1	348.8	363.0
	040009	283.8	306.3	321.9	327.4	350.7	361.8
	040010	283.4	300.5	315.3	320.5	334.9	343.1
	040011	307.3	320.7	341.0	353.7	369.2	386.4
	040012	297.3	309.8	323.5	330.7	344.1	357.1
	040013	293.8	313.8	331.3	342.6	356.3	371.3
	040014	311.6	327.5	340.4	348.6	368.5	383.9
	040015	300.2	320.5	332.1	334.2	352.9	365.0
	040016	282.5	298.8	310.9	317.8	334.5	347.2
	040017	303.5	324.0	346.7	346.4	361.4	375.5
	040018	297.0	318.0	338.9	346.9	359.3	373.2
	040019	308.2	332.4	348.7	366.6	369.9	381.5
	040020	290.2	308.8	325.0	339.0	345.0	343.9
	040021	299.4	315.8	336.4			
	040022	277.1	296.2	316.8	317.7	340.8	361.1
Non-Clay	Mean	296.2	314.7	331.9	337.0	352.4	364.5
	SD	10.8	11.7	12.3	14.3	12.0	14.1
	n	22	22	22	16	16	16

Statistics: Anova + L.S.D. tests. (Two-Sided) * P<=5%; ** P<=1%; *** P<=0.1%;

SD = standard deviation

n = number of animals

Appendix IV Body Weights - Individual Data with Means - cont'd

Study : BENTONITE-01030

Species: Rat

Sex : Male

B o d y W e i g h t (g)

M A L E S	Animal No.	Day -15	Day -8	Day -1	Day 6	Day 13	Day 20
Quartz	050001	301.8	319.5	337.5			
DQ 12	050002	283.9	303.1	308.5			
	050003	322.2	335.1	358.9			
	050004	304.5	321.1	344.0			
	050005	292.4	312.4	325.8			
	050006	291.7	311.5	330.0	335.0	350.3	362.1
	050007	312.2	334.9	358.8	363.1	382.8	395.3
	050008	293.8	308.6	325.8	323.7	341.9	360.2
	050009	277.5	293.1	311.8	317.8	332.6	334.7
	050010	275.7	288.9	302.4	306.7	321.4	334.9
	050011	304.6	328.2	331.8	347.1	357.6	373.7
	050012	301.1	319.8	325.1	340.8	357.2	370.0
	050013	294.8	308.8	313.9	327.2	351.3	365.0
	050014	312.2	334.0	341.5	358.4	373.1	383.9
	050015	300.5	313.1	318.5	331.9	347.7	363.5
	050016	283.1	296.6	306.2	320.2	329.4	341.7
	050017	303.8	318.5	344.1	341.6	359.5	370.2
	050018	294.7	311.6	329.4	339.6	351.9	357.2
	050019	303.1	314.2	328.4	341.7	352.4	361.9
	050020	295.8	306.9	324.9	338.3	343.9	350.5
	050021	300.5	321.1	342.3			
	050022	277.6	292.4	308.8	305.1	324.8	332.8
Quartz	Mean	296.7	313.3	328.2	333.6	348.6	359.9
DQ 12	SD	12.0	13.3	16.0	16.4	16.5	17.7
	n	22	22	22	16	16	16
DQ 12	060001	296.9	315.8	329.7			
+ TiO2	060002	287.1	301.9	314.8			
	060003	318.0	338.0	361.5			
	060004	305.2	328.7	343.4			
	060005	287.1	301.8	310.6			
	060006	284.0	301.5	320.6	324.5	339.5	352.0
	060007	309.3	328.0	346.6	349.9	369.2	376.8
	060008	290.6	309.8	326.3	334.4	349.5	355.4
	060009	276.6	296.5	315.0	318.2	331.8	344.2
	060010	283.7	305.9	325.5	325.3	346.2	357.1
	060011	309.3	330.3	342.2	357.6	374.2	387.4
	060012	295.1	313.1	314.4	325.0	337.4	343.7
	060013	296.9	315.8	322.9	340.3	358.1	370.7
	060014	313.1	334.1	337.1	351.6	368.6	379.6
	060015	301.2	315.6	323.3	340.4	353.2	363.0
	060016	285.7	306.8	317.1	328.6	342.9	354.3
	060017	305.0	317.6	330.2	332.8	351.3	357.1
	060018	295.9	318.1	339.1	352.5	361.7	372.3
	060019	300.3	319.4	338.5	357.3	359.7	373.6
	060020	297.3	316.8	338.7	357.9	371.2	377.9
	060021	295.7	312.9	329.7			
	060022	272.6	286.7	297.9	298.4	309.2	319.2
DQ 12	Mean	295.8	314.3	328.8	337.2	351.5	361.5
+ TiO2	SD	11.7	12.7	14.0	16.9	17.0	17.3
	n	22	22	22	16	16	16

Statistics: Anova + L.S.D. tests. (Two-Sided) * P<=5%; ** P<=1%; *** P<=0.1%;

SD = standard deviation

n = number of animals

Appendix IV Body Weights - Individual Data with Means – cont'd

Study : DENTONITE-01030

Species: Rat Sex : Male

B o d y W e i g h t (g)

M A L E S	Animal No.	Day 27	Day 34 +	Day 41 +
Control	010006	370.8		
	010007	382.7		
	010008	370.6		
	010009	347.8		
	010010	359.0		
	010011	369.1		
	010012	381.4		
	010013	384.0		
	010014	405.5		
	010015	375.7		
	010016	380.6		
	010017	382.6		
	010018	365.7	375.9	
	010019	385.8	397.1	
	010020	381.2	390.8	397.6
	010022	330.6		
Control	Mean	373.3	387.9	397.6
	SD	17.2	10.9	0.0
	n	16	3	1
TiO2	020006	377.6		
	020007	392.0		
	020008	359.9		
	020009	354.7		
	020010	359.7		
	020011	415.1		
	020012	372.3		
	020013	360.1		
	020014	413.2		
	020015	390.4		
	020016	365.6		
	020017	404.3		
	020018	380.3	396.3	
	020019	380.5	388.8	
	020020	369.1	383.7	395.9
	020022	367.4		
TiO2	Mean	378.9	389.6	395.9
	SD	19.3	6.3	0.0
	n	16	3	1
Clay	030006	361.5		
	030007	380.8		
	030008	346.5		
	030009	341.1		
	030010	341.5		
	030011	393.1		
	030012	368.6		
	030013	367.0		
	030014	383.6		
	030015	393.7		
	030016	351.5		
	030017	379.7		
	030018	372.9	387.3	
	030019	366.4	385.2	
	030020	365.3	387.9	404.3
	030022	344.5		
Clay	Mean	366.1	386.8	404.3
	SD	17.5	1.4	0.0
	n	16	3	1

Statistics: Anova + L.S.D. tests. (Two-Sided) * $P \leq 5\%$; ** $P \leq 1\%$; *** $P \leq 0.1\%$;
 SD = standard deviation n = number of animals
 + Day 34 and 41: Rats of the subgroup used for the HPRT assay; sacrifices of
 animal nos. 16-20 have been spread over 3 weeks for organisatory reasons

Appendix IV Body Weights - Individual Data with Means - cont'd

Study : BENTONITE-01030

Species: Rat Sex : Male

Body Weight (g)

M A L E S	Animal No.	Day 27	Day 34 +	Day 41 +
Non-Clay	040006	357.4		
	040007	384.0		
	040008	376.6		
	040009	374.2		
	040010	352.5		
	040011	396.7		
	040012	368.3		
	040013	385.9		
	040014	395.2		
	040015	382.0		
	040016	359.6		
	040017	392.0		
	040018	390.5	406.6	
	040019	402.5	412.2	
	040020	360.2	368.2	381.2
	040022	377.3		
Non-Clay	Mean	378.4	395.7	381.2
	SD	15.4	24.0	0.0
	n	16	3	1
Quartz DQ 12	050006	376.7		
	050007	406.3		
	050008	368.5		
	050009	350.5		
	050010	342.5		
	050011	384.2		
	050012	376.9		
	050013	377.6		
	050014	397.1		
	050015	368.8		
	050016	353.6		
	050017	382.9		
	050018	369.3	377.2	
	050019	370.9	381.9	
	050020	366.9	377.1	392.7
	050022	346.0		
Quartz DQ 12	Mean	371.2	378.7	392.7
	SD	17.4	2.7	0.0
	n	16	3	1
DQ 12 + TiO ₂	060006	364.2		
	060007	390.9		
	060008	368.2		
	060009	359.7		
	060010	376.2		
	060011	399.2		
	060012	351.1		
DQ 12 + TiO ₂	060013	374.7		
	060014	389.2		
	060015	373.2		
	060016	369.9		
	060017	366.8		
	060018	385.9	394.2	
	060019	380.5	394.8	

	060020	394.1	408.4	419.6
	060022	333.9		
DQ 12 + TiO2	Mean	373.6	399.1	419.6
	SD	16.9	8.0	0.0
	n	16	3	1

Statistics: Anova + L.S.D. tests. (Two-Sided) * P<=5%; ** P<=1%; *** P<=0.1%;

* Day 34 and 41: Rats of the subgroup used for the HPRT assay; sacrifices of animal nos. 16-20 have been spread over 3 weeks for organisatory reasons

Appendix V Enzyme and Total Protein Levels in BALF (individual data)

Study : BENTONITE-01030 Species: Rat Sex: Male

Individual Data Listing with Means

M A L E S		Day 3	Day 3	Day 3
	Animal No.	LDH U/l	8-GLU U/l	Total Protein mg/l
Control	A 010001	43	0.3	73
	A 010002	58	0.3	89
	A 010003	54	0.3	88
	A 010004	39	0.2	67
	A 010005	49	0.4	81
	A 010021	44	0.2	84
Control	Mean	48	0.3	80
	SD	7	0.1	9
	n	6	6	6
TiO2	B 020001	56	0.2	92
	B 020002	89	0.4	117
	B 020003	68	0.2	85
	B 020004	79	0.2	106
	B 020005	40	0.2	74
	B 020021	48	0.2	91
TiO2	Mean	63	0.2	94
	SD	19	0.1	15
	n	6	6	6
Clay	C 030001	226	1.7	447
	C 030002	447	2.2	511
	C 030003	319	1.6	540
	C 030004	719	2.6	770
	C 030005	436	3.7	643
	C 030021	204	1.7	434
Clay	Mean	392***	2.2***	557***
	SD	190	0.8	129
	n	6	6	6
Non-Clay	D 040001	137	1.1	243
	D 040002	114	0.8	172
	D 040003	123	0.0	156
	D 040004	150	0.5	172
	D 040005	85	0.6	147
	D 040021	114	0.7	160
Non-Clay	Mean	121	0.8	175
	SD	22	0.2	34
	n	6	6	6
Quartz DQ 12	E 050001	433	3.6	646
	E 050002	384	2.6	530
	E 050003	366	3.5	556
	E 050004	599	3.5	602
	E 050005	383	3.2	517
	E 050021	314	3.6	462
Quartz DQ 12	Mean	413***	3.3***	552***
	SD	99###	0.4###	65###
	n	6	6	6

DQ 12 + TiO2	F	060001	346	2.7	419
	F	060002	335	4.3	538
	F	060003	419	4.2	581
	F	060004	516	3.2	504
	F	060005	354	3.3	542
	F	060021	461	2.2	445
DQ 12 + TiO2	Mean	405***	3.3***	505***	
	SD	73###	0.8###	62###	
	n	6	6	6	

Statistics: Anova + Tukey's tests. * $P \leq 5\%$; ** $P \leq 1\%$; *** $p \leq 0.1\%$; (vs. Control)
 ### $p \leq 0.1\%$; {DQ12 and DQ12/TiO2 vs. Non-Clay}

Appendix V Enzyme and Total Protein Levels in BALF (individual data) -- cont'd

Study : BENTONITE-01030

Species: Rat

Sex: Male

Individual Data Listing with Means

M A L E S	Animal No.	Day 28 LDH U/l	Day 28 β-GLU U/l	Day 28 Total Protein mg/l
Control	A 010006	28	0.2	81
	A 010007	35	0.1	94
	A 010008	21	0.1	95
	A 010009	33	0.2	80
	A 010010	37	0.2	80
	A 010022	34	0.2	100
Control	Mean	31	0.2	88
	SD	6	0.1	9
	n	6	6	6
TiO2	B 020006	44	0.3	100
	B 020007	35	0.2	95
	B 020008	45	0.2	98
	B 020009	31	0.2	91
	B 020010	31	0.1	70
	B 020022	37	0.2	100
TiO2	Mean	37	0.2	92
	SD	6	0.1	11
	n	6	6	6
Clay	C 030006	50	0.2	131
	C 030007	65	0.3	136
	C 030008	40	0.2	124
	C 030009	57	0.3	133
	C 030010	53	0.2	142
	C 030022	46	0.2	122
Clay	Mean	52	0.2	131
	SD	9	0.1	8
	n	6	6	6
Non-Clay	D 040006	136	1.2	233
	D 040007	97	1.0	223
	D 040008	130	0.9	199
	D 040009	88	0.6	154
	D 040010	130	0.9	199
	D 040022	120	1.2	197
Non-Clay	Mean	117	1.0	201**
	SD	20	0.2	27
	n	6	6	6
Quartz DQ 12	E 050006	425	6.1	847
	E 050007	252	2.1	947
	E 050008	464	8.0	893
	E 050009	430	8.1	842
	E 050010	446	7.0	805
	E 050022	552	11.3	931
Quartz DQ 12	Mean	428***	7.1***	877***
	SD	98###	3.0###	55###
	n	6	6	6
DQ 12 + TiO2	F 060006	548	6.8	876
	F 060007	419	9.0	848

F 060008	476	9.0	981
F 060009	566	14.1	1033
F 060010	355	6.3	732
F 060022	626	8.1	943

DQ 12 + TiO2	Mean	498***	8.9***	902***
	SD	101###	2.8###	107###
	n	6	6	6

Statistics: Anova + Tukey's tests. * $P \leq 5\%$; ** $P \leq 1\%$; *** $p \leq 0.1\%$; (vs. Control)
 ### $p \leq 0.1\%$; (DQ12 and DQ12/TiO2 vs. Non-Clay)

Appendix VI Differential Cell Count in BALF (individual data)

TABLE OF DIFFERENTIAL CELL COUNT

Wistar WU, males			RECOVERY PERIOD AFTER LAST INSTILLATION					
			3					
			LEUKOCYTE NUMBER [cells]	MACRO- PHAGES [%]	EOSINO- PHIL. PMNs [%]	NEUTRO- PHIL. PMNs [%]	LYMPHO- CYTES [%]	Cell Viabi- lity [%]
Group	Dose [mg]	Animal						
Vehicle Control	0	1	577500	99.8	0.0	0.0	0.3	95
		2	998438	99.3	0.0	0.5	0.3	95
		3	1237500	99.8	0.0	0.3	0.0	99
		4	871875	99.8	0.0	0.0	0.3	95
		5	740625	98.8	0.0	0.0	1.3	97
		21	881250	98.5	0.0	0.3	1.3	95
TiO2	5	1	1650000	95.3	0.0	2.0	2.8	97
		2	1155000	92.3	0.0	3.5	4.3	98
		3	927500	98.3	0.0	1.5	0.3	98
		4	1340000	96.5	0.0	1.8	1.8	96
		5	1076250	92.0	0.0	6.0	2.0	99
		21	937500	95.0	0.3	2.8	2.0	99
Clay	5	1	8890000	35.0	0.0	59.8	5.3	97
		2	6370000	40.5	0.0	55.0	4.5	99
		3	5655000	36.0	0.3	62.5	1.3	100
		4	7755000	37.3	0.0	59.8	3.0	97
		5	7350000	25.3	0.0	73.3	1.5	99
		21	6780000	41.8	0.0	55.0	3.3	100
Non-Clay	5	1	4800000	74.8	0.0	20.5	4.8	95
		2	2681250	79.0	0.0	16.5	4.5	99
		3	3675000	81.5	0.5	16.0	2.0	99
		4	5145000	88.3	0.0	10.0	1.8	100
		5	3360000	83.8	0.0	12.3	4.0	96
		21	4257500	81.5	0.0	15.3	3.3	98
DQ 12	3.9	1	14195000	24.8	0.0	73.0	2.3	97
		2	7560000	35.5	0.0	63.0	1.5	100
		3	9975000	27.0	0.3	71.0	1.8	98

		4		8850000	41.3	0.0	57.0	1.8	97
		5		9440000	31.3	0.0	67.8	1.0	99
		21		10850000	24.8	0.0	72.3	3.0	99
DQ 12 + TiO2	5	1		9600000	32.3	0.0	66.5	1.3	98
		2		5475000	30.5	0.0	68.5	1.0	98
		3		5175000	35.0	0.3	63.3	1.5	97
		4		8887500	34.3	0.3	64.0	1.5	99
		5		8400000	36.0	0.0	61.3	2.8	100
		21		6020000	44.0	0.0	51.0	5.0	99

Appendix VI Differential Cell Count in BALF (individual data) – cont'd

TABLE OF DIFFERENTIAL CELL COUNT

Wistar WU, males			RECOVERY PERIOD AFTER LAST INSTILLATION					
			28					
			LEUKOCYTE NUMBER {cells}	MACRO- PHAGES {%}	EOSINO- PHIL PMNs {%	NEUTRO- PHIL PMNs {%	LYMPHO- CYTES {%}	Cell Viabi- lity {%}
Group	Dose {mg}	Animal						
Vehicle Control	0	6	647500	99.8	0.0	0.0	0.3	97
		7	862500	100.0	0.0	0.0	0.0	99
		8	428750	99.8	0.0	0.0	0.3	100
		9	389063	100.0	0.0	0.0	0.0	98
		10	333125	97.5	0.0	1.5	1.0	99
		22	380000	98.5	0.0	1.3	0.3	99
TiO2	5	6	446250	95.0	0.0	4.8	0.3	96
		7	375000	93.5	0.0	5.5	1.0	99
		8	503125	96.0	0.0	3.5	0.5	98
		9	464063	97.0	0.0	2.3	0.8	100
		10	468125	97.3	0.0	2.3	0.5	99
		22	625000	96.5	0.0	3.0	0.5	99
Clay	5	6	1096875	95.5	0.0	4.3	0.3	94
		7	1743750	82.0	0.0	17.5	0.5	100
		8	771875	85.8	0.0	13.5	0.8	98
		9	952500	89.3	0.0	10.5	0.3	89
		10	1190625	78.0	0.0	21.3	0.8	98
		22	1530000	80.8	0.0	18.5	0.8	98
Non-Clay	5	6	2940000	62.3	0.0	36.0	1.8	99
		7	1800000	60.0	0.0	37.3	2.8	99
		8	2372500	73.0	0.5	25.5	1.0	97
		9	1321875	67.0	0.0	31.3	1.8	99
		10	2184375	57.5	0.0	41.5	1.0	99
		22	2025000	55.0	0.0	41.3	3.8	99

DQ 12	3.9	6	26250000	27.0	0.0	72.3	0.8	99
		7	9187500	43.5	0.3	52.8	3.5	98
		8	20625000	23.3	0.0	75.3	1.5	99
		9	19775000	45.3	0.0	54.3	0.5	100
		10	25687500	27.0	0.0	71.8	1.3	100
		22	33250000	36.3	0.0	62.8	1.0	100
DQ 12 + TiO ₂	5	6	35200000	33.8	0.0	65.3	1.0	100
		7	22400000	36.5	0.0	63.0	0.5	100
		8	23250000	42.5	0.0	56.3	1.3	100
		9	31325000	36.3	0.0	62.3	1.5	99
		10	17906250	37.8	0.0	61.3	1.0	100
		22	41625000	33.8	0.0	64.8	1.5	99

Appendix VII Anova and Tukey's Test (Tables for LDH, β -Glucuronidase and Total Protein)

Anova-Test with TUKEYS Option

Analysis of Variance Procedure
Class Level Information

Class Levels Values

GRUPPE 6 Clay Control DQ 12 DQ 12 + TiO2 Non-Clay TiO2

Number of observations in data set = 36

1Anova-Test with TUKEYS Option

Analysis of Variance Procedure

Dependent Variable: LDH

Source	Pr > F	DF	Sum of Squares	Mean Square	F Value
Model	0.0001	5	976433.47222222	195286.69444444	22.53
Error		30	260012.16666667	8667.07222222	
Corrected Total		35	1236445.63888889		

	R-Square	C.V.	Root MSE
LDH Mean	0.789710	38.74114	93.09711178
	240.3055556		

Source	Pr > F	DF	Anova SS	Mean Square	F Value
GRUPPE	0.0001	5	976433.47222222	195286.69444444	22.53

1Anova-Test with TUKEYS Option

Analysis of Variance Procedure

Tukey's Studentized Range (HSD) Test for variable: LDH

NOTE: This test controls the type I experimentwise error rate, but generally has a higher type II error rate than REGWQ.

Alpha= 0.05 df= 30 MSE= 8667.072
Critical Value of Studentized Range= 4.301
Minimum Significant Difference= 163.48

Means with the same letter are not significantly different.

Tukey Grouping	Mean	N	GRUPPE
A	413.17	6	DQ 12
A			
A	405.17	6	DQ 12 + TiO2
A			
A	391.83	6	Clay
B			
B	120.50	6	Non-Clay
B			
B	63.33	6	TiO2
B			
B	47.83	6	Control

Appendix VII Anova and Tukey's Test (Tables for LDH, β -Glucuronidase and Total Protein)

continued

Anova-Test with TUKEYS Option

Analysis of Variance Procedure
Class Level Information

Class	Levels	Values

GRUPPE	6	Clay Control DQ 12 DQ 12 + TiO2 Non-Clay TiO2

Number of observations in data set = 36

Anova-Test with TUKEYS Option

Analysis of Variance Procedure

Dependent Variable: LDH

Source	Pr > F	DF	Sum of Squares	Mean Square	F Value
Model	0.0001	5	976433.47222222	195286.69444444	22.53
Error		30	260012.16666667	8667.07222222	
Corrected Total		35	1236445.63888889		
		R-Square	C.V.	Root MSE	
		LDH Mean			
		0.789710	38.74114	93.09711178	
		240.30555556			

Source	Pr > F	DF	Anova SS	Mean Square	F Value
GRUPPE	0.0001	5	976433.47222222	195286.69444444	22.53

Anova-Test with TUKEYS Option

Analysis of Variance Procedure

Tukey's Studentized Range (HSD) Test for variable: LDH

NOTE: This test controls the type I experimentwise error rate, but generally has a higher type II error rate than REGWQ.

Alpha= 0.01 df= 30 MSE= 8667.072
Critical Value of Studentized Range= 5.242
Minimum Significant Difference= 199.22

Means with the same letter are not significantly different.

Tukey Grouping	Mean	N	GRUPPE
A	413.17	6	DQ 12
A			
A	405.17	6	DQ 12 + TiO2
A			
A	391.83	6	Clay
B	120.50	6	Non-Clay
B			
B	63.33	6	TiO2
B			
B	47.83	6	Control

Appendix VII Anova and Tukey's Test (Tables for LDH, β -Glucuronidase and Total Protein)

continued

Anova-Test with TUKEYS Option

Analysis of Variance Procedure
Class Level Information

Class Levels Values

GRUPPE 6 Clay Control DQ 12 DQ 12 + TiO2 Non-Clay TiO2

Number of observations in data set = 36

Anova-Test with TUKEYS Option

Analysis of Variance Procedure

Dependent Variable: LDH

Source	Pr > F	DF	Sum of Squares	Mean Square	F Value
Model	0.0001	5	976433.47222222	195286.69444444	22.53
Error		30	260912.16666667	8667.07222222	
Corrected Total		35	1236445.63888889		

	R-Square	C.V.	Root MSE
LDH Mean	0.789710	38.74114	93.09711178
	240.30555556		

Source	Pr > F	DF	Anova SS	Mean Square	F Value
GRUPPE	0.0001	5	976433.47222222	195286.69444444	22.53

Anova-Test with TUKEYS Option
Analysis of Variance Procedure

Tukey's Studentized Range (HSD) Test for variable: LDH

NOTE: This test controls the type I experimentwise error rate, but generally has a higher type II error rate than REGWQ.

Alpha= 0.001 df= 30 MSE= 8667.072
Critical Value of Studentized Range= 6.469
Minimum Significant Difference= 245.87

Means with the same letter are not significantly different.

Tukey Grouping	Mean	N	GRUPPE
A	413.17	6	DQ 12
A	405.17	6	DQ 12 + TiO2
A	391.83	6	Clay
B	120.50	6	Non-Clay
B	63.33	6	TiO2
B	47.83	6	Control

Appendix VII Anova and Tukey's Test (Tables for LDH, β -Glucuronidase and Total Protein)
continued

Anova-Test with TUKEYS Option

Analysis of Variance Procedure
Class Level Information

Class	Levels	Values

GRUPPE	6	Clay Control DQ 12 DQ 12 + TiO2 Non-Clay TiO2

Number of observations in data set = 36

1Anova-Test with TUKEYS Option

Analysis of Variance Procedure

Dependent Variable: GLU

Source	Pr > F	DF	Sum of Squares	Mean Square	F Value
Model	0.0001	5	63.86555556	12.77311111	49.94
Error		30	7.67333333	0.25577778	
Corrected Total		35	71.53888889		

	R-Square	C.V.	Root MSE
GLU Mean	0.892739	29.84723	0.50574478
	1.69444444		

Source	Pr > F	DF	Anova SS	Mean Square	F Value
GRUPPE	0.0001	5	63.86555556	12.77311111	49.94

1Anova-Test with TUKEYS Option

Analysis of Variance Procedure

Tukey's Studentized Range (HSD) Test for variable: GLU

NOTE: This test controls the type I experimentwise error rate, but generally has a higher type II error rate than REGWQ.

Alpha= 0.05 df= 30 MSE= 0.255778
Critical Value of Studentized Range= 4.301
Minimum Significant Difference= 0.8881

Means with the same letter are not significantly different.

Tukey Grouping	Mean	N	GRUPPE
A	3.3333	6	DQ 12
A			
A	3.3167	6	DQ 12 + TiO2
B	2.2500	6	Clay
C	0.7500	6	Non-Clay
C			
C	0.2833	6	Control
C			
C	0.2333	6	TiO2

Appendix VII Anova and Tukey's Test (Tables for LDH, β -Glucuronidase and Total Protein)

continued

Anova-Test with TUKEYS Option

Analysis of Variance Procedure
Class Level Information

Class Levels Values

GRUPPE 6 Clay Control DQ 12 DQ 12 + TiO2 Non-Clay TiO2

Number of observations in data set = 36

1Anova-Test with TUKEYS Option

Analysis of Variance Procedure

Dependent Variable: GLU

Source	Pr > F	DF	Sum of Squares	Mean Square	F Value
Model	0.0001	5	63.86555556	12.77311111	49.94
Error		30	7.67333333	0.25577778	
Corrected Total		35	71.53888889		
		R-Square	C.V.	Root MSE	
		GLU Mean			
		0.892739	29.84723	0.50574478	
		1.69444444			

Source	Pr > F	DF	Anova SS	Mean Square	F Value
GRUPPE	0.0001	5	63.86555556	12.77311111	49.94

1Anova-Test with TUKEYS Option
Analysis of Variance Procedure

Tukey's Studentized Range (HSD) Test for variable: GLU

NOTE: This test controls the type I experimentwise error rate, but generally has a higher type II error rate than REGWQ.

Alpha= 0.01 df= 30 MSE= 0.255778
Critical Value of Studentized Range= 5.242
Minimum Significant Difference= 1.0823

Means with the same letter are not significantly different.

Tukey Grouping	Mean	N	GRUPPE
A	3.3333	6	DQ 12
A			
B	3.3167	6	DQ 12 + TiO2
B			
B	2.2500	6	Clay
C	0.7500	6	Non-Clay
C			
C	0.2833	6	Control
C			
C	0.2333	6	TiO2

Appendix VII Anova and Tukey's Test (Tables for LDH, β -Glucuronidase and Total Protein)
continued

Anova-Test with TUKEYS Option

Analysis of Variance Procedure
Class Level Information

Class Levels Values

GRUPPE 6 Clay Control DQ 12 DQ 12 + TiO2 Non-Clay TiO2

Number of observations in data set = 36

1Anova-Test with TUKEYS Option
Analysis of Variance Procedure

Dependent Variable: GLU

Source	Pr > F	DF	Sum of Squares	Mean Square	F Value
Model	0.0001	5	63.86555556	12.77311111	49.94
Error		30	7.67333333	0.25577778	
Corrected Total		35	71.53888889		
		R-Square	C.V.	Root MSE	
		GLU Mean			
		0.892739	29.84723	0.50574478	
		1.69444444			

Source	Pr > F	DF	Anova SS	Mean Square	F Value
GRUPPE	0.0001	5	63.86555556	12.77311111	49.94

1Anova-Test with TUKEYS Option
Analysis of Variance Procedure

Tukey's Studentized Range (HSD) Test for variable: GLU

NOTE: This test controls the type I experimentwise error rate, but generally has a higher type II error rate than REGWQ.

Alpha= 0.001 df= 30 MSE= 0.255778
Critical Value of Studentized Range= 6.469
Minimum Significant Difference= 1.3357

Means with the same letter are not significantly different.

Tukey Grouping	Mean	N	GRUPPE
A	3.3333	6	DQ 12
A			
A	3.3167	6	DQ 12 + TiO2
A			
A	2.2500	6	Clay
B	0.7500	6	Non-Clay
B			
B	0.2833	6	Control
B			
B	0.2333	6	TiO2

Appendix VII Anova and Tukey's Test (Tables for LDH, β -Glucuronidase and Total Protein)

continued

Anova-Test with TUKEYS Option

Analysis of Variance Procedure
Class Level Information

Class Levels Values

GRUPPE 6 Clay Control DQ 12 DQ 12 + TiO2 Non-Clay TiO2

Number of observations in data set = 36

Anova-Test with TUKEYS Option
Analysis of Variance Procedure

Dependent Variable: PRO

Source	Pr > F	DF	Sum of Squares	Mean Square	F Value
Model	0.0001	5	1641684.666666667	328336.933333333	75.57
Error		30	130347.333333333	4344.911111111	
Corrected Total		35	1772032.000000000		
		R-Square	C.V.	Root MSE	
		PRO Mean			
		0.926442	20.13725	65.91593973	
		327.3333333			

Source	Pr > F	DF	Anova SS	Mean Square	F Value
GRUPPE	0.0001	5	1641684.666666667	328336.933333333	75.57

Anova-Test with TUKEYS Option
Analysis of Variance Procedure

Tukey's Studentized Range (HSD) Test for variable: PRO

NOTE: This test controls the type I experimentwise error rate, but generally has a higher type II error rate than REGWQ.

