Biological Interaction of Inhaled
Mineral Fibers and Cigarette Smoke
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Battelle Press, 1989

Edited Transcript of the Discussions of Day 1

Chairman: Dr. F. J. C. Roe

Papers by the following authors were discussed in this session: Roe, Wehner, Martonen, Sterling, Pott, Ferin, Davis, Churg, Muhle, Gerde, and Scholander.

DR. ROE: Ladies and gentlemen, I will call the discussion to order. During the first hour or so we will talk about the papers which were presented this morning. I think it is only right to give you an opportunity for questions and comments on each of these talks and, obviously, I gave the first talk, so I must offer you the chance of criticizing and commenting on what I said. Regarding the maximum loading for dust inhalation, I believe we should take into consideration the nature of the dust, especially its density, and not only the mass of the dust. Basically I am saying that the dust quantity, whether it is, say, 2 to 3 mg/m^3 or 16 mg/m^3, would be a function of the density of the dust. Too little attention has been paid to this in the past. In relation to overload, there is a "magic" number for maximal dust loads. The maximum is between 1 and 2 mg, up to 4 mg of dust/g of lung tissue. Knowing the minute volume and the exposure time, and assuming the deposition rate, one can estimate the aerosol concentration. Obviously, one of the basic factors for these estimates is the density.

DISCUSSANT: We have worked with asbestos fibers and titanium dioxide, and found an overloading of lung clearance even with "inert" particles. With cytotoxic particles such as asbestos, of course, this overloading occurs at much lower concentrations.

I will first explain what happens with inert particles with respect to overloading the lung clearance mechanism because we do not have the compounding effect of cytotoxicity here. Macrophages have a limited capacity for phagocytizing particles; you can fill them up to about 50%. This is approximately the point at which alveolar clearance becomes severely retarded, and the movement of the macrophages becomes slower and slower. Also, at this point

they start to form clusters, so that aggregated macrophages are seen in tissue slides.

Alveolar clearance can be retarded from a half-time of 80 days to half-times of up to 500 days, even by inert particles, if you fill up the macrophages by more than 50% of their capacity.

DISCUSSANT: One thing I think you should take into consideration, at least based on our observations, when you are making your calculations: We found that there is at least one subpopulation of pulmonary macrophages that does not phagocytize particles, and this subpopulation may contribute from 25 to 35% of the total population. I do not know whether it has to do with receptors or whether the nonphagocytizing macrophages are simply immature or old.

DR. ROE: One very interesting thing that I came across when we were looking at the effects of inhaled cigarette smoke was that, early in the study, we observed macrophages in the lungs, in the region of the terminal bronchioles, which did not stain positively for iron. When we then looked again at a later time in the study, not necessarily after more smoke exposure, many macrophages were found to stain positively for iron. The question is, from where did the iron come? There is, for practical purposes, no iron in cigarette smoke. It is not coming from there—and in any case, even if it were, it does not stain immediately—so it seems that the iron is picked up perhaps as a result of absorption of fluid that is getting into the lung by transudation from the blood, and it is therefore a secondary phenomenon. This suggests that pulmonary macrophages phagocytize particles other than those that stimulate their proliferation. This may be a variable which, perhaps, is relevant to interaction.

DISCUSSANT: Following up on both those comments, the lung should certainly not be regarded as a static organ.

DISCUSSANT: In the case of inert particles, one does not see many new macrophages.

DISCUSSANT: What defines an inert particle?

DISCUSSANT: That is a good question. In our view, there is no such thing as an inert particle. All particles impact on the lung, although not necessarily because they are cytotoxic.

DISCUSSANT: "Inert" is really like "insoluble"; it is a relative concept.

DR. ROE: It is obviously easier to detect inertia amongst colleagues than among particles.

DISCUSSANT: I find this discussion a bit disquieting because it assumes that one can extrapolate from animal studies directly to

humans. A review of data on inhalation of dust by human subjects does not lead to any concrete conclusions that smoking interferes with dust inhalation. Some investigators have reported that smoking suppresses particle clearance, and others have reported that it stimulates it.

Cohen used a magnetometric technique to determine the elimination of dust that was *not* labeled radioactively, and I understand that this technique is mostly used with radioactive dust. Cohen's subjects inhaled large quantities of ferric oxide and then were magnetized and the regions of magnetism recorded. I am sure that most of you are familiar with this kind of technique. However, there is a serious difficulty with the interpretation of Cohen's results.

What happened in Cohen's case was that when he subjected smokers who had swallowed or inhaled iron particles to strong magnetic forces, these particles traveled in the mucus stream within the lung and formed large clusters. The reason that smokers in the Cohen study did not eliminate metal dust as quickly as nonsmokers do was because these clusters of metal were too large to be moved.

Cohen's experiment was simulated by Reinstein, and the whole subject was discussed at the meeting of the Magnetometric Society, which was an acrimonious and noisy affair. In my opinion, Cohen's experiment should not be considered as proof that smoking can affect the elimination of dust from the lungs.

Over the years, a number of investigators have measured, by magnetometric techniques, dust elimination from the lungs in smoking and nonsmoking individuals who were occupationally exposed to welding and mining dust. These investigators either found no difference, or refrained from publishing their failure to find any difference, because they believed that such publications would not be of interest to the general population. Everybody assumes that smoking interferes with the elimination of dust, so any study that does not support this assumption is suspect and may not be accepted for publication.

There are other questions that need to be addressed: Are all types of dust eliminated in the same way? Maybe smoking does interfere with elimination of the kind of dust to which asbestos workers are exposed but not with that of other dusts with which it is safer to experiment.

DR. ROE: We seem to be rapidly getting into the field of metal analysis.

DISCUSSANT: I agree. The kind of data I am talking about should rightly be considered in context with metal analysis.

DISCUSSANT: I should like to comment on one thing Dr. Muhle said. He claimed that one does not get macrophage influx in lung

tissue following exposure to innocuous dusts. I believe this is largely true over a certain range of doses, but not entirely true. We have looked at the comparison between titanium dioxide as an innocuous dust and asbestos and quartz. With 10 mg/m^3, there was a massive influx of macrophages with asbestos and quartz, and none with titanium dioxide. When we did the same experiment with 50 mg/m^3, there was, after 6 to 8 weeks, quite a large influx of macrophages, even with the titanium dioxide. Fifty mg/m^3 is, of course, a very high dose. Nevertheless, it is important to recognize that even with this otherwise innocuous material, macrophage influx can occur.

DR. ROE: On the subject of anatomical models of the respiratory tree, I remember papers published in the 1950s in which there were models of the respiratory tract, and not just the lower respiratory tract but of the upper respiratory tract as well. As far as I recall, the findings using these models were no different from those which were presented today. Be that as it may, I ask myself: Is it reasonable to expect a silicone surface to be a valid model for a warm, living, moist surface, which is changing all the time with respiration, and which has several specialized layers of surface fluid? Is it reasonable to expect such a simple model to tell one very much of importance?

DR. WEHNER: Not being a specialist in that field, my gut reaction would be that such a model would probably be better than no model at all.

DISCUSSANT: The whole concept of using such a model is to try to develop something that simulates a biological entity. By definition, a model can never be perfect, regardless of what the model intends to simulate. Ideally, I would have conducted my experiments with human replica casts. We still propose to do that. The advantage of human replica casts is that they have anatomical features, such as cartilaginous rings, which simulate realistically the conditions in the human body. However, when we did our experiments, we did not have access to replica casts.

DR. ROE: Could you describe what you mean by a replica cast?

DISCUSSANT: A replica cast is made from the lung of a cadaver. A replica cast is a cast made from a biological organ.

DR. ROE: In terms of the respiratory tree shape, clearly, a replica cast is ideal. However, it is important to ask: With what is it lined?

DISCUSSANT: We make the replica cast from lungs of the cadaver, then we line the inner surface of those replica casts with silicone oil to simulate the mucus lining, so that the particles will be

deposited in various locations and will not become re-entrained in the air flow.

In my view, these models are therefore suitable surrogates. The reason for using these models is that if one wishes to identify factors affecting deposition in the human lung, one can do appropriate tests with them.

One never measures deposition; one infers deposition from subsequent clearance data. One can then estimate tracheobronchial deposition by what has been cleared within, say, 20 hours, and subsequently one can measure pulmonary clearance in terms of gross compartmental clearance.

The advantage of latex replica casts is that one can simulate breathing patterns and study microscopic deposition patterns. One will not be able to quantify or even observe microscopic deposition patterns in the human lung, because one will need a high-resolution instrument capable of focusing on a three-dimensional, branching, tree-like network.

I do not advocate, and never have advocated, that deposition experiments be conducted using only models. Modeling should be regarded as complementary to human tests.

In my view, models are an integral part of an inhalation-exposure protocol, and I think they are definitely realistic in the sense that, by coating them appropriately, and by making the walls thin enough so that they can indeed expand and contract on inhalation and expiration when we introduce chrysotile dust, we are engaging surrogate modeling to the state of the art we have today within the limits of practicality.

DR. ROE: You said you used 100% smoke. Did you make allowances for the fact that smokers only inhale the more dilute smoke?

DISCUSSANT: Yes.

DR. ROE: And did you control such variables as humidity and temperature, etc.?

DISCUSSANT: We are doing that. I was educated by a very wise professor, Dr. Werner Stöber, who told me that science is error, error, error. Being an engineer, I try to approach problems very simply and then add complexities, rather than look at an overall problem which is too hopelessly complex for a solution.

