

BIOPERSISTENCE OF SYNTHETIC MINERAL FIBERS AS A PREDICTOR OF CHRONIC INHALATION TOXICITY IN RATS

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In December 1997 the European Commission (EC) adopted Directive 97/69/EC (O.J. L 343/19 of 13 December 1997) in which criteria were established for the classification and labeling of synthetic mineral fibers. This directive was derived based upon an extensive program evaluating current scientific knowledge on fiber pathogenicity and its relationship to the biopersistence of long fibers. Within this context, the biopersistence of fibers longer than 20 μm was found to be a good predictor of the lung burden and early pathological changes in chronic inhalation studies with fibers as well as of the tumor response in chronic intraperitoneal studies with fibers. The analysis that provided the scientific basis for the relationship of biopersistence to the chronic inhalation results is presented in detail. Proportional odds regression techniques were used to determine the relationship between both inhalation and intratracheal instillation biopersistence clearance half-times and the collagen deposition at the broncho-alveolar junction as determined following 24 mo in chronic inhalation toxicity studies. The results indicate all the indicators of biopersistence considered are equally good predictors of the early long-term change that occurs in the lung in response to more durable fibers. This change, the collagen deposition at the broncho-alveolar junction, is a precursor of interstitial fibrosis, which has been shown to be associated with tumor response in fiber-exposed animals. The results show that the clearance half-times set in the EC directive are within the baseline for this parameter.

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In December 1997 the European Commission (EC) adopted Directive 97/69/EC (O.J. L 343/19 of 13 December 1997; European Commission, 1997) in which criteria were established for the classification and labeling of synthetic mineral fibers. This directive was derived based upon an extensive program evaluating current scientific knowledge on fiber pathogenicity and its relationship to the biopersistence of long fibers. Within this context, the biopersistence of fibers longer than 20 μm was found to be a good predictor of the lung burden and early pathological changes in chronic inhalation studies with fibers as well as the tumor response in chronic intraperitoneal studies with fibers. These results were presented in a preliminary form in an internal report to the European Chemicals Bureau (1997b; ECB/TM/15(97)). This article summarizes the report and expands the analysis that provided the scientific basis for the relationship of biopersistence to the chronic inhalation results. A parallel publication presents the basis for the relationship of biopersistence to the chronic intraperitoneal (ip) results (Bernstein et al., 2001).

The association that long fibers (20–50 μm) have with both lung and peritoneal disease as opposed to shorter ball-milled fibers (3 μm or less) was reported as early as 1951 (Vorwald et al., 1951). In one of the earlier studies investigating the biopersistence of synthetic mineral fibers (SMF), Hammad (1984) found that fibers <5 μm in length had the longest retention following short-term inhalation, with longer fibers clearing more rapidly and fibers >30 μm in length clearing very rapidly. He proposed that clearance of mineral wools is a result of biological clearance and the elimination of fibers by dissolution and subsequent breakage. However, there was no relationship of these phenomena to long-term toxicological effects.

Early chronic inhalation studies of fibers were often performed without consideration of the respirability of the fibers in the rat and without preserving the length distribution of the fibers. As commercial insulation synthetic mineral fibers are usually thick and long, investigators would often grind the fibers to produce a more respirable fraction. This process frequently pulverized the rat respirable long fiber fraction.

In 1988, a series of well-designed chronic inhalation studies on synthetic mineral fibers was initiated at the Research and Consulting Company Ltd. (Itingen, Switzerland) by the Thermal Insulation Manufacturers Association, TIMA (Hesterberg et al., 1993, 1995; Mast et al., 1995a, 1995b; McConnell et al., 1994, 1995). These studies were the first of their kind to take into account the respirability of mineral fibers in the rats and the importance of fiber length. The results of the studies indicated that the more soluble fibers tested showed little or no pathogenic response, while less soluble fibers showed some response.

To further investigate this, a protocol was developed for the evaluation of the biopersistence of SMF (Bernstein et al., 1994; Musselman et al., 1994), and all the fibers from the chronic TIMA studies were evaluated

for their biopersistence. Subsequently, additional studies were performed on some of the synthetic mineral fibers included in this analysis (Bernstein et al., 1996; Hesterberg et al., 1998). In these early studies, there was no standardization of the statistical methods for determining the clearance half-times of the fibers tested. This resulted in values that were not always comparable between studies, as these protocols were not standardized.

To resolve this difficulty and permit the determination of the relationship of biopersistence to toxicity, standardized biopersistence protocols were developed at the European Chemicals Bureau (ECB) ("Ispra Protocols") and were subsequently published as interim protocols (Bernstein & Riego-Sintes, 1999). In parallel, all interested parties who had performed either inhalation or intratracheal instillation biopersistence studies using protocols roughly following those developed at the ECB were offered the opportunity to submit such data for a unified data analysis. The procedures for data analysis used are outlined here and follow those described in the Ispra Biopersistence Protocols. The results were compiled in the EC report ECB/TM/11(97) (European Chemicals Bureau, 1997a), and only results included in this report were used in the analysis of biopersistence to chronic toxicity.