Alpha= 0.05 df= 30 MSE= 4344.911
Critical Value of Studentized Range= 4.301
Minimum Significant Difference= 115.75

Means with the same letter are not significantly different.

Tukey Grouping	Mean	N	GRUPPE
A	557.50	6	Clay
A	552.17	6	DQ 12
A	504.83	6	DQ 12 + TiO2
B	175.00	6	Non-Clay
B	94.17	6	TiO2
B	80.33	6	Control

Appendix VII Anova and Tukey's Test (Tables for LDH, β -Glucuronidase and Total Protein)

continued

Anova-Test with TUKEYS Option

Analysis of Variance Procedure
Class Level Information

Class Levels Values

GRUPPE 6 Clay Control DQ 12 DQ 12 + TiO2 Non-Clay TiO2

Number of observations in data set = 36

1Anova-Test with TUKEYS Option

Analysis of Variance Procedure

Dependent Variable: PRO

Source	Pr > F	DF	Sum of Squares	Mean Square	F Value
Model	0.0001	5	1641684.66666667	328336.933333333	75.57
Error		30	130347.333333333	4344.91111111	
Corrected Total		35	1772032.00000000		

	R-Square	C.V.	Root MSE
PRO Mean	0.926442	20.13725	65.91593973
	327.33333333		

Source	Pr > F	DF	Anova SS	Mean Square	F Value
GRUPPE	0.0001	5	1641684.66666667	328336.933333333	75.57

1Anova-Test with TUKEYS Option

Analysis of Variance Procedure

Tukey's Studentized Range (HSD) Test for variable: PRO

NOTE: This test controls the type I experimentwise error rate, but generally has a higher type II error rate than REGWQ.

Alpha= 0.01 df= 30 MSE= 4344.911
Critical Value of Studentized Range= 5.242
Minimum Significant Difference= 141.06

Means with the same letter are not significantly different.

Tukey Grouping	Mean	N	GRUPPE
A	557.50	6	Clay
A			
A	552.17	6	DQ 12
A			
A	504.83	6	DQ 12 + TiO2
B			
B	175.00	6	Non-Clay
B			
B	94.17	6	TiO2
B			
B	80.33	6	Control

Appendix VII Anova and Tukey's Test (Tables for LDH, B-Glucuronidase and Total Protein)
continued

Anova-Test with TUKEYS Option

Analysis of Variance Procedure
Class Level Information

Class	Levels	Values
GRUPPE	6	Clay Control DQ 12 DQ 12 + TiO2 Non-Clay TiO2

Number of observations in data set = 36

1Anova-Test with TUKEYS Option

Analysis of Variance Procedure

Dependent Variable: PRO

Source	Pr > F	DF	Sum of Squares	Mean Square	F Value
Model	0.0001	5	1641684.66666667	328336.933333333	75.57
Error		30	130347.333333333	4344.91111111	
Corrected Total		35	1772032.00000000		
R-Square					
C.V.					
Root MSE					
PRO Mean					
0.926442					
327.33333333					
20.13725					
65.91593973					

Source	Pr > F	DF	Anova SS	Mean Square	F Value
GRUPPE	0.0001	5	1641684.66666667	328336.933333333	75.57

1Anova-Test with TUKEYS Option

Analysis of Variance Procedure

Tukey's Studentized Range (HSD) Test for variable: PRO

NOTE: This test controls the type I experimentwise error rate, but generally has a higher type II error rate than REGWQ.

Alpha= 0.001 df= 30 MSE= 4344.911
Critical Value of Studentized Range= 6.469
Minimum Significant Difference= 174.09

Means with the same letter are not significantly different.

Tukey Grouping	Mean	N	GRUPPE
A	557.50	6	Clay
A	552.17	6	DQ 12
A	504.83	6	DQ 12 + TiO2
B	175.00	6	Non-Clay
B	94.17	6	TiO2
B	80.33	6	Control

Appendix VII Anova and Tukey's Test (Tables for LDH, B-Glucuronidase and Total Protein)

continued

Anova-Test with TUKEYS Option

Analysis of Variance Procedure
Class Level Information

Class Levels Values

GRUPPE 6 Clay Control DQ 12 DQ 12 + TiO2 Non-Clay TiO2

Number of observations in data set = 36

1Anova-Test with TUKEYS Option

Analysis of Variance Procedure

Dependent Variable: LDH

Source	Pr > F	DF	Sum of Squares	Mean Square	F Value
Model	0.0001	5	1348055.88888889	269611.17777778	79.80
Error		30	101356.00000000	3378.53333333	
Corrected Total		35	1449411.88888889		
		R-Square	C.V.	Root MSE	
		LDH Mean			
		0.930071	29.97000	58.12515233	
		193.9444444			

Source	Pr > F	DF	Anova SS	Mean Square	F Value
GRUPPE	0.0001	5	1348055.88888889	269611.17777778	79.80

1Anova-Test with TUKEYS Option

Analysis of Variance Procedure

Tukey's Studentized Range (HSD) Test for variable: LDH

NOTE: This test controls the type I experimentwise error rate, but generally has a higher type II error rate than REGWQ.

Alpha= 0.05 df= 30 MSE= 3378.533
Critical Value of Studentized Range= 4.301
Minimum Significant Difference= 102.07

Means with the same letter are not significantly different.

Tukey Grouping	Mean	N	GRUPPE
A	498.33	6	DQ 12 + TiO2
A			
A	428.17	6	DQ 12
B	116.83	6	Non-Clay
B			
B	51.83	6	Clay
B			
B	37.17	6	TiO2
B			
B	31.33	6	Control

Appendix VII Anova and Tukey's Test (Tables for LDH, β -Glucuronidase and Total Protein)
continued

Anova-Test with TUKEYS Option

Analysis of Variance Procedure
Class Level Information

Class Levels Values

GRUPPE 6 Clay Control DQ 12 DQ 12 + TiO2 Non-Clay TiO2

Number of observations in data set = 36

1Anova-Test with TUKEYS Option

Analysis of Variance Procedure

Dependent Variable: LDH

Source	Pr > F	DF	Sum of Squares	Mean Square	F Value
Model	0.0001	5	1348055.88888889	269611.17777778	79.80
Error		30	101356.00000000	3378.53333333	
Corrected Total		35	1449411.88888889		

R-Square	C.V.	Root MSE
LDH Mean		
0.930071	29.97000	58.12515233
193.94444444		

Source	Pr > F	DF	Anova SS	Mean Square	F Value
GRUPPE	0.0001	5	1348055.88888889	269611.17777778	79.80

1Anova-Test with TUKEYS Option

Analysis of Variance Procedure

Tukey's Studentized Range (HSD) Test for variable: LDH

NOTE: This test controls the type I experimentwise error rate, but generally has a higher type II error rate than REGWQ.

Alpha= 0.01 df= 30 MSE= 3378.533
Critical Value of Studentized Range= 5.242
Minimum Significant Difference= 124.39

Means with the same letter are not significantly different.

Tukey Grouping	Mean	N	GRUPPE
A	498.33	6	DQ 12 + TiO2
A			
A	428.17	6	DQ 12
B	116.83	6	Non-Clay
B			
B	51.83	6	Clay
B			
B	37.17	6	TiO2
B			
B	31.33	6	Control

Appendix VII Anova and Tukey's Test (Tables for LDH, β -Glucuronidase and Total Protein)

continued

Anova-Test with TUKEYS Option

Analysis of Variance Procedure
Class Level Information

Class	Levels	Values

GRUPPE	6	Clay Control DQ 12 DQ 12 + TiO2 Non-Clay TiO2

Number of observations in data set = 36

Anova-Test with TUKEYS Option

Analysis of Variance Procedure

Dependent Variable: LDH

Source	Pr > F	DF	Sum of Squares	Mean Square	F Value
Model	0.0001	5	1348055.88888889	269611.17777778	79.80
Error		30	101356.00000000	3378.53333333	
Corrected Total		35	1449411.88888889		

	R-Square	C.V.	Root MSE
LDH Mean	0.930071	29.97000	58.12515233
	193.94444444		

Source	Pr > F	DF	Anova SS	Mean Square	F Value
GRUPPE	0.0001	5	1348055.88888889	269611.17777778	79.80

Anova-Test with TUKEYS Option

Analysis of Variance Procedure

Tukey's Studentized Range (HSD) Test for variable: LDH.

NOTE: This test controls the type I experimentwise error rate, but generally has a higher type II error rate than REGWQ.

Alpha= 0.001 df= 30 MSE= 3378.533
Critical Value of Studentized Range= 6.469
Minimum Significant Difference= 153.51

Means with the same letter are not significantly different.

Tukey Grouping	Mean	N	GRUPPE
A	498.33	6	DQ 12 + TiO2
A	428.17	6	DQ 12
B	116.83	6	Non-Clay
B	51.83	6	Clay
B	37.17	6	TiO2
B	31.33	6	Control

Appendix VII Anova and Tukey's Test (Tables for LDH, β -Glucuronidase and Total Protein)

continued

Anova-Test with TUKEYS Option

Analysis of Variance Procedure
Class Level Information

Class	Levels	Values
GRUPPE	6	Clay Control DQ 12 DQ 12 + TiO2 Non-Clay TiO2

Number of observations in data set = 36

Anova-Test with TUKEYS Option

Analysis of Variance Procedure

Dependent Variable: GLU

Source	Pr > F	DF	Sum of Squares	Mean Square	F Value
Model	0.0001	5	474.27916667	94.85583333	33.63
Error		30	84.62833333	2.82094444	
Corrected Total		35	558.90750000		

	R-Square	C.V.	Root MSE
GLU Mean	0.848583	57.42109	1.67956674
	2.92500000		

Source	Pr > F	DF	Anova SS	Mean Square	F Value
GRUPPE	0.0001	5	474.27916667	94.85583333	33.63

Anova-Test with TUKEYS Option

Analysis of Variance Procedure

Tukey's Studentized Range (HSD) Test for variable: GLU

NOTE: This test controls the type I experimentwise error rate, but generally has a higher type II error rate than REGWQ.

Alpha= 0.05 df= 30 MSE= 2.820944
Critical Value of Studentized Range= 4.301
Minimum Significant Difference= 2.9494

Means with the same letter are not significantly different.

Tukey Grouping	Mean	N	GRUPPE
A	8.8933	6	DQ 12 + TiO2
A	7.1000	6	DQ 12
B	0.9667	6	Non-Clay
B	0.2333	6	Clay
B	0.2000	6	TiO2
B	0.1667	6	Control

Appendix VII Anova and Tukey's Test (Tables for LDH, β -Glucuronidase and Total Protein)

continued

Anova-Test with TUKEYS Option

Analysis of Variance Procedure
Class Level Information

Class Levels Values

GRUPPE 6 Clay Control DQ 12 DQ 12 + TiO2 Non-Clay TiO2

Number of observations in data set = 36

1Anova-Test with TUKEYS Option

Analysis of Variance Procedure

Dependent Variable: GLU

Source	Pr > F	DF	Sum of Squares	Mean Square	F Value
Model	0.0001	5	474.27916667	94.85583333	33.63
Error		30	84.62833333	2.82094444	
Corrected Total		35	558.90750000		
		R-Square	C.V.	Root MSE	
		GLU Mean			
		0.848583	57.42109	1.67956674	
		2.92500000			

Source	Pr > F	DF	Anova SS	Mean Square	F Value
GRUPPE	0.0001	5	474.27916667	94.85583333	33.63

1Anova-Test with TUKEYS Option

Analysis of Variance Procedure

Tukey's Studentized Range (HSD) Test for variable: GLU

NOTE: This test controls the type I experimentwise error rate, but generally has a higher type II error rate than REGWQ.

Alpha= 0.01 df= 30 MSE= 2.820944
Critical Value of Studentized Range= 5.242
Minimum Significant Difference= 3.5942

Means with the same letter are not significantly different.

Tukey Grouping	Mean	N	GRUPPE
A	8.8833	6	DQ 12 + TiO2
A			
A	7.1000	6	DQ 12
B	0.9667	6	Non-Clay
B			
B	0.2333	6	Clay
B			
B	0.2000	6	TiO2
B			
B	0.1667	6	Control

Appendix VII Anova and Tukey's Test (Tables for LDH, B-Glucuronidase and Total Protein)

continued

Anova-Test with TUKEYS Option

Analysis of Variance Procedure
Class Level Information

Class Levels Values

GRUPPE 6 Clay Control DQ 12 DQ 12 + TiO2 Non-Clay TiO2

Number of observations in data set = 36

1Anova-Test with TUKEYS Option

Analysis of Variance Procedure

Dependent Variable: GLU

Source	Pr > F	DF	Sum of Squares	Mean Square	F Value
Model	0.0001	5	474.27916667	94.85583333	33.63
Error		30	84.62833333	2.82094444	
Corrected Total		35	558.90750000		
		R-Square	C.V.	Root MSE	
		GLU Mean			
		0.848583	57.42109	1.67956674	
		2.92500000			

Source	Pr > F	DF	Anova SS	Mean Square	F Value
GRUPPE	0.0001	5	474.27916667	94.85583333	33.63

1Anova-Test with TUKEYS Option

Analysis of Variance Procedure

Tukey's Studentized Range (HSD) Test for variable: GLU

NOTE: This test controls the type I experimentwise error rate, but generally has a higher type II error rate than REGWQ.

Alpha= 0.001 df= 30 MSE= 2.820944
Critical Value of Studentized Range= 6.469
Minimum Significant Difference= 4.4358

Means with the same letter are not significantly different.

Tukey Grouping	Mean	N	GRUPPE
A	8.8833	6	DQ 12 + TiO2
A			
A	7.1000	6	DQ 12
B	0.9567	6	Non-Clay
B			
B	0.2333	6	Clay
B			
B	0.2000	6	TiO2
B			
B	0.1667	6	Control

Appendix VII Anova and Tukey's Test (Tables for LDH, β -Glucuronidase and Total Protein)

continued

Anova-Test with TUKEYS Option

Analysis of Variance Procedure
Class Level Information

Class Levels Values

GRUPPE 6 Clay Control DQ 12 DQ 12 + TiO2 Non-Clay TiO2

Number of observations in data set = 36

ANova-Test with TUKEYS Option

Analysis of Variance Procedure

Dependent Variable: PRO

Source	Pr > F	DF	Sum of Squares	Mean Square	F Value
Model	0.0001	5	4691373.58333333	938274.71666667	360.70
Error		30	78037.16666667	2601.23888889	
Corrected Total		35	4769410.75000000		
		R-Square	C.V.	Root MSE	
PRO Mean		0.983638	13.34849	51.00234199	
382.08333333					

Source	Pr > F	DF	Anova SS	Mean Square	F Value
GRUPPE	0.0001	5	4691373.58333333	938274.71666667	360.70

ANova-Test with TUKEYS Option

Analysis of Variance Procedure

Tukey's Studentized Range (HSD) Test for variable: PRO

NOTE: This test controls the type I experimentwise error rate, but generally has a higher type II error rate than REGWQ.

Alpha= 0.05 df= 30 MSE= 2601.239
Critical Value of Studentized Range= 4.301
Minimum Significant Difference= 89.564

Means with the same letter are not significantly different.

Tukey Grouping	Mean	N	GRUPPE
A	902.17	6	DQ 12 + TiO2
A			
A	877.50	6	DQ 12
B	200.83	6	Non-Clay
B			
C	131.33	6	Clay
C			
C	92.33	6	TiO2
C			
C	88.33	6	Control

Appendix VII ANova and Tukey's Test (Tables for LDH, β -Glucuronidase and Total Protein)
continued

ANova-Test with TUKEYS Option

Analysis of Variance Procedure
Class Level Information

Class Levels Values

GRUPPE 6 Clay Control DQ 12 DQ 12 + TiO2 Non-Clay TiO2

Number of observations in data set = 36

1Anova-Test with TUKEYS Option

Analysis of Variance Procedure

Dependent Variable: PRO

Source	Pr > F	DF	Sum of Squares	Mean Square	F Value
Model	0.0001	5	4691373.58333333	938274.71666667	360.70
Error		30	78037.16666667	2601.23888889	
Corrected Total		35	4769410.75000000		
		R-Square	C.V.	Root MSE	
		PRO Mean			
		0.983638	13.34849	51.00234199	
		382.08333333			

Source	Pr > F	DF	Anova SS	Mean Square	F Value
GRUPPE	0.0001	5	4691373.58333333	938274.71666667	360.70

1Anova-Test with TUKEYS Option

Analysis of Variance Procedure

Tukey's Studentized Range (HSD) Test for variable: PRO

NOTE: This test controls the type I experimentwise error rate, but generally has a higher type II error rate than REGWQ.

Alpha= 0.01 df= 30 MSE= 2601.239

Critical Value of Studentized Range= 5.242

Minimum Significant Difference= 109.14

Means with the same letter are not significantly different.

Tukey Grouping	Mean	N	GRUPPE
A	902.17	6	DQ 12 + TiO2
A			
A	877.50	6	DQ 12
B	200.83	6	Non-Clay
B			
C	131.33	6	Clay
C			
C	92.33	6	TiO2
C			
C	88.33	6	Control

Appendix VII Anova and Tukey's Test (Tables for LDH, B-Glucuronidase and Total Protein)
continued

Anova-Test with TUKEYS Option

Analysis of Variance Procedure

Class Level Information

Class Levels Values

GRUPPE 6 Clay Control DQ 12 DQ 12 + TiO2 Non-Clay TiO2

Number of observations in data set = 36

1Anova-Test with TUKEYS Option

Analysis of Variance Procedure

Dependent Variable: PRO

Source	Pr > F	DF	Sum of Squares	Mean Square	F Value
Model	0.0001	5	4691373.583333333	938274.716666667	360.70
Error		30	78037.166666667	2601.23888889	
Corrected Total		35	4769410.75000000		

R-Square	C.V.	Root MSE
0.983638	13.34849	51.00234199
382.0833333		

Source	Pr > F	DF	Anova SS	Mean Square	F Value
GRUPPE	0.0001	5	4691373.583333333	938274.716666667	360.70

1Anova-Test with TUKEYS Option

Analysis of Variance Procedure

Tukey's Studentized Range (HSD) Test for variable: PRO

NOTE: This test controls the type I experimentwise error rate, but generally has a higher type II error rate than REGWQ.

Alpha= 0.001 df= 30 MSE= 2601.239
 Critical Value of Studentized Range= 6.469
 Minimum Significant Difference= 134.7

Means with the same letter are not significantly different.

Tukey Grouping	Mean	N	GRUPPE
A	902.17	6	DQ 12 + TiO2
A			
A	877.50	6	DQ 12
B			
B	200.83	6	Non-Clay
B			
B	131.33	6	Clay
B			
B	92.33	6	TiO2
B			
B	88.33	6	Control

Appendix VIII Epithelial Cells in BALF (individual data with means)

TABLE OF EPITHELIAL CELLS PER 200 TOTAL CELLS

Wistar WU	RECOVERY PERIOD AFTER LAST INSTILLATION
males	3

	Group					
	Vehicle Control	TiO2	Clay	Non-Clay	DQ 12	DQ 12 + TiO2
	Dose [mg/animal]					
	0	5	5	5	3.9	5
EPITHELIAL CELLS [per 200 cells]						
Animal						
1	0	0	2	3	3	3
2	0	1	1	1	0	2
3	1	1	1	1	0	0
4	6	4	0	0	0	0
5	8	0	0	0	0	0
21	0	0	5	0	0	0
MEAN	2.5	1.0	1.5	0.8	0.5	0.8
SD	3.6	1.5	1.9	1.2	1.2	1.3
n	6	6	6	6	6	6

Wistar WU males	RECOVERY PERIOD AFTER LAST INSTILLATION					
	28					
	Group					
	Vehicle Control	TiO2	Clay	Non-Clay	DQ 12	DQ 12 + TiO2
Dose [mg/animal]						
0						
5						
5						
5						
3.9						
5						
EPITHELIAL CELLS [per 200 cells]						
Animal						
6	4	0	0	0	0	0
7	9	7	15	7	0	0
8	4	17	0	5	0	0
9	16	11	1	7	0	1
10	19	18	14	0	0	0
22	4	0	1	5	0	0
MEAN	9.3	8.8	5.2	4.0	0.0	0.2
SD	6.7	7.9	7.3	3.2	0.0	0.4
n	6	6	6	6	6	6

Appendix IX Terminal Body and Lung Wet Weights (BAL Rats: individual data)

Study : BENTONITE-01030 Species: Rat Sex : Male

Individual Data Listing with Means				
M A L E S	Animal No.	Day 3 TermBW g	Day 3 Rat Lung g	Day 3 rel. Lung g/kg
Control	A 010001	335.8	1.248	3.718
	A 010002	335.1	1.103	3.291
	A 010003	358.2	1.334	3.726
	A 010004	350.2	1.123	3.206
	A 010005	341.7	1.178	3.451
	A 010021	341.2	1.196	3.505
Control	Mean	343.7	1.197	3.483
	SD	8.9	0.085	0.214
	n	6	6	6
TiO2	B 020001	341.5	1.206	3.533
	B 020002	327.0	1.217	3.721
	B 020003	361.5	1.264	3.497
	B 020004	348.0	1.243	3.571
	B 020005	318.4	1.287	4.042
	B 020021	339.0	1.248	3.680
TiO2	Mean	339.2	1.244	3.674
	SD	15.2	0.030	0.200
	n	6	6	6
Clay	C 030001	317.5	2.790	8.789
	C 030002	305.2	2.430	7.961
	C 030003	322.7	2.929	9.076
	C 030004	323.4	3.069	9.489
	C 030005	293.2	2.611	8.905
	C 030021	301.3	2.758	9.154
Clay	Mean	310.6**	2.764***	8.896***
	SD	12.5	0.226	0.517
	n	6	6	6
Non-Clay	D 040001	351.2	1.510	4.299
	D 040002	310.7	1.371	4.411
	D 040003	349.5	1.506	4.309
	D 040004	338.9	1.597	4.713
	D 040005	328.9	1.457	4.429
	D 040021	331.9	1.571	4.733
Non-Clay	Mean	335.2	1.502**	4.482***
	SD	15.0	0.081	0.193
	n	6	6	6
Quartz DQ 12	E 050001	340.4	1.935	5.684
	E 050002	313.4	1.587	5.063
	E 050003	362.8	1.998	5.506
	E 050004	343.2	2.003	5.835
	E 050005	324.2	1.688	5.207
	E 050021	344.2	1.906	5.537
Quartz DQ 12	Mean	338.0	1.853***	5.472***
	SD	17.2	0.174##	0.290###
	n	6	6	6
DQ 12 + TiO2	F 060001	328.0	1.634	4.983
	F 060002	321.8	1.730	5.376
	F 060003	365.7	1.966	5.377
	F 060004	351.2	1.872	5.331
	F 060005	317.4	1.795	5.655
	F 060021	327.1	1.739	5.317
DQ 12 + TiO2	Mean	335.2	1.790***	5.340***
	SD	19.0	0.117#	0.215###
	n	6	6	6

significantly different compared to vehicle controls

* p < 0.05; ** p < 0.01; *** p < 0.001 (Tukey's test)

p < 0.05; ## p < 0.01; ### p < 0.001 (DQ12 and DQ12/TiO2 vs. Non-clay)

SD = standard deviation n = number of animals

Appendix IX Terminal Body and Lung Wet Weights (BAL Rats: individual data) – c'd

Study : BENTONITE-01030 Species: Rat Sex : Male

Individual Data Listing with Means				
M A L E S	Animal No.	Day 28 TermBW g	Day 28 Rat lung g	Day 28 rel. lung g/kg
Control	A 010006	376.8	1.337	3.548
	A 010007	389.0	1.465	3.765
	A 010008	376.4	1.328	3.527
	A 010009	353.8	1.261	3.563
	A 010010	363.7	1.321	3.632
	A 010022	339.1	1.358	4.006
Control	Mean	366.5	1.345	3.673
	SD	18.1	0.067	0.184
	n	6	6	6
TiO2	B 020006	384.0	1.478	3.850
	B 020007	397.2	1.439	3.622
	B 020008	369.1	1.378	3.733
	B 020009	354.1	1.369	3.866
	B 020010	361.4	1.364	3.774
	B 020022	372.2	1.370	3.682
TiO2	Mean	373.0	1.400	3.755
	SD	15.6	0.047	0.095
	n	6	6	6
Clay	C 030006	368.5	2.091	5.673
	C 030007	381.1	2.224	5.835
	C 030008	351.9	2.040	5.798
	C 030009	348.7	1.784	5.117
	C 030010	345.9	2.135	6.173
	C 030022	350.9	2.025	5.772
Clay	Mean	357.8	2.050***	5.728***
	SD	13.9	0.149	0.344
	n	6	6	6
Non-Clay	D 040006	365.9	1.490	4.073
	D 040007	385.4	1.666	4.324
	D 040008	380.1	1.411	3.713
	D 040009	377.1	1.320	3.500
	D 040010	359.0	1.528	4.257
	D 040022	370.1	1.476	3.903
Non-Clay	Mean	374.3	1.482	3.962
	SD	9.8	0.116	0.319
	n	6	6	6
Quartz DQ 12	E 050006	383.1	2.452	6.400
	E 050007	412.0	2.693	6.536
	E 050008	376.3	2.297	6.104
	E 050009	358.9	2.063	5.747
	E 050010	341.6	2.195	6.427
	E 050022	351.1	2.498	7.116
Quartz DQ 12	Mean	370.5	2.366***	6.388***
	SD	25.5	0.227###	0.457###
	n	6	6	6
DQ 12 + TiO2	F 060006	371.9	2.344	6.303
	F 060007	394.5	2.459	6.233
	F 060008	373.7	2.717	7.270
	F 060009	362.7	2.471	6.813
	F 060010	380.9	2.249	5.903
	F 060022	337.5	2.128	6.306
DQ 12 + TiO2	Mean	370.2	2.395***	6.472***
	SD	19.2	0.204###	0.488###
	n	6	6	6

significantly different compared to vehicle controls

* p < 0.05; ** p < 0.01; *** p < 0.001 (Tukey's test)

p < 0.05; ## p < 0.01; ### p < 0.001 (DQ12 and DQ12/TiO2 vs. Non-clay)

SD = standard deviation n = number of animals

Appendix X Unmerged Histopathological Findings

LESIONS	TREATMENT	INCIDENCE OF LESIONS (ANIMAL REFERENCES)						
		Males						
		0.3 ml Saline	5 mg TiO ₂	5 mg Clay	5 mg Non- Clay	3.9 mg DQ 12	3.9 + 1.1 mg DQ12 + TiO ₂	
LUNGS		(5)	(5)	(5)	(5)	(5)	(5)	
No abnormality detected		010011						
		010012						
		010013						
		010014						
		010015						
Multifocal alveolar accumulation of particle-laden macrophages	slight		020011		040011			
			020012		040012			
			020013		040014			
			020014		040015			
			020015					
	moderate			030011	040013	050011	060011	
				030012		050012	060012	
				030013		050013	060013	
				030014		050014	060014	
				030015		050015	060015	
Score Expanded Totals		5		5		5	5	
Multifocal interstitial accumulation of particle-laden macrophages	very slight		020012		040011			
			020013		040012			
			020014		040015			
			020015					
	slight				040013	050011	060011	
					040014	050012	060012	
						050013	060013	
						050014	060014	
						050015		
	moderate						060014	
Score Expanded Totals		4			5	5	5	
Focal interstitial accumulation of particle-laden macrophages	very slight		020011					
Score Expanded Totals		1						
Multifocal interstitial mononuclear cell infiltration	very slight		020012					
			020014					
	slight			030011	040011			
				030012	040012			
				030013	040013			
				030014	040014			
				030015	040015			
	moderate					050011	060011	
						050012	060012	
						050013	060013	
						050014		
						050015		
	severe						060013	
							060014	
Score Expanded Totals		2	5	5	5	5	5	

Figures in parentheses represent the number of animals from which this tissue was examined microscopically

The absence of a numeral indicates that the lesion specified was not identified

*** Continued

Appendix X Unmerged Histopathological Findings – cont'd

LESIONS	TREATMENT	INCIDENCE OF LESIONS (ANIMAL REFERENCES)					
		Males					
		0.3 ml Saline	5 mg TiO ₂	5 mg Clay	5 mg Non-Clay	3.9 mg BQ 12	3.9 mg BQ 12 + TiO ₂
LUNGS		(5)	(5)	(5)	(5)	(5)	(5)
Focal interstitial mononuclear cell infiltration	very slight		020011				
			020015				
Score Expanded Totals			2				
Multifocal alveolar granulocyte infiltr.	very slight			030011	040012		
				030012	040014		
				030013			
				030014			
				030015			
	slight				040011	050011	
					040013	050013	
					040015		
	moderate					050012	060011
						050014	060012
						050015	060013
							060014
							060015
Score Expanded Totals				5	5	5	5
Multifocal interstitial histiocytic granulomata	slight						060011
	moderate			030011			
				030012			
				030013			
				030014			
				030015			
Score Expanded Totals				5			1
Focal interstitial histiocytic granulomata	moderate					050014	
Score Expanded Totals						1	
Multifocal bronchiolo-alveolar hyperplasia	very slight			030013	040013		
				030014	040014		
				030015			
	slight			030011	040011	050011	060011
				030012	040012	050012	060012
					040015	050013	060013
						050014	060014
						050015	060015
Score Expanded Totals				5	5	5	5
Focal bronchiolo-alveolar hyperplasia	very slight		020015				
Score Expanded Totals			1				
Multifocal goblet cell hyperplasia, bronchioles	slight			030012		050012	060015
Score Expanded Totals				1		1	1
Multifocal interstitial fibrosis	very slight				040011	050011	060011
					040012	050013	060012
					040013		060015
					040014		
					040015		
	slight			030011		050012	060013
				030012		050014	060014
				030013		050015	
				030014			
				030015			
Score Expanded Totals				5	5	5	5