In our *first* test we introduced an aerosol into a replica cast that was lined with silicone, but the interior was *not* humidified. I am quite aware of the effects of hygroscopic growth and the effects of aerosols on pulmonary deposition and have published numerous peer-reviewed papers on this subject.

In our *next* generation of models, we have developed a bifurcating, three-dimensional model in which the temperature and relative

humidity of the upper human airways *are* simulated. This successful engineering model is the culmination of a 2-year research effort that is described in a 40-page publication.

DR. ROE: I thank you very much. I apologize for attacking you, but it was worth it to get the additional information.

DISCUSSANT: Do you have any information about the deposition patterns in the distal lung, in the alveolar regions, either based on calculations or on what you may have seen? Secondly, based on the hot spots in the airways, and with respect to development of tumors, the first thing I thought of is when particles deposit in the distal lungs, they probably do not go anywhere for a certain period of time, because fluid flows are notoriously slow at the alveolar duct bifurcations. However, at the bifurcations of airways, all sorts of things are going on: ciliary activity, mucus flow, etc. I would think that the particles which deposit there probably do not stay there very long. I would like to hear your comments on this.

DISCUSSANT: I wanted to talk about that in my presentation this morning, but I ran out of time. I have two transparencies which I would like to show or at least enter into a discussion this afternoon. They show a fiber deposition model in which we have compared fiber deposition with experiments described in a recent publication in the *Journal of Aerosol Science* by a Japanese author who has developed a bifurcation unit which simulates other areas of the human bronchial tree. He has quantitated, experimentally, the extent to which enhanced deposition of fibers also occurs in other areas.

Now, that has never been observed in humans *in vivo*, for obvious reasons, but I think that bifurcation enhancement of fibers can occur throughout bronchial and alveolar passages, and the extent to which it happens I think we can explain by the few extra models I am prepared to present at the technical session this afternoon.

DISCUSSANT: There was the issue of clearance.

DISCUSSANT: Professor Hofmann and I were trying to take into account factors affecting clearance in the upper bronchi. There are experimental observations of reduced clearance in areas of bifurcations of cats and dogs, but I am not quite certain whether the extent to which inhibited clearance occurred explains the increased risk in terms of underlying airway cells being exposed longer to carcinogens, thereby experiencing (a higher degree of) toxic effects.

Reduced clearance at bifurcations definitely will increase exposure of those cells relative to other airway cells. My cigarette-smoke experiments indicate that clearance is impaired at bifurcations, and this is a factor that has to be considered. In terms of the

cloud-settling effect, I want to introduce something brand-new by saying that submicron particles are actually deposited at bifurcations, rather than in more distal airways and are reaching bifurcations later, after being carried up there in the mucociliary stream.

DR. ROE: The major problem that I have with this model is that it really does not simulate smoking. The human inhales the smoke bolus, holds it in his oral cavity for a moment, during which time the characteristics of the aerosol are changing, and then he inhales a part of that bolus further into the lung. To make the matter even more complex, the depth of inhalation and the speed of inhalation vary from smoker to smoker. The model is more applicable to a passive smoking scenario or an ambient air scenario than to active smoking.

DISCUSSANT: As was pointed out in the instructions to the speakers, the oral presentations had to be limited to 20 minutes, and more detailed descriptions had to be relegated to the manuscripts. But since you brought up this question, let me explain what we did.

We introduced the smoke into the oropharyngeal cavity by means of a standard 35-ml puff, drawn over a 2-second period, and then we simulated that pause. That is the way the smoke was introduced.

Now, there is another issue: when a smoker puffs, it is not a concentrated stream that enters the mouth. There is some leakage or dilution occurring around the lips. Unfortunately, there is no way of quantifying the extent of this dilution. We simply lit the cigarette, loosely placed it in position, and then smoked it, using an electronic smoking device under conditions in which dilution was allowed to occur. We just hoped that the venturi effect would simulate what was happening physiologically. By these means, we tried to simulate actual smoking as closely as possible. We also added that oropharyngeal pause specifically in an attempt to take mixing into account.

With regard to cloud-settling, I believe that this effect only occurs in the upper bronchi, because it is only there that the cigarette bolus has not been diluted sufficiently to have single-particle settling. As that bolus travels further toward the deep lung, the volume of the airways increases dramatically and relatively rapidly. The bolus is therefore going to be diluted so much that a cloud-settling effect will no longer occur.

DISCUSSANT: I want to make a comment on the issue of turbulent versus laminar flow. You didn't mention what impact turbulent flow has on the hypothesis of the cloud-settling effect.

DISCUSSANT: Once again, I didn't have time to talk about that. I have six or seven transparencies here that show the mechanism of how the hodgepodge developed, but I would like to specifically

effect of smoking and occupational exposure was unchallengeable. A lot of assumptions have been made based on that premise.

Now, I believe, we are being told that we should not build up huge theories on the assumption that this is, in fact, correct, and that it is possible under certain circumstances that smoking can be somewhat "protective" against some forms of dust, at least that is what I understand from this discussion. Does anybody want to disagree with me?

DISCUSSANT: I think you overstated that, particularly when you bring in words like "protective" in terms of smoking. I personally take offense at that. I could probably build a case that driving race cars is protective of one's dying of cancer.

DISCUSSANT: I do not see how you can be offended by data. The fact of the matter is that smokers have an absolutely lower prevalence of disease than nonsmokers, especially in occupational asthma and in pneumoconiosis. These problems (observations) do show up, and they are real, and one has to deal with them. The fact is that certain occupational lung diseases are more frequent among nonsmokers than among smokers. These are scientific data, and they *have* been ignored but *shouldn't* be ignored.

DISCUSSANT: Regarding the term "protective." We are simplifying greatly, not only in experiments where we are modeling and in experiments where we use animals but, it seems to me, also in experiments where we use human beings, where we come to some simplified conclusions that do not take into account all the variables which are encountered when we do a clinical study like the ones mentioned here.

Here is a small example of what I am trying to say: we know that bronchitis by itself, which is produced by smoking, is in some way protective. When you use usual techniques for determining lung clearance, you will find that the clearance in a bronchitic patient is changed. Why? Because the deposition pattern is changed. When the deposition pattern is changed in bronchitis, those particles which are used to identify clearance will deposit more in ciliated airways than in the alveoli, as they do in a nonbronchitic person. Then the clearance rate of that radioactive tracer will be different.

Now, tell me, what does this mean? Does it mean that the bronchitis produced by cigarette smoke is protective? It is not. That is what you would really assume if you were to look only at the results and say, "Oh, in cigarette smokers in a certain stage of bronchitis, we find that clearance is not effective, or it is faster." You can find these types of results, but they do not mean anything if you do not analyze the whole data correctly and carefully enough to get the correct answer; otherwise, you might even say that bronchitis produced by smoking is protective.

DR. ROE: Let me just make clear that when I said the data are fragile, I meant to include exactly the point that Dr. McClellan has made and that you have made: these data on humans all contain variables which were uncontrollable at the moment and which have to be taken into account. You cannot uncritically rely on these data, particularly when you have only small numbers (e.g., lung cancer rates in non-asbestos-exposed nonsmokers versus asbestos-exposed nonsmokers). So I do not think anybody is going to disagree with you or Dr. McClellan.

DR. WEHNER: Perhaps we should stay away from attaching adjectives to the findings, such as "protective" or "beneficial," and should concentrate on characterizing the phenomena. When we investigate and describe certain phenomena, we do not have to pass value judgments or state whether the phenomena are beneficial or protective or harmful. This is the best way to avoid unnecessary controversies.

DISCUSSANT: I have no difficulty with the use of words such as "more frequent," "less frequent," whatever. I agree with that. When you start to take another step and use adjectives that carry with them values, then I have some problems.

DR. ROE: Let us go on to Dr. Pott's presentation. There are many interesting things which were said, such as that there was no multiplicative effect of combined exposures to asbestos and cigarette smoke on fibrosis. I am sure there are some questions.

DISCUSSANT: Professor Pott, there are two of several conclusions you drew for which I would really like more clarification. Firstly, you said there is no effective difference between experimental exposure by inhalation and intraperitoneal injection. As far as I am concerned, they are very different. I have a hard time understanding, in the case of intratracheal instillation, and much more so in the case of intraperitoneal injections, the role of these methods in elucidating mechanisms of pathogenicity or interaction of *inhaled* substances. Please, would you clarify that.

The second point you made was that asbestos fiber numbers relate more to asbestosis than to lung cancer. I wonder whether you have considered that asbestosis is a chronic inflammatory process and whether that could contribute to the development of tumors, whether through oxygen radicals or other mediators. It would seem to me that they are not mutually exclusive.

DISCUSSANT: First we calculated the effective dose, and I underline the word "effective." We can assume that all injected fibers become effective after intraperitoneal injection, because no processes like lung clearance are involved.

For inhalation exposures the situation is markedly different. We have seen, or other authors have found, that very few tumors developed following heavy inhalation exposure to crocidolite. More than 99% of the inhaled fibers were cleared, and a high percentage of the retained fibers was deposited in the unciliated airways. Therefore, only very few fibers were chronically embedded in the ciliated airways. This is an important point: they have to be embedded in the ciliated airways for a long period.

The other point which you mentioned concerned the number of fibers found in the lung. This number shows us the dose which is effective for asbestosis but does not show us the dose which is effective for lung cancer.