Following this phase, a request was issued to concerned parties requesting submission of chronic inhalation and ip study data for use in investigating relationships between the results of biopersistence studies and chronic fiber toxicity studies.

DATA

Biopersistence Data

The individual data of the number of fibers by size category ($<5\ \mu\text{m}$, $5\text{--}20\ \mu\text{m}$, $>20\ \mu\text{m}$, and WHO fibers) were received for analysis from the Fraunhofer-Institut für Toxikologie und Aerosolforschung, the North American Insulation Manufacturers Association and Isover Saint Gobain. The data were analyzed using nonlinear regression techniques using the statistical computer program Statistica (StatSoft, Inc., 1997). The data were fit to a double exponential, or a single exponential if the single exponential provided an equally good fit to the data. In all cases when performing the regression the loss function was weighted by the inverse of the variance and asymptotic standard errors obtained.

Chronic Inhalation Toxicity Studies

The numbers of fibers and exposure groups included in the analysis were limited by the available data on biopersistence. For the relationships of biopersistence to chronic inhalation, the chronic studies included were the original TIMA studies referred to earlier. Five fiber types were evaluated

in these studies with a total of 15 exposure groups. The fiber compositions ranged in solubility from ceramic fibers (RCF 1) to slag wool (MMVF 22). Thus the inhalation data set provided a suitable range in fiber solubility for assessing relationships of chronic toxicity response to biopersistence.

The individual fibers types included in the inhalation analyses presented in this report were as follows: RCF 1 (ceramic), MMVF 21 (stone wool), MMVF 11 (glass wool), MMVF 10 (glass wool), and MMVF 22 (slag wool).

As already mentioned, these include the results of the fiber chronic inhalation studies performed at the Research and Consulting Company Ltd (RCC), sponsored by the Thermal Insulation Manufacturers Association (TIMA). This series of studies, initiated in 1987, used protocols in which the fibers tested were preselected to be generally rat respirable and with a minimum length criteria. In addition, the lungs were examined sequentially throughout the study for lung burden, thus permitting the investigation of relationship between lung burden of specific size fractions and effects. While many other fiber inhalation studies have been performed, these have not been included due to significant differences in exposure criteria, procedure, and analysis.

The chronic inhalation studies evaluated five types of fibers using multi-dose exposures, as already listed. Two additional RCF compositions were investigated; however, biopersistence data were not available. In addition, the RCF 1a fiber evaluated by inhalation biopersistence was of the same composition as the RCF 1 tested by chronic inhalation but with significantly fewer nonfibrous particles present.

The lung burden was determined on a subset of animals at each sacrifice in the study. The lung was removed at necropsy, frozen at -70°C , and subsequently digested using low-temperature plasma ashing. The bivariate length and diameter fiber distribution were determined using scanning electron microscopy. The methods are described in the corresponding references already cited.

Tumorigenic response is usually considered the most important indicator of pathogenicity in fiber studies. However, in the chronic TIMA studies, only RCF 1 at the highest dose resulted in a statistically significantly number of lung tumors compared to the air controls. (The large number of non-fibrous particles present may in the RCF 1 sample may have contributed to its tumorigenic response.) As such, the number of tumors could not be used in determining an association with biopersistence. Fortunately, Dr. Jorg Chevalier (Experimental Pathology Service, Muttentz/Basel, Switzerland), the pathologist who initially read these studies, included a number of other indices of early pathogenic response. One of these, collagen deposition at the broncho-alveolar junction is a measure of early response at the site where fibrosis can occur in response to fiber exposure. In the pathology reports, the collagen deposition was scored on a scale of 0 to 5, with 2.9 the highest observed single value in these chronic inhalation studies. The data used in this analysis are presented in the appendix.