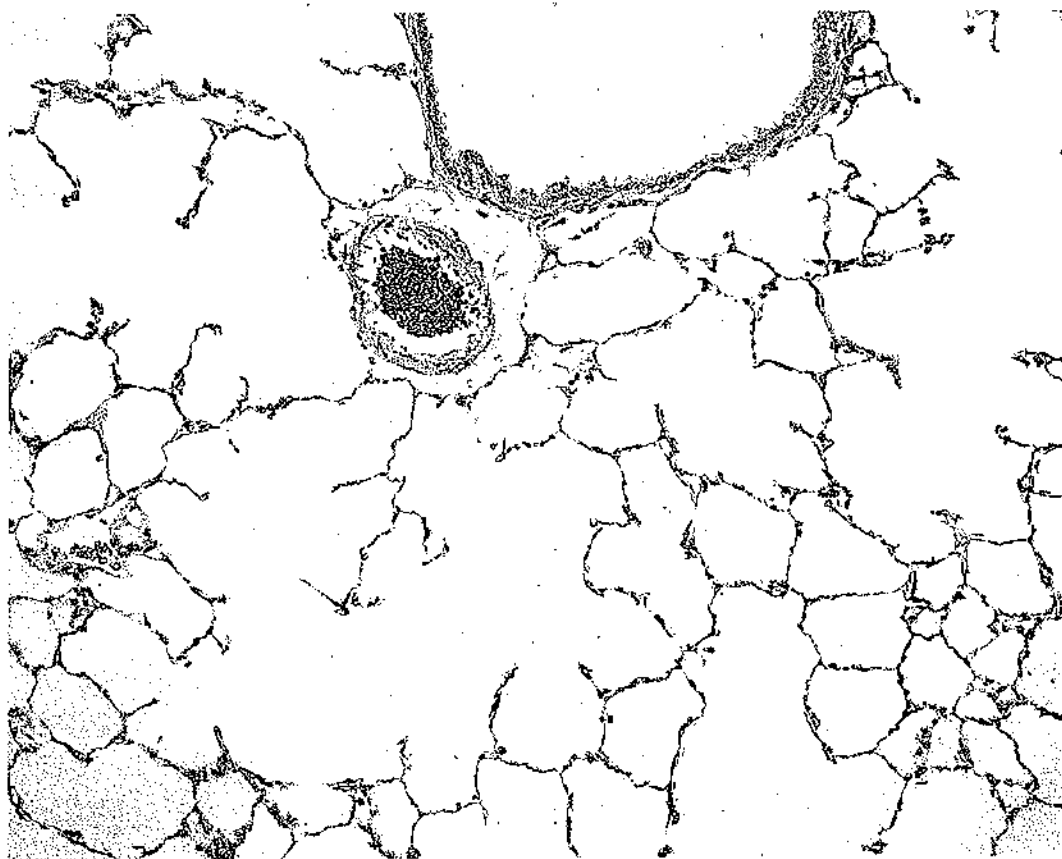
Figures in parentheses represent the number of animals from which this tissue was examined microscopically - The absence of a numeral indicates that the lesion specified was not identified ** Cont>d

Appendix X Unmerged Histopathological Findings – cont'd

LESIONS	TREATMENT	INCIDENCE OF LESIONS (ANIMAL REFERENCES)					
		Males					
		0.3 ml Saline	5 mg TiO ₂	5 mg Clay	5 mg Non- Clay	13.9 mg DQ 12	13.9 mg DQ 12 + TiO ₂
LUNGS		(5)	(5)	(5)	(5)	(5)	(5)
Multifocal alveolar lipoproteinosis							
slight							060013
moderate						050011	060011
						050012	060012
						050013	060014
						050014	060015
severe						050015	
Score Expanded Totals						5	5
Focal alveolar lipoproteinosis							
slight					040013		
Score Expanded Totals					1		
Focal foreign-body granuloma							
slight			020011				060011
Score Expanded Totals			1				1
Focal osseous metaplasia							
slight			020014	030012			
Score Expanded Totals			2	2			
Multifocal haemorrhage							
slight				030012			
Score Expanded Totals				030014			
Focal haemorrhage				2			
slight				030013			060014
Score Expanded Totals				1			1
LUNG ASSOCIATED LYMPH NODES		(5)	(5)	(5)	(5)	(5)	(5)
No abnormality detected		010011					
		010012					
		010013					
		010015					
Accumulation of particle-laden macrophages							
very slight			020013		040015		
slight			020011	030012	040011		
			020012	030013	040012		
			020014		040013		
			020015		040014		
moderate				030011		050011	060011
				030014		050012	060013
				030015		050013	060014
						050015	060015
severe						050014	060012
Score Expanded Totals			5	5	5	5	5
Lymphoid hyperplasia							
slight			020012	030011	040011		
				030015	040013		
moderate				030014		050011	060011
						050012	060012
						050013	060015
						050014	
severe							060014
Score Expanded Totals			1	3	2	5	5
Mast-cell infiltration							
moderate			010014				
Score Expanded Totals			1				

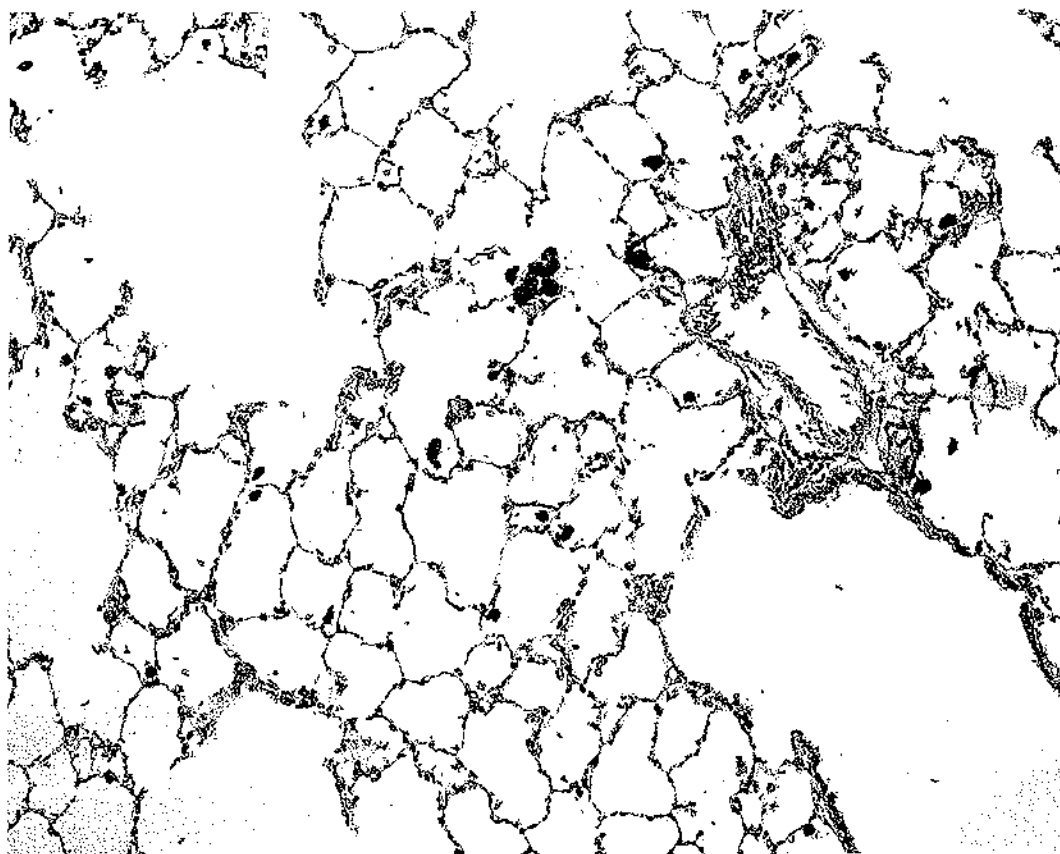
Figures in parentheses represent the number of animals from which this tissue was
examined microscopically
The absence of a numeral indicates that the lesion specified was not identified
*** Listing Complete ***

Appendix XI Photographs of Histopathological Findings



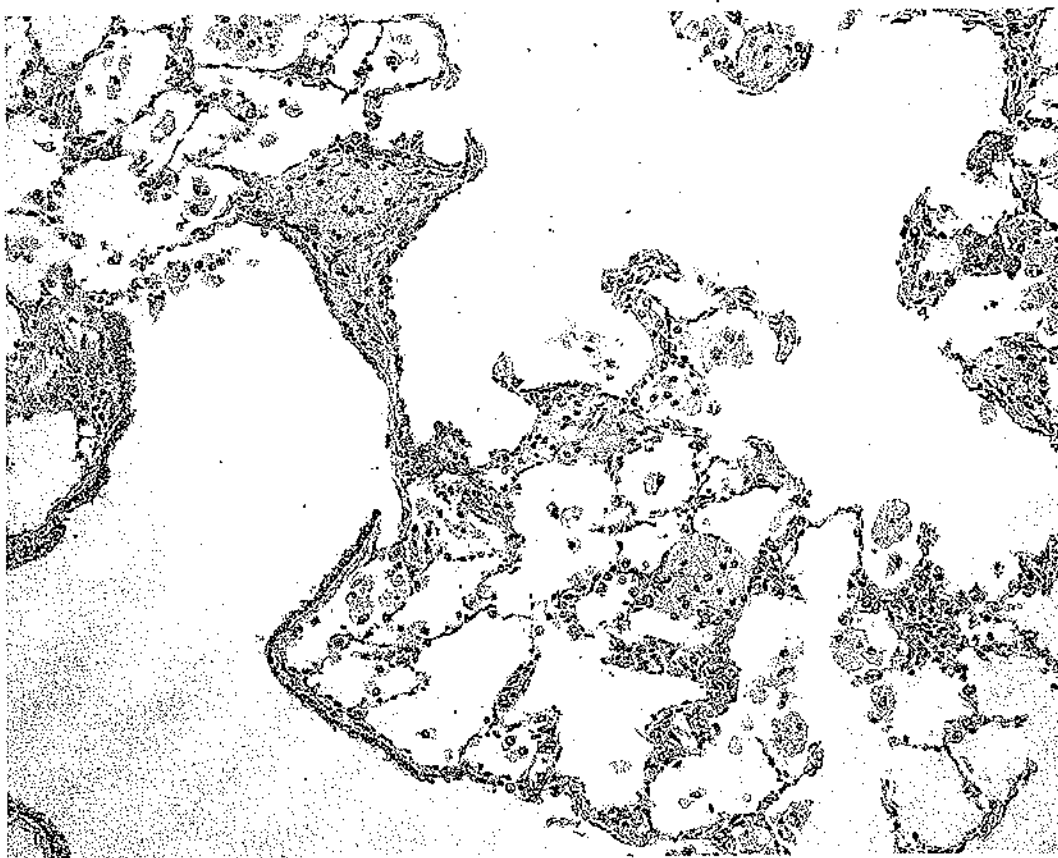
Animal 010013 (Control): no abnormalities detected, Masson-Trichrome (MT) staining, x 200

Appendix XI Photographs of Histopathological Findings -- cont'd



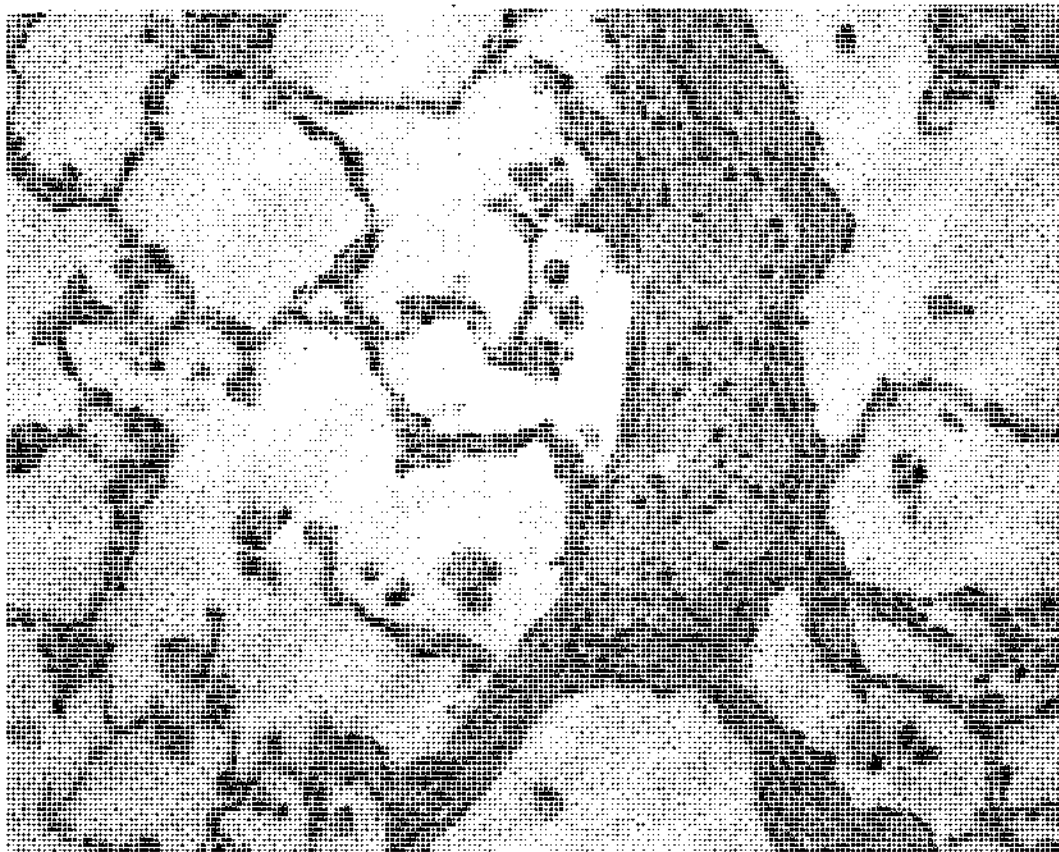
Animal 020011 (TiO₂): accumulation of particle-laden macrophages, no interstitial fibrosis, MT staining, x 200

Appendix XI Photographs of Histopathological Findings – cont'd



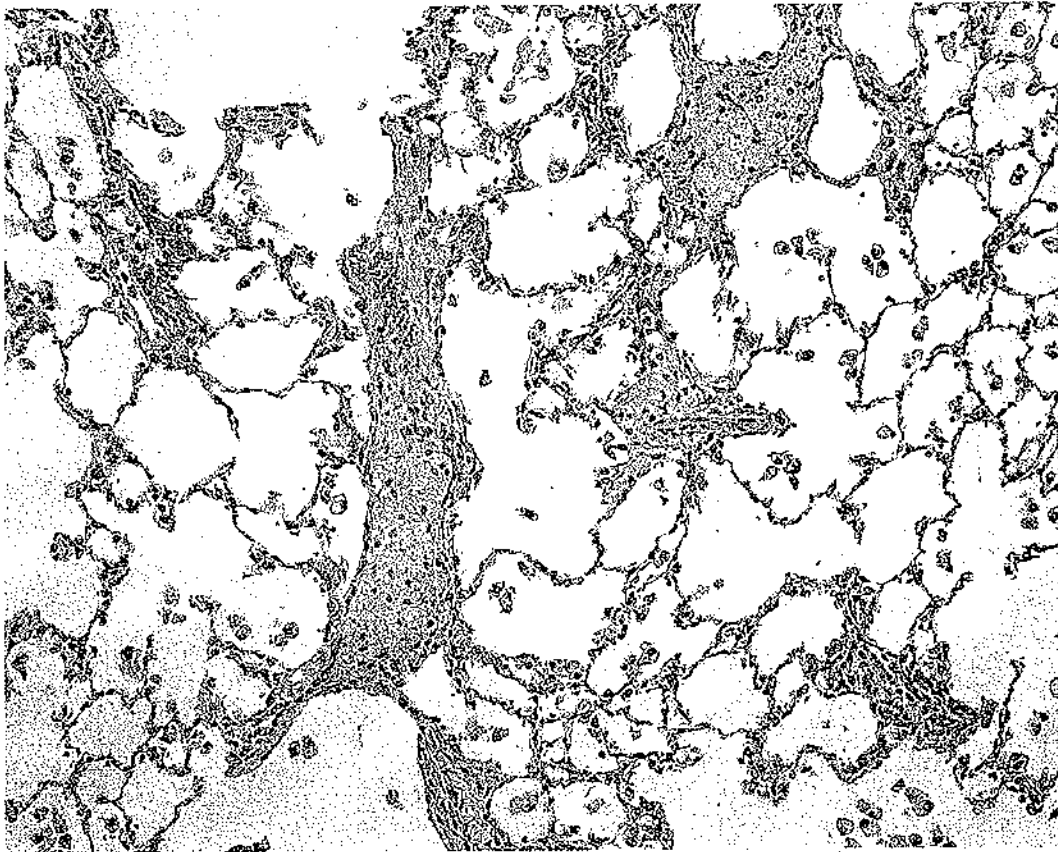
Animal 030014 (Clay): interstitial fibro-histiocytic granulomata, MT staining, x 200

Appendix XI Photographs of Histopathological Findings -- cont'd



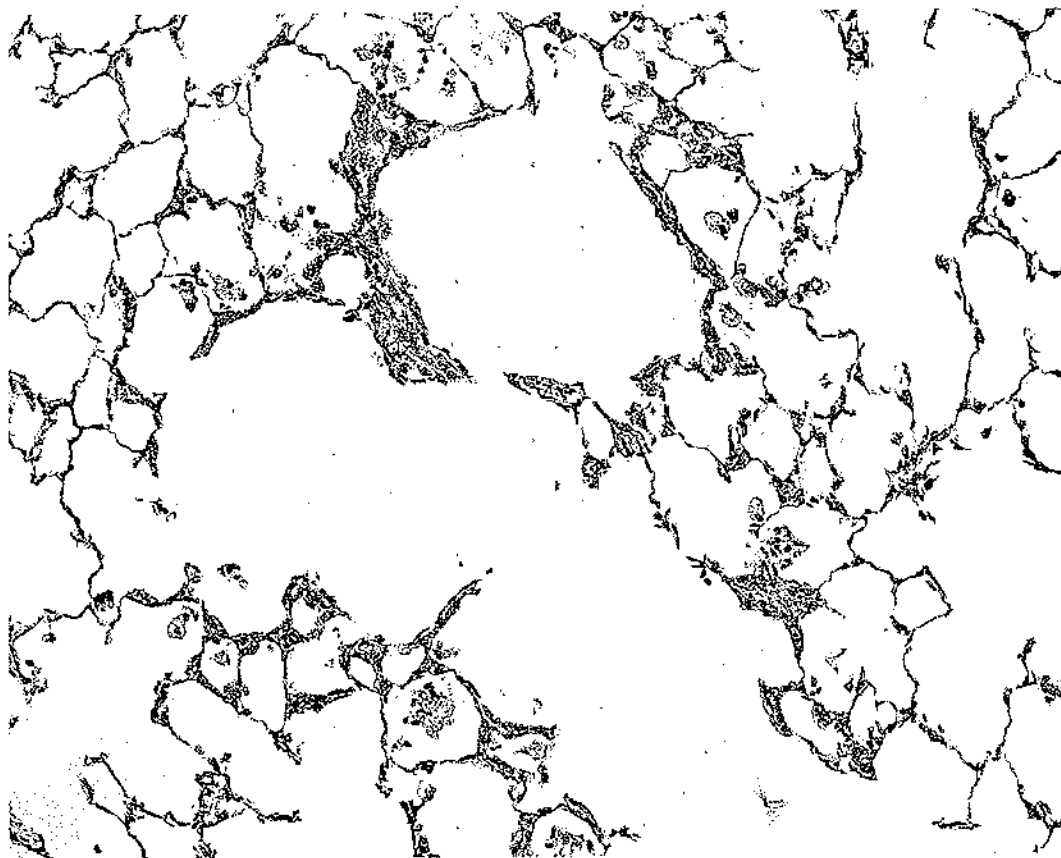
Animal 030014 (Clay): Interstitial fibro-histiocytic granulomata (partially surrounded by collagen), MT staining, x 400

Appendix XI Photographs of Histopathological Findings -- cont'd



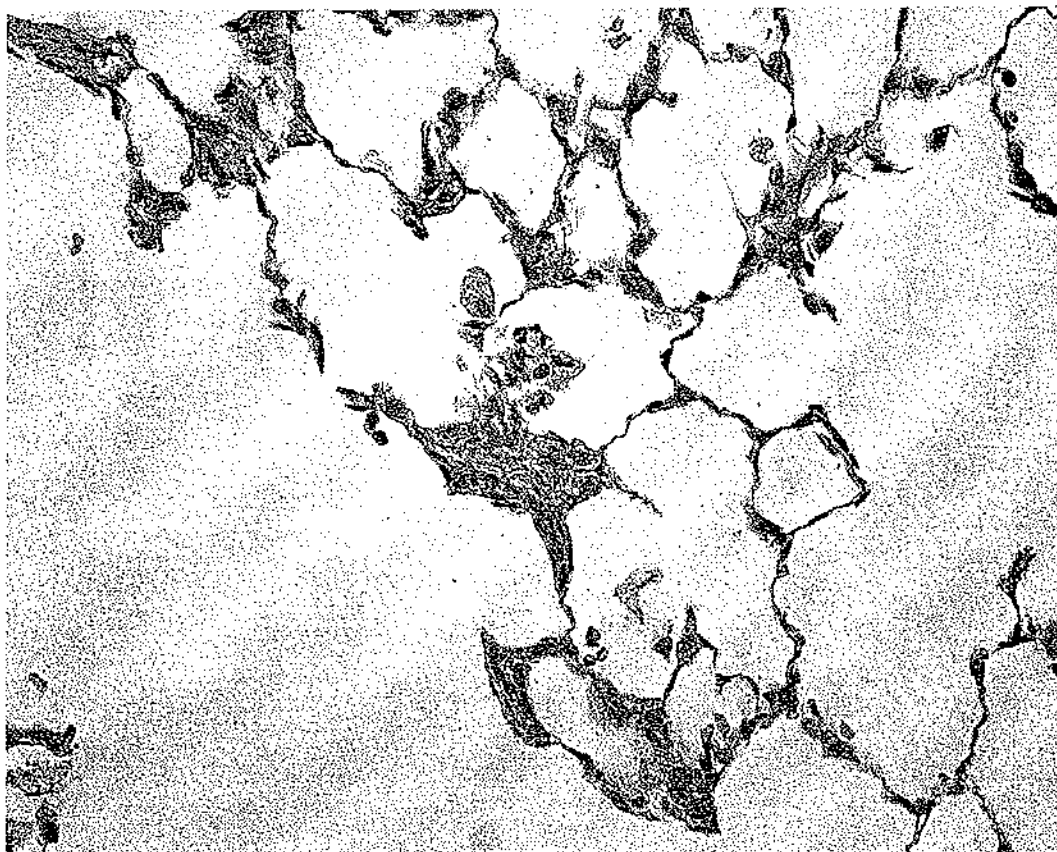
Animal 030014 (Clay): interstitial fibro-histiocytic granulomata, HE staining, x 200

Appendix XI Photographs of Histopathological Findings – cont'd



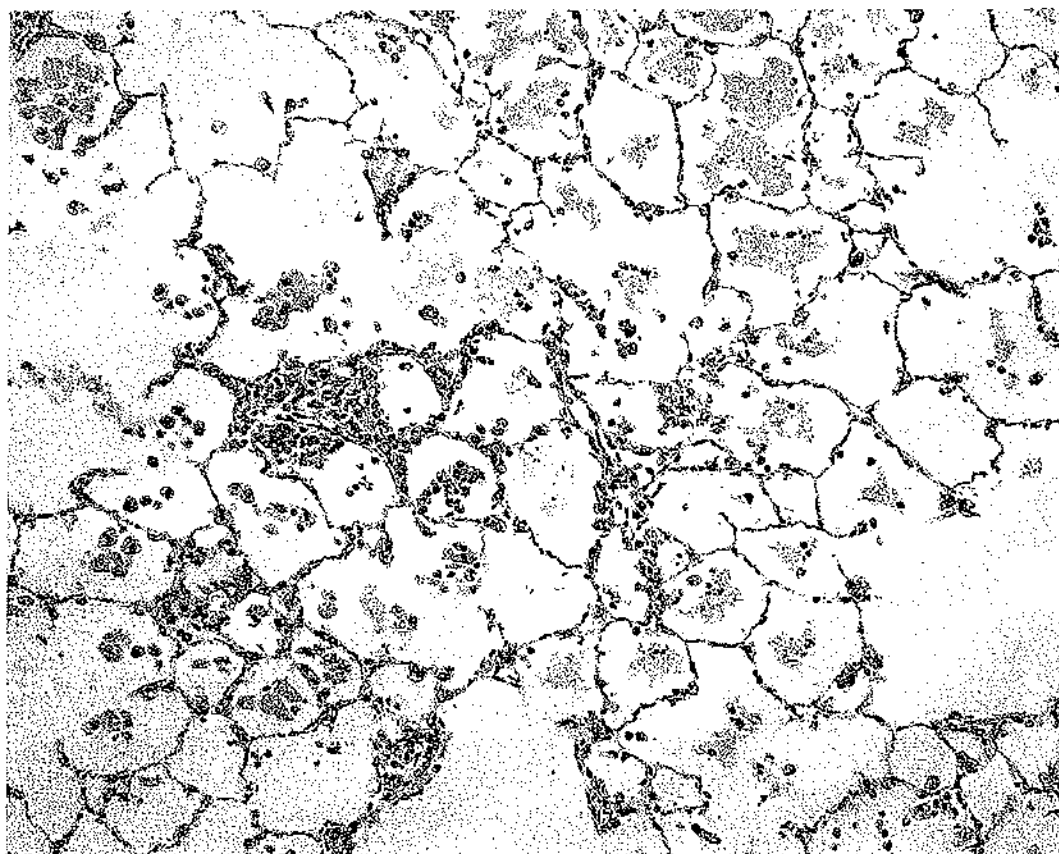
Animal 040011 (Non-Clay): minimal interstitial fibrosis and inflammation, MT staining, x 200

Appendix XI Photographs of Histopathological Findings – cont'd



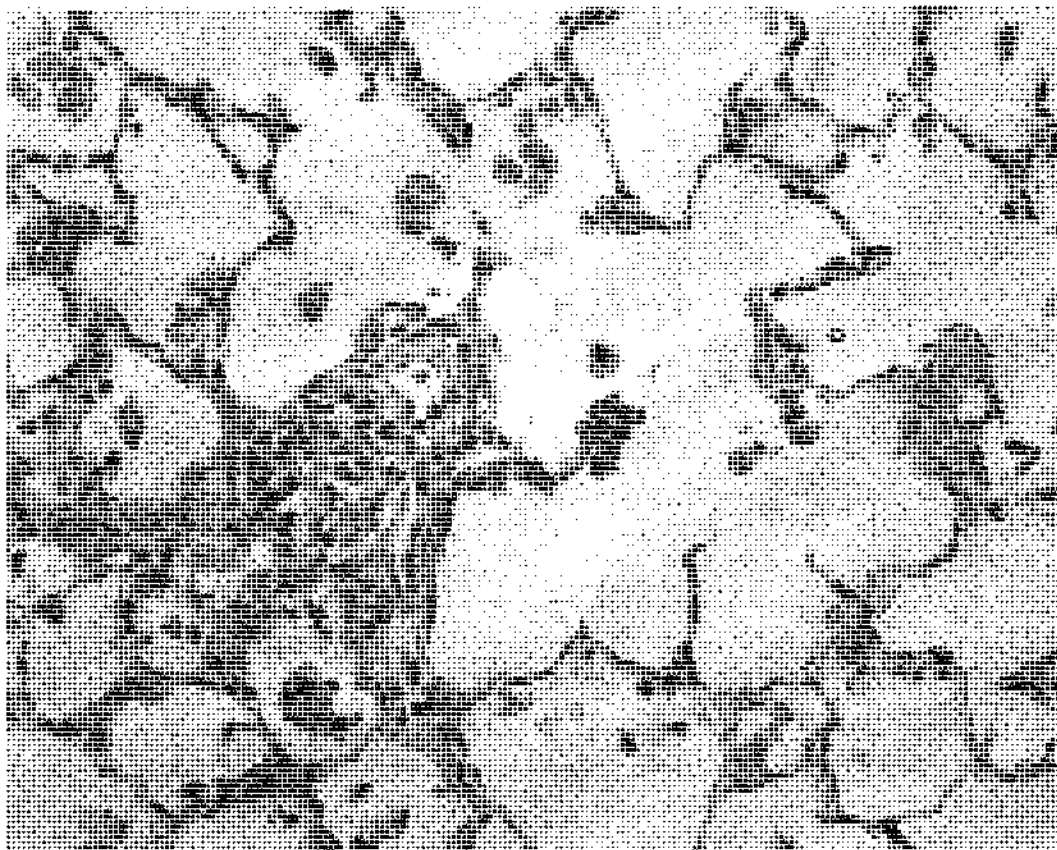
Animal 040011 (Non-Clay): minimal interstitial fibrosis and inflammation, MT staining, x 400