I mentioned that about 95% of all human lung cancers occur in the relatively small area of the ciliated airways and only 5% in the large area of the unciliated airways. For this calculation, at the moment, we can disregard the 5%. We have to find an explanation for the high lung-cancer incidence in the small areas of the ciliated airways. When we count the fibers, we find that the very small number of fibers that are chronically implanted in the ciliated airways are responsible for the tumor incidence, and about 99%—certainly more than 90%—are retained in the unciliated airways. After we have determined the number of fibers in the lung of an asbestos worker who died, we have to decide whether his lung tumor was caused by asbestos or not. (Incidentally, I believe that the number of fibers found in the lung of a deceased worker is not a correct measure for the carcinogenic potency which was active 20 or 30 years before.) In Germany we have to decide, on the basis of the number of fibers in the lungs of the dead asbestos worker, whether the worker's family is entitled to compensation for his lung cancer. I think this is the wrong criterion, especially in view of the low, or the relatively low, durability of chrysotile asbestos in the human lung.

I hope I have answered your questions.

DISCUSSANT: I would like to take issue with you on one of your points. You stated correctly, in general, that 95% of lung cancers occur in the larger airways. However, that is not true in the asbestos situation, where many (if not the majority) of cancers occur peripherally.

Secondly, it is well known that the risk of developing lung cancer in individuals who have asbestosis is twice that of asbestos-exposed individuals who do not have asbestosis. Given that background, I would find it difficult to accept your hypothesis on this fiber contact with the larger ciliated airways and either deposition, impaction or contact, or uptake of fibers by those cells, being the major factor responsible for asbestos-related lung cancer. If we are talking about the question of the synergism of smoking with asbestos, certainly

the question of whether products of tobacco smoke may interact with larger airway cells is important, and I accept that this interaction may well occur in this case. But I do not think that this necessarily applies to the asbestos situation. In fact, if fibers are retained in the interstitial compartment of the lung, certainly away from the airways, it is very likely that any interaction that is going on there will occur in an entirely different area from the one you described.

I was wondering what comment you might have on that. I might add that several other speakers have also concentrated on the role of the airways, and I frankly wonder whether this really applies in the asbestos situation.

DR. ROE: Before you answer, I would like to ask Dr. Davis for a comment.

DISCUSSANT: I was, in fact, going to follow this up. We have been debating for years the point of whether asbestos-related lung cancers in humans are peripheral or central, and I do not think there is a definite opinion. Some people say one thing, some people say another. In experiments with animals, however, asbestos-related tumors are always peripheral. I've seen hundreds of them, and not one was in a major bronchial tube.

Because we are discussing so much the deposition in the major bronchial tubes, I think the tendency was to assume that certainly some aspects related to tumors in human beings are central. I was going to ask to discuss a theory as to why this should be so. Why should you have asbestos-related tumors in the main bronchial tubes in humans and not with experimental animals?

DR. ROE: In my written paper I addressed the same issue. Conventional thinking is that the epithelial changes in the main airways are the precursors of smoking-associated cancers. The cancers that are present in excess in smoking asbestos workers, are they really the same type of cancers as those in unexposed smokers, or are they two different populations of cancers?

Now, in a nonsmoking asbestos worker, do you see the epithelial changes in the main airways that have been described in smokers? Interaction has to mean something quite different when you have two different target sites in the respiratory system, from when you have only one target. This is something which we might want to discuss.

Now, the issue is complicated by the misuse of the terms, or the variable use of the terms "central" and "peripheral," because to some people, central means the main bronchus and the next one or two generations. But in airway terms, you can have up to 35 generations of airway and you are still in a reasonably big airway in humans, a much bigger airway than even the trachea of a laboratory rodent. So what does "central" and what does "peripheral" mean?

DISCUSSANT: There are additional questions, not only whether it is main bronchus or peripheral, but also which lobe. Two-thirds of the cigarette-smoke-induced tumors are in the main bronchus; two-thirds of the tumors in asbestos workers are in the lower lobe and "peripheral lung."

DISCUSSANT: Yes, of course, in the older literature, in which asbestosis was quite commonly described, most of these cancers were described as adenocarcinomas.

DISCUSSANT: I think it would be interesting—and possibly this has been done—to look at the difference or similarities between the lung cancers of persons who were exposed to asbestos and who smoke and those who do not. Has this comparison been made?

DISCUSSANT: There were only four or five nonsmokers in that cohort. This is just too low a number.

DISCUSSANT: Dr. Churg has the numbers; he has done the studies.

DISCUSSANT: The only numbers I have come from the literature. The notion that adenocarcinoma is most prevalent in asbestos workers is well ingrained. However, when you go back to the original data which make that claim, it really does not hold up. These data show a slight increase in adenocarcinoma in asbestos workers, but this is statistically no different from the non-asbestos-exposed controls. I went back 2 or 3 years ago to find what I could in the literature concerning cases with controls. I was unable to find any evidence that there is a preferential type of carcinoma seen in those with asbestos exposure.

DISCUSSANT: Firstly, I am very sorry that I am not a pathologist, but my arguments were regarding lung cancer in man and not lung cancer in rats. We do not know anything about the combined effect of cigarette smoke and asbestos in rats because we do not have any experimental data.

There may be more peripheral lung cancer cases than only 5%. It is not so important whether we say we have only 5% or as much as 40%. In relation to the surface area, it is a very small percentage, because only 1%, I believe, of the total surface area is ciliated, and 99% is unciliated, and this 99% is peripherally located. We can assume that, at maximum, 50% of the cancers are located in the unciliated 99% area, and 50% may be in the ciliated 1% area. Thus, there is a large difference between these two areas. The other significant difference is that the large 99% surface area contains the bulk of retained fibers. I think there is no doubt about this fact. This may be different in the case of a short inhalation exposure to a high concentration of fibers, when a high deposition in the upper

airways occurs. But what I am discussing is that long-term persistence of fibers in the bronchial walls. When we count the number of fibers in the lung, at least 90% or more of these fibers are from the area of the unciliated airways, and this high number is not a correct measure for the carcinogenic potency of the fibers.

DR. ROE: You have made your point very clearly.

Now, may we go back to Dr. Churg? There is a loose end there. I believe you stated, in effect, and I know there are publications on this, that men were dying from asbestosis before the age of 50. I would suggest that the data do exist for preferential existence of lung cancers in the lower lobes and for adenocarcinoma, unless there have been changes, and pathologists are now using different criteria than in years past.

When exposure to asbestos is reduced and men live longer, then it seems as though the location of the lesion shifts closer to the center, and perhaps the type of lesion changes, too. The data which you examined, perhaps from the United States, probably are fairly recent. So, historically, there may have been a change.

DISCUSSANT: I think "historically" is something different. The question is, how far back are you referring to? You said the data exist, so how far back are you going?

DR. ROE: In the United Kingdom, the data go back at least to the 1930s and 1940s.

DISCUSSANT: I argue that it does not stand up to any kind of sound analysis.

DR. ROE: I still think that some of the questions remain unanswered. If you have the same target tissue, and if you accept the data for bronchial epithelial changes as being the precursors of bronchial carcinoma, do you see these changes in asbestos-exposed people or not? Do you get these metaplastic and dysplastic changes at the sites where the fibers are actually or theoretically lodged in the basement membrane?

DISCUSSANT: I don't have data on human experiments. In our rats, we observed squamous cancers that I am sure were alveolus-derived, because we get squamous metaplasia in the alveolar region.

DISCUSSANT: Certainly. In humans, the cell type provides very little good correlation; currently, anyway.

DISCUSSANT: I would like to ask Professor Pott whether he actually said that intratracheal instillation is a good model for inhalation exposure. If he did, I find that very hard to swallow.

There is evidence that intratracheal instillation results in a very high and localized dose to an area. Is that not unrealistic compared to inhalation?

DISCUSSANT: I wasn't emphasizing intratracheal instillation but, rather, intraperitoneal injection for testing the carcinogenicity of fibers. However, intratracheal instillation of dusts may also be a suitable method for clarifying some questions, e.g., for examining the hypothesis that fibers lodge in increasing numbers in the bronchial mucosa as a consequence of inflammatory lesions. As mentioned, only 10 μg of the fibrous dusts were given per instillation; this is not a large amount. Certainly, this method of administration is unrealistic in principle. However, results observed after exposure by nonphysiological routes are not necessarily irrelevant. We are sometimes forced to use nonphysiological methods to elucidate important biological responses. The carcinogenicity of crocidolite and man-made mineral fibers is a good example of the need to use unrealistic routes of administration.

Although several inhalation experiments were performed with man-made mineral fibers, their results were ambiguous as far as tumor induction is concerned. More to the point, only a few critics took notice of the fact that only four of these inhalation studies contained a positive control group that received appropriate fibers: those which do not split up, thereby multiplying their numbers in the body, as chrysotile does. Crocidolite fibers are suitable for a positive control group because they are generally durable and very carcinogenic in humans. Nevertheless, they did not induce tumors in inhalation experiments as expected.

These findings are remarkable. In two of the four studies, no lung tumor or mesothelioma occurred; in one experiment, two lung tumors and one mesothelioma were found in 57 rats; and in the fourth study, 1 of 50 rats developed a pulmonary adenocarcinoma. These sparse effects show clearly that the test system was not sufficiently sensitive to detect the strong carcinogenicity of crocidolite fibers in humans. Therefore, we cannot expect that the system is more sensitive for man-made mineral fibers, and we must expect false-negative results here, too.

In contrast, the serosal tests—especially the intraperitoneal test—demonstrate a high degree of carcinogenicity and clear dose-response relationships after injection of crocidolite and some other natural and man-made mineral fibers. However, some fiber types were not carcinogenic, or only slightly carcinogenic, after intraperitoneal injection. This leads to the conclusions that negative results from an appropriately conducted intraperitoneal test exclude (reasonably well) an unacceptable cancer risk to humans.