The parameter "collagen deposition at the broncho alveolar junction" was described according to distribution, severity, and morphologic character. The distribution was described as focal, multifocal, or diffuse. Severity scores for this parameter were assigned as follows:

- Grade 0 was characterized as no remarkable changes (refers to a normal lung).
- Grade 1 was characterized by minimal, just detectable, very few, very small foci of collagen deposition (usually 1 or 2 foci). A lesion of this severity was not considered to be sufficient to apply grade 4 in the Wagner scoring system (McConnell et al., 1984).
- Grade 2 was characterized by slight, fairly easily detected, few, small foci of collagen deposition. Lesions of this severity represented the lowest level of grade 4 in the Wagner scoring system. Grade 4 in the Wagner scale represents the lowest grade designated as fibrotic change.
- Grade 3 was characterized by moderate, easily detected foci of collagen deposition in considerably enlarged areas at the bronchiolar-alveolar junction. Lesions of this severity also represented grade 4 in the Wagner scoring system.
- Grade 4 was characterized by marked, obvious or extensive foci of collagen deposition extending from the bronchiolar-alveolar junction into the interstitium of more peripheral parts of the lung parenchyma. Lesions of this severity also represented grade 4 in the Wagner scoring system.
- Grade 5 was characterized by severe, widespread collagen deposition with consolidation at the bronchiolar-alveolar junction, sometimes with interlobular linking. Lesions of this severity represented grade 4 to 5 in the Wagner scoring system.

Statistical Methods

Analysis of the Association Between Biopersistence and Chronic Effect As mentioned earlier, the collagen deposition at the broncho-alveolar junction used in this analysis was scored on a scale of 0 to 5, with 2.9 the highest average value for one group in these chronic inhalation studies. The average value was determined as the arithmetic mean of all scores. In the original EC report to the ECB, the association between collagen deposition and biopersistence half-time was determined by fitting a logistic curve. As the logistic curve takes on values between 0 and 1, the collagen deposition scores were normalized by dividing by 2.9 and the following logistic function fitted using nonlinear regression:

$$\text{Collagen deposition} = (\exp\{b_0 + b_1[\ln(\text{NoFib}_{24})]\}) / (1 + \exp\{b_0 + b_1[\ln(\text{NoFib}_{24})]\})$$

where collagen deposition is the collagen deposition at the broncho-alveolar junction; NoFib_{24} is the number of fibers longer than 20 μm remaining in the lung at the end of 24 mo of exposure; and b_0 and b_1 are the

regression coefficients. The loss function used in this regression analysis was that of ordinary least squares as defined by $(\text{observed} - \text{predicted})^2$.

Fitting a logistic curve by nonweighted least squares, however, assumes that all observed means have the same variance and does not take into account that the variance should depend on the predicted value. Note that the mean score is always nonnegative. Therefore, if a value near zero is predicted, then the observation should have a smaller variance: A non-negative variable can only have an expectation of 0 if it is always 0 and hence has variance 0. Similarly, a variable that is always not greater than 1 can only have an expectation of 1 if it also has variance 0. Therefore, to properly fit the data using logistic regression, the logistic curve should have been fitted using weighted least squares, where the distance between a point and the curve is weighted much more heavily whenever the predicted curve is 0 or 1. This implies that the curve, if properly fitted, will not become 0 collagen score at lower values of fiber number and will not become 1 collagen score at higher values of fiber number as was the case in the analysis presented in the report to the ECB.

From consideration of biological response, the step function type of curve that was derived in the original ECB report is not in full coherence with the mechanistic theories that have been put forward in understanding fiber carcinogenesis. Collagen deposition at the broncho-alveolar junction is a precursor of what later develops into fibrosis. Fibrosis is considered by most investigators to be either a necessary or at least a parallel event in a continuum that can eventually lead to tumors. Thus it would be unreasonable to expect that the curve will plateau at a maximum collagen value seen in these studies.

A more appropriate method for fitting the data is to use the proportional odds model, which tries to take full account of the upper and lower bounds for the data. This model effectively superimposes multiple logistic functions, one for each level of the observed collagen deposition score. The proportional odds model assumes that for rat i , for which the predictor variable has size x_i , there is a hidden variable z_i that has a logistic distribution with mean $-\alpha x_i$ and variance 1. This hidden variable determines the collagen score of the rat. The observed collagen score is:

$$\begin{aligned} 0 & \text{ if } z_i \leq \xi_1 \\ 1 & \text{ if } \xi_1 \leq z_i \leq \xi_2 \\ 2 & \text{ if } \xi_2 \leq z_i \leq \xi_3 \\ 3 & \text{ if } \xi_3 \leq z_i \end{aligned}$$

(If higher levels were observed there would be more intercepts.) The unknown intercepts ξ_1 , ξ_2 , and ξ_3 and the parameter α are estimated from the observed collagen scores y_i by maximum likelihood. This model implies

that for given x the probability π_j to observe a y that is less or equal to j fulfills the equation

$$\log\left(\frac{\pi_j}{1 - \pi_j}\right) = \xi_{j+1} + ax$$

It follows that for a given x we have

$$\log\frac{\pi_{j_2}}{1 - \pi_{j_2}} = \log\frac{\pi_{j_1}}{1 - \pi_{j_1}} + \xi_{j_1+1} - \xi_{j_2+1}$$

Hence the name proportional odds. If we want to determine the importance of the variable x for the prediction of the collagen score, we compare the model just described to the model where x is omitted from the mean of the logistic regression, i.e., where we assume that the hidden variable has mean 0 for all rats.