Appendix XI Photographs of Histopathological Findings – cont'd



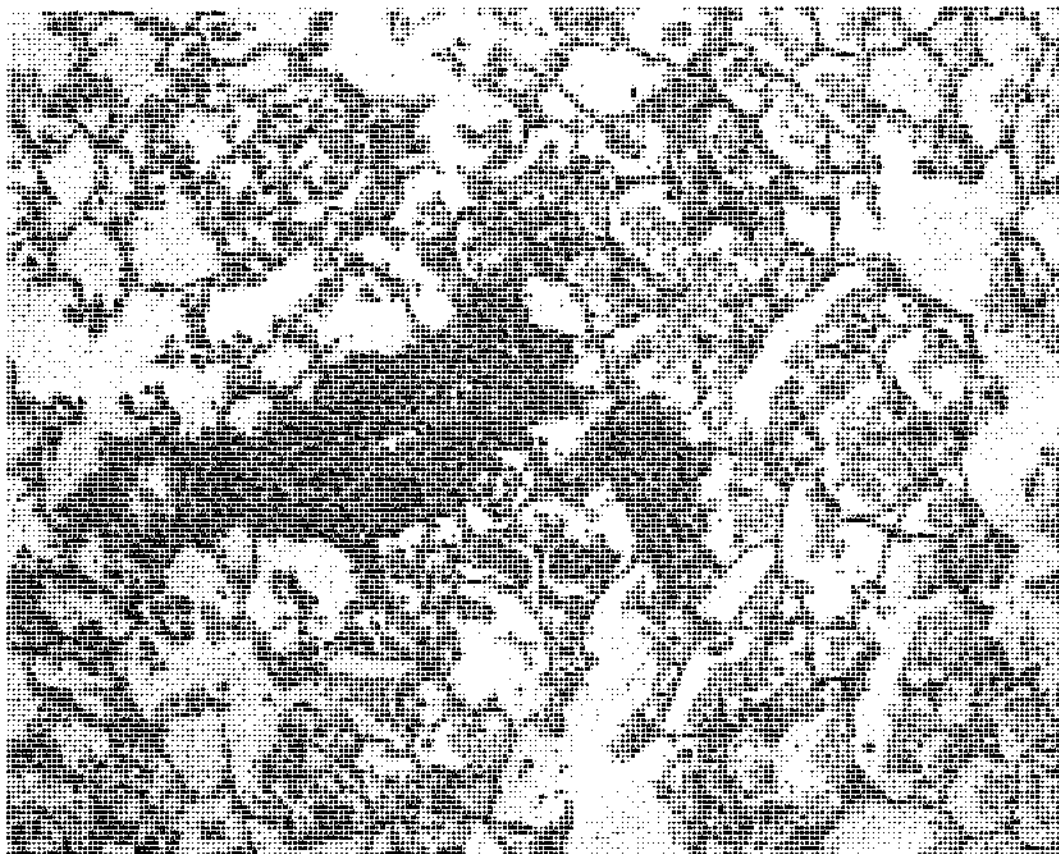
Animal 050015 (DQ12): alveolar lipoproteinosis, inflammatory cell infiltration (interstitial, intra-alveolar, perivascular), MT staining, x 200

Appendix XI Photographs of Histopathological Findings – cont'd



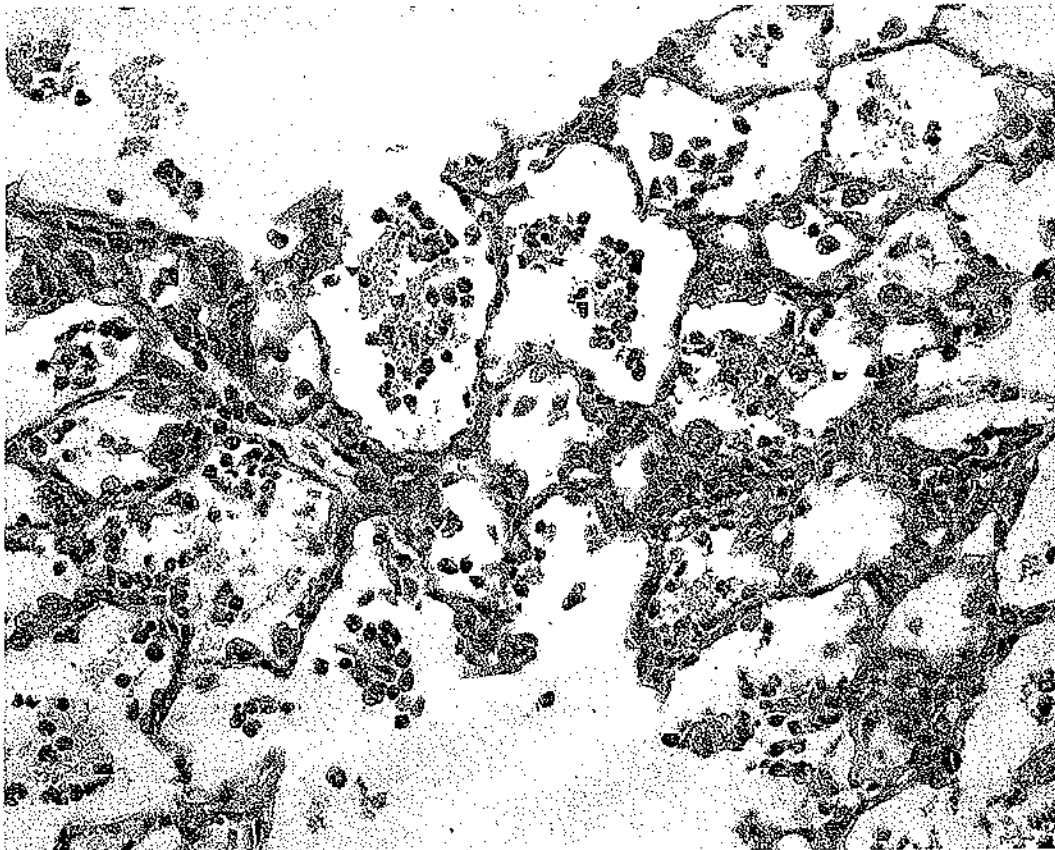
Animal 050015 (DQ12): minimal to slight interstitial fibrosis, slight focal interstitial mononuclear cell infiltration, MT staining, x 400

Appendix XI Photographs of Histopathological Findings – cont'd



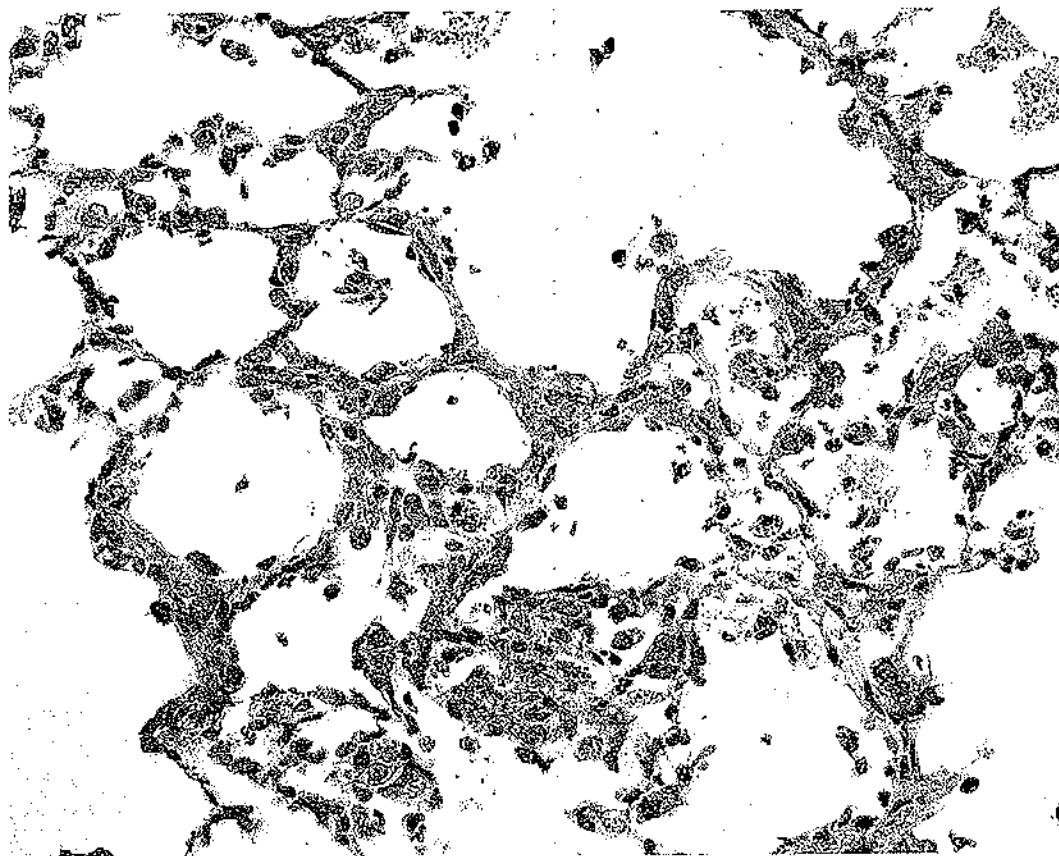
Animal 050015 (DQ12): alveolar lipoproteinosis, marked perivascular inflammatory cell infiltration, HE staining, x 200

Appendix XI Photographs of Histopathological Findings – cont'd



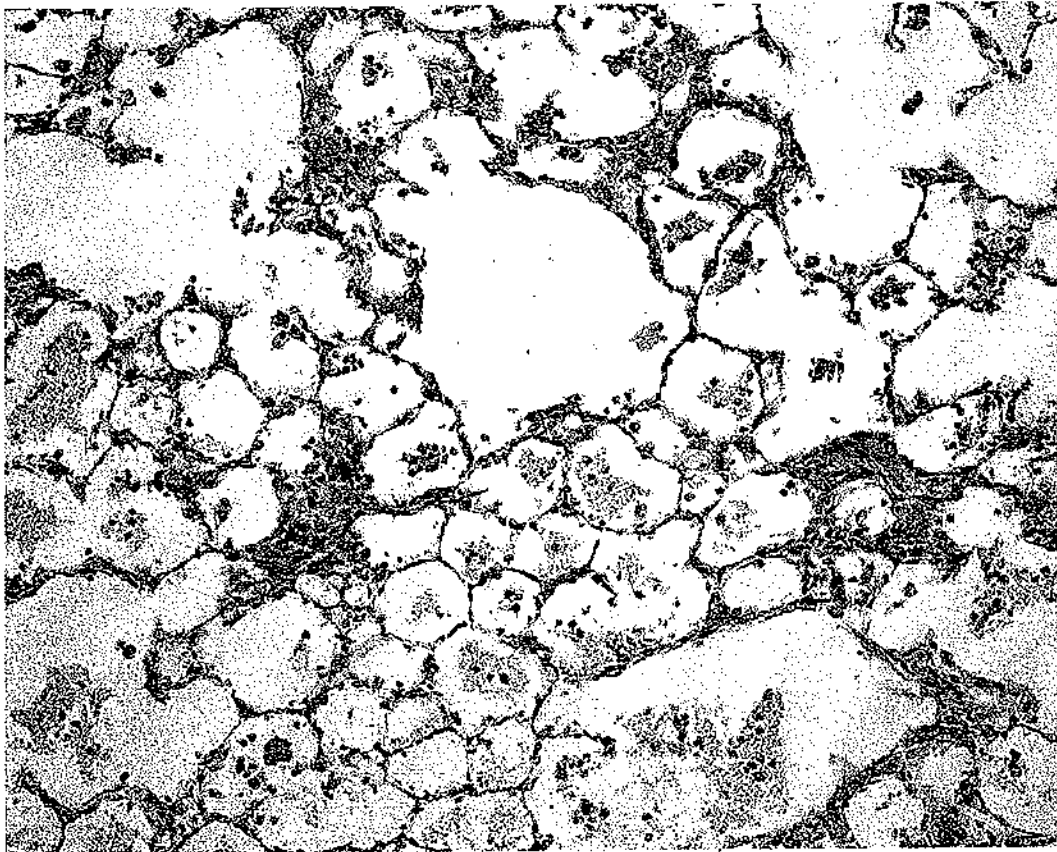
Animal 060011 (DQ12 + TiO₂): interstitial and alveolar inflammatory cell infiltration, accumulation of particle-laden macrophages, alveolar lipoproteinosis, HE staining, x 400

Appendix XI Photographs of Histopathological Findings – cont'd



Animal 060011 (DQ12 + TiO₂): multifocal slight interstitial fibrosis, interstitial and alveolar inflammatory cell infiltration, accumulation of particle-laden macrophages, alveolar lipoproteinosis, multifocal minimal bronchiolo-alveolar hyperplasia, MT staining, x 400

Appendix XI Photographs of Histopathological Findings – cont'd



Animal 060011 (DQ12 + TiO_2): multifocal slight interstitial fibrosis, interstitial and alveolar inflammatory cell infiltration, accumulation of particle-laden macrophages, alveolar lipoproteinosis, MT staining, x 200

Appendix XII Determination of Epithelial Cells in the Isolated Fraction

Number of Isolated Cells ($\times 10^6$)			
	Mean	SD	n
Saline	6.7	2.1	5
TiO ₂	9.3	5.5	5
Clay	12.7**	2.9	5
Non-clay	11.6**	1.5	5
DQ 12 Quartz	44.0**	27.3	5
DQ 12 Quartz + TiO ₂	33.2*	29.5	5

significantly different compared to controls (t-test)

Plating Efficiency ($\times 10^5$; from initially seeded 1×10^6 cells)			
	Mean	SD	n
Saline	2.3	0.5	5
TiO ₂	2.5	1.1	5
Clay	1.9	0.8	5
Non-clay	1.7	0.5	5
DQ 12 Quartz	1.3**	0.4	5
DQ 12 Quartz + TiO ₂	1.4*	0.6	5

significantly different compared to controls (t-test)

SD = standard deviation

n = number of animals

Appendix XII Determination of Epithelial Cells in the Isolated Fraction – cont'd

Alkaline Phosphatase Positive Epithelial Cells (%)			
	Mean	SD	n
Saline	15.2	4.2	5
TiO ₂	24.3	21.2	5
Clay	12.9	6.1	5
Non-clay	13.6	6.4	5
DQ 12 Quartz	14.3	9.0	5
DQ 12 Quartz + TiO ₂	11.3	5.4	5

Additional Evaluation: Epithelial Cells with Lamellar Bodies Detected after 48-Hour Culture (%)			
	Mean	SD	n
Saline	67.8	12.0	5
TiO ₂	68.8	15.5	5
Clay	52.0	21.3	5
Non-clay	54.8	18.5	5
DQ 12 Quartz	22.6	16.1	5
DQ 12 Quartz + TiO ₂	19.6	11.5	5

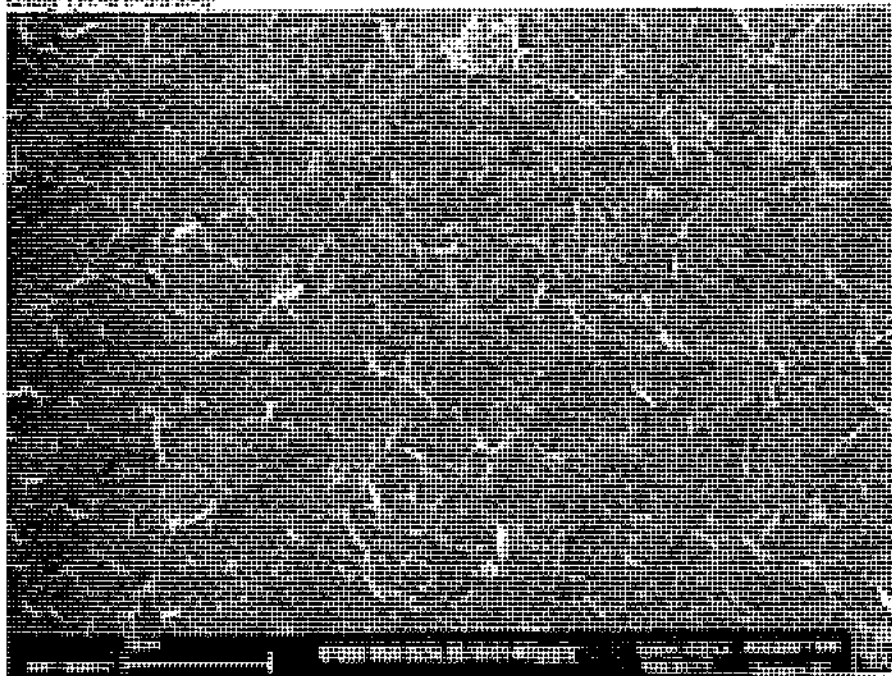
Evaluation after 48 hours resulted in higher percentages of 50-70% of detected epithelial cells. The lower values in the quartz DQ 12 groups reflect initial high percentages of inflammatory cells which have died in the meantime.

SD = standard deviation
n = number of animals

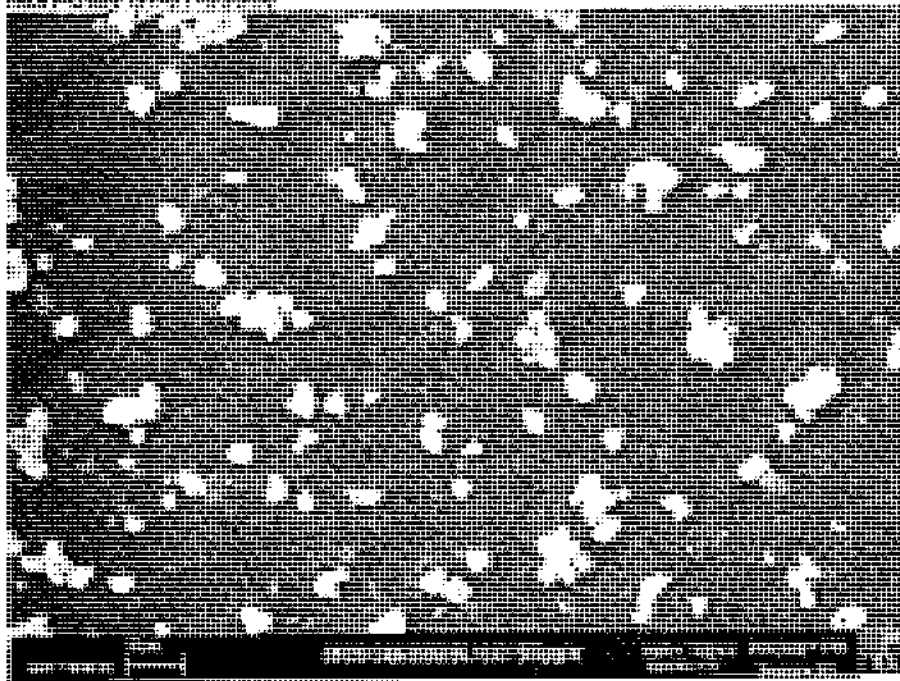
Note: The additional evaluation reported in the last table was not done in compliance with GLP principles.

Appendix XIII SEM Photographs of Test Items

Clay (H-41-3574)



Clay (H-41-3575)



Appendix XIV Study Plan Amendments**Form for Study Plan Amendment**

Study No.: 02G01030 page 1

Date: 25-Oct-01

Amendment No.: 1

A.

Subject: MMAD Page 5, Table 1, lines 4 and 14

Page 6, lines 19 and 24

Change: Delete: MMAD: about 1 μm , GSD: about 1.5
MMAD: about 1.4 μm , GSD: about 1.8
The MMAD is 1.4 μm (GSD: 1.8 μm).
The MMAD of the particles is 1.1 μm (GSD: 1.6 μm).

Reason: MMADs are not relevant for i.tr. study.

B.

Subject: Test Item Page 5, Table 1, line 11

Change: Add to read: particle size: 1 - 10 μm (Malvern laser particle sizer)

Reason: Gives more information

C.

Subject: Reference Item Page 5, Table 1, last line

Change: Delete last 3 words: ... and # 5.

Reason: Statement valid only for group 4.

D.

Subject: SEM Page 6, lines 9 and 10

Change: Delete last part of sentence: ... and scanning electron microscopy (SEM) conducted by Bob Hamilton on no fewer than 1000 particles per sample.

Reason: SEM analysis not yet done cont'd

Appendix XIV Study Plan Amendments – cont'd

Form for Study Plan Amendment

Study No.: 02G01030
Date: 25-Oct-01 cont'd ---> page 2
Amendment No.: 1

E.

Subject: DQ12 quartz Page 6, line 14
Change: Delete fourth word: ultrafine
Reason: The used DQ12 fraction is a fine fraction (not ultrafine)

F.

Subject: Acclimatization Page 8, line 3
Change: Delete: one week - write: seven weeks
Reason: Original start date has been shifted

G.

Subject: Cell viability Page 10, table 3
Change: Include in first column, fifth line: Cell viability
Reason: Cell viability is one of the parameters.

H.

Subject: Particle suspension Page 11, line 2
Change: Substitute *aqueous solution* by: ... *aqueous suspension*.
Before treatment of rats dry sodium chloride was added to the suspension to achieve 0.9% NaCl (= saline vehicle)
Reason: Actually, with insoluble particles it is a suspension. Treatment of rats has to be done with the vehicle saline.

cont'd

Appendix XIV Study Plan Amendments – cont'd

Form for Study Plan Amendment

Study No.: 02G01030

Date: 25-Oct-01

cont'd ----> page 3

Amendment No.: 1

I.

Subject: Distribution of test item Page 11, line 7

Change: *Substitute to enable an even distribution by: ... achieve a more even distribution.*

Reason: Better wording

J.

Subject: Instillation procedure Page 11, line 9

Change: Add to read: ... with CO₂, hanging in a vertical position.

Reason: Gives more details.

Approval - Signature:

signed October 26, 2001

Study Director

(Fraunhofer ITA)

Dr. Otto Creutzenberg

Date

signed October 26, 2001

Head, QAU

(Fraunhofer ITA)

Dr. Madhukar Ketkar

Date

signed November 1, 2001

Sponsor's Project Monitor

(SMI)

Prof. Dr. Günter Oberdörster

Date

Appendix XIV Study Plan Amendments – cont'd**Form for Study Plan Amendment**

Study No.: 02G01030

Date: 26-Oct-01

Amendment No.: 2

Subject: BAL Page 11, line 23

Change: Alter to read: two lavages of 5 ml each

Reason: Adaptation of lavage volume to males of higher age (study start has been shifted)

Approval - Signature:Study Director
(Fraunhofer ITA)

signed October 26, 2001

Dr. Otto Creutzenberg

Date

Head, QAU
(Fraunhofer ITA)

signed October 26, 2001

Dr. Madhukar Ketkar

Date

Sponsor's Project Monitor
(SMI)

not applicable

Prof. Dr. Günter Oberdörster

Date

Appendix XIV Study Plan Amendments – cont'd**Form for Study Plan Amendment**

Study No.: 02G01030

Date: Jul-25-03

Amendment No.: 3

Subject: Genotoxicity Page 12, last paragraph

Supplement: After last word (...colonies.): As an alternative genotoxic assay the evaluation of micronuclei in alveolar macrophages was done. Results of this investigation will be included to the final report as a supplement (upon request of sponsor; see e-mail from 10-Jul-03)

Reason: Results of the micronucleus test contribute to an assessment of different genotoxic potential of test items tested

Approval - Signature:

Study Director
(Fraunhofer ITEM) signed July 25, 2003
Dr. Otto Creutzenberg Date

Head, QAU
(Fraunhofer ITEM) signed July 25, 2003
Dr. Madhukar Ketkar Date

Sponsor's Project Monitor
(SMI) not applicable
Prof. Dr. Günter Oberdörster Date

Supplement to Fraunhofer ITEM Study No. 02G01030: Micronuclei in Pulmonary Alveolar Macrophages

Note: This part of the report has been done not in compliance with GLP.

Introduction

The micronucleus (MN) assay is a widely used and accepted test for detection of genotoxicity. Counting of MN is predominantly done with bone marrow polychromatic erythrocytes (PCE) or in vitro cell lines.

Various authors have reported that the micronucleus assay can be applied successfully on pulmonary alveolar macrophages (PAM), e.g. to detect a genotoxic potential after inhalative or instillative uptake of smokes or dusts. MN are counted in PAMs isolated by bronchoalveolar lavage (BAL).

Regarding their origin PAMs are formed in the bone marrow from stem cells. After reaching the lung, only a small fraction of these initial population is self-renewing and divides in the alveoli. Most investigators consider that this fraction is capable of a few divisions at maximum.

Das et al. (1994) reported a significant increase of MN in rat PAM after inhalation of mosquito coil smoke. One day or 7 days after exposure they found the same value of approx. 9 MN per 1000 PAM (controls: 1.5 MN) and concluded that this was due to the transient nature of PAMs. MN recorded after 7 days represent the damages during the cell cycle, not a cumulative damage for the entire period of exposure.

Balansky and Agostini et al. (1992; 1992; 1993; 1993) detected increased MN levels in PAMs after exposure of rats to cigarette smoke. In humans, however, no increase was observed. This finding could reflect the very low rate of mitoses of PAM in the alveolar lumen. Evidence for micronuclei in rat alveolar macrophages after instillative treatment with crystalline silica dust has been reported by Wang et al. (1997) and Leigh et al. (2000).

Evaluation of study 02G01030

Based on these findings it seemed reasonable to analyze the BAL cytospides of the above bentonite study for presence of micronuclei. Cytospides have been prepared 3 and 28 days after end of instillative treatment of animals. Cytospides of controls and groups 5 (positive control: DQ 12 quartz) and 6 (DQ12 quartz supplemented with titanium dioxide) have been evaluated and are shown in the Table below. In groups 5 and 6 means in the range of 7-10 MN per 1000 PAM were determined. Compared to the control group all four means are significantly increased.

These values are similar to those reported by Leigh et al. (2000) after administration of 2.5 mg Min-U-Sil quartz; they found 11 MN per 1000 PAM 5 days after instillation (controls: 3-4 MN per 1000 PAM; see figure below).

Conclusion

Significantly increased micronuclei numbers in alveolar macrophages are induced after quartz DQ12 treatment of rat lungs. In contrary, the MN index of the non-clay sample ('old quartz') is in the range of control values. Both indices are persistent over the 4-week post-observation period.

The clay sample (non quartz-containing) showed an initial high, significant value which returned to control levels within 4 weeks. This transience of effect was also observed for BAL parameters.

Table of micronucleus numbers

BENTONITE - 02G01030

TABLE OF MICRONUCLEI IN POLMONARY ALVEOLAR MACROPHAGES (MN per 1000 PAM)

Wistar WU, males	Group						
	Vehicle						
	Control	TiO2	Clay	Non-Clay	DQ 12	DQ 12 + TiO2	
	Dose (mg)						
	0	5	5	5	3.9	5	
RECOVERY PERIOD AFTER AFTER LAST INSTILLATION							
3	MEAN	4.3	3.0	*# 9.8	4.5	* 8.7	*# 9.5
	STD	2.7	1.4	4.1	1.6	3.9	2.3
	N	6	6	6	6	6	6
28	MEAN	3.7	2.2	2.7	4.5	* 7.1	*# 7.7
	STD	2.0	0.8	1.8	1.5	2.2	2.9
	N	6	6	6	6	6	6

significantly different compared to vehicle controls * p<0.05; ** p<0.01; *** p<0.001 (student's t-test)

significantly different compared to vehicle controls # p<0.05; ## p<0.01; ### p<0.001 (Tukey's test)

significantly different compared to vehicle controls + p<0.05; ++ p<0.01; +++ p<0.001 (Dunnett's test)

significant difference between Non-Clay and DQ12:

student's t-test: * (day 3); * (day 28)

Tukey's test: no

Dunnett's test: no

significant difference between Non-Clay and DQ12 + TiO2:

student's t-test: ** (day 3); * (day 28)

Tukey's test: no

Dunnett's test: + (day 3) and + (day 28)

Literature

D'Agostini F, Bonatti S, Oddera S, De Flora S

Micronuclei in human alveolar macrophages

Respiration 59 (Suppl 1), 35-37 (1992)

Balansky RM, Blagoeva PM, Mircheva ZI, De Flora S

Coclastogenicity of ethanol with cigarette smoke in rat erythroblasts and anticlastogenicity in alveolar macrophages

Cancer Lett 72, 183-189 (1993)

Balansky RM, D'Agostini F, Zancacchi P, De Flora S

Protection by N-acetylcysteine of the histopathological and cytogenetical damage produced by exposure of rats to cigarette smoke

Cancer Lett 64, 123-131 (1992)

De Flora S, Izzoti A, D'Agostini F, Rossi GA, Balansky RM
Pulmonary alveolar macrophages in molecular epidemiology and chemoprevention of cancer
Environm Health Persp 99, 249-252 (1993)

Das RK, Sahu K, Dash BC
Induction of chromosome aberrations and micronuclei in pulmonary alveolar macrophages of rats following inhalation of mosquito coil smoke
Mutation Res 320, 285-292 (1994)

Leigh J, Bonin A, Wang H
In vivo genotoxicity of crystalline silica as evidenced by micronuclei in pulmonary alveolar macrophages: Low-dose study
Inhalation Tox 12 (Suppl 3), 199-204 (2000)

Wang H, Leigh J, Bonin A, Peters M
Silica induced micronuclei in pulmonary alveolar macrophages in vivo
Proc. Inhaled Particles VIII: Ann Occup Hyg 41 (Suppl. 1), 434-439 (1997)

Figure 2 shows results from paper of Leigh et al. (2000)

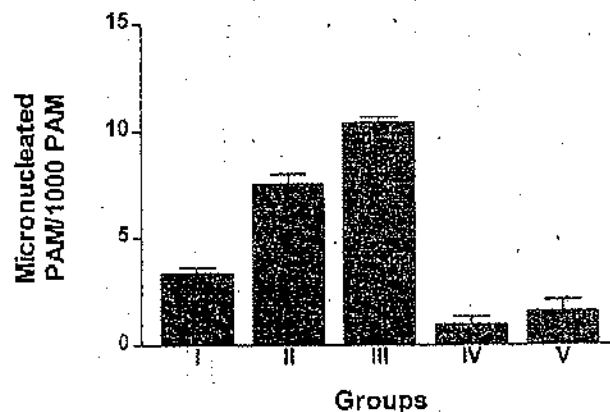


FIGURE 2. Proportion of micronucleated PAM in BALF from dust- or saline-instilled rats (mean $\pm$ SEM). I, crystalline silica, 0.025 mg in 0.5 ml saline; II, crystalline silica, 0.25 mg in 0.5 ml saline; III, crystalline silica, 2.5 mg in 0.5 ml saline; IV, TiO_2 2.5 mg in 0.5 ml saline; V, saline, 0.5 ml.