With regard to the carcinogenic risk assessment for humans, we can conclude that nonphysiological intraperitoneal tests with

fibers produce more relevant results than inhalation studies. Some people argue that positive results from injection studies with fibers must be confirmed by inhalation studies before serious health concerns are justified. However, our society does not accept a carcinogenic risk from fibers that may equal 50% of the risk of highly carcinogenic crocidolite, but which cannot be reliably detected by inhalation studies.

DR. ROE: We now come to Dr. Ferin's paper. I am calling for comments on Dr. Ferin's paper.

DISCUSSANT: Talking about a multiplicative interaction, it is only Selikoff, in his evaluation of the combined effects of smoking and asbestos exposure, who comes up with numbers which suggest multiplicative interaction. Other data dealt with other dusts and came up with numbers indicative of something between additive and multiplicative effects. In terms of mechanism, there are numerous possible ways in which the effects of particles and smoke might interact additively, and there are also numerous ways in which they might interact multiplicatively.

The question to Dr. Ferin is, at the mechanistic level, would it make any difference—would an effect of cigarette-smoking on clearance make any difference, in your opinion, if it were an additive risk factor rather than a so-called synergistic, multiplicative type? Would cigarettes also be able to act in a way that would appear to be additive and still show some deleterious effect on asbestos lung-cancer outcome?

DISCUSSANT: The simple answer would be, "No." Basically, I started by considering the difference between mesothelioma and asbestosis. Mesothelioma is no additional risk factor in an asbestosis case. It does not matter whether smoke plus asbestos exposures are additive or multiplicative. That was really the basic outcome. I tried to emphasize and again suggested the hypothesis that basically the difference is in the dose.

DR. ROE: Is there anybody who seriously believes that intrapleural injection is of any value for the biological evaluation of dusts for lung-cancer potential or other kinds of health risk?

DISCUSSANT: This is not pertinent to that question, but I want to get something off my chest concerning the supposed interaction between tobacco-smoking and asbestos exposure regarding lung cancer.

We are trying to wrestle with a very complex system, in which all sorts of factors are interacting: clearance, as has been discussed here at length today. It seems to me that no one has tried to determine whether there actually is an interaction using some simpler

system, such as intraperitoneal or intrapleural injections. In other words, if you can show the interaction between asbestos fibers and some product of tobacco smoke in a simple system, then maybe you might reasonably hope to demonstrate it in a more complex system.

DR. ROE: I suppose this is a generic problem. However difficult it is to have a model which has a realistic end point, once you have established this end point, you can go back and say, "Well, if we can reproduce the right end point, if we had looked earlier or done the thing more simply, is there a cheaper, earlier marker we can use as a surrogate for the realistic end point?" However, unless one is sure that the end point one uses is really a marker for the realistic end point, there will always be doubt concerning the validity of the work.

In the past, I have been involved with people in the tobacco industry who said, "We do not want to do a long-term study. It costs too much and is too difficult." Instead, they have fiddled away, doing short-term tests, and then when they find that they can distinguish between two different products in a short-term test, they come to the toxicologist and ask, "What does this mean? Which is the safer product?" When I have been in this situation, I have had to say I haven't the remotest idea, because there is no point of reference. I can't relate the end point in the short-term test to any meaningful tobacco-associated disease. Thus, in my view, one must start with a realistic end point and then work backwards to simpler approaches. Trying to proceed in the opposite direction simply does not work.

I should like to ask another question. Do people believe that intrapleural injection is a good model for predicting mesothelioma?

DISCUSSANT: I am sorry. I want to take up the first question first: "What is a good model for lung tumors?," which I do not think we have answered. I would like to suggest that the value there is in a negative outcome rather than in a positive outcome. I suggest that negative results in injection studies may provide reasurrance even though the model is artificial and the end point is not realistic. If one tests a dust sample by injecting it into the body cavity, and it does not produce tumors, I would be very surprised indeed if it would do so in lung tissue. By contrast, it is more difficult to interpret positive results in such tests.

DISCUSSANT: I would like to say that we should not confuse two aspects of experimental testing. One is to determine a mechanism. For example, in this case you would say: "Injection into the intrapleural cavity is so far from what is happening in man, why bother?" For that purpose, obviously this is not a good test. But if you are talking about some really basic question, such as: "Is this compound capable of producing a tumorigenic response or not?

That is a new compound about which we do not know anything yet." In that case, if the test system produces tumors even in this artificial situation, it answers the question it was designed to answer, and therefore it was a good test.

We thus have to determine what we really want to ask of a test. If, for example, you were to say, "Oh, injection into the pleural cavity produced tumors; that means the same will happen by inhalation," that, of course, would be absolute nonsense. But we could say: "Maybe the results warrant additional experimentation," if the findings are relevant to a more realistic situation.

DR. ROE: I imagine there is general agreement on this.

DISCUSSANT: I would like to ask an open question. Certainly we have some ideas of where the dust particles or fibers go. Can anybody tell me where intraperitoneally injected fibers go? Do they land at the bottom of the peritoneum?

DISCUSSANT: What is the bottom of the peritoneum?

DISCUSSANT: Do we know what happens to them?

DISCUSSANT: They end up in aggregates or granulomas that can be located anywhere within the peritoneal cavity.

DISCUSSANT: You are talking about an intraperitoneal injection, not intrapleural?

DISCUSSANT: Yes, but I believe that roughly the same thing happens in the pleural cavity.

DISCUSSANT: But the macrophages pick up very small particles, and they go to the lymph nodes.

DISCUSSANT: That is right, but macrophages which pick up larger fibers tend to form aggregates and to end up as granulomas. We have found that a large number of short fibers get in the lymph nodes, but I believe that the longer fibers do not go in the lymph nodes; the bulk of the longer fibers go to the omentum, and also to the liver and the diaphragm, and so on.

I believe, in principle, we have a good analogy between the carcinogenicity of some dusts after intraperitoneal injection and after administration into the lungs. There are positive effects in both locations for all types of asbestos and for erionite. Perhaps there is sufficient evidence for carcinogenicity following bronchial and intraperitoneal administrations. We have two positive studies, and there is limited evidence in epidemiological studies. There are positive results after both intraperitoneal injection and after intratracheal instillation of cadmium and nickel compounds.

DISCUSSANT: I believe that this is a very controversial area, if you do not mind my saying so. We recently reviewed the epidemiological evidence for man-made mineral fibers and found some positive associations, but there is also a lot of negative evidence. You believe that the positive result in an intrapleural or intraperitoneal study is telling you something important about the hazard of a given test agent to which humans are exposed by inhalation, and I personally am very doubtful about this.

DISCUSSANT: We were having a little discussion yesterday. We do not own an inhalation facility, and therefore we see this problem a little differently from others. I believe that everything that you test in some intact animal has some biological relevance. I refer to the experiments that Chris Wagner's group conducted and the number of tumors produced by inhalation of asbestos minerals.

DISCUSSANT: Have you ever produced a mesothelioma by inhalation of asbestos?

DISCUSSANT: Yes.

DISCUSSANT: It is extremely rare. It is a very difficult experiment. If we had left the resolution of the asbestos problem to the results of inhalation studies, we would still be scratching our heads. It is nice to have an inhalation facility, and it is nice to do these inhalation experiments, but they are time-consuming and they are expensive, and there is only a finite number of facilities where individuals with the proper expertise are capable of conducting them appropriately. They may all be here in this room.

DISCUSSANT: Has anybody actually compared a washed fiber with a fiber on which polycyclic aromatic hydrocarbons (PAH) have been adsorbed.

DISCUSSANT: Yes.

DISCUSSANT: What happened?

DISCUSSANT: Absolutely the same outcome. Dr. Nolan had done this with his crocidolites. He showed that, whether a fiber is contaminated with trace metals or contaminated with PAH, it produced the same number of tumors.

DISCUSSANT: So why should one be so interested in adsorption when it comes to the lungs?

DISCUSSANT: Because it is a nice, simple model. It is a nice, simplistic approach to a complex problem. It is nice to have a passive carrier that transports PAH. It is a very attractive hypothesis. Why not?

DISCUSSANT: I believe that we ought to be very careful about your interpretation of intrapleural injection studies and, perhaps, even more about intraperitoneal studies, because there are several differences between the two injection studies with erionite, and erionite in intrapleural studies is not showing nearly the toxicity that chrysotile showed by inhalation. Inhalation of erionite produced almost 100% mesotheliomas. In intrapleural studies, it produced marginally more than crocidolite. In fact, when you inject it intraperitoneally, as I understand it, you got less—

DISCUSSANT: No.

DISCUSSANT: No, you get 95% with erionite.

DISCUSSANT: One of the points that I did not mention in my presentation this morning, simply because of insufficient time, was that we have recently completed a dose-response study using erionite. I did not receive the data in time to include them in my slides, but now I have the analyzed data. We observed exactly the same dose response, and erionite was somewhat more carcinogenic than chrysotile. In other words, erionite was the most carcinogenic dust, but only by a relatively small amount.

You have this complete anomaly: here is a dust (erionite) that is capable of producing mesotheliomas following inhalation but that is only slightly better than anything else at producing mesotheliomas following injection. This is one of the great mysteries. What is it about erionite that makes it particularly able to produce mesotheliomas by inhalation?

DISCUSSANT: The dose response to which you are referring is by which route of administration?