The procedure LOGISTIC of the SAS statistical package was used for analysis.

Analysis of the Biopersistence Clearance Data As described in the analysis report, the data were fit using nonlinear regression to a double-exponential clearance function. The double-exponential function is of the form,

$$\text{Percent fiber remaining} = a_1\{\exp[-b_1(\text{Time})]\} + a_2\{\exp[-b_2(\text{Time})]\}$$

and was fitted to the data using nonlinear regression (StatSoft, Inc., 1997), with the loss function weighted by the inverse of the variance (Neter et al., 1990).

For each curve two clearance half-times are obtained, one for the coefficient b_1 and another for coefficient b_2 as follows:

$$T_{1/2}^{(1)} = \ln 2/b_1 \quad \text{and} \quad T_{1/2}^{(2)} = \ln 2/b_2$$

These clearance half-times often correspond to a "faster" clearance phase followed by a "slower" clearance phase. The fast clearance phase is considered to represent the clearance of fibers longer than 20 μm from the tracheobronchial tree and of shorter fibers from both the tracheobronchial tree and rapid macrophage clearance from the alveoli. For shorter fibers, the slow phase is thought to represent clearance by dissolution from fibers that have accumulated in either microgranulomas or the bronchial-associated lymphoid tissue and lymph nodes. For fibers longer than 20 μm the slow clearance phase is considered to represent the clearance of those fibers that are too long to be removed by the macrophages. The removal of these fibers is thought to be primarily by dissolution in the

fluid milieu of the lung. It is these fibers that are thought to have the greatest potential in producing disease.

The average time a fiber exists before dissolution is proportional to the half time $T_{1/2}^{(1)}$ or $T_{1/2}^{(2)}$, depending on whether it is a fast or slower dissolvable fiber. Hence, the average time in the mixture is proportional to the combined weighted clearance times ($W - T_{1/2}$) determined by summing the product of each half-time weighted by its coefficient a_x as follows:

$$W - T_{1/2} = \frac{a_1}{a_1 + a_2} \times T_{1/2}^{(1)} + \frac{a_2}{a_1 + a_2} \times T_{1/2}^{(2)}$$

The "fast" and "slow" clearance functions and the weighted clearance function are illustrated in Figure 1. The solid line represents the double-exponential function that fits the clearance data (shown as diamond points). The fast clearance phase is shown by the dashed line and a slow clearance phase is shown by the dotted line.

In those cases where a single exponential explained as much or more variance in the data when compared to a double exponential, then only a single exponential was used to determine the half-time. In addition, if a double exponential was fitted with the a_2 coefficient and was not statistically different from zero (this coefficient provides an index of what percentage of the clearance range the second exponential is explaining), then in this case the half-time was also determined from a single exponential was used.

In the following analyses, both the weighted clearance half-times and slow clearance half-times were examined for their ability to predict the outcome of the chronic studies.

RESULTS

In assessing the relationship of fiber biopersistence to chronic inhalation toxicity, initially all classes of fiber length were considered. These included fibers less than 5 μm in length, WHO fibers ($L > 5 \mu\text{m}$ and $D < 3 \mu\text{m}$), fibers 5–20 μm in length, and fibers $> 20 \mu\text{m}$ in length. The category of fibers $> 20 \mu\text{m}$ in length was considered to be above the limit at which fibers could be fully phagocytized and removed by macrophages. The authors realize that the actual limit is most likely in a range below 20 μm .

The relationship of pathological response to the number of long fibers in the lung in the chronic studies was first considered. Following this we examined the relationship of biopersistence half-time to the number of long fibers in lung, and subsequently we examined the ability of the biopersistence half-times to predict the collagen score in the chronic studies. Half-times from both inhalation biopersistence and intratracheal instillation biopersistence were considered. With inhalation biopersistence, both the weighted half-times and the "slow-phase" half-time of the fibers longer than 20 μm