Suitability of Etoposide as a Positive Control

Etoposide is an aneugenic agent

- complexing with topoisomerase II and DNA
- enhancing double-strand and single-strand cleavage of DNA and reversibly inhibiting religation
- blocking cell cycle in S-phase and G2-phase

Etoposide is

- insoluble in water
- yields a small-sized particle suspension (approx. < 5 µm) in saline after ultrasonication
- can be phagocytized by macrophages
- induced a strong inflammatory response as documented by high PMN values

Five rats received 0.5 mg etoposide each on two consecutive days (total dose: 1 mg) by intratracheal instillation and were sacrificed one day later. Analysis of micronuclei in alveolar macrophages:

Animal no.	Micronuclei per 1000 macrophages
1	10
2	9
3	11
4	12
5	14
Mean	11.2
SD	1.9
n	5

For comparison: vehicle controls: approx. 4 per 1000; quartz DQ12: 7-9.5 per 1000

Conclusion:

Etoposide can be used as an effective positive control in the micronucleus test on alveolar macrophages

Sorptive Minerals Institute

Attachment 6

A Retrospective Cohort Mortality Study of Males Mining and Milling Attapulgitic Clay

Richard J. Waxweiler, PhD, Ralph D. Zumwalde, MS, Gregory O. Ness, MS, and David P. Brown, MS

To evaluate the possible health effects of occupational exposure to a nonasbestos mineral fiber, a cohort of 2,302 males employed for at least 1 month between 1940 and 1975 at an attapulgitic (clay fiber) mining and milling facility was followed through 1975. A significant deficit of mortality (SMR = 43, 90% CI 23-76) from nonmalignant respiratory disease (NMRD) was observed for the cohort based on age-, calendar year-, and race-specific rates for U.S. males. A marked deficit of NMRD was seen regardless of presumed dust exposure level, induction-latency period, or duration employed. A statistically significant excess of mortality from lung cancer was observed among whites (SMR = 193, 90% CI 121-293), but a deficit occurred among nonwhites (SMR = 53, 90% CI 21-112). Lung cancer risk in either race was not altered substantially with presumed dust exposure level, induction-latency period, or duration employed with one exception—those employed for at least 5 years in high-exposure-level jobs.

Key words: nonasbestos fibers, carcinogenicity, fibrogenicity, clay fibers

INTRODUCTION

The carcinogenicity and fibrogenicity of asbestos are well established in both animals and humans. Substantial experimental evidence has been published and recently reviewed [Harington, 1981] supporting the theory that the carcinogenicity, and possibly the fibrogenicity, of asbestos and of other durable fibers is related to the fiber dimensions [Pott et al, 1976; Stanton et al, 1981; Wagner, 1980; Davis et al, 1978]. Stanton et al [1981] assessed the carcinogenicity in rats of a wide variety of durable mineral fibers. They found that the probability of developing pleural sarcoma correlated well with exposure to fibers that measured $< 0.25 \mu\text{m}$ in diameter and $> 8 \mu\text{m}$ in length. High positive correlations for occurrence of pleural sarcoma also were demonstrated for exposure to fibers with diameters up to $1.5 \mu\text{m}$ and lengths $> 4 \mu\text{m}$. Data from other investigators corroborate Stanton's findings at approximately the same dimensions [Harington, 1981].

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If the fiber dimension hypothesis is correct and can be extended to man, then prevention of disease resulting from exposure to man-made fibers may be possible by restriction of fiber size during production. To assess the fiber dimension hypothesis, the National Institute for Occupational Safety and Health (NIOSH) undertook cohort mortality and industrial hygiene studies of clay fiber (attapulgite) miners and millers, because most of the natural fibers associated with this industry were not in the long, thin categories hypothesized to be carcinogenic or fibrogenic [Zumwalde, 1977].

Attapulgite clay, commonly called attapulgite after its principal mineral component, is a crystalline, hydrated, magnesium-aluminum silicate with a unique chain structure that gives it unusual colloidal and sorptive properties. "Attapulgite is a commercial designation for materials that consist of asbestiform and platy palygorskite" [National Research Council, 1984]. Attapulgite is also the principal member of a group of sorptive clays including sepiolite and montmorillonite and known collectively as fuller's earth [Haden and Schwint, 1967] because of their original use in cleaning or "fulling" wool.

Attapulgite has a wide variety of industrial applications: colloidal grades are used in paint and drilling muds, and sorptive grades find use as clarifying agents, animal litter, pesticide carriers, components of no-carbon copy papers, and pharmaceutical carriers. U.S. attapulgite is mined and milled only in the Georgia-Florida area of the U.S. (900,000 tons/year). Structurally similar clays, including attapulgite from Spain and palygorskite from the U.S.S.R., consist of much longer fibers [Huggins et al, 1962]. As mined in the U.S., attapulgite clay contains: 70-80% attapulgite; 10-15% montmorillonite, sepiolite, and other clays; 4-8% quartz; and 1-5% calcite or dolomite [Haden and Schwint, 1967].

PLANT AND EXPOSURE DESCRIPTIONS

One of the major attapulgite companies in the U.S. was selected for study. Production at the plant started around 1920, and mostly granular (large particle) grades of attapulgite were produced. Although products of fine particle sizes were more common after 1940, fine materials are always produced to some degree in the processing of attapulgite clay. The clay is strip mined, hauled to the plant, then crushed to half-inch granules. Some products require extrusion as an intermediate step. All products are then rotary dried, reduced in grinding mills, size-classified by sifting, and either bagged or shipped in bulk.

In 1976, exposure levels for total and respirable personal dust samples were measured among three groups of employees during the industrial hygiene survey [Zumwalde, 1977] (Table I); group A workers were exposed to the initial process in milling the wetted clay, group B workers were exposed to the clay after it had been dried and ground, and group C workers were exposed to the clay after it had been sized for bagging or bulk shipment. In general, group A was exposed to less respirable and total dust than the other groups. Time-weighted average (TWA) free silica exposures and trace metal analyses (Cd, Co, Cr, Fe, Mn, Ni, Zn) from airborne samples were usually near the lower limits of detection. Iron was the only trace metal found in any appreciable quantity (1-2.4%). All TWA concentrations for respirable dust were below the Mine Safety and Health Administration nuisance dust standard of 5 mg/m³.

TABLE I. Time-Weighted Average Exposures to Airborne Dust by Mill Job Type

	Job type ^a	No. of samples	Geometric mean (mg/m ³)	Geometric standard deviation
Total dust	A	6	1.75	3.95
	B	5	15.90	1.85
	C	27	11.92	2.41
MSHA standard			10.0	
Respirable dust	A	11	0.45	2.95
	B	11	1.60	2.03
	C	66	1.15	2.26
MSHA standard			5.0	

^aJob definitions: A, initial process, milling wetted clay; B, intermediate process, exposed to clay after drying and grinding; C, final process, exposed to clay after sizing.

To determine fiber size characteristics, 15 airborne samples of total dust were randomly selected from the 200 collected within the mill. Measurements made by transmission electron microscopy (TEM) at $\times 20,000$ on approximately 460 fibers (after high-energy ultrasound dispersion) indicated attapulgite clay fiber lengths of 0.1–2.5 μm with a geometric mean of 0.52 μm , and diameters of 0.02–0.1 μm , with a geometric mean of 0.06 μm . In addition, fiber aspect ratios (length to diameter) were generally $>10:1$. It was noted during the TEM analysis that attapulgite clay fibers had an affinity to agglomerate into jagged particles ranging in diameters from 0.5 to 5.0 μm . No other types of fibrous minerals ($>3:1$ aspect ratio) were observed in either airborne or bulk mine samples based on analysis by both selected area electron diffraction and energy dispersive X-ray analysis [Zumwalde and Dement, 1977].

METHODS

The study cohort was limited to the 2,302 males who had worked either in the mine or mill of this attapulgite facility for at least 1 month any time between January 1, 1940, and December 31, 1975. The cohort members were identified from microfilm copies that we made of company personnel records. In most cases, data initially missing on race, sex, date of birth, or work history were later supplied by the company management or the Social Security Administration. Fifty males ($<2\%$ of the cohort) for whom race could not be determined were assumed to be white.

The vital status determination as of December 31, 1975, of each of the 2,302 cohort members was based on information from the Social Security Administration, Internal Revenue Service, state bureaus of motor vehicles, state vital statistics offices, and other sources. Death certificates were obtained, and the underlying cause of death was coded by a qualified nosologist in the revision of the International Classification of Diseases (ICD) in effect at the time of death and converted to 7th revision ICD codes. This method was used to maintain comparability with procedures used to determine cause-specific death rates for the U.S. male population.

Person-years at risk (PYAR) were begun for each employee upon the latter of two events; January 1, 1940, or completion of 1 month of employment at the facility.

PYAR were terminated on the date of death or, for persons alive or lost to follow-up, on December 31, 1975. This date was chosen for the persons lost to follow-up to minimize any chance of overestimating risk; consequently, we might have slightly underestimated risk. The race-, sex-, and cause-specific expected deaths, based on U.S. mortality rates, were adjusted for 5-year age- and calendar-specific periods using a modified life-table analysis program [Waxweiler et al, 1983].

Standardized mortality ratios (SMR) were calculated to represent the observed number of deaths as a percentage of the expected number. Ninety percent confidence intervals (CI) for the SMRs were based on a Poisson distribution for the observed frequency [Armitage, 1971].

Further analyses were carried out by dust exposure level. Milling jobs corresponding to groups B and C in Table I were considered to be high-exposure jobs relative to group A. Jobs in the mill, such as maintenance, that required occasional work among groups B and C were classified as intermittent-exposure jobs. Mining jobs were not sampled for dust levels but were included in the analyses with group A jobs based on our judgment of very little exposure. There were no historical industrial hygiene data available, and the dust levels undoubtedly varied over time. However, for purposes of the epidemiologic analysis, we assumed that the same jobs were always the one with high or intermittent exposures.

For these analyses, risk (PYAR) and induction-latency period began when an employee entered an exposed job, and duration of employment refers to time spent in exposed jobs. For instance, consider a person who worked first in a group A job, then in a maintenance (intermittent-exposure) job. In the first overall analysis, his PYAR and induction-latency began when he began the group A job and his duration of employment included all jobs. In the intermittent-exposure analysis, his PYAR and induction-latency began with his maintenance job, and his duration of employment included only his maintenance job.

RESULTS

All but 140 (6%) of the cohort members were successfully traced until December 31, 1975 (Table II). Of the 315 reported to be deceased, death certificates could not be located for 19 (6%). A smaller proportion of death certificates could not be located for whites, 3%, than for nonwhites, 9%. The 2,302 cohort members (1,235

TABLE II. Vital Status of Cohort of Male Attapulgitte Miners and Millers as of December 31, 1975

	All exposure levels		Intermittent-exposure		High-exposure		Intermittent high-exposure	
	Whites	Nonwhites	Whites	Nonwhites	Whites	Nonwhites	Whites	Nonwhites
Alive	1,021	826	185	231	141	599	320	756
Deceased	151	164	42	88	18	79	59	139
Missing death certificates	5	14	1	7	0	8	1	11
Lost to follow-up	63	77	11	32	8	45	19	70
Total	1,235	1,067	238	351	167	723	398	965
Person-years at risk	20,297	18,426	4,650	8,395	2,150	10,415	6,784	16,498

white and 1,067 nonwhite) contributed 38,723 PYAR to the study; an average of 17 years per member. A much smaller proportion of the whites, 398 of 1,235 (32%), than of the nonwhites, 965 of 1,067 (91%), had worked in intermittent- or high-exposure jobs. Few persons, regardless of race, had worked in both an intermittent- and a high-exposure job. For instance, only four white males ($238 + 164 - 398 = 4$ in Table II) were included both in the intermittent-exposure cohort and in the high-exposure cohort.

The 315 deaths observed in the study were 80% of expected (Table III). Overall mortality was less favorable among whites (151 observed and 157.8 expected) than among nonwhites (164 observed and 235.9 expected). The low overall mortality among the nonwhites was due mostly to very favorable mortality patterns for tuberculosis, all malignant neoplasms combined, circulatory system diseases, and NMRD. With the exception of all malignant neoplasms, whites experienced fewer observed than expected deaths for these same causes, but the deficits were not as great.

Among the entire cohort, statistically significant excess risk was not seen for any cause. The excess risk for liver cancer was not quite significant (SMR = 250, 90% CI 99-526). Slight, statistically insignificant excesses were found for cancer of the stomach overall (SMR = 120, 90% CI 52-237) and for cancer of the intestines among whites (SMR = 160, 90% CI 55-366). A large discrepancy was observed between the two races for mortality from lung cancer. Whites exhibited almost a twofold excess risk (SMR = 193, 90% CI 121-293), nonwhites a 40% deficit (SMR = 53, 90% CI 21-112).

For selected causes, associations between mortality and duration of employment were investigated after limiting the analysis to the period of 10 or more years since initial employment at the facility (Table IV). The excess of stomach cancer, based on six cases, was not statistically significant and showed no relationship to duration of employment. The liver cancer excess was essentially eliminated after 10 years since first employment, precluding meaningful analysis by duration of employment.

Mortality from lung cancer was strikingly different between whites and nonwhites and thus is presented separately for the two racial groups (Table IV). After 10 years induction-latency, whites exhibited nearly a twofold ratio of observed to expected deaths, which was statistically significant. However, no trend by duration of employment was evident. Among nonwhites, on the other hand, there was a substantial deficit in risk for those working < 5 years and a slight excess risk for those working longer. A statistically significantly low SMR (41; 90% CI 19-77) was seen for NMRD. The deficit in mortality from NMRD was independent of duration of employment.

Further analysis of lung cancer by calendar period of risk indicated no trends, despite the fact that no such deaths were observed prior to 1960. Furthermore, younger (< 65 years) persons appeared to be at no different relative risk (17 observed vs. 14.8 expected) than older persons (4 observed vs. 2.8 expected). Similar analyses for stomach cancer and NMRD also found no trends by age or calendar period.

Analyses of employees exposed to intermittent levels of dust combined with those exposed to high levels revealed no statistically significant excesses overall; however, the numbers of expected deaths were usually quite small (Table V). Mortality from NMRD in these subcohorts was much lower than expected. Excess lung cancer deaths were limited to the whites. This anomalous finding was partially clarified by dividing the high-exposure results for lung cancer further by duration

TABLE III. Cause-Specific Mortality Among Male Attapulgitte Miners and Millers 1940-1975*

Cause of death	ICD (7th Rev)	White		Nonwhite		Total		SMR
		Obs	Exp	Obs	Exp	Obs	Exp	
All causes		151	157.8	164	235.9	315	393.7	80
Tuberculosis	(1-19)	1	1.8	2	8.6	3	10.4	29
All malignant neoplasms (MN)	(140-205)	31	28.0	18	33.5	49	61.5	80
Buccal cavity and pharynx	(140-148)	0	0.9	0	1.4	0	2.3	
Digestive System	(150-159)	9	8.2	8	11.6	17	19.8	86
Esophagus	(150)	1	0.6	0	2.1	1	2.7	
Stomach	(151)	2	1.7	4	3.3	6	5.0	120
Intestines	(152,153)	4	2.5	1	2.1	5	4.6	109
Rectum	(154)	0	1.0	0	0.9	0	1.9	
Liver and biliary tract	(155,156A)	2	0.7	3	1.3	5	2.0	250
Pancreas	(157)	0	1.5	0	1.8	0	3.3	
Other digestive system	(158,159)	0	0.1	0	0.2	0	0.3	
Lung	(162,163)	16	8.3	5	9.4	21	17.7	119
Larynx	(161)	0	0.4	1	0.6	1	1.0	
Lymphatic and hematopoietic tissue	(200-205)	2	3.0	2	2.5	4	5.5	73
Other malignancies		4	7.2	2	8.0	6	15.2	39
Vascular lesions of CNS	(330-334)	11	10.3	21	21.4	32	31.7	101
Circulatory system diseases	(400-468)	62	66.9	47	75.6	109	142.5	76
Nonmalignant respiratory system disease	(470-527)	4	7.8	5	13.0	9	20.8	43
Acute upper respiratory infection	(470-475)	0	0.0	0	0.1	0	0.1	
Influenza and pneumonia	(480-483, 490-493)	1	3.7	3	9.1	4	12.8	31
Bronchitis	(500-502)	0	0.5	0	0.4	0	0.9	
Other nonmalignant respiratory disease	(510-527)	3	3.6	2	3.4	5	7.0	71
Accidents	(800-962)	17	16.1	20	22.9	37	39.0	95
All other causes		17	25.0	32	52.8	49	77.8	63
Unknown cause	(780-783, 795)	3	1.9	5	7.8	8	9.7	83
Missing death certificates		5		14		19		

*Obs, observed; Exp, expected; SMR = 100 (obs/exp).

TABLE IV. Cause-Specific Mortality by Duration of Employment Among Attapulgitte Miners and Millers After 10 Years Since First Employment by Duration of Employment*

Cause of Death	Duration of Employment						SMR
	1 Month-4.9 Years		≥ 5 Years		Total		
	Obs	Exp	Obs	Exp	Obs	Exp	
All causes	166	186.8	99	121.6	265	308.4	86
Stomach cancer	3	2.4	3	1.9	6	4.2	143
Intestinal cancer	2	2.3	2	1.6	4	3.9	103
Liver cancer	2	1.0	0	0.7	2	1.7	
Lung cancer	12	9.9	9	5.9	21	15.8	133
Whites	10	4.3	6	2.9	16	7.2	222
Nonwhites	2	5.6	3	3.0	5	8.6	58
Nonmalignant respiratory system disease	4	10.2	3	6.8	7	17.0	41
Missing and unknown cause	17	4.4	6	2.9	23	7.4	311
Person-years at risk	14,193		7,174		21,366		

*Obs, observed deaths; Exp, expected deaths; SMR = 100 (obs/exp).

TABLE V. Mortality From Selected Causes Among Attapulgitte Miners and Millers Exposed to Intermittent and High Levels of Respirable Dust*

Cause of death	Intermittent exposure				High exposure			
	White		Nonwhite		White		Nonwhite	
	Obs	Exp	Obs	Exp	Obs	Exp	Obs	Exp
All causes	42	49.1	88	120.3	18	18.4	79	120.6
Stomach cancer	1	0.6	3	1.8	1	0.2	1	1.6
Lung cancer	4	2.5	3	4.9	3	1.0	3	4.7
Circulatory system diseases	19	22.1	27	39.9	8	7.9	19	37.4
Nonmalignant respiratory system disease	1	2.6	1	6.8	1	0.9	3	6.4

*Obs, observed deaths; Exp, expected deaths.

worked. Among those with >5 years of employment in high-exposure jobs, there were five observed deaths from lung cancer versus 1.62 expected (SMR = 309, 90% CI 122-649). Both whites (two observed, 0.45 expected) and nonwhites (three observed, 1.18 expected) contributed to this excess.

Because of particular a priori interest in lung cancer and digestive system cancer and the slight excesses of observed deaths for lung and stomach cancer in Tables IV and V, further analyses of these sites were performed. Experience in both intermittent- and high-exposure jobs was considered simultaneously, and risk was examined by periods of duration worked in such jobs and induction-latency period (Table VI). The only statistically significant excess in this analysis was for lung cancer among whites after 20 years induction-latency (six observed, two expected; SMR = 300, 90% CI 131-592). The comparable cell for nonwhites had a decreased risk for lung cancer. An examination of risk of mortality from stomach cancer among those who had worked in either intermittent- or high-exposure jobs uncovered no statistically significant excesses (Table VII).

TABLE VI. Observed and Expected Deaths From Lung Cancer by Induction-Latency Period and Duration Worked in High- and Intermittent-Exposure Jobs*

Induction latency (years)		Duration					
		1 Month-4.9 years		≥ 5 years		Total	
		Obs	Exp	Obs	Exp	Obs	Exp
0-9.9	White	0	0.4	0	0.1	0	0.5
	Nonwhite	0	0.6	0	0.1	0	0.7
10-19.9	White	1	0.8	0	0.3	1	1.1
	Nonwhite	0	1.5	1	0.4	1	1.9
≥ 20	White	3	1.2	3	0.7	6	2.0
	Nonwhite	2	3.7	2	2.0	4	5.7
Total	White	4	2.5	3	1.1	7	3.5
	Nonwhite	2	5.8	3	2.6	5	8.3
	All	6	8.2	6	3.6	12	11.9

*Obs, observed deaths; Exp, expected deaths.

TABLE VII. Observed and Expected Deaths From Stomach Cancer by Induction-Latency Period and Duration Worked in High- and Intermittent-Exposure Jobs*

Induction Latency (years)	Duration				Total	
	1 Month-4.9 Years		≥ 5 Years			
	Obs	Exp	Obs	Exp	Obs	Exp
0-9.9	0	0.52	0	0.06	0	0.58
10-19.9	2	0.76	1	0.23	3	0.99
≥ 20	1	1.10	2	1.00	3	2.10
Total	3	2.37	3	1.30	6	3.67

*Obs, observed deaths; Exp, expected deaths.

DISCUSSION

A substantial body of literature supports the concepts that fiber dimensions influence their respirability, deposition, carcinogenicity, and perhaps fibrogenicity. Respiratory tract and gastrointestinal tract deposition of fibrous aerosols in humans is dependent on a number of fiber characteristics, the most important being aerodynamic diameter [Timbrell, 1973; Dement and Harris, 1979]. Most airborne attapulgite clay fibers are found in jagged agglomerated particles; thus only a minor portion would be expected to be respirable. However, essentially all of the less common individual airborne fibers (0.1 μm in diameter) would be respirable, probably penetrating beyond the upper airways and into the smaller airways and alveoli [Bignon et al, 1978; Timbrell, 1973]. One individual exposed to airborne clay fibers and who had lung fibrosis was found to have clay fibers in his alveolar spaces [Bignon et al, 1978].

Fibrogenicity of fibers is thought to be influenced by fiber length. Both inhalation and pleural injection studies indicate that long fibers are more fibrogenic than shorter ones or particles [Davis et al, 1978; National Research Council, 1984]. Wagner [1980] has postulated that pulmonary fibrosis is a possible hazard for humans when exposures occur to fibers < 2 μm in diameter and 10-50 μm in length. Our results appear to be consistent with this hypothesis. Airborne attapulgite fibers measured were short, 0.1-2.5 μm in length, and there was no excess of NMRD mortality. In fact, even after a 10-year induction-latency period, a striking deficit of

NMRD was seen ($SMR = 41$, 90% CI 19–77), which did not vary by duration of employment.

Carcinogenicity of fibers also has been shown experimentally to reflect the importance of fiber dimensions [Harington, 1981; Pott et al, 1976; Stanton et al, 1981; Wagner, 1982]. Until now, the direct evaluation of the carcinogenicity of attapulgite has depended on experimental data alone. In making such an evaluation, it is important to be aware of the strikingly different fiber sizes between U.S. attapulgite (short fibers) and attapulgite from Spain or palygorskite from the U.S.S.R. (long fibers). Pott et al [1974] demonstrated the tumorigenicity of palygorskite from the U.S.S.R. Stanton et al [1981] found minimal sarcoma induction after pleural implantation of two different samples of U.S. attapulgite, both of which consisted mainly of short, thin fibers. These results were consistent with their overall findings that the tumorigenicity of various types of fibers implanted pleurally in rats appeared to be correlated with the number of fibers less than $1.5 \mu m$ in diameter and greater than $4.0 \mu m$ in length. Furthermore, the tumorigenicity appeared to be most highly correlated to the number of fibers $<0.25 \mu m$ in diameter and $>8.0 \mu m$ in length [Stanton et al, 1981]. The diameters of airborne attapulgite fibers measured at our study plant fell into this range, but the lengths were much shorter ($<2.5 \mu m$). Nevertheless, it is still open to question whether the experimental results of Stanton et al can be extrapolated to the inhalation of fibers (and the cocarcinogenic effect of cigarette smoke) and subsequent development of epithelial tumors in humans.

We found a slight excess of stomach cancer after a 10-year induction-latency period but no statistically significant associations by exposure level or duration. This slight excess could not be explained by local rates for stomach cancer mortality [Riggan et al, 1983].

Our epidemiologic results for lung cancer were mixed. White employees experienced approximately a twofold excess of lung cancer that increased slightly but not substantially with induction-latency period, duration of employment, or exposure level. Nonwhite employees, on the other hand, experienced an overall lung cancer risk approximately half that expected based on U.S. rates. The pattern of local rates for lung cancer for white and nonwhite males did not account for this distinct difference in lung cancer risk. Deficits of lung cancer mortality among nonwhite employees were seen for all subcategories of duration of employment, induction-latency period, or exposure level except among those who worked at least 5 years in intermittent- and high-exposure jobs or at least 5 years in high-exposure jobs alone. Neither of these latter two excesses was statistically significant. However, the excess lung cancer mortality among all employees, regardless of race, who worked at least 5 years in high-exposure jobs was significant (5 observed vs 1.62 expected; $SMR = 309$, 90% CI 122–649).

Cigarette smoking must be considered as a potential explanation of the lung cancer pattern that we observed. No data on cigarette smoking were available for the cohort, but for whites and nonwhites the deficits of mortality from NMRD and circulatory system disease certainly do not appear to be consistent with an increased prevalence of cigarette smoking. Furthermore, among the current work force of white males and nonwhite males, there are, respectively, 44% and 46% current cigarette smokers, 21% and 11% exsmokers, and 35% and 44% nonsmokers (J. Gamble, personal communication). Despite the fact that smoking patterns may change over time in a dynamic cohort, and that cigarette smoking is a strong risk factor for lung cancer, it is doubtful that smoking explains any $SMRs >200$ [Steenland et al, 1984].

In addition to the lack of cigarette smoking data for the cohort, other limitations of this study must be noted, including 1) an inability to confirm the complete enumeration of the cohort with an independent source of data, 2) persons lost to follow-up, 3) missing death certificates, 4) missing race on 50 persons, 5) lack of longitudinal person-specific dosimetry, 6) few expected deaths beyond 20 years induction-latency, 7) few persons who worked for long durations at the facility, and 8) use of the general U.S. population for comparison. Because of these limitations, our findings should be interpreted as neither strongly supportive of nor contradictory of an association between the lung carcinogenicity of U.S. attapulgite, or short thin fibers in general, and lung cancer risk in humans. Given the importance of this cohort in its potential to further the understanding of fiber carcinogenicity and fibrogenicity, it is important that smoking data be gathered on this cohort and that mortality follow-up be extended prospectively in the future to increase our statistical power and observation of risk at longer induction-latency periods.

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SORPTIVE MINERALS INSTITUTE

June 3, 2014

Via electronic submission

OSHA Docket Office
Docket No. OSHA-2010-0034,
U.S. Department of Labor, Room N-2625,
200 Constitution Avenue, NW
Washington, DC 20210

Docket Number: **OSHA-2010-0034**

**Re: Post-Hearing Submission of the Sorptive Minerals Institute (SMI);
Occupational Safety and Health Administration's Proposed Rule on Occupational
Exposure to Respirable Crystalline Silica**

On April 2, 2014, Richard Brown, provided oral testimony on behalf of the Sorptive Minerals Institute (SMI) in regard to OSHA's Proposed Rule on Occupational Exposure to Respirable Crystalline Silica. During the question and answer period following his testimony, Mr. Brown was asked several questions that he indicated would require additional research. This letter provides SMI's response to these questions.

World Health Organization Report

Janet Carter from OSHA asked Mr. Brown to comment on studies cited in the World Health Organization's (WHO) 2005 report titled "Bentonite, Kaolin and Selected Clay Minerals" in which it was noted that silicosis was found in bentonite workers. It is important to note that this report is careful to state that "*There are important limitations in the data on the health effects of clays in humans. Most studies are case reports or case series (bentonite) or cross-sectional in design (kaolin). Smoking data are not available in most of the studies. In many studies, exposure characterization, both qualitatively and quantitatively, is incomplete.*" This statement is particularly important in reference to the specific study in the report to which Ms. Carter was referring, Phibbs et al., 1971. This early study was flawed in three key areas: a failure to recognize that many of the workers employed by the bentonite industry at that time had recently come from the copper mining industry in Butte, Montana and had a history of exposure to hard rock mining and processing occupational conditions; a failure to account for exposure characterization while the workers who were studied worked in the bentonite industry; and a failure to adequately account for the effect of smoking on these workers. These failures make it impossible to draw any meaningful conclusions from the Phibbs study.

SMI submitted comments to WHO shortly after the 2005 report was released noting significant short comings in the research that the report relied upon and asking WHO to

correct the report accordingly. Specifically, SMI told WHO that the studies it presented had failed to properly characterize the mineral materials that they purported to study. SMI stated that *“It is fundamental to the study of the toxicology of any mineral material that the identity and characteristics of the mineral be correctly and accurately defined. Where the identity and characteristics of the mineral material studied are not precisely known the study results cannot be properly interpreted because the causative agent is not identified. At a minimum the following mineral characteristics must be accurately defined for any mineral sample used in toxicological testing:*

Particle size distribution

Particle morphology

Mineralogical composition

Bulk chemical composition

Surface chemistry (to determine the presence of surficial contaminants such as transition metals and organic compounds)

Crystallinity (Murata ratio)

Surface charge

Surface reactivity

Other characteristics may also need to be defined depending on the type of mineral being studied.”

In its comments on the 2005 report, SMI also noted that, in some of the studies cited, the mineral materials used had been purposely altered during the testing without taking into account how this might affect the study outcome. In response to this SMI stated *“It is critical that the process used to create the mineral material samples used for testing not alter the properties of the material or, where they are altered, that the new properties of the material be fully defined and distinguished from those of the original material. If this is not done then there is no way to accurately link the results obtained with the material used.”*

A copy of SMI's comments on the 2005 WHO report is included here as “Appendix 1”.

2013 Study

Janet Carter also asked Mr. Brown if he was aware of and could comment on a 2013 study which showed genetic damage and lipid peroxidation in workers that were occupationally exposed to bentonite particles. After learning that Mr. Brown was not aware of this study Ms. Carter agreed to provide a reference for it. Upon reviewing this study (Huang, et al. (2013) *Genetic damage and lipid peroxidation in workers occupationally exposed to organic bentonite particles.*), SMI finds that it failed to provide any meaningful information pertinent to the current rulemaking for the following reasons:

1. While the study authors spent considerable time characterizing the study subjects they failed to even minimally characterize the base mineral materials (“Bentonite”) that were being used at the three factories where the study subjects worked or the product (organically modified bentonite) that was being produced at two of the factories. They also failed to characterize the surface properties of either material. As previously stated, physicochemical and mineralogical characterization of test materials is extremely important when trying to establish toxicological profiles. OSHA has also recognized the importance of material surfaces in determining toxicity when it stated in its Notice of Proposed Rulemaking *“there is considerable evidence to support the hypothesis that surface*

activity of crystalline silica particles plays an important role in producing disease”. This failure makes it impossible to causally link the findings of the study with a particular mineral material.