DISCUSSANT: Intraperitoneal injection.

DISCUSSANT: How did you compare your dose, by mass?

DISCUSSANT: By mass, but we also have fiber number data.

DISCUSSANT: Because we have used fiber numbers, it appears to us that, based on fiber numbers as the criterion, injected fibers are much more inclined to induce mesothelioma in the pleura, and certainly in much shorter time. There seems to be the following ranking order: chrysotile, crocidolite, and erionite.

DISCUSSANT: I think you are right there. Our data are not yet ready for presentation, but the erionite sample was very similar in fiber size and fiber number to UICC crocidolite.

DISCUSSANT: We used fibers/unit mass for crocidolite, but both crocidolite and erionite fibers were far fewer than chrysotile fibers, fewer by several orders of magnitude.

DISCUSSANT: From where did Chris Wagner get that erionite specimen?

DISCUSSANT: I believe from Rome in Oregon.

DISCUSSANT: Rome in Oregon, yes.

DISCUSSANT: He had two samples: one from Rome and one from somewhere else, and he had very similar results with them.

DISCUSSANT: I want to get back to Dr. Ferin's hypothesis that cancer is caused by increased asbestos exposure on account of decreased airway clearance, and the emphasis in Dr. Pott's presentation on the local effect of fibers on the airways and, of course, to the problem for the clinician: the fact that we only seem to see carcinomas in patients with asbestosis. A recent paper from Mt. Sinai describes about 130 patients with pulmonary carcinomas following asbestos exposure; every one of them had some degree of asbestosis. That is 100% of 130 patients—

DISCUSSANT: That is based on pathologic examination?

DISCUSSANT: On pathologic examination, right. These findings are quite convincing regarding the association between asbestosis and lung cancer. So the issue is really: How does asbestosis affect the incidence of lung cancer? How does it affect the carcinogenicity of fibers? How does it affect the development of cancer in the central airways? (And most tumors do develop in the central airways and not in the parenchyma.) If these data are true, why does this happen? Is it because there is concurrent fiber deposition in central airways? Why does asbestosis show such a strong association with lung cancer in the central airways? What is the mechanism here? If it were simply one of fiber deposition in the central airways, one wouldn't expect to see such a strong association with peripheral airway disease and asbestosis.

DISCUSSANT: I do not remember the details of this Mt. Sinai paper, but I doubt that it said that fibrosis was confirmed pathologically. Were these 130 tumors all centrally located? What portion was peripheral?

DISCUSSANT: I do not remember.

DISCUSSANT: I do not think it is central.

DISCUSSANT: That seems to be a rather significant issue.

DISCUSSANT: One of the things with which I am a little uncomfortable: you are really talking about the numerator, because you are talking about pathologic diagnosis and you really do not have a

population control with which to compare your results. I am sure most of us in this room appreciate that a pathologic diagnosis and a clinical diagnosis are based on X rays, which oftentimes can be at variance. We really do not have anything with which to compare these findings to determine whether or not many of those patients had clinical asbestosis. I believe that this is an important point.

DISCUSSANT: Take, for instance, the population of patients with asbestosis in the studies from Great Britain, in which the dose causing pneumoconiosis was certified by fiber count in all of those patients. The incidence of lung cancer in the certified asbestosis patients was extremely high, about 25 to 30%. Thus, the incidence of lung cancer in patients with asbestosis is extremely high as opposed to the incidence of lung cancer in a comparable population of workers who have the same asbestos fiber counts in their lungs, but who do not have asbestosis. We all agree that there is a population of workers with identical exposure who have very high fiber counts; some of them have asbestosis, and some do not. It is the ones who have asbestosis who seem to get the lung cancer, and the longer they have had asbestosis, the greater the incidence of lung cancer in that population. Therefore, fiber count does not seem to me to be the issue. The issue is asbestosis.

DR. ROE: I am coming back to Dr. Pott now.

DISCUSSANT: I only wanted to add that we, too, did not find any difference in the carcinogenicity of erionite and crocidolite after intraperitoneal injection. Up to now I do not have any explanation for the high mesothelioma rate in rats following erionite inhalation, as reported by Wagner et al. Did you see the slides? There should be some carcinomas too, because we have heard that, in Turkey, the lung-cancer incidence was high, too, not only the incidence of mesothelioma.

DISCUSSANT: I am not saying we should have seen lung cancers following our injection studies. We certainly didn't. I never saw Dr. Wagner's slides. I wish I had seen them.

DISCUSSANT: I have seen them. The animals died too soon to get lung carcinomas. They were exposed to the dust for a year, and most of them died within 14 months. That is only 2 months after termination of the dust exposures and too soon for lung carcinomas to develop.

DISCUSSANT: The Turkish data show an appreciable incidence of lung cancer in males. There were, of course, a lot of mesotheliomas, many more than one would expect with asbestosis. If these data are representative, erionite can be expected to produce more

mesotheliomas than lung cancers. It could be, I agree, that we sometimes get deaths from mesothelioma in intrapleurally inoculated animals within approximately 370 days. However, we certainly never see lung cancer in a rat at that age following asbestos exposure. It could be that erionite is peculiarly different from asbestos in that it is much more mesotheliogenic than it is carcinogenic for the lung.

DISCUSSANT: Most of us here would agree with this statement. The challenge is to find out why.

DISCUSSANT: We do not have many data, but we have some ideas and some preliminary experiments with erionite, because it is a fiber constructed so very differently from asbestos.

DISCUSSANT: I would like to comment on the question of fibrosis in relation to asbestosis and lung cancer. In experimental animals, we oftentimes see fibrosis. Animals—for example, dogs exposed to plutonium—develop pulmonary fibrosis, radiation pneumonitis, or a variety of nonstochastic changes that influence the incidence of lung cancer. This merely reinforces what Dr. Roe said earlier. Many factors influence the incidence of lung cancer. We have seen lung cancer in rats following chronic silica inhalation. Admittedly, it was a high dose of silica, but the lesions were epidermoid carcinomas, and at least one of them was metastasizing.

So, yes, I believe that there is an association between fibrosis and lung cancer in laboratory animals. We have at least some evidence to suggest that. You can see this broad association of fibrosis with that wide variety of nonstochastic changes that affect the incidence of lung cancer.

DISCUSSANT: The point I was making is that the risk of lung cancer in a population of people who do not have asbestosis may be relatively low compared to that in people with the same number of fibers who do have asbestosis. The fiber counts would be the same in both populations, but the risk would be much different because fibrosis predisposes to cancer development.

DISCUSSANT: Do we have definite data to support this conclusion? It is conceivable that the individuals who do not have asbestosis have a lower fiber count.

DISCUSSANT: We see people who do not have asbestosis with fiber counts that are equivalent to those with asbestosis, is that correct?

DISCUSSANT: Yes, but that is very hard to approach. The best data are from Dr. Wagner.

DISCUSSANT: How was the fiber count done? Was it done from a sample of the lung?

DISCUSSANT: Yes.

DISCUSSANT: So here we have that problem which Dr. Martonen already mentioned. If you analyze a sample of lung tissue and extrapolate from that sample to the total lung, your conclusions may be correct, depending on the size of the sample. But we are emphasizing now the local aspects of the events which happened, from deposition up to the local changes. Therefore, when I mentioned that dose would be the important factor, I meant the dose at certain local spots.

For example, if there are changes in the ciliary epithelium of the airways which focally affect the function of the ciliary escalator, it is possible that the total clearance may not be so much affected as it is affected in some spots. In that case, the dose becomes much larger in these spots.

You can then also ask the question: "Why is it so rare that lung carcinoma after asbestos exposure usually occurs on only one side? Why on that one side? Why, if lung cancer is such a common thing, why doesn't it occur suddenly in 10 places in the lung?"

DR. ROE: Everybody is aware of the diagnostic problems with asbestosis and of the fact that it is usually just a clinical diagnosis, made using radiology. It is insensitive and often misleading, compared with diagnosis based on pathology. The pathologic evalution must be performed appropriately and is more reliable when based on an autopsy rather than on a small tissue sample. But I perceive in the published literature that experimentalists sometimes are very glib in using the term "asbestosis," when, in fact, all they have seen is a lesion in the vicinity of the terminal bronchioles that I call cuboidal metaplasia of the alveolar epithelium. And without any serious evidence, even of fibrois, some people publish papers and illustrate lesions which they call "asbestosis." They use the term simply because the animal happens to have been exposed to asbestos, and not because the lesion suits pathological criteria for the diagnosis of asbestosis. The literature is polluted by this sort of misleading thing. In fact, you can produce exactly the same lesion by exposing animals, for example, to nitrogen dioxide.

Am I correct, or do people disagree with me as to the use of the term?

DISCUSSANT: I do not believe that you produce the same lesion with nitrogen dioxide as you do with asbestos. You do get an alveolar change in the acini with nitrogen dioxide, and you eventually get emphysema, beautiful emphysema, from it, but I do not think it mimics what you see in asbestosis; you don't see the fibrosis there.

DR. ROE: I am not disagreeing with you. I am saying that the literature is polluted by papers where the lesion illustrated does not really include fibrosis, and yet, because the animals have been exposed to asbestos and have some sort of lesion, people put the term "asbestosis" underneath.

DISCUSSANT: I agree with you entirely. I think that in order to use the term asbestosis, you do need evidence of excess fibrosis, particularly in the alveolar walls. Many experimentally induced lesions do justify using the term asbestosis, but in many other papers the term is used for trivial lesions.