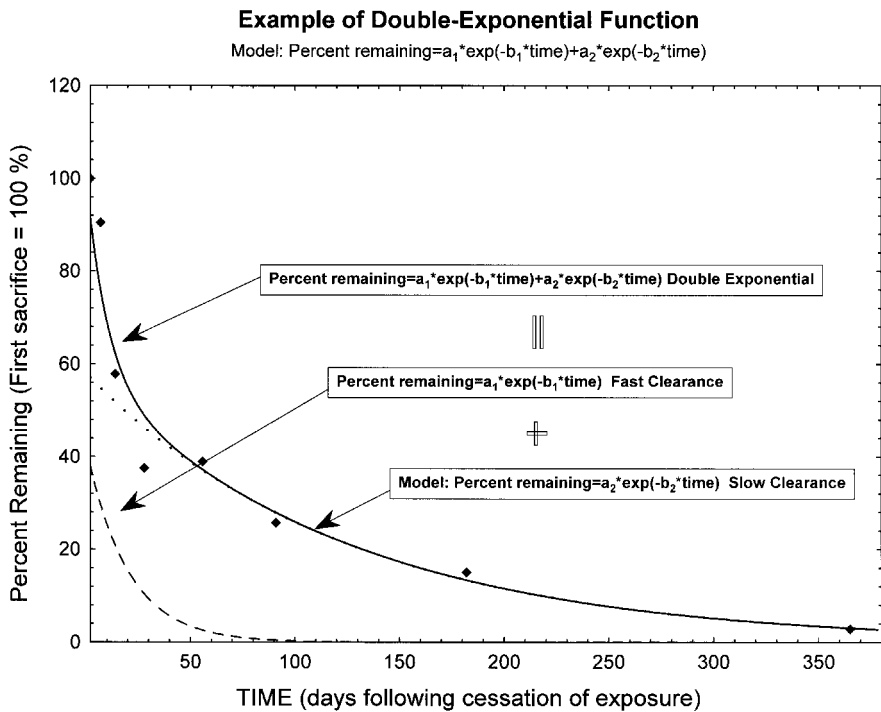


FIGURE 1. The “fast” and “slow” clearance functions and the weighted clearance function are illustrated. The solid line represents the double-exponential function that fits the clearance data (shown as diamond points). The fast clearance phase is shown by the dashed line and a slow clearance phase is shown by the dotted line. The average time a fiber exists before dissolution is proportional to the half time $T_{1/2}^{(1)}$ or $T_{1/2}^{(2)}$, depending on whether it is a fast or slower dissolvable fiber. Hence, the average time in the mixture is proportional to the combined weighted clearance times ($W - T_{1/2}$) determined by summing the product of each half-time weighted by its coefficient a_x as follows:

$$W - T_{1/2} = \frac{a_1}{a_1 + a_2} \times T_{1/2}^{(1)} + \frac{a_2}{a_1 + a_2} \times T_{1/2}^{(2)}$$

are presented. With intratracheal instillation, the clearance half-times of both the WHO fibers and the fibers longer than 20 μm are presented.

Relationship of the Number of Fibers With Lengths Greater Than 20 μm in the Lung to Pathological Response After Chronic Inhalation Exposure

Of the fiber length categories $<5 \mu\text{m}$, $5\text{--}20 \mu\text{m}$, and $>20 \mu\text{m}$, only the number of fibers longer than 20 μm remaining in the lung was found to provide a statistically significant relationship to the collagen score, and as such only the results for these long fibers are presented. As already discussed, while tumorigenic response is usually considered the most important indicator of pathogenicity in fiber studies, due to the data set containing only one synthetic mineral fiber that produced tumors in the chronic inhalation studies, the collagen deposition at the broncho-alveolar junc-

tion, which is a measure of early response at the site where fibrosis can occur in response to fiber exposure, was used as a surrogate indicator of early pathological response.

Using the proportional odds model, we examined the relationship of the number of fibers with $L > 20\text{ }\mu\text{m}$ in the lung following 24 mo of exposure in the chronic inhalation studies to the collagen deposition at the broncho-alveolar junction following 24 mo of exposure. A statistically significant relationship was found, which is summarized in Figure 2 and Table 1. (Note: The number of fibers was divided by 1,000,000 to have smaller numbers.) While we fitted the collagen deposition to the number of fibers itself and not the logarithm, the plot is in a logarithmic scale for clarity. Figure 2 displays the average collagen scores for the groups of six rats which received the same fibers in the same dose. (Note that the model predicts the same collagen score for each rat in such a group.)

The model fitting information in Table 1 shows that with the three measures of fit used by the program, the fit is always much worse (i.e., the measure of fit is larger) if the number of fibers is omitted from the model. It