2. No attempt was made to segregate the effect of the organic modifier, per se, from that of the modified bentonite, the base bentonite or any of the mineral components in the base bentonite. As a result, there is no way to determine which material is causally linked to the reported results.
3. Perhaps most critically, the study was specifically focused on organically modified bentonite, not respirable crystalline silica which is the subject of this rule making.

SMI Exposure Data for Respirable Crystalline Silica

Dave O'Connor from OSHA asked if SMI had crystalline silica exposure data for its facilities and, if so, if SMI would be willing to share that data with OSHA. Richard Brown agreed to consult with the SMI membership and provide the available data.

The exposure data provided below is from the twenty-three SMI production facilities that are regulated by the Mine Safety and Health Administration. The exposure figures for each job description listed below represent an average of the data gathered by MSHA during its regular inspections of SMI member facilities over the past five years (2009 to the present). The data has been prepared in consultation with MSHA and verified by the MSHA Laboratory in Pittsburgh, PA. Please note that MSHA calculates its exposures on a time weighted average (TWA). They provided SMI with the formula to convert the raw data they provided to TWA values.

We have included all test results regardless of the number of samples for each job classification and the level of exposure. We are enclosing a spreadsheet with the raw data from MSHA (“Appendix 2”).

<u>Job Description</u>	<u>TWA Respirable CS Exposure - 8 hrs.</u> <u>µg/m³</u>
Equipment Operator – 24 Samples	6.41 µg/m ³
Kiln / Dryer Operator – 32 Samples	20.38 µg/m ³
Bagging and Packaging – 54 Samples	34.35 µg/m ³
Mechanics / Utility Workers – 21 Samples	8.02 µg/m ³
Truck Driver – 7 Samples	1.72 µg/m ³
Lab Technician – 3 Samples	30.11 µg/m ³
Supervisor – 2 Samples	11.64 µg/m ³

Safe Use Determination

Anna Laura Bennett from OSHA asked if SMI would provide a copy of the Safe Use Determination (SUD) that it had obtained from the California Office of Health Hazard Assessment. This is attached as “Appendix 3”.

TOXNET Hazardous Substances Data Bank Search

In addition to the reference to the Haung et al. study, OSHA also provided SMI with the results of a National Library of Medicine's TOXNET system, Hazardous Substances Data Bank (HSDB) search conducted on the term “Bentonite” and various synonyms. A review of the studies cited in this thirty seven page report reveals that they do not

contribute information meaningful to the subject of the proposed rulemaking for the following reasons:

1. Many of the studies do not deal or are not related to respirable exposure.
2. All of the cited studies focus on bentonite, not the Occupational Exposure to Respirable Crystalline Silica, which is the subject of the proposed rulemaking. SMI's work has been focused on crystalline silica from sorptive minerals. After significant research, SMI found that bentonite was the only sorptive mineral from which crystalline silica (quartz) of respirable size could be obtained without potentially altering the surface properties of the quartz particles. Water dispersion of undried chunks of sodium bentonite using low shear mixing allows respirable size particles of quartz to be concentrated through repeated settling and centrifugation without the need for dispersants or other chemical treatments that might alter the particles' surfaces. It is for this reason that samples of quartz from bentonite produced in this fashion, and maintained in aqueous suspension, were used in the Fraunhofer studies that SMI has previously reported to OSHA.
3. Most of the studies fail to even minimally characterize the materials being studied. As previously noted, this characterization is critical making it impossible to draw a causative relationship with the results obtained. To date, the Fraunhofer Institute study, "Toxicity of a Quartz with Occluded Surfaces in a 90-Day Intratracheal Instillation Study in Rats" is the only study that includes a detailed characterization of the test materials including DQ12 and occluded quartz. This study found that quartz with occluded surfaces had a significantly lower and non-progressive toxicity profile after intratracheal instillation in rats with follow-up for up to 90 days than DQ12 crushed quartz.

Hochella Study

During his testimony, Mr. Brown discussed the results of an unpublished research study conducted for SMI by Hochella and Muryama in 2010. This work shows that the surface of quartz grains taken from samples used in the Fraunhofer studies consisted of an amorphous alumino-silicate coating. The amorphous nature of this coating distinguishes it from the crystalline aluminosilicate structure of the montmorillonite mineral that makes up the bulk of the bentonite from which the quartz grains were obtained. A copy of this study is attached here as "Appendix 4".

SMI appreciates this opportunity to respond to the questions posed by OSHA during our oral testimony and to provide the enclosed materials.

Sincerely,



Lee Coogan
Executive Director
Sorptive Minerals Institute



Kathryn McMahon
Counsel to Sorptive Minerals Institute

Appendix 1

December 27, 2005

Director, International Program on Chemical Safety
World Health Organization (WHO)
20 Avenue Appia
1211 Geneva 27
Switzerland

Re: WHO Environmental Health Criteria 231
Bentonite, Kaolin and Selected Clay Minerals

Dear Mr. or Madam Director:

Earlier this year the World Health Organization published the final version of its monograph on Environmental Health Criteria of Bentonite, Kaolin and Selected Clay Minerals. In a note included in the monograph readers were requested to report errors in the monograph to you so that they could be corrected in corrigenda. We are taking this opportunity to write to you to report several such errors.

The Sorptive Minerals Institute (SMI) is a national trade organization in the United States representing the producers and marketers of absorbent clay products, including clay-based pet litters containing bentonite. Over the past 20 years SMI has actively pursued information that would shed light on the potential for human health hazard from the clay minerals that its member companies utilize. This effort has enabled SMI, in concert with many highly regarded scientific researchers from around the world, to develop a large database of information on clay and crystalline silica mineralogy, physics, chemistry and toxicology. It is with this background that we provide the following comments (Note: all page references refer to the "pdf" version of the monograph).

1. Cristobalite In Bentonite

There are numerous instances through out the monograph where it is stated that the silica mineral form cristobalite is present in bentonite. The first of these may be found in the first paragraph of Section 1.1 on Page 1 which states "In addition to montmorillonite, bentonite may contain feldspar, cristobalite, and crystalline quartz." Other statements about the presence of cristobalite in bentonite occur on pages 65, 86 and 114. These statements are incorrect.

In fact, it would be extraordinarily rare to find cristobalite present in bentonite clay. Bentonite is thought to have most commonly been formed by devitrification of a specific type of volcanic ash in salty water. Most of the commercial bentonite marketed around the world is thought to have been formed in this manner during the Tertiary, Cretaceous and Jurassic periods, from 30 to 175 million years ago. Other methods of bentonite formation include hydrothermal alteration of *in situ* volcanic ash or feldspathic parent rock.

In all cases, the process involves dissolution of the parent material in liquid water, over time, and *in situ* re-precipitation of daughter materials including montmorillonite, crystalline and amorphous silica, gypsum, feldspar, mica and other minerals. Since water is necessarily involved, this process must occur at relatively low temperatures. Cristobalite is a high temperature polymorph of quartz requiring temperatures in excess of 1470 °C to form. It is only metastable at normal temperatures and pressures and, given sufficient time, will eventually change to quartz. Since bentonite is formed by dissolution of the parent material in water and re-precipitation of the clay and associated minerals it would be impossible to form cristobalite during this process. As a result, the only situation where cristobalite could be found in bentonite would be the very rare circumstance where both a deposit of cristobalite containing material and a deposit of bentonite were eroded, transported, and re-deposited together by water to form a composite secondary deposit.

It is now well known that the material in bentonite previously thought to be cristobalite is actually a hydrous form of amorphous silica known as "Opal-CT". Opal-CT produces a few X-ray scattering effects occurring in areas near some of the X-ray diffraction lines used to identify cristobalite. This similarity has often caused Opal-CT to be confused with cristobalite. To avoid this confusion, Miles (1994) ¹ combined two standard National Institutes of Occupational Safety and Health (NIOSH) procedures, 7500 ² and 7601 ³, to enable the positive differentiation of Opal-CT and cristobalite. In 1998 Smith ⁴ conducted an exhaustive survey of the existing scientific literature and developed a straightforward nomenclature and classification for the opal forms. Smith found that very few analytical laboratories are aware of the ambiguity between opal and cristobalite in the literature and, as a result, routinely fail to distinguish between these materials.

Based upon these facts we request that the WHO monograph be modified to: 1) acknowledge that only opal, and not cristobalite, naturally occurs in bentonite, and 2) remove all references indicating the presence of cristobalite in bentonite.

2. Illness Due To Bentonite-Based Litter

At Section 6.2.1 on page 78 a single reference is given where a sick (profoundly anemic) cat was known to eat bentonite-based cat litter. A reference is also given which notes that pica (the eating of non-food items) in cats is caused by anemia. Though the causality of the cat's illness is noted as being uncertain there is an implication inherent in this section that the bentonite-based cat litter had something to do with it. Although the popular (internet-based) literature has numerous anecdotal references concerning clay-based litter causing illness in cats our review of the scientific literature has never found a single instance where this has been independently verified. Further, our communications with numerous veterinary experts in this field have failed to find anyone who could confirm that they have seen a case where clay-based litter caused illness in a cat. In all cases where illness was claimed, consumption of the litter was merely a symptom of the illness and not the causative agent. The mere fact that the Hornfeldt & Westfall citation is included in the monograph presents a situation of guilt by inference.

For these reasons we request that: 1) that a stronger statement be included to note that there is no scientifically verifiable data showing that eating of bentonite-based litter will cause illness in cats, and 2) that the citation be removed from any future publications of the monograph.

3. Mineral Identification and Characterization Requirement

In the introduction to Section 6, page 59, referring to the toxicological studies cited, the monograph notes that "...it is often not known what it was precisely that was studied and the impurities present in the material used." The monograph goes on to admonish the reader that, because of this, the results of the studies must be carefully interpreted. Unfortunately, this statement is completely inadequate to enable any but the most sophisticated readers to properly establish the value of the studies that are presented. It is fundamental to the study of the toxicology of any mineral material that the identity and characteristics of the mineral be correctly and accurately defined. Where the identity and characteristics of the mineral material studied are not precisely known, the study results cannot be properly interpreted because the causative agent is not identified. At a minimum the following mineral characteristics must be accurately defined for any mineral sample used in toxicological testing:

- Particle size distribution
- Particle morphology
- Mineralogical composition
- Bulk chemical composition
- Surface chemistry (to determine the presence of surficial contaminants such as transition metals and organic compounds)
- Crystallinity (Murata ratio)
- Surface charge
- Surface reactivity

Other characteristics may also need to be defined depending on the type of mineral being studied. For this reason we request that a statement be added to alert the reader to the fact that the results of cited studies must be viewed as highly suspect unless the mineral identity of the test material used has been determined.

4. Modification of Mineral Properties

It is critical that the process used to create the mineral material samples used for testing not alter the properties of the material or, where the materials are altered, that the new properties of the material be fully defined and distinguished from those of the original material. If this is not done, there is no way to accurately link the results obtained with the material used.

In Section 6.1.1 at page 68 the results of a study by Schreider et al., 1985, are presented where gross lung lesions and mild fibrosis were found 90 days after intratracheal instillation of montmorillonite that had previously been heated to 750°C. It is noted that this was done "...to collapse the crystal structure of the montmorillonite and inactivate any traces of silica..." No mention is made of the

fact that as a direct result of this process, the resulting material used in the tests was no longer montmorillonite. Absent this information the reader is forced to draw the false conclusion that montmorillonite caused the resulting abnormalities. Only a very sophisticated reader who is knowledgeable in clay mineralogy would be able to correctly interpret these results.

As a result, we request that: 1) a statement be included in the corrigenda strongly stating that the material tested was no longer montmorillonite due to alteration by the thermal treatment used, and 2) that any future publications of the monograph be corrected to either eliminate this reference or to strongly state that the material tested was no longer montmorillonite due to the thermal treatment that it underwent.

5. Use of Bentonite for Animal Feed

Section 6.2.1 presents the results of four studies by Wilson in the early 1950's where adverse effects were reported when bentonite was included in the diet of laboratory mice. The citation acknowledges that only limited details were provided in the studies and that some of the subject animals had previously been administered a known carcinogen. No mention was made, however, of any of the large body of more recent scientific studies (1960's forward) on the use of bentonite as a feed supplement or animal feed additive for cattle, sheep, pigs, horses, fish, shrimp, etc., where bentonite has been found to have significant animal health benefits such as increased weight gain, prevention of scours (uncontrolled diarrhea) and sequestering of mycotoxin and heavy metal contaminants in the gut. As a result the monograph leads the reader to an incorrect and biased view of the health effects of ingestion of bentonite.

For this reason we request: 1) a statement be included in the corrigenda noting the long term, beneficial use of bentonite in animal, fish and shellfish feed, and 2) that any future publications of the monograph either delete the Wilson references or include references to the more recent body of work citing the results of the use of bentonite in animal, fish and shellfish feed.

Finally, in order to alert the research and regulatory communities to the problems that we have identified and begin the process of correcting them, we request that this letter, and any response to it, be posted on the WHO web site and that a link to the letter be appended to the on-line version of this monograph that is available for public viewing through the WHO web site.

The SMI appreciates this opportunity to comment on the World Health Organization's monograph on Environmental Health Criteria of Bentonite, Kaolin and Selected Clay Minerals. Please contact me at lcoogan@navista.net if any additional information is needed in order to act upon our requests.

Sincerely,

Lee Coogan
Executive Director
The Sorptive Minerals Institute
www.sorptive.org

References

- ¹ Miles, W.J. (1994). Crystalline Silica Analysis of Wyoming Bentonite by X-ray Diffraction After Phosphoric Acid Digestion. *Anal. Chim. Acta*, 286, pp. 97-105.
- ² NIOSH Analytical Method 7500. "2/15/84", Silica, Crystalline by VIS.
- ³ NIOSH Analytical Method 7601. "8/15/94", 4th ed., Silica, Crystalline by VIS.
- ⁴ Smith, D.K. (1998). Opal, Cristobalite and Tridymite: Non-Crystallinity versus Crystallinity, Nomenclature of the Silica Minerals and Bibliography. *Powder Diffraction*, 13, pp. 1-18.

Appendix 2

SMI Production Facilities: Respirable Crystalline Silica Exposure
2009 - March 2014

SAMPLE_NO	MINE_ID	SAMPLE DATE	Flow Rate (Lpm)	START_TIME	END_TIME	Sample time (Minutes)	ug Quartz	JOB_DESCRIPTION	TWA
2009043127	3200459	4/15/2009	1.7	7:08	14:58	470	44	BAGGING/PACKAGE	55.07
2009043128	3200459	4/15/2009	1.7	7:09	15:01	472	35	BAGGING/PACKAGE	43.62
2009043129	3200459	4/15/2009	1.7	7:09	15:09	480	27	BAGGING/PACKAGE	33.09
2009043130	3200459	4/15/2009	1.7	7:11	15:06	475	52	BAGGING/PACKAGE	64.40
2010018681	3200459	2/22/2010	1.70	19:30	7:05	695	14	Front-end Loader Ope	11.85
2010018682	3200459	2/22/2010	1.70	19:32	7:07	695	6	Kiln/Dryer Operator	5.08
2010053379	3200459	5/26/2010	1.7	7:05	15:20	495	20	Bagging Operator	23.77
2010053380	3200459	5/26/2010	1.7	7:10	15:10	480	22	Bagging Operator	26.96
2010053381	3200459	5/26/2010	1.7	7:12	15:30	498	29	Bagging Operator	34.25
2010053382	3200459	5/26/2010	1.7	7:15	15:05	470	ANP	Supervisor, Co. Offi	#VALUE!
2010118415	3200459	12/15/2010	1.7	7:00	15:30	510	14	Utility Man	16.15
2010118416	3200459	12/15/2010	1.7	7:00	15:30	510	23	Bagging Operator	26.53
2010118417	3200459	12/15/2010	1.7	7:00	15:30	510	66	Bagging Operator	76.12
2011044937	3200459	5/10/2011	1.7	7:00	17:00	600	26	Bagging Operator	25.49
2011044938	3200459	5/10/2011	1.7	7:00	17:00	600	57	Bagging Operator	55.88
2011044939	3200459	5/10/2011	1.7	7:00	17:00	600	15	Bagging Operator	14.71
2011044940	3200459	5/10/2011	1.7	7:00	17:00	600	39	Bagging Operator	38.24
2012021320	3200459	2/29/2012	1.7	6:50	15:00	490	7	Bagging Operator	8.40
2012021321	3200459	2/29/2012	1.7	6:55	15:00	485	17	Bagging Operator	20.62
2012021322	3200459	2/29/2012	1.7	7:00	15:00	480	21	Bagging Operator	25.74
2013005562	3200459	1/8/2013	1.7	7:05	15:05	480	186	Kiln/Dryer Operator	227.94
2013005563	3200459	1/8/2013	1.7	7:09	13:58	409	6	Mechanic	8.63
2013027297	3200459	4/3/2013	1.7	6:54	15:00	486	13	Kiln/Dryer Operator	15.73

2009093893	3900049	9/29/2009	1.7	7:40	16:13	513	0	Kiln/Dryer Operator	0.00
2009093894	3900049	9/29/2009	1.7	7:45	14:38	413	0	Pump, Slurry Mix Ope	0.00
2010006645	3900049	1/12/2010	1.7	7:00	18:45	705	0	Pump, Slurry Mix Ope	0.00
2010006646	3900049	1/12/2010	1.7	7:00	18:45	705	0	Kiln/Dryer Operator	0.00

SMI Production Facilities: Respirable Crystalline Silica Exposure
2009 - March 2014

2010031626	4800057	3/31/2010	1.70	6:05	14:00	475	109	Bagging Operator	134.98
2010031627	4800057	3/31/2010	1.70	6:10	13:55	465	59	Bagging Operator	74.64
2010031628	4800057	3/31/2010	1.70	6:00	14:35	515	53	Bagging Operator	60.54
2010064979	4800057	7/8/2010	1.7	6:00	13:58	478	77	Bagging Operator	94.76
2012083090	4800057	9/26/2012	1.7	14:05	23:30	565	64	Bagging Operator	66.63
2012083091	4800057	9/26/2012	1.7	14:00	23:30	570	64	Bagging Operator	66.05

2009106761	4800245	11/5/2009	1.7	6:00	15:26	566	12	Bagging Operator	12.47
2009106762	4800245	11/5/2009	1.72	6:05	15:32	567	13	Bagging Operator	13.33
2009106763	4800245	11/5/2009	1.7	6:15	15:36	561	18	Bagging Operator	18.87
2013006115	4800245	1/23/2013	1.7	5:50	15:30	580	0	Bagging Operator	0.00
2013006116	4800245	1/23/2013	1.7	5:36	14:07	511	0	Mechanic	0.00
2013006117	4800245	1/23/2013	1.7	7:00	18:58	718	7	Kiln/Dryer Operator	5.73

2009106960	4800594	11/6/2009	1.7	6:20	13:52	452	35	Bagging Operator	45.55
2009106961	4800594	11/6/2009	1.72	6:12	14:58	526	55	Bagging Operator	60.79
2011013116	4800594	2/10/2011	1.70	5:12	14:50	578	13	Bagging Operator	13.23
2011013117	4800594	2/10/2011	1.70	5:25	16:55	690	ANP	Front-end Loader Ope	#VALUE!
2011013118	4800594	2/10/2011	1.70	5:45	14:55	550	ANP	Bulldozer Operator	#VALUE!
2011013119	4800594	2/10/2011	1.70	7:45	16:00	495	0	Forklift Operator	0.00
2013008732	4800594	1/30/2013	1.7	5:55	13:50	475	0	Mechanic	0.00
2013008733	4800594	1/30/2013	1.7	5:12	16:50	698	9	Kiln/Dryer Operator	7.58
2013008734	4800594	1/30/2013	1.7	6:08	16:05	597	13	Bagging Operator	12.81

2012082855	4800607	9/26/2012	1.7	6:00	16:00	600	ANP	Tractor Operator	#VALUE!
2012082856	4800607	9/26/2012	1.7	5:00	16:50	710	ANP	Bulldozer Operator	#VALUE!

SMI Production Facilities: Respirable Crystalline Silica Exposure
2009 - March 2014

2010041292	4800888	4/20/2010	1.72	5:20	15:04	584	ANP	Bulldozer Operator	#VALUE!
2010041293	4800888	4/20/2010	1.68	5:25	15:50	625	ANP	Scraper Operator	#VALUE!
2010041294	4800888	4/20/2010	1.7	5:29	15:55	626	ANP	Scraper Operator	#VALUE!
2010041295	4800888	4/20/2010	1.7	5:29	15:55	626	17	Road Grader Operator	15.97

2009106975	4800070	11/9/2009	1.7	7:42	15:45	483	24	Kiln/Dryer Operator	29.23
2009106976	4800070	11/9/2009	1.7	7:52	15:50	478	23	Bagging Operator	28.30
2013013298	4800070	2/13/2013	1.7	7:55	15:57	482	14	Bagging Operator	17.09
2013013299	4800070	2/13/2013	1.7	7:50	15:50	480	20	Kiln/Dryer Operator	24.51
2013013300	4800070	2/13/2013	1.7	7:35	15:50	495	26	Mechanic	30.90
2014017895	4800070	3/11/2014	1.7	8:00	16:00	480	39	Packaging Operator	47.79
2014017896	4800070	3/11/2014	1.7	8:00	16:00	480	0	Packaging Operator	0.00
2014017897	4800070	3/11/2014	1.7	8:00	16:00	480	8	Packaging Operator	9.80
2014017898	4800070	3/11/2014	1.7	8:00	16:00	480	11	Packaging Operator	13.48

2011061307	4801016	6/28/2011	1.70	6:37	15:34	537	ANP	Drill Helper	#VALUE!
2011061308	4801016	6/28/2011	1.71	6:25	15:38	553	ANP	Drill Helper	#VALUE!

2010009941	4801405	1/27/2010	1.7	4:45	15:00	615	16	Bagging Operator	15.30
2010009942	4801405	1/27/2010	1.7	4:50	15:00	610	11	Bagging Operator	10.61

2009015068	4002968	2/7/2009	1.7	22:05	6:00	475	9	DRYER/KILN OPER	11.15
2009015069	4002968	2/7/2009	1.7	22:06	6:25	499	21	DRYER/KILN OPER	24.76

SMI Production Facilities: Respirable Crystalline Silica Exposure
2009 - March 2014

2009068547	4002968	7/11/2009	1.7	22:00	6:12	492	21	DRYER/KILN OPER	25.11
2009068548	4002968	7/11/2009	1.7	22:03	6:14	491	0	DRYER/KILN OPER	0.00
2011079369	4002968	8/22/2011	1.7	6:00	18:07	727	12	Kiln/Dryer Operator	9.71
2011079370	4002968	8/22/2011	1.7	6:01	18:04	723	0	Utility Man	0.00
2011079371	4002968	8/22/2011	1.7	7:30	16:32	542	0	Forklift Operator	0.00
2011079372	4002968	8/22/2011	1.7	7:31	16:33	542	8	Bagging Operator	8.68
2011079379	4002968	8/22/2011	1.7	5:56	18:12	736	20	Front-end Loader Ope	15.98
2011079380	4002968	8/22/2011	1.69	6:01	18:22	741	17	Kiln/Dryer Operator	13.58
2011079381	4002968	8/22/2011	1.71	7:24	16:05	521	0	Forklift Operator	0.00
2011079382	4002968	8/22/2011	1.7	7:30	16:10	520	9	Bagging Operator	10.18
2012074108	4002968	8/26/2012	1.7	5:59	17:35	696	5	Kiln/Dryer Operator	4.23
2012074109	4002968	8/26/2012	1.7	6:02	17:25	683	0	Utility Man	0.00

2013003469	4800602	1/16/2013	1.7	3:55	16:10	735	25	Hammer Mill Operator	20.01
2013003470	4800602	1/16/2013	1.7	6:55	14:50	475	37	Bagging Operator	45.82
2014002914	4800602	1/15/2014	1.7	8:00	16:55	535	ANP	Bagging Operator	#VALUE!
2014002915	4800602	1/15/2014	1.7	7:45	16:00	495	5	Ball Mill Operator	5.94

2010067528	4103033	7/20/2010	1.7	7:45	15:50	485	13	Crusher Oper/Worker	15.77
2010067529	4103033	7/20/2010	1.7	7:50	15:55	485	9	Bagging Operator	10.92
2010067530	4103033	7/20/2010	1.7	7:55	16:00	485	ANP	Laborer, Bullgang	#VALUE!
2012044127	4103033	5/17/2012	1.7	6:56	18:41	705	54	Flotation/Concentrat	45.06
2012044128	4103033	5/17/2012	1.7	6:58	9:18	140	ANP	Hammer Mill Operator	#VALUE!
2012044129	4103033	5/17/2012	1.7	7:02	14:56	474	0	Laborer, Bullgang	0.00
2012074181	4103033	8/29/2012	1.7	6:55	15:58	543	19	Flotation/Concentrat	20.58

2010004197	4800603	1/13/2010	1.70	8:00	15:55	475	ANP	Scraper Operator	#VALUE!
2010004198	4800603	1/13/2010	1.70	7:45	16:05	500	8	Bulldozer Operator	9.41

SMI Production Facilities: Respirable Crystalline Silica Exposure
2009 - March 2014

2009016023	0402638	2/4/2009	1.71	19:00	4:50	590	7	DRY SCREENING	6.94
2009016024	0402638	2/4/2009	1.72	17:00	3:00	600	0	DRY SCREENING	0.00
2013093159	0402638	12/11/2013	1.7	7:28	14:51	443	17	Cleanup Man	22.57
2013093160	0402638	12/11/2013	1.7	5:14	14:43	569	0	Sampler, Dust	0.00
2013093161	0402638	12/11/2013	1.7	5:21	14:31	550	7	Dry Screen Plant Ope	7.49

2011103669	4406882	11/9/2011	1.7	7:00	15:00	480	0	Sampler, Dust	0.00
2011103670	4406882	11/9/2011	1.7	7:00	15:00	480	18	Kiln/Dryer Operator	22.06
2011103671	4406882	11/9/2011	1.7	7:00	15:00	480	17	Ball Mill Operator	20.83
2012092594	4406882	10/30/2012	1.7	15:00	22:58	478	0	Hammer Mill Operator	0.00
2012092595	4406882	10/30/2012	1.7	15:00	22:55	475	20	Lab Technician	24.77
2012092596	4406882	10/30/2012	1.7	15:00	23:00	480	19	Supervisor, Co. Offi	23.28
2013064533	4406882	8/18/2013	1.7	6:58	18:45	707	10	Mechanic	8.32
2013064534	4406882	8/18/2013	1.7	7:00	18:40	700	0	Mechanic	0.00
2013064535	4406882	8/18/2013	1.7	7:02	18:48	706	11	Dry Screen Plant Ope	9.17
2013064536	4406882	8/18/2013	1.7	7:04	16:45	581	0	Laborer, Bullgang	0.00
2013064537	4406882	8/18/2013	1.7	7:06	18:48	702	0	Dry Screen Plant Ope	0.00

2010115890	2200035	12/8/2010	1.7	7:26	16:20	534	ANP	Truck Driver	#VALUE!
2010115891	2200035	12/8/2010	1.7	7:05	15:05	480	ANP	Truck Driver	#VALUE!
2010115892	2200035	12/8/2010	1.7	7:10	15:10	480	0	Truck Driver	0.00

2009069335	0900114	7/15/2009	1.7	7:15	15:30	495	42	BAGGING/PACKAGING	49.91
2009069336	0900114	7/15/2009	1.7	7:05	15:15	490	5	BAGGING/PACKAGE	6.00
2009069337	0900114	7/15/2009	1.7	7:50	15:50	480	ANP	TRUCK DRIVER	#VALUE!

SMI Production Facilities: Respirable Crystalline Silica Exposure
2009 - March 2014

2009069338	0900114	7/15/2009	1.7	7:45	15:50	485	7	DRYER/KILN OPER	8.49
2011081207	0900114	8/30/2011	1.7	7:53	15:03	430	18	Hammer Mill Operator	24.62
2011081208	0900114	8/30/2011	1.68	7:35	14:58	443	16	Utility Man	21.50
2011081209	0900114	8/30/2011	1.69	7:49	15:05	436	ANP	Kiln/Dryer Operator	#VALUE!
2011114953	0900114	12/14/2011	1.7	6:59	15:00	481	33	Kiln/Dryer Operator	40.36
2011114954	0900114	12/14/2011	1.7	6:50	15:30	520	50	Lab Technician	56.56
2011114955	0900114	12/14/2011	1.7	6:50	15:24	514	9	Laborer, Bullgang	10.30
2012035865	0900114	4/18/2012	1.7	7:00	15:00	480	28	Bagging Operator	34.31
2012035866	0900114	4/18/2012	1.7	7:07	18:00	653	10	Lab Technician	9.01
2012035867	0900114	4/18/2012	1.7	7:19	17:00	581	0	Truck Driver	0.00
2013080767	0900114	10/30/2013	1.7	6:26	14:57	511	27	Utility Man	31.08
2013080768	0900114	10/30/2013	1.7	6:50	15:03	493	36	Bagging Operator	42.95
2013080769	0900114	10/30/2013	1.69	6:44	15:01	497	48	Bagging Operator	57.15
2013080770	0900114	10/30/2013	1.7	6:48	15:04	496	16	Mechanic	18.98

2009029535	0402964	3/18/2009	1.71	6:10	14:04	474	0	TRUCK DRIVER	0.00
2009029536	0402964	3/18/2009	1.72	6:05	14:09	484	10	TRUCK DRIVER	12.01
2009029537	0402964	3/18/2009	1.7	6:15	16:06	591	8	BACKHOE OPER	7.96
2009029538	0402964	3/18/2009	1.71	6:20	13:10	410	8	DRY SCREENING	11.41

2013001559	2200044	1/9/2013	1.7	7:01	15:16	495	21	Bagging Operator	24.96
2013001560	2200044	1/9/2013	1.69	7:15	15:15	480	0	Bagging Operator	0.00

2010003623	4800612	1/13/2010	1.70	7:00	15:09	489	37	Bagging Operator	44.51
2010003624	4800612	1/13/2010	1.70	6:50	15:06	496	34	Kiln/Dryer Operator	40.32
2010003625	4800612	1/13/2010	1.70	7:04	15:11	487	77	Bagging Operator	93.01

SMI Production Facilities: Respirable Crystalline Silica Exposure
2009 - March 2014

2010008922	4800611	1/26/2010	1.71	6:53	14:54	481	34	Kiln/Dryer Operator	41.34
2010008923	4800611	1/26/2010	1.70	6:59	14:58	479	30	Bagging Operator	36.84
2010008924	4800611	1/26/2010	1.71	7:05	15:00	475	18	Bagging Operator	22.16
2013025672	4800974	3/19/2013	1.7	6:50	16:30	580	ANP	Bulldozer Operator	#VALUE!