DISCUSSANT: Is it necessary to have asbestos fibers within the fibrous tissue in order to call the lesion asbestosis?

DISCUSSANT: Philosophically, I am not sure whether this is necessary. However, in reality, you almost invariably do find fibers.

DISCUSSANT: With respect to your comment, I think one really has to define the terms. In our case, in 1984 we were looking at the earliest lesions of asbestosis, and we demonstrated that the earliest lesions begin 48 hours after just a 1-hour exposure, using a technique called ultrastructural morphometry.

Clearly, that isn't asbestosis, and we didn't mean to imply that it was. However, it is a lesion that is irreversible, because 1 month later, after the 1-hour exposure, that lesion has expanded, and we are presuming that ultimately it progresses, particularly with continuing exposure.

We called it "the earliest form of asbestosis," but we did not mean to imply that it met the textbook criteria for fully developed asbestosis. As a matter of fact, if you looked at those bifurcations, you wouldn't be able to recognize any changes unless you did an ultrastructural morphometric study, using transmission electron microscopy. It thus really depends on whether it is mechanistic, or whether someone just exposed some animals and subsequently tried to diagnose or label what was going on. I therefore believe that the intent is as important as the label.

DR. ROE: When people define their terms, I have less of a problem, but there are some papers in which the terms are not defined. The experiment has been conducted by a nonpathologist, and the slides were subsequently shown to a pathologist, who hears that there has been exposure to asbestos, and he labels it asbestosis because he sees a lesion there. It is a very poor use of terminology. I am not criticizing work where terms have been properly defined.

DISCUSSANT: I would like to respond to the question of whether there is inconsistency in the proposed hypothesis regarding the

dose. If the clinical evidence shows that there is asbestosis in almost all cases of carcinoma after asbestos exposure, I do not think there is an inconsistency there; quite the opposite.

If this hypothesis should be proven, then a depressed clearance mechanism will result in more fibers, and if the dose has an effect on asbestosis, which I believe it has, then one shouldn't be surprised to also find asbestosis and cancer.

Of course, as I emphasized, this is only the first step in a sequence of events. Even identical exposures and focally identical fiber retention do not mean an identical pathological response. There is an additional chance to develop a cancer in that spot.

As for the fiber numbers, I have already responded. I believe that a tissue sample—even a small sample—gives some indication of exposure, but it is not necessarily indicative of focal changes where either asbestosis or scar changes occur, or cancer starts.

DISCUSSANT: One more point. You can expose two animals or two humans to high concentrations of asbestos. Very little fibrosis may develop in one of them, while the other may respond with severe fibrosis. This is, I suppose, a genetically determined difference. The point is, one has to recognize that, in announcing theories on dose-response relationships, there are also other factors involved, perhaps genetic factors.

DISCUSSANT: If the effects of asbestos on the airways are local, one would anticipate a greater concentration of asbestos fibers in the central airways where these tumors develop. I do not think that is where you find most of the fibers; you find them in the respiratory bronchioles and not in the central airways.

I am just being a devil's advocate—I am saying: "Look, if your hypothesis postulates that cancer development is due to a direct effect on the central airways, show me that data that demonstrate that the fibers that caused it are there." There are other explanations for why patients with asbestosis develop central tumors, but as far as data are concerned that show that there are more fibers, I do not know of any.

DR. ROE: I think the weakness of what you are saying is that within any part of the lung there are perhaps 30 different types of cells, and unless you know which is the target cell or cells, I do not think it is realistic to say: "This cubic centimeter of lung contains so many fibers, therefore the risk is so much." You have to look at the structure of the cells, and you have to know more than we know now.

DISCUSSANT: Sure; absolutely. But I think that needs to be pointed out.

DISCUSSANT: From what I hear, fiber count appears to emerge as an important variable. Is there a standardized way, a protocol, by which fiber count is determined which says something about sampling procedure and counting procedure, so that you can compare fiber counts from one study with fiber counts from another study?

DISCUSSANT: We can study the fibers in the whole lung in the rat, but you can't do that in humans.

DISCUSSANT: Even if you can, it does not help. There was a meeting in Oxford, I suppose 3 years ago, which resulted in the creation of standard samples that were sent to various laboratories for analysis. All participating laboratories in the study did reasonably well in identifying samples as having high or low fiber counts; however the absolute numbers ranged all over hell.

DISCUSSANT: You have to get the appropriate materials. Pathologists select materials that interest them—usually lesions of various kinds. So, in terms of some statistical sampling of the pulmonary structure, that is out to begin with; secondly, people use light microscopes. We saw a lot of photographs, photomicrographs, light microscopic analyses, and pulmonary tissues with fibers. It is archaic. You only see the tip of the iceberg, depending on fiber type and other factors. Generally, fibers observed by light microscopy can vary in length over four orders of magnitude.

In terms of the instrumentation, we prefer using transmission units, analytical electron microscopes, and diffraction chemistry by one of the energy-dispersive spectrometry methods. You can go along and count, and you can count very well, and you come up with numbers, and the numbers represent a great range, and the ranges reflect low, moderate, or heavy exposure concentrations, and we have some numbers to support that. It is not as bad as some people would have it.

DR. ROE: Anybody who has been there knows that you are right.

I have to go on now and ask specifically for any questions on Dr. Davis's presentation.

DISCUSSANT: There are various studies that show that chrysotile has relatively fast clearance. You have also pointed them out. Do you have any idea why chrysotile is so different from other fibers, especially with respect to fast clearance?

DISCUSSANT: Well, I was speculating this morning that, when we use the term "clearance," we simply mean it disappears from the lung tissue. I was suggesting, in the case of chrysotile, that a lot of it actually dissolves in the lung tissue, in addition to the mechanical

clearance. Certainly you can show that chrysotile dissolves very easily in a number of very mild chemical solutions. At the present time, we and, I think, a lot of other people, are trying to get general data on the rate of chrysoltile dissolution in lung tissue, but results are not yet available.

DISCUSSANT: We, too, have a lot of problems. We have seen that fibers split in the lungs, and therefore the number of fibers increases. Over a period of 1 to 2 years, you will get many more fibers at the end than at the beginning, but they are very thin. We have subjected them to analytical transmission electron microscopy.

DISCUSSANT: We found that almost no chrysotile remained in the lungs of 3-year-old rats. We have not done the same detailed studies at 2 years. We looked at 18 months and, in that case, not much chrysotile had cleared. There was about 20 or 30 μg of chrysotile in the whole rat lung, which can still amount to a lot of fibers. But I agree with you, you get a separation of chrysotile fibers into fibrils, so the first effect is an increased number of fibers.

DISCUSSANT: I wonder whether that is not the so-called "healthy-rat" effect. Rats on study that survive for 3 years usually are the ones that did not develop asbestosis and no longer have any chrysotile fibers in their lungs. You are therefore looking at a very skewed population of animals. They are survivors.

DISCUSSANT: To a certain extent that is true, but the fact that they have survived does not mean that they have nice, clean, healthy lungs. They very often have the most severe degree of asbestosis that you have ever seen.

DR. ROE: I would like to comment on this because the terms "healthy-rat effect" and "healthy-worker effect" have been misused today. Let us be perfectly clear. The healthy-worker effect refers to a population of people who are fit to work and are, therefore, fitter than a population that is unfit to work. Consequently, the former population tends to experience a lower incidence of certain diseases than the latter.

The term "healthy-rat effect" has been used in a totally different sense and also wrongly, in my view. If you want rats or hamsters to live longer, you restrict their feed intake. They not only live longer, they get less cancers of all sorts, including cancer of the lung. Hypocalorically maintained rats also develop fewer endocrine dysfunctions and fewer endocrine tumors.

Now, if you want to use the term "healthy-rat effect," that is the sort of thing you ought to be talking about. If you are not age-standardizing the data in your studies, then you should do so. To

undertake a study and end up with different incidences of this or that, and then to say it might have been because the animals lived longer than the controls is a position in which you should not be caught. You should conduct your study in such a way that your data are age-standardized, using the appropriate statistics for fatal lesions and incidental lesions. However, it is not always possible to do this.

DISCUSSANT: Can I come back and say, quite simply, that of course we have control populations that usually live slightly longer than the asbestosis population. Sometimes there is no difference. But it is never the other way around. The controls never die first.

DISCUSSANT: I have a question regarding intraperitoneally injected rats. Were those mesotheliomas fatal or nonfatal? Did the rats die and did you then find the mesotheliomas incidentally, or were they diagnosed before the animals died?

DISCUSSANT: Do you mean, were they actually fatal or potentially fatal? They were potentially fatal 100%, yes, but we try to diagnose mesotheliomas in the animals when they are still alive. This can be readily done because peritoneal mesotheliomas are associated with ascites, which results in abdominal distension.

DISCUSSANT: Did you have to euthanize them?

DISCUSSANT: That is right. That does not affect the diagnosis.

DISCUSSANT: Did only your animals with parenchymal tumors have asbestosis or did all animals have asbestosis?

DISCUSSANT: By and large, yes. There is a very good correlation between what I think you can genuinely call asbestosis and the presence of pulmonary tumors, usually in the same animals, but certainly in the same population. We have just discussed that there is very good evidence that lung cancer in asbestos workers is associated with the presence of asbestosis. We can therefore say, yes, that is scar cancer.

Now, asbestosis is always, by definition, parenchymal or peripheral; it does not occur in the main bronchial tubes. So if the statement about a close relationship with scar cancers is correct, all asbestos tumors in human beings ought to be peripheral. The discussion earlier indicated that this certainly was not the case. So we have a lot of anomalies to tie up.