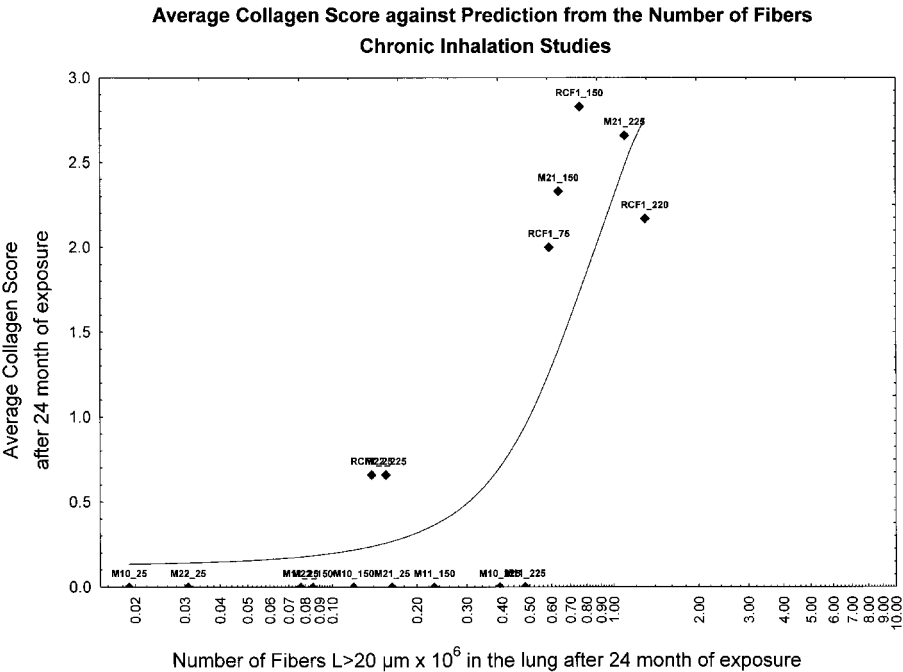


FIGURE 2. Plot of the relationship of the number of fibers with $L > 20\text{ }\mu\text{m}$ in the lung following 24 mo of exposure in the chronic inhalation studies to the collagen deposition at the broncho-alveolar junction following 24 mo of exposure as determined using the proportional odds model. The collagen deposition was fitted to the number of fibers itself and not the logarithm; however, the plot is in a logarithmic scale for clarity. The high chi-square for covariates of 64.9 implies that the influence of the number of fibers on the collagen score is highly significant (a p value of .0001).

TABLE 1. Long fibers and collagen deposition at broncho-alveolar junction in the chronic inhalation studies

A. Response profile							
	Ordered value	Collagen score	Total number observed				
	1	0	60				
	2	1	4				
	3	2	20				
	4	3	12				
B. Model fitting information and testing global null hypothesis beta = 0							
	Criterion	Intercept only	Intercept and covariates	Chi-square for covariates			
	AIC	200.476	137.582				
	SC	204.01	142.294				
	-2 LOG L	194.476	129.582	64.894 with 1 df (p = .0001)			
C. Analysis of maximum likelihood estimates							
Variable	df	Parameter estimate	Standard error	Wald chi-square	Pr > chi-square	Standardized estimate	Odds ratio
INTERCP1	1	2.5807	0.4352	35.1687	.0001		
INTERCP2	1	2.9931	0.4679	40.9165	.0001		
INTERCP3	1	5.5819	0.7977	48.9677	.0001		
NOFIBERS	1	-5.3549	0.8311	41.515	.0001	-2.2795	.005
D. Association of predicted probabilities and observed responses							
Concordant = 81.90%				Somers D = 0.689			
Discordant = 13.00%				Gamma = 0.727			
Tied = 5.20%				Tau-a = 0.482			
(193 pairs)				c = 0.845			

Note. Goodness of fit for individual rats: $R^2 = .668$. For comparison we performed a simple linear regression of the CHV scores on the number of fibers. This gave an $R^2_{lin-reg}$ of .64. A simple linear regression on the logarithm of the number of fibers gave an $R^2_{lin-reg}$ of .51.

therefore is clear that the number of fibers is a useful predictor of the collagen score. The high chi-square for covariates of 64.9 implies that the influence of the number of fibers is highly significant (a p value of .0001). The analysis of maximum likelihood estimates in Table 1 shows that increasing the number of fibers decreases the probability to have a low collagen score. The odds ratio of .005 means that the odds for a score of j or less decreases to 0.5% if the number of fibers increases by 1,000,000.

In addition to the output produced by the program, we calculated an R^2 measure of fit. Following arguments by Zheng and Agresti (2000), we calculated:

$$R^2 = 1 - \frac{\Sigma(\hat{y}_i - y_i)^2}{\Sigma(y_i - \bar{y})^2}$$

where y_i is the score of the i th rat, while $\hat{y}_i$ is the predicted score for the i th rat which is produced from the model. Here we use $\Sigma(\hat{y}_i - y_i)^2/(n - 1)$ as an estimate of $E[\text{var}(Y|X)]$ and $\Sigma(y_i - \bar{y})^2/(n - 1)$ as an estimate of $\text{var}(Y)$; compare Zheng and Agresti (2000, section 3). If each rat had exactly the predicted score, then we would have an R^2 of 1. Note that for the kind of response variables considered here, an R^2 of 1 is not always mathematically attainable, because all observed y_i must be integers. So R^2 should be interpreted with this in mind.