Description	No. of Samples	avg µg/m ³
Kiln/Dryer/Mill/Screen Operator	32 Samples	20.38
Bagging / Packaging	54 Samples	34.35
Equipment Operator	24 Samples	6.41
Mechanic / Utility Worker	21 Samples	8.02
Lab Technician	3 Samples	30.11
Truck Driver	7 Samples	1.72
Supervisor / Office	2 Samples	11.64

Appendix 3

Office of Environmental Health Hazard Assessment



Winston H. Hickox
Secretary for
Environmental
Protection

Joan E. Denton, Ph.D., Director
Headquarters • 301 Capitol Mall, Rm. 205 • Sacramento, California 95814-4308
Oakland Office • Mailing Address: 1515 Clay Street, 16th Floor • Oakland, California 94612



Gray Davis
Governor

May 20, 1999

Mr. S. Lee Coogan
Executive Director
Sorpitive Minerals Institute
818 Connecticut Avenue, NW
Washington, District of Columbia 20006

Dear Mr. Coogan:

On behalf of the Office of Environmental Health Hazard Assessment, I am pleased to inform you of our decision to grant the Sorptive Minerals Institute a safe use determination for crystalline silica in packaged sorptive mineral-based pet litter, pursuant to our authority under Section 12104 of Title 22 of the California Code of Regulations.

Please find enclosed a copy of the notice, as it will appear in the *California Regulatory Notice Registry*, on June 4, 1999. If you would like to discuss any issue concerning the safe use determination further, please call me at (916) 324-7572

Sincerely,

George V. Alexeeff, Ph.D., D.A.B.T.
Deputy Director for Scientific Affairs

Enclosure

cc: Laurie E. Nelson
Randlett/Nelson Associates
1110 Second Street
Sacramento, California 95814

GVA:ke

California Environmental Protection Agency



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**CALIFORNIA ENVIRONMENTAL PROTECTION AGENCY
OFFICE OF ENVIRONMENTAL HEALTH HAZARD ASSESSMENT**

**SAFE DRINKING WATER AND TOXIC ENFORCEMENT ACT OF 1986
(PROPOSITION 65)**

**NOTICE TO INTERESTED PARTIES
JUNE 4, 1999**

**ISSUANCE OF A SAFE USE DETERMINATION FOR CRYSTALLINE SILICA
IN SORPTIVE MINERAL-BASED PET LITTER**

The California Environmental Protection Agency's Office of Environmental Health Hazard Assessment (OEHHA), as the lead agency for the implementation of the Safe Drinking Water and Toxic Enforcement Act of 1986 (Proposition 65), has received a request from the Sorptive Minerals Institute (SMI, Washington, DC) that OEHHA grant a safe use determination for packaged sorptive mineral-based pet litter containing crystalline silica, pursuant to its authority under Section 12104 of Title 22 of the California Code of Regulations (22CCR). The products that are the subject of the request are clay-based pet litters that are purchased and poured by the consumer, used by the pet, disposed of by the consumer, and replenished by the consumer. Crystalline silica (airborne particles of respirable size) has been listed under Proposition 65 as a chemical known to the State to cause cancer since October 1, 1988.

In accordance with the process set forth in Section 12104(f) of 22CCR, public comment on this request was solicited on November 27, 1998, particularly with respect to the potential for exposure to airborne particles of crystalline silica of respirable size that may result from the use of pet litter commercially produced from clay-containing mineral deposits as in packaged sorptive mineral-based pet litter. A public hearing was convened on the last day of the 30-day comment period, January 6, 1999, in Sacramento, California.

Based on the results of a screening level assessment using testing data submitted by SMI on the amounts of respirable crystalline silica produced from the normal use of both conventional and scoopable pet litter products, and a review of the comments submitted by the public, OEHHA has decided to issue a safe use determination to SMI for crystalline silica in mineral-based pet litter. The essential elements and results of this screening level assessment are described below.

SMI provided testing data on 12 conventional pet litters and nine scoopable pet litters originating from clay deposits from different parts of the country (termed East and West Coast litters by SMI). Briefly, the key data consisted of laboratory measurements over time of respirable dust ($<10\ \mu\text{m}$) generated from activities related to the use of pet litter including initial pouring, clump removal (in the case of scoopable pet litter) and replenishment. Respirable dust collected from the different activities was further evaluated by SMI for quartz content. Quartz was assumed to be the only form of crystalline silica in pet litter. Data provided by the requester also included

parameters on normal use in terms of time and amount used by the average consumer. In accordance with the process set forth in Section 12104(f) of 22CCR, OEHHA has previously deemed the submitted data adequate and appropriate for performing this screening level assessment.

The primary sources of exposure to dust containing crystalline silica from the use of conventional pet litter are from the filling of the litter pan and subsequent disposal following use. Using the data generated during 10 pound pour tests measuring the respirable dust generated over time (up to three minutes), the quartz content of the respirable dust, and the annual use and frequency of activities related to the use of conventional pet litters, average yearly exposure levels were estimated for each of the 12 products for which testing data were submitted. The testing data indicate that the average concentration of exposure to respirable crystalline silica from the use of conventional litter ranges from 0.0007 to 0.01 $\mu\text{g}/\text{m}^3$.

The primary sources of exposure to dust containing crystalline silica from the use of scoopable pet litter are from the filling of the litter pan, clump removal, replenishment of removed litter, and subsequent disposal following use. Data provided by SMI for scoopable pet litters included measurements over time (up to $\sim 2\frac{1}{2}$ minutes) of respirable dust generated from 10 pound pour tests; clump removal tests and tests of clump removal with replenishment of litter. Parameters on the annual use and frequency of activities related to the use of scoopable pet litters were also provided. Using this information, average yearly exposure levels were estimated for each of the nine products for which testing data were submitted. The testing data indicate that the average concentration of exposure to respirable crystalline silica from the use of scoopable litter ranges from 0.002 to 0.06 $\mu\text{g}/\text{m}^3$.

A number of factors may tend to increase or decrease estimates of exposure relative to the approach used to develop the exposure levels described above. We believe, on the whole, that the assumptions made are likely to have resulted in overestimates of exposure levels for the average user of pet litter.

For the estimation of risk of cancer from the exposure to crystalline silica, readily available potency estimates in the scientific literature were used. Estimates derived from epidemiological studies were considered to be most appropriate for this screening evaluation of carcinogenic risk. Goldsmith *et al.* (1995; Scand J Work Environ Health 21(Suppl 2):104-7) has developed estimates of risk based upon the increase in lung cancers observed in epidemiological studies of gold miners in South Africa and diatomaceous earth workers in California. Cancer slope factors derived from these studies ranged from 6.8×10^{-7} to 1.85×10^{-5} for exposure to 1 $\mu\text{g}/\text{m}^3$ silica dust and were derived using occupational exposure assumptions of 40 years of employment, eight hour workshifts, 50 hour workweeks, and 50 workweeks per year. Based on these estimates, concentrations associated with excess cancer risk of one in 100,000 would range from 0.54 to 15 $\mu\text{g}/\text{m}^3$ silica dust. Active research is being conducted with respect to the relationship between silicosis and lung cancer in humans, the contribution of reactive oxygen species to the development of malignancy, and the "biological activity" of crystalline silica. Additional data and increased confidence in information regarding crystalline silica's mode of action in the

induction of human malignancy will likely lead to reductions in cancer potency estimates. Thus we expect that the upper end of the screening cancer slope factors presented above represent a "worst case" estimates of the true low dose cancer slope factor for crystalline silica present in clay-based pet litter.

With the assumption that a consumer uses a single product throughout his/her lifetime, the highest estimate produced is an annual time weighted average exposure of $0.06 \mu\text{g}/\text{m}^3$ quartz dust for a scoopable litter product. This exposure level is approximately nine-fold lower than the lowest concentration ($0.54 \mu\text{g}/\text{m}^3$) associated with one in 100,000 persons exposed derived from occupational epidemiological studies. Thus, the estimated exposure of the average user of either conventional or scoopable pet litter to crystalline silica under the conditions used to produce this assessment corresponds to an excess cancer risk of less than one in 100,000, and would not trigger the Proposition 65 warning requirement.

As stated in 22CCR, Section 12104 (a) and (k), this safe use determination is specific to the requester, SMI, and for crystalline silica in packaged sorptive mineral-based pet litter as used in accordance with the facts presented in the request, and is advisory only. Moreover, the issuance of a safe use determination does not affect the authority of the Attorney General, district attorneys, certain city attorneys and any other person in the public interest to prosecute violations of the Act pursuant to Section 25249.7 nor does it affect the responsibility of courts to interpret the Act and apply the provisions of the Act to particular facts.

Questions regarding this notice should be directed to:

Ms. Cynthia Oshita
Office of Environmental Health Hazard Assessment
301 Capitol Mall, 2nd Floor, Room 205
Sacramento, California 95814
Fax No.: (916) 327-1097

Telephone: (916) 445-6900

Appendix 4

The Nature of Naturally Occurring Quartz Surfaces as Observed by High Resolution Transmission Electron Microscopy

A Report to the Sorptive Minerals Institute (SMI), 1155 Fifteenth St., NW, Suite
500, Washington, DC 20005

Principal Investigators:

Prof. Michael F. Hochella, Jr.
Department of Geosciences
4044 Derring Hall, Virginia Tech
Blacksburg, VA 24061-0420

and

Prof. Mitsuhiro Murayama
Institute for Critical Technology and Applied
Science
Virginia Tech
Blacksburg, VA 24061

July 20, 2010

Introduction

This report contains the results of research conducted by Michael Hochella and Mitsu Murayama, carried out exactly as described in the proposal submitted by the same two scientists to SMI on May 20, 2010. In this work, we have used high resolution transmission electron microscopy (HRTEM), selected area electron diffraction (SAED), and energy dispersive spectroscopy (EDS), all performed using a FEI Titan 300 TEM operated at 200 kV. The sample studied was SMI-supplied occluded quartz (sample R-01-353B2). This is exactly the same sample referred to as Quartz Isolate 2001 which was the basis of the intratracheal instillation toxicology studies in rats, published as Miles et al. in the journal *Inhalation Toxicology* (v. 20, pp. 981-993, 2009). This sample has remained in slurry form since it was extracted by centrifugal methods from natural-moisture Wyoming Newcastle bentonite for the Miles et al. (2009) study. For the purposes of this present TEM study, the slurry, as received from SMI, was diluted with distilled water, and a drop of the suspension was placed on a Cu TEM lacey carbon grid. Excess suspension was wicked away with an absorbent tissue, and the water film remaining was allowed to air dry in a dust free environment before insertion into the TEM.

Results and Discussion

Figure 1 shows two types of TEM images at lower magnification of a representative field of particles. The image on the left is a TEM-bright field (TEM-BF) image, formed from the primary beam. The centers of the particles are dark because they are too thick for the electron beam, even at 200 keV, to penetrate. The light gray background with white holes is a part of the carbon film support upon which the particles are resting. The particle this is circled, with a clearly defined border, contains Si and O, with lesser amounts of Al, Mg, Fe, and K, as shown by the energy dispersive X-ray spectroscopy (EDS) spectra just below the image (the Cu is from x-rays scattering from the Cu TEM grid, and is not part of the sample). The overall nature of this bentonite sample, it is likely that this is a clay grain, likely illite considering the observed chemistry. The image to the right is a scanning TEM-high angle annular dark field (STEM-HAADF) image which is good for precise (high spatial resolution) EDS analysis, with the particle brightness dependant on the average atomic

number of the particle. The area circled in this image consists of primarily Si and O, with much less Al, and minor amounts of K and Fe. This core of this grain is mostly likely quartz, with Al, K, and Fe in the surrounding film (see Figures 2 and 3).

Figure 2 shows the bright field (image formed by the primary beam) and dark field (image formed by the (100) diffracted beam) of a typical particle in this sample. The core of the particle is α -quartz as positively identified by the ring pattern of four quartz diffraction lines. The diffuse, partly spotted rings suggests that the quartz crystallites are very small (nanometer scale) in a somewhat preferred orientation, and otherwise randomly oriented. The EDS spectrum shows that in the core, the major elements are only Si and O, as expected. Acicular quartz crystalites appear to protrude into the irregular, lighter contrast coating which is an amorphous alumino-silicate with lesser amounts of Fe, Mg, and Ca. Notes that the "swiss-cheese" background in both images is the amorphous carbon film supporting the sample.

Figure 3 shows a high resolution image of the interface between a crystalline portion of a particle, with (100) lattice spacings visible, and the amorphous film on the surface (below the white dashed line; no fringing). These samples are susceptible to electron beam damage (not unusual for quartz), so that only a portion of the (100) lattice fringing remains visible after image collection. Although not visible in these images, the interface between amorphous and crystalline areas seems to be atomically sharp. This was apparent before the particle was affected by electron beam damage.

Conclusions

A detailed, state-of-the-art TEM study of SMI-supplied occluded quartz (sample R-01-353B2) showed that the sample contains clays and quartz crystals. The quartz particles are made up of multiple and minute crystals which are, as a group, coated by an amorphous alumino-silicate which also contains minor amounts of alkaline and alkaline earth elements, as well as iron.

Figure 1. The majority of the particles were covered by a hazy film best seen in the STEM-HAADF image to the right, but also apparent in the TEM-BF image to the left. The core parts of these particles showed evidence of crystallinity (Kikuchi patterns were visible); the surface films did not. EDS analysis indicated the dominance of Si and O in all particles, with other elements present as explained in the text.

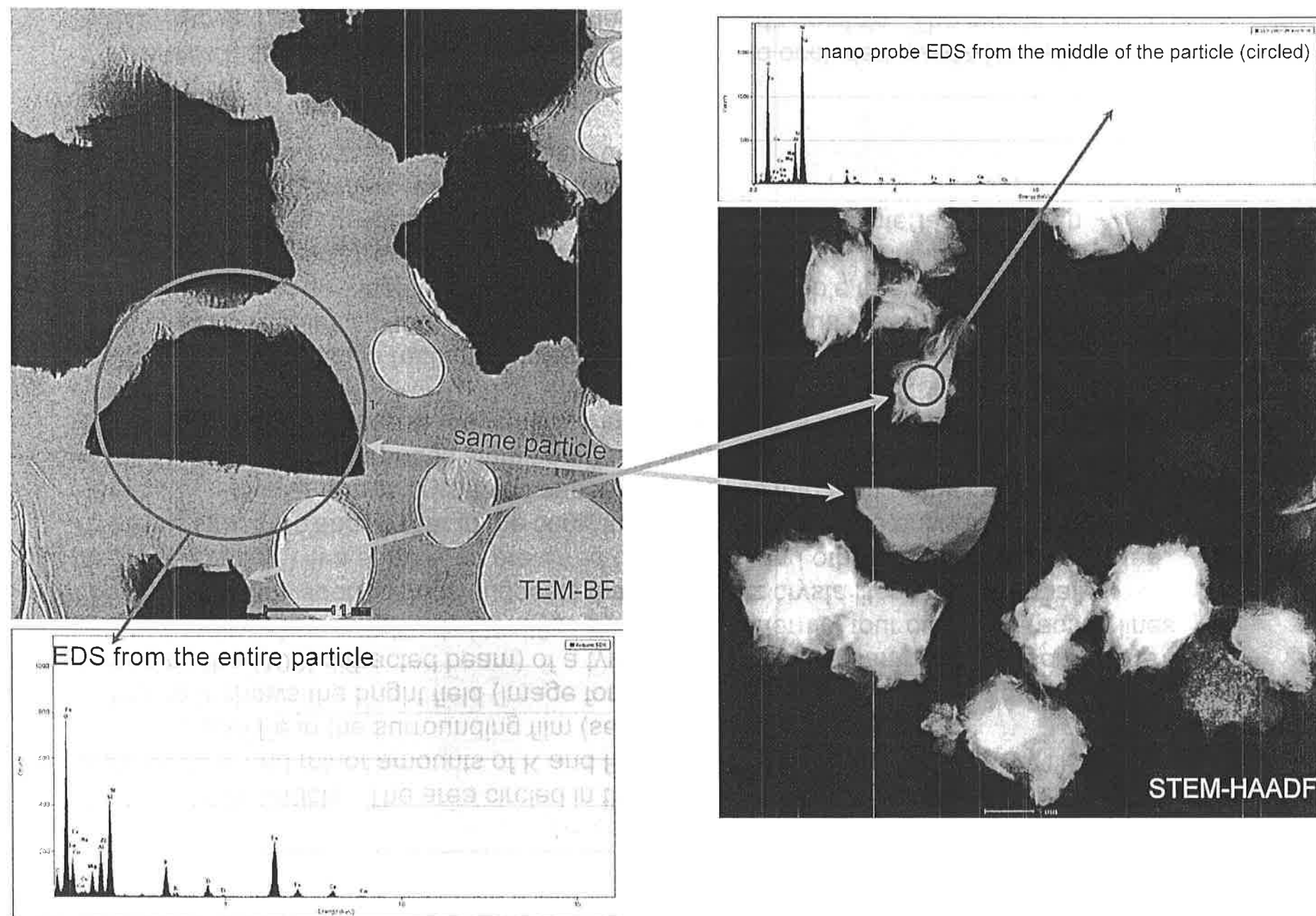


Figure 2. The typical morphology of particles in the sample. The diffraction pattern in the upper left shows that the dark particle core is made up of multiple, very small crystals of α -quartz. The surrounding film appears to be amorphous aluminosilicate with other elements.

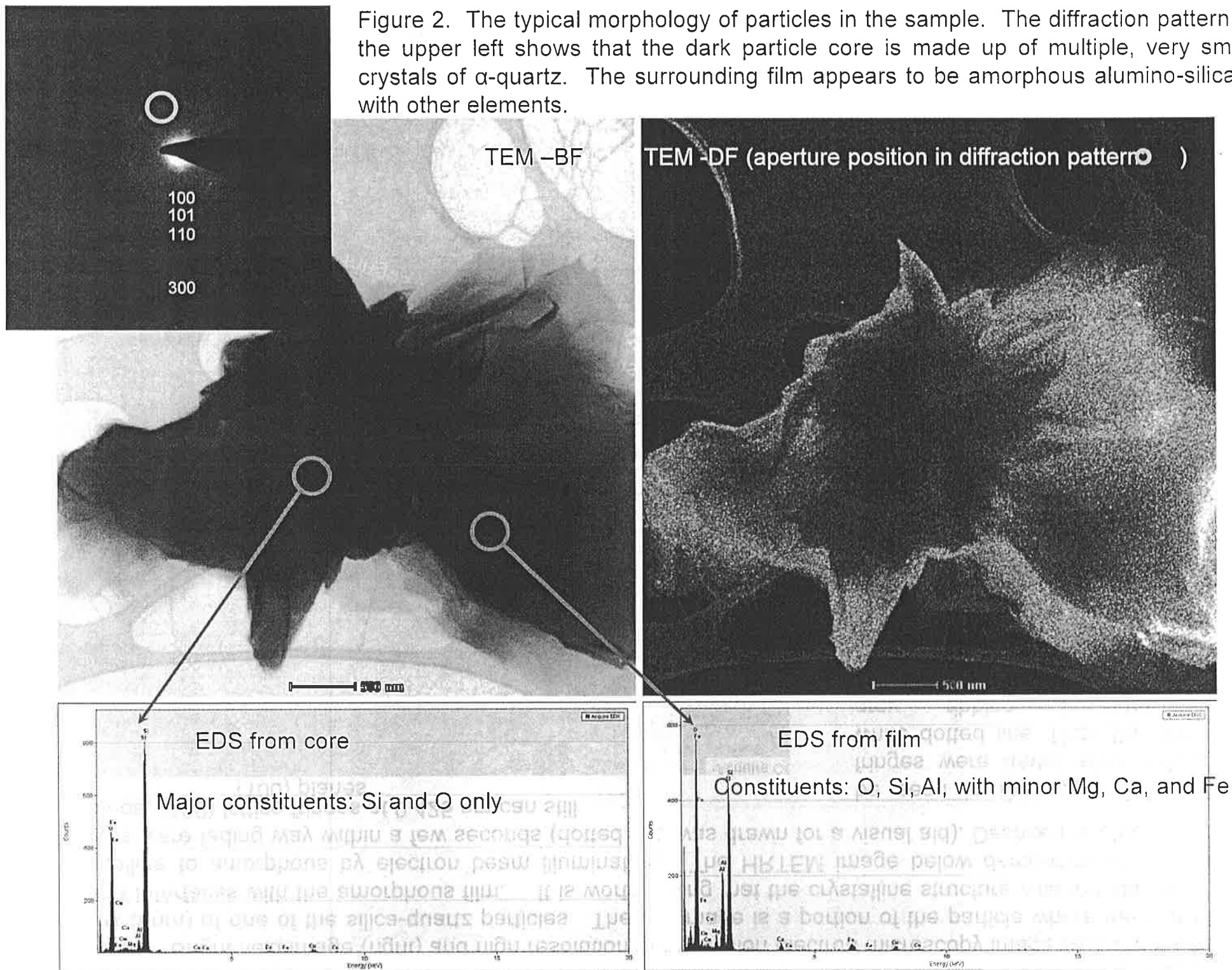
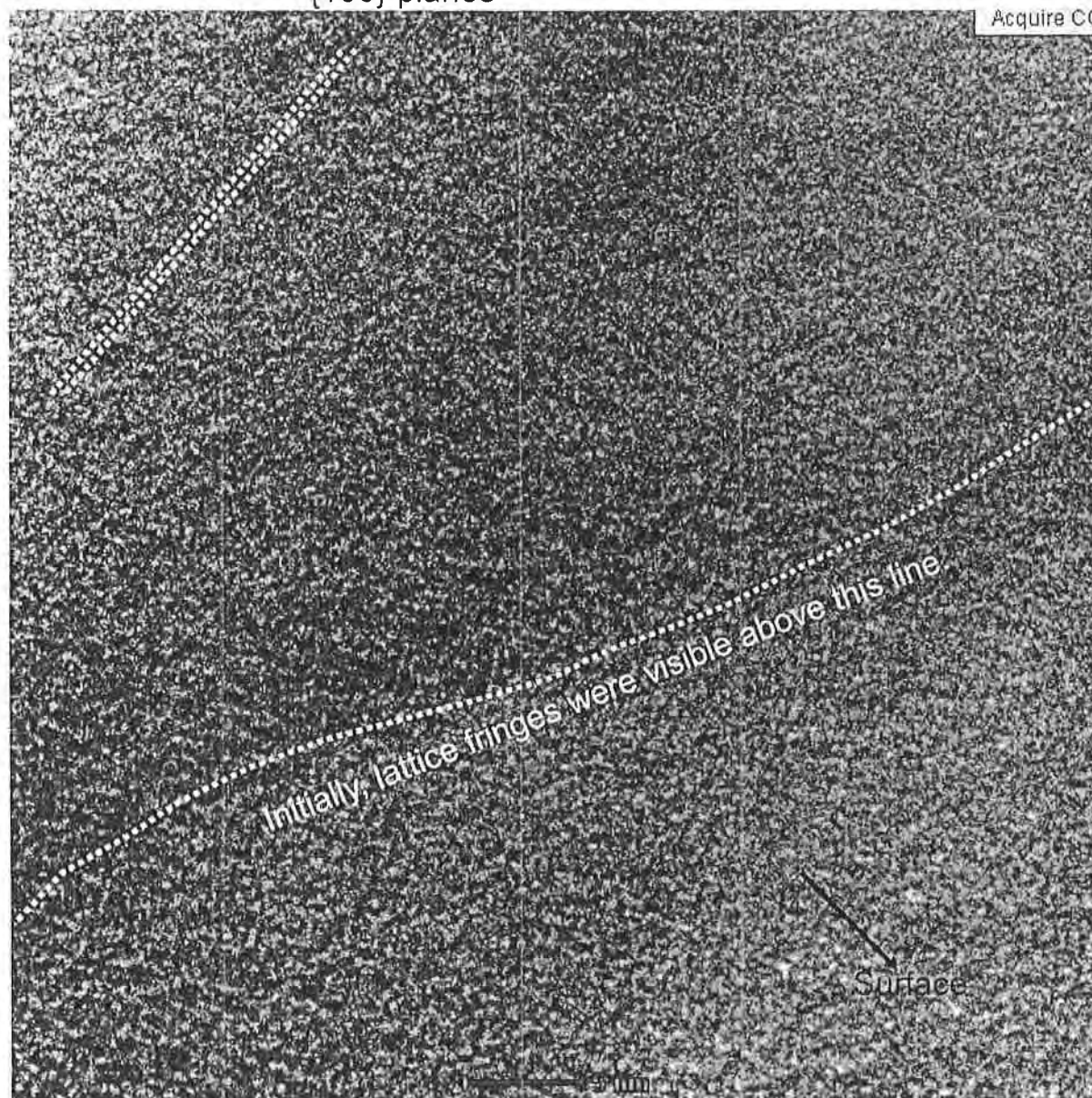


Figure 3. Bright field image (right) and high resolution transmission electron microscopy image (left; the red scale bar is 5 nm) of one of the silica-quartz particles. The left image is a portion of the particle where the crystalline quartz interfaces with the amorphous film. It is worth noting that the crystalline structure was transformed from crystalline to amorphous by electron beam illumination. The HRTEM image below demonstrates that lattice fringes were fading away within a few seconds (dotted line was drawn for a visual aid). Despite the electron beam damage, (100) lattice fringes at 0.425 nm can still
{100} planes



be seen in the upper left. Lattice fringes were never seen below the white dotted line. Note that the light gray ribbing in the right photomicrograph is the lacey carbon support structure on the Cu grid.



**BEFORE THE UNITED STATES DEPARTMENT OF LABOR
OCCUPATIONAL SAFETY AND HEALTH ADMINISTRATION**

**OCCUPATIONAL EXPOSURE
TO CRYSTALLINE SILICA;
PROPOSED RULE
78 Federal Register 56274-504**

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**POST-HEARING BRIEF SUBMITTED ON BEHALF OF
SORPTIVE MINERALS INSTITUTE**

August 18, 2014

Submitted by counsel:

Kathryn M. McMahon
Epstein Becker & Green, P.C.
1227 25th Street, N.W.
Washington, DC 20007
202.861.5331

I. STATEMENT OF SMI POSITION

Enhanced regulation of exposures to crystalline silica in the sorptive minerals industry is neither warranted nor legally permissible under sections 3(8) and 6(b) of the Occupational Safety and Health Act (OSH Act), 29 U.S.C. §§ 652(8); 655(b). The risk assessment conducted by OSHA, and the studies underpinning it, while correctly identifying exposure to crystalline silica from industrial sources as posing a significant risk of material impairment to health, do not purport to analyze exposures experienced in the sorptive minerals industry. That assessment, therefore, is not applicable to silica existing in sorptive minerals, and certainly does not represent the best available evidence of the potential risk associated with exposure to silica in sorptive clays.

Silica exists in sorptive clays in *only* one of two forms -- either as amorphous silica (Opal-CT) or in the form of geologically ancient, occluded quartz -- *neither of which pose the health risk identified and studied in OSHA's risk assessment*. OSHA's risk assessment does not assess the risk of ancient quartz found in sorptive clays. To the extent OSHA studied "aged" silica at all, it recognized the significantly lower toxicity potential of this material from the type of freshly fractured quartz that is the focus of the agency's risk assessment. Critically, however, the "aged" silica studied -- aged for 60 days or less -- does not accurately reflect the risk of *geologically ancient*, (occluded) silica formed millions of years ago found in sorptive clays.

The body of work presented by SMI to OSHA in this rulemaking accurately assesses the consequences of exposure to silica existing in sorptive clays, and simply does not support application of OSHA's proposed standard to this industry. This evidence, which is the best available evidence of the risk likely to be associated with exposures to the type of silica existing in the sorptive minerals industry, does not provide a basis to support a finding of "significant risk of material impairment of health" from sorptive minerals industry exposures. In fact, it shows that it is not "reasonably necessary or appropriate" to regulate this form of silica. Accordingly, OSHA has not met its burden under sections 3(8) and 6(b) of the OSH Act.

Moreover, OSHA has made no attempt to conduct an assessment of the feasibility of compliance with the silica rule by the sorptive minerals industry. Neither the Preliminary Initial Regulatory Feasibility Analysis (PIRFA) presented during the Small Business Regulatory Enforcement and Fairness Act (SBREFA) process, nor the updated Final Economic and Regulatory Feasibility Analysis, identify the sorptive minerals industry as an industry affected by this rulemaking. Long-standing federal court precedent prohibits OSHA from applying a regulation to an industry without first assessing the economic and technological feasibility of compliance with the rule by that industry. Accordingly, application of a final silica rule to the sorptive minerals industry is impermissible.