DISCUSSANT: Diffuse interstitial fibrosis increases the incidence of lung cancers in a fair number of people. Then you get both central and peripheral tumors, and maybe it has nothing to do with this, or there may be a common mechanism.

DISCUSSANT: And any cause of fibrosis increases—

DISCUSSANT: Any diffuse fibrosis; collagen disease, for example. There was a good article in *Thorax*, I suppose about 5 years ago, showing this. The author was mostly looking at fibrosis, alveolitis, and collagen disease. They all were associated with an increased incidence of all types of tumors. My recollection is about a ninefold increase, and there were both central and peripheral tumors.

DISCUSSANT: We do not know, but I do not believe that the number of tumors associated with interstitial fibrosis is quite as great as the ones with asbestosis. I see a lot of these patients, and we certainly see an inordinate increase in lung cancer, but I think the ones with significant asbestosis have a greater increase of lung cancer.

DISCUSSANT: My point is that diffuse scarring predisposes for lung cancer, peripheral and central, and that predisposition is not specific to asbestos.

DISCUSSANT: Right. We can conclude from your statement that just diffuse fibrosis alone would not explain the carcinogenicity of asbestosis, and I think it is more than that. I think we all agree on that.

DISCUSSANT: In your study, such concepts as fiber length and fiber diameter appear as quite variable. Is there a standardized way in which one samples fibers to determine length and diameter?

DISCUSSANT: Well, there is a recommended way for industrial use. In our studies, we followed this procedure fairly closely. We collect fibers on Nuclepore® filters, but otherwise the procedure we use is the same as that recommended. The procedure for counting fibers, of course, is also standardized.

DISCUSSANT: So, if somebody else repeats the study, his numbers should be comparable to your numbers.

DISCUSSANT: Provided that he uses the same collection techniques, deposition techniques, and the same initial dust count. It is losing these variables that people forget to allow for. They tend to talk about a cloud of chrysotile or a cloud of crocidolite, assuming that they are identical and, of course, they are not. It depends on how you generate the aerosols. They are tremendously variable from two different origins. If the fibers are properly counted and sized, you should see no difference; but differences will always be there.

DISCUSSANT: You may have noticed that there was a discrepancy between your amosite clearance data and those of Dr. Churg.

I believe you reported that for both the uncut and the cut amosite, there was very little clearance from the lung compared to that for chrysotile. Dr. Churg's data seemed to show that amosite clearance was fairly nominal compared to that for the other types of asbestos. I was wondering whether you might comment or have any idea why there might be a difference.

DISCUSSANT: No, I have not. We were simply comparing four dust samples, and our data did seem consistent. We observed much greater clearance in this period of 6 months with both chrysotile samples, but both the long-fiber samples cleared more quickly than the short-fiber samples. As far as other reports on amosite clearance are concerned, one is often in this position. We just cannot explain these discrepancies.

DR. ROE: Are there specific comments on Dr. Churg's paper?

DISCUSSANT: I have one comment regarding the fibers that are retained in the alveolar wall. How sure were you that you did not scrape off the epithelium during the administration procedure and that the epithelium just grew back over the inoculated dust? There is good evidence that this has happened in the case of chrysotile.

DISCUSSANT: That is a difficult problem. A number of people have said, and I think it is probably true, that one does get some ulceration. They are convinced that this is the major mechanism for penetration of fibers into the interstitium. It may be true. We did not look at each epithelial specimen.

Of course, the smoke is still augmenting the necrosis.

DR. WEHNER: I am surprised by your low carboxyhemoglobin values, 5%. Normally one reads about 10, up to 40, even 50%. In our animals we have observed anywhere from 10 to 40%, depending on the smoke concentration. I wonder, do you have an explanation for your low COHb values? Are they perhaps typical of the guinea pig?

DISCUSSANT: I do not know whether it is particular to the guinea pig. You realize, of course, that anytime you change the geometry in the smoke administration you are going to change what's going into the animal.

All I can tell you is, we did run this against a set of nonexposed controls.

DR. ROE: When were the blood samples collected?

DISCUSSANT: Immediately after exposure. They smoke their 10 cigarettes, and then the animals are sacrificed, following collection of the blood samples.

DISCUSSANT: I was wondering whether you would clarify a point that I thought I heard earlier. It was said that smoking increases the penetration of the asbestos particles, and some of the speakers have discussed impaired clearance caused by cigarette-smoking. I am just not clear on this. When you say "increased penetration," do you find that smoking causes the particles to deposit further down the airways, down the smaller airways, or do you mean that more fibers find their way through the epithelial layer?

DISCUSSANT: I am referring to literal penetration into tissue rather than distal penetration into the lung. The material goes from the airways into the interstitial space.

We have not looked at distribution. Because we were using intratracheal instillation, that may be a bit treacherous. Two things are really separate: one is clearance, or bulk removed, whichever way you want to measure it; the other is penetration of the epithelium as seen under the microscope. This penetration is increased in the smoker's lung samples that are collected from the same area of the respiratory tree.

DISCUSSANT: I wanted to hear Dr. Churg's comments on the macrophage storage phenomenon. You showed some photomicrographs of macrophages in which amosite was nicely stored. Was that observed by light microscopy?

DISCUSSANT: No. We simply took a macrophage pellet, dissolved it, and processed it just as if it were a tissue sample.

DISCUSSANT: I see. So it is not actually quantified by TEM.

DISCUSSANT: Well, the quantification comes from the dissolved pellet. We are not looking at individual macrophages.

DR. ROE: Incidentally, one part of the so-called overloading phenomenon is associated with the alveolar macrophages which aggregate in some alveoli. The storage of particles in these macrophages constitutes a part of the lung burden of particles. For some reason, these macrophages lose the capability to move normally and start to aggregate in alveoli, usually in alveolar ducts near the terminal bronchioles. These macrophages obviously are, in some way, functionally impaired. They may have phagocytized some fibers and really act as depots, staying in the alveoli for long periods.

DISCUSSANT: One could argue that we happened to pick just the right combination of smoke and asbestos dust to kick the animal into overload, but I would rather not believe that. I think overload is very real.

DR. ROE: Smoke particles also contribute to the overload. If you have a combination of asbestos and cigarette smoke and measure only the asbestos in the lung, you do not reach that magic overload number of 2 mg/g of lung tissue because you are analyzing only for asbestos, but there is also some contribution from the cigarette smoke.

Are there specific questions for Dr. Muhle?

DISCUSSANT: I believe one of your results was that the clearance of crocidolite was impaired twofold, and that chrysotile was unaffected, is that right? I am really intrigued by possible mechanisms that may be operative. Normally, I would think that cigarette smoke might have an impact on macrophage mobility or phagocytosis, but that this should differ for different forms of asbestosis is very interesting. Do you have any explanation?

DISCUSSANT: I speculate about this in my paper, but I do not really know whether my speculation is correct. I am referring to the results of Dr. Wagner, who also found, after exposing rats to chrysotile, a steady state after 3 months. According to the same study, during a 24-month exposure to crocidolite, an almost linear increase in retained fibers was observed. Possibly, the deposition mechanism may be different in the upper airways for both fibers. For chrysotile, it is more the clearance in the upper airways, and this may not be impaired by the cigarette smoke. Whereas with crocidolite, we may have higher deposition in the deep lung. That is one possible explanation.

DISCUSSANT: I wonder whether there is not another explanation, namely that cigarette smoke can impair mechanical clearance of both types of asbestos. In addition, chrysotile undergoes simple chemical dissolution, which is not affected by cigarette smoke. I wonder whether that is partly the explanation.

DISCUSSANT: It is possible.

DISCUSSANT: I would like to take that one step further. What if the rate of dissolution is sufficiently high that a two- or threefold increase in, let us say, macrophage retention is simply trivial by comparison? One is then dealing with a situation in which the retention of crocidolite is effectively doubled. Let us assume that the retention of chrysotile is also doubled. If special mechanisms operate, the rate of those mechanisms may be so fast that one cannot get above that baseline except by an extraordinary increase in retention. In other words, if smoking were to lead to retention of 100 times as much chrysotile, one might detect it. But if smoking leads to the retention of only two or three times as much chrysotile

in smoke-exposed subjects as in nonsmokers, one would not be able to see it, because its rate of dissolution in the lung is too rapid.

DISCUSSANT: If the dissolution is very high, then, obviously, the pathological response is completely different. There are examples for materials other than asbestos: one is zinc oxide, and another is silica. Experiments with silica—silica in a form which had very small particles—produced inflammation, edema, and so on, that led to the death of most rats. But those which survived no longer had any silica in the lungs, because it had translocated from the lungs to other tissues, which means clearance was complete. On the other hand, most of the rats died!

Zinc oxide is relatively soluble in the lung and will be cleared extremely fast, not through macrophages but probably by dissolution.

Permeability is an additional factor. Say that some substance increases the permeability of the originally tight epithelium; it may, in this way, enter the interstitial space. In those circumstances, obviously, the pathological response would be different.

DR. ROE: Surely the gaps between some cells are not that large. The chance of an asbestos fiber actually hitting a gap is not going to be that great.

DISCUSSANT: It can go through the cell. It does not have to go through the gap.

DISCUSSANT: Exactly. So the gaps are irrelevant.

DISCUSSANT: Not in asbestos penetration. I said there was increased penetration. Some substances change the permeability of the otherwise tight epithelium by either increasing the gap or affecting the epithelial cells themselves so that the penetration is increased. This means that the permeability and the absorption are increased. In that case, the compounds disappear from the lung; they are cleared, but not by the type of clearance that we are discussing mostly: namely, clearance by alveolar macrophages.