The R^2 value for the proportional odds model fit is slightly larger than that of the linear fit, and both are seemingly not large. This, however, is

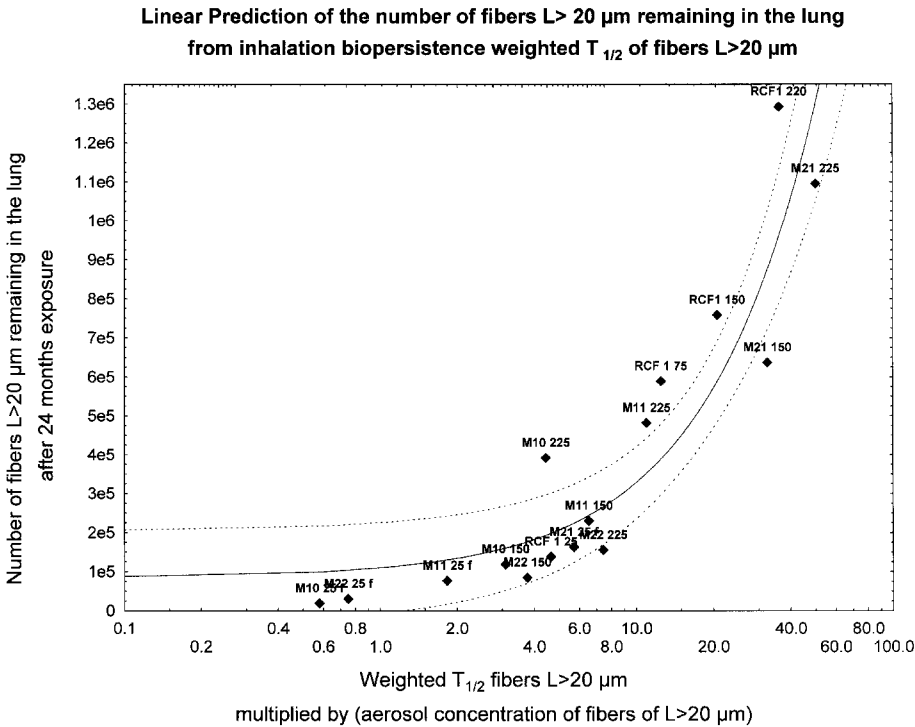


FIGURE 3. Plot of the linear prediction of the number of fibers with $L > 20 \mu\text{m}$ remaining in the lung after 24 mo of exposure in the chronic inhalation studies from the weighted $T_{1/2}$ of fibers $L > 20 \mu\text{m}$ as determined in the inhalation biopersistence studies. The weighted $T_{1/2}$ was found to be a good predictor, explaining 83% of the variance in fiber number ($L > 20 \mu\text{m}$) in the lung. The relationships shown in these figures are linear; however, for clarity the half-times were plotted on a logarithmic scale.