For these reasons, OSHA should include an explicit exception or “carve-out” for the sorptive minerals industry in the scope of the final rule.¹

II. FACTUAL BACKGROUND

A. The Nature of Sorptive Clays

Sorptive clays mined and processed by SMI members are a specific and discreet subset of all clays, formed between 5.2 million and 110 million years ago. As explained in Comments submitted by SMI on February 11, 2014 to OSHA’s Proposed Occupational Health Standard on Silica (hereinafter “SMI Comments”) and supportive materials submitted to the docket, these clays exist in only a few geologic deposits in the country, including, specifically:

- o the Monterey formation of California (5.2 - 9.2 Ma)
- o the Porters Creek formation of the Mississippi valley (61 – 66 Ma)
- o the Twiggs fullers earth of the southeastern US (13.8 – 38 Ma)
- o the Meigs fullers earth of the southeastern US (11.6 – 13.8 Ma)
- o the Wyoming or Western-type sodium bentonite deposits (90 – 110 Ma)
- o the calcium bentonite deposits of north-central Florida (11.6 – 13.8 Ma) and
- o the fullers earth deposits of eastern Virginia. (11.6 – 20.4 Ma)

A comprehensive description of the geology and mineralogy of sorptive clays has been submitted to the docket. See SMI Comments; April 2, 2014 Testimony of Richard Brown,

¹ Note that silica in sorptive clays is already regulated under the existing silica standard and, effectively, under the nuisance dust standard as well. SMI is not arguing that the existing regulatory scheme should be altered, but simply that the industry should not be subject to the enhanced regulation OSHA is proposing for occupational exposures to crystalline silica.

Chairman of SMI Technical Committee at OSHA Hearing on Proposed Silica Rule (hereinafter “SMI Hearing Testimony”); and June 3, 2014 SMI Post-Hearing Information Submission (hereinafter “SMI Post-Hearing Submission”).

The unique character of sorptive clays, primarily their absorbent and adsorbent properties, renders them extremely useful in a wide range of consumer, commercial and industrial applications. While these clays may be most well-known for their use in pet litter, they also are used in cosmetics, pharmaceuticals and animal feeds as well as many other products and applications.

B. Silica in Sorptive Clays

Silica exists in one of two forms in sorptive clays, either as Opal-CT, an amorphous form of silica, or as quartz, a crystalline form of silica. While both forms consist of silicon dioxide, Opal-CT lacks the long range periodicity and patterned crystallinity of crystalline silica. Opal-CT is sometimes referred to as paracrystalline because of its limited, short-range ordering of six-member Si-O rings in a disordered, amorphous matrix.² The chemistry of Opal-CT, a hydrated, amorphous form of silica, is also distinguishable from that of anhydrous crystalline silica. It is estimated that approximately 5 to 25 percent of the silica found in sorptive clays mined and processed by the sorptive minerals industry is in the Opal-CT or amorphous form.³ OSHA correctly recognizes that the amorphous form of silica does not present the risk posed by the crystalline form, and, therefore, does not propose to regulate this form of silica.

In addition to Opal-CT, sorptive clays commonly contain crystalline silica. *Id.* This crystalline silica exists solely in the form of occluded quartz. It is noteworthy that of the three common polymorphs of crystalline silica, quartz, cristobalite, and tridymite, only quartz can form or stably exist at the low temperatures (< about 150°C) at which sorptive clays can form or

² Smith, D.K., Opal, cristobalite, and tridymite: Noncrystallinity versus crystallinity, nomenclature of the silica minerals and bibliography, *Powder Diff.* 13:1, 2-19.

³ Elzea, J.M., I.E. Odom, and W.J. Miles Distinguishing well-ordered opal-CT and opal-C from high temperature cristobalite by X-ray diffraction, *Analytica Chimica Acta*, 286, 107-116 (1994).
Miles, W.J., and R.D. Hamilton Chemical Methods of Analysis for Crystalline Silica, A Critical Review, *Analytica Chimica Acta*, 286, 3-7 (1994).
Miles, W. J. Crystalline silica analysis of Wyoming bentonite by X-ray diffraction after phosphoric acid digestion, *Analytica Chimica Acta*, 286, pp. 97-105 (1994).
Industry sources

exist. Quartz, therefore, is the only crystalline form of silica that is known to naturally exist in sorptive clays.

C. The Nature and Surface of Geologically Ancient Quartz in Sorptive Clays.

Quartz occurs in sorptive clays as a result of several natural processes. (See SMI Comments for a description of these processes.) Regardless of the method of occurrence, however, all quartz in sorptive clay deposits is demonstrably different from the forms of crystalline silica which are the subject of OSHA's risk assessment supporting the proposed rule. Unique to the quartz in sorptive clays is an amorphous aluminosilicate rind bound at an atomic level to a crystalline silicon dioxide core.

Quartz in sorptive minerals has aged over millions to many tens of millions of years in a clay matrix and has had, literally, eons to interact and become equilibrated with the ions, chemicals and other materials in its environment. Critically, as detailed in evidence submitted to the record by SMI in this rulemaking,⁴ the result of this equilibration is the development of particles with geologically ancient, occluded surfaces – notably amorphous in nature – that shroud the crystalline quartz. While surface occlusions of quartz from other sources may consist of adventitious metal and non-metal ions, such as aluminum, magnesium, sodium, iron, carbon compounds and other materials, the occluded surface of quartz from sorptive clays notably consists of amorphous aluminosilicate. The work submitted by SMI shows that the crystalline core of the geologically ancient, occluded quartz found in sorptive clays is not exposed, but completely shrouded by an amorphous aluminosilicate rind which, importantly, is integrally connected to the quartz core at an atomic level.⁵ That is, the amorphous rind is an intrinsic part of the quartz particle itself rather than simply a distinct coating.

The work cited and included in the record by SMI shows the significant difference in particle surface between the geologically ancient, occluded quartz found in sorptive clays and the non-occluded crystalline silicon dioxide materials, such as freshly fractured quartz and other polymorphs that are the focus of OSHA's rule making. These materials occur as single crystal

⁴ See Wendlandt R.F., (2007) *et al.* Surface coatings on quartz grains in bentonites and their relevance to human health, *Appl Geochem* 22:2290-2306; Hearing Testimony of Richard Brown, SMI Technical Committee Chairman, April 2, 2014 describing 2010 unpublished report by Hochella and Murayama (The Nature of Naturally Occurring Quartz Surfaces as Observed by High Resolution Transmission Electron Microscopy); and the study by Miles, *et al.* (2008) (Physicochemical and Mineralogical Characterization of Test Materials used in 28-Day and 90-Day Intratracheal Instillation Toxicology Studies in Rats, *Inhalation Toxicology* 20:981-983 (See SMI February 11, 2014 Comments; Exhibit 2).

⁵ *Id.* Hochella and Murayama (2010)

particles with highly crystalline and highly chemically reactive pure silicon dioxide surfaces. They have surfaces that are sharply angular in nature compared to the highly irregular amorphous aluminosilicate-covered surfaces of the geologically ancient occluded quartz found in sorptive clays. The fundamental surface characteristics of the geologically ancient quartz particles contained in sorptive clays directly and significantly impact the toxicity potential of these particles. In sum, the occluded surface consists of an amorphous aluminosilicate rind that mediates the ability of quartz particles found in sorptive clays to interact with their environment and, therefore, their bio-availability and toxicity potential.

D. The Sorptive Minerals Industry: Mining, Processing and Packaging Sorptive Clays

1. The Nature of the Occluded Quartz is Not Altered by Industry Operations.

The membership of the Sorptive Minerals Institute consists of eleven companies that mine, process, and package sorptive clays. Industry operations can be broadly broken into three segments: (1) mining operations during which clay deposits are unearthed and removed; (2) processing operations where the mined clays undergo a two-step sequential process, including drying to remove excess water, and sizing to separate and organize the clay material by size; and (3) packaging the resulting clay product for end consumer, commercial, or industrial uses and applications.

Sorptive clays are typically found in shallow deposits and are mined using surface rather than underground mining methods. Here, surface mining consists of using scrapers and bulldozers to remove the geologic material (overburden) on top of the clay to expose the clay deposit. The crude clay deposit is then removed and stockpiled at the mine site, using scrapers, prior to being transported to production facilities for processing. Trucks haul the wet (25 – 30 percent moisture), crude clay to processing plants where the large chunks are mechanically reduced to smaller chunks, usually less than one inch in size. The clay then goes into dryers where it is mildly heated for a short time to less than 250°F (less than 121°C) to drive off water. The clay is then milled or screened to create appropriate particles sizes, from powder to gravel-size for the end use. The finished products are then packaged in paper or plastic bags, jugs, cartons or bulk sacks for shipping. The packaged products are placed on pallets and shipped via truck or railcar. Finished clays also may be shipped in bulk rail cars or truck trailers without additional packaging. Published, peer reviewed, scientific studies have proven that the type of

processing used to create sorptive mineral products does not remove or adversely affect the occlusion layer that has formed over millennia on the surface of geologically ancient occluded quartz particles from sorptive clays.⁶ Accordingly, the occluded quartz in sorptive clays remains in the same geologically ancient form, with the same occluded surfaces that it has had for eons, throughout the entire production process.

2. Employee Exposures Occurring in the Industry Do Not Warrant Regulation.

SMI members collectively employ approximately 3,500 people. Employee job titles/job descriptions associated with operations where direct or indirect exposure to raw or processed sorptive clay may occur include:

(1) Mining:

- o Front end loader operator
- o Mechanic
- o Pump/slurry mix operator
- o Bulldozer operator
- o Tractor operator
- o Scraper operator
- o Road grader operator
- o Drill helper
- o Laborer

(2) Processing

- o Kiln/dryer operator
- o Utility man
- o Hammer mill operator
- o Ball mill operator
- o Crusher operator/worker
- o Flotation/concentration operator
- o Dry screening
- o Cleanup man
- o Sampler/dust
- o Dry screen plant operator

⁶ *Id.* Wendlandt (2007).

- o Truck driver
- o Mechanic

(3) Packaging

- o Bagging/packaging
- o Bagging operator
- o Lab technician
- o Forklift operator

Exposure data collected by the MSHA from 36 SMI mining and processing facilities show the following average exposures for select jobs within the three general types of operations conducted by the industry, for the period 2009 through 2013:

o Equipment operator	6.41 ug/m ³
o Kiln/dryer operator	20.38 ug/m ³
o Bagging and packaging	34.35 ug/m ³
o Mechanics/utility workers	8.02 ug/m ³
o Truck driver	1.72 ug/m ³
o Lab technician	30.11 ug/m ³
o Supervisor	11.64 ug/m ³

While the average exposures are well below the existing PEL, the raw data associated with these exposures, which was included as part of SMI's Post-hearing Submission, show many instances of exposures above the proposed PEL. OSHA has not conducted any analysis to determine how and whether these exposures could be reduced to levels below the proposed PEL, or, if it is feasible to further reduce exposures in the industry, what the cost would be of the controls that would be necessary to achieve such reductions.

III OSHA Has Not Met Its Legal Burden Under the Occupational Safety and Health Act to Promulgate a Health Standard for Silica Applicable to the Sorptive Minerals Industry.

It is beyond dispute that OSHA has correctly determined that industrial exposure to certain types of silica can cause extremely serious, sometimes even fatal disease. In the massive rulemaking docket being compiled by the Agency, credible claims to the contrary are sparse to non-existent.

This rulemaking is no different than most in this sense: with few exceptions, most industry challenges to OSHA's health standards do not center on *whether* a material impairment of health can be caused by exposure to the chemical, but *what level or type of exposure* causes this material impairment. Frankly, industry's arguments challenging whether *their* industry's exposures pose a significant risk of material impairment of health are so common in OSHA rulemakings that they likely fall on deaf ears at an Agency deluged with arguments of this nature in every rulemaking it initiates. Indeed, the Assistant Secretary of Labor has written a book compellingly portraying the extent to which industry will go to challenge the Agency's conclusions on risk: *Doubt Is Their Product*, explains Dr. Michaels.

The Agency's skepticism is warranted, and its mission to ensure workplaces free from harm laudable. Yet, its mission and skeptical view of the credibility of industry's arguments do not give the Agency unbridled discretion to establish health standards outside the scope of its statutory authority under the OSH Act. Congress and the courts have established clear parameters within which the Agency must operate in attempting to achieve that mission. In this rulemaking, OSHA would be stepping well outside those parameters by applying the proposed silica standard to the sorptive minerals industry.⁷

A. OSHA Has Not Met Its Burden to Demonstrate Significant Risk of Material Impairment Under Section 6(b) of the OSH Act, Nor That It Is Reasonably Necessary to Apply the Proposed Silica Standard to the Sorptive Minerals Industry under Section 3(8) of the Act.

OSHA's authority to promulgate the proposed silica standard derives from Sections 3(8) and 6(b)(5) of the OSH Act. Section 6(b)(5) states, in pertinent part:

The Secretary, in promulgating standards dealing with toxic materials or harmful physical agents under this subsection, shall set the standard which most adequately assures, ***to the extent feasible, on the basis of the best available evidence, that no employee will suffer material impairment of health or functional capacity*** even if such employee has regular exposure to the hazard dealt with by such standard for the period of his working life.

29 U.S.C. § 651(b)(5) (emphasis added). Moreover, and as a threshold matter, Section 3(8) requires the Secretary of Labor, before issuing any standard, to demonstrate that application of

⁷ SMI is arguing neither that there is a "threshold level" below which exposures are not of concern, nor that the *type* of exposure occurring in the industry is not problematic. Rather, SMI's position is that the silica found in sorptive clays is fundamentally different in nature than the crystalline silica that is the focus of the agency's rulemaking, a position OSHA itself has taken regarding amorphous silica, which it has excluded from the scope of the proposed rule.

the standard to an industry is “*reasonably necessary and appropriate*” to remedy a significant risk of material health impairment in that industry. *Industrial Union Dep’t, AFL-CIO v. American Petroleum Institute*, 448 U.S. 607, 639 (1980); *United Steelworkers v. Marshall*, 647 F.2d 1189, 1251 (D.C. Cir. 1980).

The Secretary of Labor carries the burden to meet these statutory thresholds by substantial evidence on the record as a whole. 29 U.S.C. § 655(f); *see also American Fed’n of Labor v. OSHA*, 965 F.2d 962 (11th Cir. 1992). In interpreting the meaning of the substantial evidence standard as applied to OSHA’s standard setting authority, case law clarifies that the substantial evidence test requires the consideration of all evidence in the record, both supportive, as well as in conflict with the Secretary’s conclusions. *Associated Industries of New York State v. United States Dep’t of Labor*, 487 F.2d 342 (2nd Cir. 1972). While unarguably, the substantial evidence test affords the agency a fair amount of deference in making judgments and interpreting factual data, it does not give the agency unbridled discretion to establish any standard in its mission to ensure workplaces free from risk. *Industrial Union Dep’t AFL-CIO v. American Petroleum Institute*, 448 U.S. 607 (1980). In fact, the Agency is prohibited from establishing standards that rest on “faulty assumptions or [faulty] factual foundations” even in the name of safety. *Color Pigments Mfrs. Ass’n v. OSHA*, 16 F.3d 1157, 1160 (11th Cir. 1994).

SMI has submitted abundant, uncontroverted evidence to the record in this rulemaking to demonstrate that the silica in sorptive minerals exists in only two forms, either as amorphous silica that OSHA has determined does not warrant enhanced regulation, or as occluded, geologically ancient quartz with an amorphous aluminosilicate surface covering the entirety of the crystalline core.⁸ Indeed, record evidence shows that the forms of crystalline silica other than quartz that OSHA proposes to regulate -- namely, cristobalite and tridymite -- cannot form at temperatures at which sorptive clays, and the quartz they contain, are formed and exist.

The question to be answered by the Agency in finalizing the silica rule, then, is whether geologically ancient, occluded quartz found in sorptive clays poses a significant risk of material impairment or functional capacity to the workers exposed in the sorptive minerals industry for which it is reasonably necessary and appropriate to prevent that risk. The record does not support an affirmative finding to this question. Indeed, the best available evidence indicates that

⁸ *Id.* Hochella and Muryama (2010)

this is not the case, and that the silica forms found in sorptive minerals should be carved out of the enhanced regulatory scheme like OSHA has already opted to do for amorphous silica.

In proposing the silica rule, OSHA recognized that the surface of quartz plays “an important role” in disease. 78 Fed. Reg. 56310 (Sept. 12, 2013). In fact, OSHA recognized that modification of the surface characteristics of crystalline silica particles including, specifically, clay occlusions, the presence of impurities, particularly metal ions, and the changes caused by the age of the surfaces, may alter the disease potential and toxicity potency of silica by affecting the physical and chemical pathways of the mechanistic process. OSHA cites a number of studies supporting this “hypothesis,” and, on the basis of these studies, concludes that the surface activity of quartz plays a significant role in risk potential of the material. *Id.* Nevertheless, OSHA proposed to regulate all crystalline silica materials regardless of their surface characteristics because it concluded that the information and studies it had reviewed to develop the proposed rule provided insufficient science to make a clear determination about the relationship between surface activity and disease.

The scientific work submitted to the record by SMI is the evidence the agency found lacking in its study of the health effects of exposure to occluded quartz. Specifically, the work conducted by Geh in 2006⁹, and, most significantly, the *in vivo* study by Creutzenberg in 2008¹⁰, show the low toxicity potential of occluded quartz found in sorptive clays. In comparing the effects of freshly fractured quartz (DQ-12) to occluded quartz found in sorptive minerals, Creutzenberg showed that, while freshly fractured quartz produced a persistent, progressive inflammatory response and significant tissue damage, the same dose of occluded quartz from sorptive clay produced a significantly reduced response that, importantly, was not progressive. The earlier Geh *in vitro* study is consistent with the Creutzenberg findings, showing very low levels of genotoxicity, as measured by micronucleus assays, and, in fact, no increase in micronucleus formation with less than one percent quartz. This work fills the gap that OSHA identified in its proposal to support the agency’s hypothesis that the occluded nature of quartz significantly reduces disease potential.

⁹ Geh, *et al.* (2006) Genotoxic Potential of Respirable Bentonite Particles with Different Quartz Contents and Chemical Modifications in Human Lung Fibroblasts, *Inhal. Tox.* 18:405-412 (see SMI February 11, 2014 Comment, Exhibit 4).

¹⁰ Creutzenberg, *et al.* (2008) 28 Day Study: Inflammatory and Genotoxic Effects of Native or Treated Quartz Samples Following Intratracheal Instillation in the Rat, *Inhal. Tox.*, 20:995–1008 (see SMI February 11, 2014 Comment, Exhibit 5).

Section 6(b)(5) of the OSH Act requires OSHA to rely on the best available evidence in setting the silica standard. These studies, in particular the Creutzenberg study, offer the Agency the best evidence available to analyze and assess the risk posed by exposures to geologically ancient occluded quartz found in sorptive clays. While a wealth of epidemiological data would be valuable, the Supreme Court has implicitly recognized, as has the Agency, that in the absence of credible, applicable epidemiological data, animal studies may be relied on to conduct a substantial risk determination. *Industrial Union Dep't v. American Petroleum Institute*, 448 U.S. at 657 n.64 ("In other proceedings, the Agency has had a good deal of data from animal experiments on which it could base a conclusion on the significance of the risk."). In testimony during the silica Hearing, OSHA affirmed its view that animal studies would be acceptable evidence on which to assess risk.¹¹

Indeed, supportive of these relevant *in vitro* and *in vivo* studies, one epidemiological study of the sorptive clay industry has been conducted. Waxweiler, *et. al.* (1988)¹², in a NIOSH retrospective cohort study of workers in a sorptive mineral mine and processing facility in Georgia, failed to find a causal link between exposure to crystalline silica-containing sorptive clay and lung disease, stating that, “[a] significant deficit of mortality (SMR = 43, 90% CI 23-76) from nonmalignant respiratory disease (NMRD) was observed for the cohort based on age-, calendar year-, and race-specific rates for U.S. males. A marked deficit of NMRD was seen regardless of presumed dust exposure level, induction-latency period, or duration employed.”

Rather than the work provided by SMI and cited herein, however, it seems OSHA may be mistakenly relying on either epidemiological studies of workers in other types of clay industries or a group of extremely flawed studies analyzing silica exposures to bentonite workers. While sound epidemiological studies are highly useful in characterizing exposures and making risk determinations, they are so only where the studies meet two essential criteria, namely, in the first instance, where the cohort of workers studied is representative of the industrial exposures at issue; and second, where the studies themselves are conducted in a manner that is scientifically valid and sound. As described in SMI’s June 3, 2014 Post-Hearing Submission, one or both of these criteria are not true of the studies referenced by OSHA.

¹¹ Testimony of William Perry, Acting Director of Directorate of Standards, March 4, 2014, in response to questioning by Kathryn McMahon.

¹² Waxweiler, R.J., et.al. (1988) A retrospective cohort mortality study of males mining and milling attapulgite clay, *Am J Ind. Med.* 13(3):305-15.

The studies of workers in clay industries other than sorptive clays, such as the Miller/Souter and Love studies of brick-making and pottery-making operations, simply are not representative of clay products produced by the sorptive minerals industry. Sorptive clays (and the quartz contained within the clays) are not exposed to the high temperatures to which brick and pottery clays are exposed. The very high temperatures used to make brick and pottery has the potential to change the chemical and physical characteristics of the silica in the clays used in these industries and, thereby, change its toxicology and bioavailability.

The bentonite studies, including the 2013 Huang, *et al.* study and the much earlier 1971 Phibbs, *et al.* study, are essentially devoid of materials characterization, and have such fundamental scientific flaws as to not be useful in assessing risk. While OSHA has significant discretion in conducting risk assessments, it is not permitted to rely on flawed analysis or faulty assumptions. *Color Pigment Mfrs. Ass'n v. OSHA*, 16 F. 3d 1157 (1994). To rely on the flawed or misrepresentative studies seemingly relied on by OSHA, in lieu of the work submitted by SMI in this rulemaking, would certainly not be relying on the best available evidence to assess risk to workers in the sorptive minerals industry and would, therefore, be impermissible.

Finally, it should be noted that OSHA may believe it has analyzed relevant studies for the sorptive minerals industry by including in its assessment studies of risk from exposure to “aged” quartz. However, these studies do not accurately portray the risk associated with exposure to the “geologically ancient” quartz in sportive clays. The oldest of the aged quartz in the studies cited by OSHA in the preamble to the proposed rule was aged all of 60 days¹³. The geologically ancient quartz in sorptive clays has been aged from about 5.2 to 110 million years. As a result, these studies are not even in the proverbial ballpark, and it truly would be an impermissibly faulty assumption to assume that quartz aged 60 days or less can accurately portray the characteristics of quartz aged millions of years.

B. OSHA Has Not Met Its Burden to Demonstrate the Proposed Rule is Feasible for the Sorptive Minerals Industry.

1. Technological Feasibility

The OSH Act prohibits OSHA from setting exposure limits that are either economically or technologically infeasible. Accordingly, to promulgate a standard, OSHA must build a record that demonstrates the technological and economic feasibility of the proposed standard. To

¹³ Vallyathan, V., et. al. Freshly fractured quartz inhalation leads to enhanced lung injury and inflammation – Potential role of free radicals, *Am J Respir Crit Care Med* 152: 1003-1009 (1995).

demonstrate technological feasibility, OSHA must show “that modern technology has at least conceived some industrial strategies or devices which are likely to be capable of meeting the PEL and which the industries are generally capable of adopting.” *American Fed’n. of Labor v. OSHA*, 965 F.2d at 980 quoting *United Steelworkers of America v. Marshall*, 647 F.2d 1189, 1266 (D.C. Cir. 1980). It is an “undisputed principle [that] feasibility is to be tested industry-by-industry” which requires that OSHA “examine the technological feasibility of each industry individually.” *United Steelworkers of America v. Marshall*, 647 F.2d at 1301. Courts have and will remand OSHA determinations where the agency has not sufficiently analyzed the abilities of specific industries to meet proposed standards. *AFL-CIO v. OSHA*, 965 F.2d 962 (11th Cir. 1992); see also *Building & Constr. Trades Dep’t v. Brock*, 838 F.2d 1258, 1272-73; *Industrial Union Dep’t., AFL-CIO v. Hodgson*, 499 F.2d 467, 480-481 (D.C. Cir. 1974).

In *Color Pigments Mfrs. Ass’n v. OSHA*, 16 F.3d 1157 (11th Circuit 1994), for instance, the Eleventh Circuit remanded the cadmium rule as it applied to the dry color (pigment) formulating industry because OSHA had not satisfied its burden to conduct an individual analysis of the technological feasibility of the industry to comply with the standard. In that rulemaking, OSHA had in fact made an affirmative finding that the dry color formulating industry had the technological capability of meeting the standard. However, this finding was based on an analysis of workers whose jobs, while “similar or identical in some cases” to jobs in the dry color formulating industry, were “insufficiently similar” according to the Eleventh Circuit, to establish the exposure levels to which workers in the dry color formulator industry would be exposed. The court’s determination was based on the fact that the workers studied (who admittedly conducted jobs that were identical to or very similar to jobs in the dry color formulating industry) were at large companies, and workers at large companies would have “sources of and opportunities for exposure far more diverse” than that of a typical dry color formulator, the vast majority of which were small businesses. *Color Pigment Mfrs. Ass’n v. OSHA*, 16 F.3d at 1162.

Similarly, in *United Steelworkers*, the D.C. Circuit rejected OSHA’s determination regarding the feasibility of the lead standard as it applied to select industries because OSHA’s findings “lacked a detailed industry-or operation-specific analysis.” *United Steelworkers* 647 F. 2d at 1278-79. For example, as to the nonferrous foundries, OSHA analyzed “in modest detail” the specific operations of the industry, including molding, melting, and pouring, and cited a

report that provided suggestions on technology that would be necessary to reduce lead exposure for those operations. *Id.* Notwithstanding, the court remanded the rule as to the non-ferrous foundries because the agency had cited no evidence on the ability of such technology to meet any specific PEL for these operations. The court concluded that the agency had “hardly asserted, much less offered substantial evidence for, the feasibility of the PEL for these foundries.” *Id.* at 1293-94. Again, in *AFL-CIO v. OSHA*, 965 F. 2d 962, the court found that OSHA cannot rely on generic conclusions regarding whether an industry has the technological capability to meet a specific PEL based on lumping together industries with similar SIC codes to make generic conclusions about those --albeit similar -- industries. *Id.* at 980-81. Thus, it is well established that OSHA cannot meet its burden to demonstrate technological feasibility by making overbroad generalities as to feasibility, or by grouping large categories of industries together without a specific explanation of why findings for the group adequately represent the exposures and technological capabilities of each industry in that group.

Here, OSHA has not even attempted to analyze the exposures or engineering control capabilities available in the sorptive minerals industry. The rulemaking is simply absent of any analysis of the sorptive minerals industry whatsoever. OSHA’s Final Economic and Regulatory Feasibility Analysis supports the proposed rule by providing an analysis of the impact of the rule on those industries to which it would be applied. Specifically, Chapters 3 and 4 (“Profile of Impacted Industries”; “Technological Feasibility”) identify those industries that are covered by the rule, and provide a technological feasibility assessment of the impact of the rule on those covered industries. The Profile of Impacted Industries does not mention the sorptive minerals industry. The impact of the rule on the sorptive minerals industry, let alone whether compliance with the rule would be technologically feasible, simply is not analyzed by OSHA.

This is not a case where OSHA can argue that it has met its burden by including an industry sector analysis for a “similar industry” to the sorptive minerals industry. The only purportedly similar industry analyzed by OSHA is the structural clay industry sector, which includes the “brick and structural clay tile manufacturing industry (NAICS Code 327121); the ceramic wall and floor tile manufacturing industry (NAICS 327122); and the “other structural clay product manufacturing” industry (NAICS 327123). The sorptive minerals industry, which mines, processes and packages sorptive clays is nothing like the structural clay products industries identified by OSHA for various reasons, most importantly, because the processes that

clays in the structural clay manufacturing industries undergo exposes these clays to much higher heat, a factor known to cause physicochemical changes to and fracturing of the quartz particles. Structural clays then, if they become freshly fractured, could cause the type of exposures OSHA has identified in its risk assessment as posing harm. As explained, sorptive clays do not undergo this same sort of heat exposure, thereby rendering them critically different from structural clays from a risk standpoint.

2. Economic Feasibility

The Agency's burden to demonstrate the economic feasibility of a rule is similarly industry-specific. To demonstrate economic feasibility, OSHA must "demonstrate a reasonable likelihood that these costs [of a rule] will not threaten the existence or competitive structure of an industry, even if it does portend disaster for some marginal firms." *AFL-CIO v. OSHA*, 965 F.2d at 982 *quoting United Steelworkers of Am. v. Marshall*, 647 F.2d 1189, 1272 (D.C. Cir. 1980). Indeed, courts have found that "it would seem particularly important not to aggregate disparate industries [by industry sector groupings] when making a showing of economic feasibility." *Id.* This is because reliance on average estimates of costs can be extremely misleading in assessing the impact of a particular standard on individual industries. *Id.* For these reasons, reviewing courts universally have rejected OSHA's generic economic analyses showing the ability of industry sectors to survive promulgation of a rule, and remanded rules based on this sort of generic analysis to the agency to either develop industry-specific data or except those industries from coverage of the rule. *Id.*

As with the technological feasibility analysis, OSHA has provided absolutely no analyses or findings -- specific or otherwise -- of the economic feasibility of compliance with the silica rule by the sorptive minerals industry. Chapters 5 and 6 of the Final Economic and Regulatory Flexibility Analysis address the economic impact and feasibility of the proposed standard. There is no mention of the sorptive minerals industry. Accordingly, because OSHA has completely failed to meet its burden to demonstrate the economic and technological feasibility of the sorptive materials industry to comply with the proposed silica rule, it would be impermissible to include this industry within the scope of the rule.¹⁴

¹⁴ The jurisdictional boundary of OSHA's authority to regulate the sorptive minerals industry is not pertinent to this analysis. Wherever those boundaries lie between OSHA and MSHA is irrelevant in that, if OSHA otherwise has authority to promulgate a rule covering a portion of the sorptive minerals industry, the Agency failed to meet an essential prerequisite to apply the silica rule to any segment of this industry. To the extent the Agency does not have

C. Conclusion

For the aforementioned reasons, OSHA should include the scientific work provided by SMI in this rulemaking in its risk assessment, recognize the clear distinction between ancient occluded quartz found in sorptive clays and the forms of crystalline silica which are the focus of OSHA's risk assessment and rulemaking, and explicitly exclude the sorptive minerals industry from coverage under the final revised silica standard.

authority over segments of this industry, SMI's participation in this rulemaking remains highly relevant because we have provided critically important information to understand the unique nature of sorptive clays and risks in the industry that will be relevant to MSHA as it moves forward in its silica rulemaking process.