DISCUSSANT: Am I missing something? If I were going to attack something with the intention of creating a penetrating wound, I would choose a straight thing for a weapon. How does the penetration theory account for chrysotile, a wiggly thing?

DISCUSSANT: With chrysotile?

DISCUSSANT: Yes. Some people have proposed that penetration is an important factor. How is it going to happen with chrysotile, which is wiggly?

mucus cover. Then they analyzed the surfactant content and found some three to five promille of surfactant lipids there.

The distribution, of course, is not clear from that article, but the authors also tried to redistribute this three to five promille of surfactant lipids within the mucus layer and found that the rate of penetration of highly lipophilic substances does not differ that much. These substances will get trapped within the lipids as soon as they hit a lipid phase. Of course the model is highly dependent on the existence of a continuous mucus layer and its lipid content. On the other hand, the consequences of a missing lipid-aqueous mucus layer are implicitly predicted by the model and should be as interesting. This should result in more localized and considerably higher cellular doses of lipophilic substances at places in the epithelium that are not covered by mucus.

DISCUSSANT: The list of carcinogens which have been identified in tobacco smoke is quite long. Do you think it is justifiable to confine interest in the adsorption theory to polycyclic aromatic hydrocarbons? Do you think some of the other classes of substances ought to be looked at? And is it just a matter of time, is it just a matter of habit, or is there some other justification for selecting PAH?

DISCUSSANT: Well, not being a pathologist, I cannot judge whether PAH is a good choice, but our model should apply to essentially any highly lipophilic substances. Most lipophilic substances of higher molecular weight would probably behave rather similarly. If they have a partition coefficient of, say 10^6 or 10^7, they should behave roughly in the same manner.

DISCUSSANT: Some people would think that nitrosamines are important. How would they behave?

DISCUSSANT: I guess their average molecular weights are smaller, and their lipid-aqueous partition coefficients also are lower. Probably the model is not as applicable to those substances, or, rather, to the assumption that a major resistance to mass transfer of genotoxic substances lies within the mucus layer. But again, this limitation is as interesting. The lower the lipid-aqueous partition coefficient, the more rapid the uptake by the epithelium will be. The mechanism for this is the same as that for the accumulation of lipophilic substances in body fat, known to all toxicologists.

DISCUSSANT: Your model depends on a fiber nosing down into a thick—I believe it is a thick—mucus layer. Have you had any data or done any modeling on how fibers orient themselves in a cilia-driven mucus layer?

DISCUSSANT: I think it was Woodworth et al. who took biopsies of the upper bronchial tree and found fibers—I think even chrysotile fibers—that were partly phagocytized also by the epithelial cells. I believe many of these cells were squamous metaplasia cells. Fibers started to become phagocytized even by the epithelial cells themselves, but whether this is sufficient to account for fibers really penetrating the mucus layer in this fashion, I suppose no one could tell today.

DISCUSSANT: I was trying to get a picture of how the fiber could begin penetration. When the fiber is floating on the mucus, how could it nose in?

My other question: Did you measure the specific surface area of your fibers? I think it would be very interesting to see your graphs normalized to specific surface area.

DISCUSSANT: You mean in the adsorption of PAH?

DISCUSSANT: Just the total available surface area of your glass fibers and of your asbestos fibers, using nitrogen adsorption or a similar technique.

DISCUSSANT: I believe the average specific surface areas differ, roughly, by a factor of 100 between the rock wool samples and the asbestos fibers.

DISCUSSANT: And that was the difference between the surface areas of your two fiber types?

DISCUSSANT: Roughly, yes.

DISCUSSANT: So, what you are saying is, when you added water vapor, the water molecules were filling the cracks in the asbestos fiber and reducing the available surface area.

DISCUSSANT: Well, I think this desorption started even before there was a complete monomolecular layer of water on the fiber surfaces. Water molecules seemed to compete for roughly the same active sites on the fiber surface as the hydrocarbons.

DISCUSSANT: One of the things that cigarette smoke is known to do is to disrupt pulmonary surfactant or a phospholipid mucous membrane. To what extent would that action of cigarette smoke sort of blow a hole in your theory? Is the functional integrity of that phospholipid membrane very important for your hypothesis to work, or could it be that it would work even for a partial injury?

DISCUSSANT: I think if you translate the lipid content of the central airways into a number of monomolecular layers, you would obtain some seven or eight monolayers, continuous layers of lipids,

even in the central airways. Of course, if you go below one continuous layer, then the fiber hypothesis would run into trouble, but not the model for the protective properties of the undisturbed lipid-aqueous mucus layer.

DISCUSSANT: I would like to know whether we have direct evidence that tumors appear at bifurcation sites. There are some pathologists who say that there are no such data. We have an assumption that this is where they are appearing. I do not take that position.

DISCUSSANT: I will ask you—and perhaps the others will, too—whether you are looking at humans. By the time you first see a lesion, it extends way beyond the small area of the bifurcation site. It would be a bold person who would say exactly where it did arise.

There is even a further problem. When you have tumors which you discover rather late, they spread toward the hilar area of the lung. So, when we were talking earlier about peripheral and central tumors, the fact is that tumors always spread towards the center. For this reason, the first diagnosis of even a peripherally arising tumor may be of a centrally placed shadow seen in the chest X ray.

Now, there are articles in the literature on populations of people with lung cancer of whom a series of X rays had been taken previously—diagnostic X rays, screening X rays—in which no lesions had been discovered. And there are claims that, when these same X rays were re-examined retrospectively by someone who knew the end result, they often found a lesion which was much more peripheral than the one that was eventually discovered. So all the discussion that we had earlier as to whether tumors are peripheral or central is clouded by the difficulty or impossibility of knowing where they arise.

DISCUSSANT: My question is more specific. I am talking about bifurcations versus the bronchial tubes.

DR. ROE: The answer must be that there cannot possibly be any reliable information on that. Auerbach and his colleagues provide information on the distribution of precancerous lesions and, I believe, reported some tendency for tumors to occur near bifurcations. However, that does not prove that "full-blooded" cancers arise perferentially at bifurcations. There is a report, based on the use of a fluorescent technique, on the distribution of epithelial changes that are not detectable by other means. According to those who have used this technique, it seems that many of the early lesions are *not* at bifurcations but in other areas of the airways. There is no big, long study that used that technique in humans, but that technique is going to be the one, I believe, that is going to answer your question.

I think it is very important. I do not think there are enough data yet to warrant any conclusion.

In some rat studies where there has been very heavy exposure to tobacco smoke, the squamous metaplasia has gone all the way down the trachea. There was no special tendency for it to occur at bifurcations.

DISCUSSANT: I have one point. I wanted to take up the statement that was made in the paper by Dr. Mossman on penetration of fibers in areas of squamous metaplasia. Would such an area be covered by a layer of mucus?

DISCUSSANT: I do not know.

DISCUSSANT: Is there any information regarding electrical charges on asbestos fiber? Does the adsorption of certain chemicals produce a charge which affects the orientation, so that this fiber—if it develops a charge—becomes polar? Could it then change its orientation vis-à-vis the airway and penetrate the cell because of a change in charge? Such phenomena—if they exist—may be related to subsurface qualities. Is there anything known about the impact of charges on the orientation of fibers?

DISCUSSANT: Variation in the amount of adsorbed water vapor might significantly affect surface charge.

DR. ROE: Last question, last comment.

DISCUSSANT: We have gone into a considerable degree of detail thinking about the environment in these airways. We envision curly fibers and straight fibers floating placidly along this relatively uniform tube in this warm, moist environment. Something we need to remember in our catalog of factors that can perturb that environment is the fact that mucus layers are, at least partially, discontinuous.

I think it is reasonable to assume that the ciliated surfaces could probably be bare in places. Areas of metaplasia were just mentioned; I think it is reasonable to assume that those could be bare, at least for part of the time. So we can accept the probability that fibers might encounter the cells directly.

But there is one factor that has not been mentioned: one of the characteristics of smokers is that they have a great tendency to cough. The more they smoke, the more they cough; and the longer they smoke, the more they cough. We know quite a bit about the physiology of the cough, and it is an awesome thing to behold on a microscopic level.

During coughing, the glottis is closed, and tremendous pressures are generated. The epithelia of large airways are then exposed to

violent bursts of air passing over their surfaces. These are surely potentially damaging. They might even strip off the epithelium.

As you proceed peripherally, these flow-limiting segments go peripherally and stop at some point that is determined by the elastic properties of the lung. But at that point, the number of airways is so large that the mass flow and the shear force must be fairly small.

The reason why we cough is because—I am being teleological, of course—we can develop enough shear force along that epithelium to strip mucus, to shake it loose. I can imagine that if there is a discontinuity in the epithelial surface—e.g., metaplasia, whatever—that this discontinuity would be a focus of shear force too, an irritating focus.

Therefore, I believe that it is useful to crank those factors into our thinking, too, when we are cogitating about forces that are perturbing these airways.

DISCUSSANT: There probably is an orientation effect on the deposition of long, straight fibers at bifurcations. They would deposit like javelins.

DISCUSSANT: It is much more likely during a cough, during exhalation than during inhalation. That is the point I was trying to make. If you envision projectiles going down the airways and attacking, you'd like to think of it happening during inhalation. I am suggesting you might also want to think of it during exhalation. You could make a much better case for it.

DR. ROE: Any further comments? If not, this concludes the discussions for today. Thank you very much.