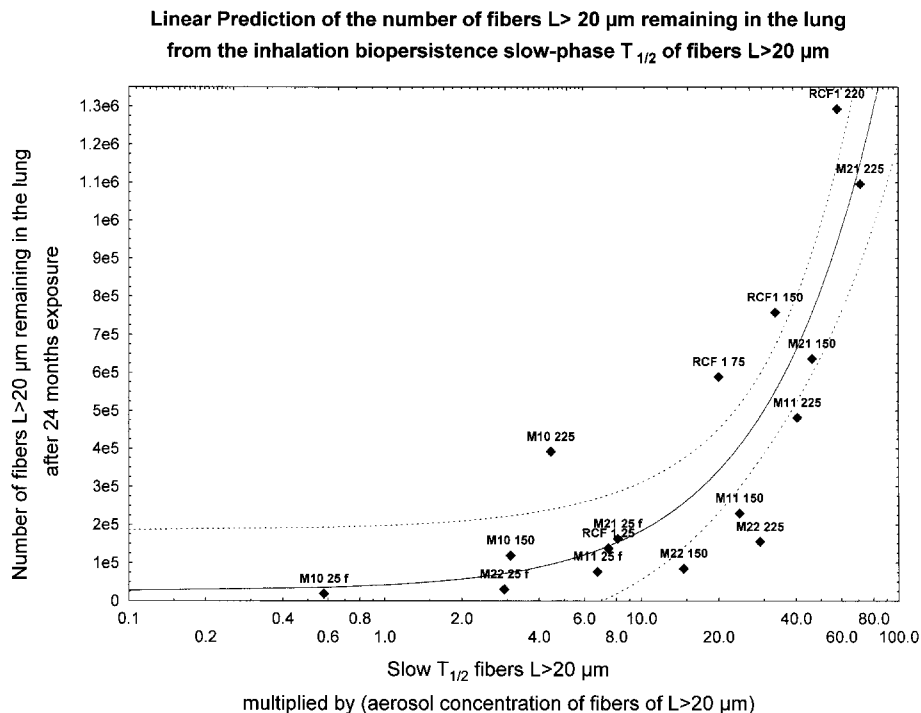


FIGURE 4. Plot of the linear prediction of the number of fibers with $L > 20 \mu\text{m}$ remaining in the lung after 24 mo of exposure in the chronic inhalation studies from the “slow-phase” $T_{1/2}$ of fibers with $L > 20 \mu\text{m}$ as determined in the inhalation biopersistence studies. The “slow-phase” $T_{1/2}$ was found to be a good predictor, explaining 86% of the variance in fiber number ($L > 20 \mu\text{m}$) in the lung. The relationships shown in these figures are linear; however, for clarity the half-times were plotted on a logarithmic scale.

due to the fact that our R^2 measures the prediction of the scores of the single rats, not the averages. It is obvious from Figure 2 that the predicted values follow more closely the observed values in the proportional odds model fit than the linear fit, especially with fiber number below 0.6×10^6 .

Relationship of the Number of Fibers With Lengths Greater Than $20 \mu\text{m}$ in the Lung to the Biopersistence Clearance Half-Time

The relationship of the clearance half-time of the fibers that are longer than $20 \mu\text{m}$ remaining in the lung of the rats as determined in the inhalation biopersistence assay to the number of fibers longer than $20 \mu\text{m}$ remaining in the lung following chronic inhalation was then examined.

Both the weighted $T_{1/2}$ and the “slow-phase” $T_{1/2}$ were considered as predictors of the number of fibers remaining in the lung following chronic inhalation. In the chronic inhalation studies, three different exposure concentrations were used with each fiber. If the clearance kinetics are determined by the biopersistence measurements, then there should be three parallel clearance curves for each fiber. In order to simulate this, the clear-

TABLE 2. Linear prediction of long fibers in the lung with weighted $T_{1/2}$ of long fibers

A. Analysis of variance					
Source	df	Sum of squares	Mean square	F value	Prob > F
Model	1	1.91E+12	1.91E+12	70.151	.0001
Error	14	3.8E+11	2.72E+10		
C Total	15	2.29E+12			
Root MSE	164793.2	R-square	.8336		
Dep Mean	391487.5	Adj R-sq	.8217		
C.V.	42.09411				
B. Parameter estimates					
Variable	df	Parameter estimate	Standard error	T for H ₀ : Parameter = 0	Prob > T
INTERCEP	1	78293	55637.97	1.407	.1812
weighted $T_{1/2}$	1	18378	2194.224	8.376	.0001

ance half-times were normalized by multiplying the actual clearance half time by the ratio of the number of fibers in the aerosol with $L > 20 \mu\text{m}$ divided by 115 [normalized $T_{1/2} = T_{1/2}$ (aerosol concentration $L > 20 \mu\text{m}$)/115]. The concentration was divided by 115 to have observed values in the range between 0 and 100.

TABLE 3. Linear prediction of long fibers in the lung with “slow-phase” $T_{1/2}$ of long fibers

A. Analysis of variance					
Source	df	Sum of squares	Mean square	F value	Prob > F
Model	1	1.96E+12	1.96E+12	85.455	.0001
Error	14	3.2169E+11	2.2978E+10		
C Total	15	2.29E+12			
Root MSE	151584.609	R-square	.8592		
Dep Mean	391487.5	Adj R-sq	.8492		
C.V.	38.72017				
B. Parameter estimates					
Variable	df	Parameter estimate	Standard error	T for H ₀ : Parameter = 0	Prob > T
INTERCEP	1	11282	55926.07	0.202	.843
weighted $T_{1/2}$	1	13167	1424.39	9.244	.0001

As shown in Figures 3 and 4, both the weighted $T_{1/2}$ and the “slow-phase” $T_{1/2}$ are good predictors of the number of long fibers remaining in the lung after 24 mo of exposure in the chronic studies, explaining 83 and 86% of the variance in fiber number in the lung, respectively (Tables 2 and 3). (There were no individual data available; the number of fibers in the lung is one observed value for each combination of substance and dose.) The relationships shown in these figures are linear; however, for clarity the half-times were plotted on a logarithmic scale.

While it is interesting that the biopersistence clearance half-time of the long fibers can predict the number of long fibers remaining in the lung following a 2-yr chronic inhalation exposure, of primary importance is whether these parameters can predict the pathological response in the lung.

Relationship of Biopersistence Half-Times to Pathological Response

Inhalation Biopersistence As presented earlier, the clearance half-time values of the long fibers as determined from the short-term bioper-

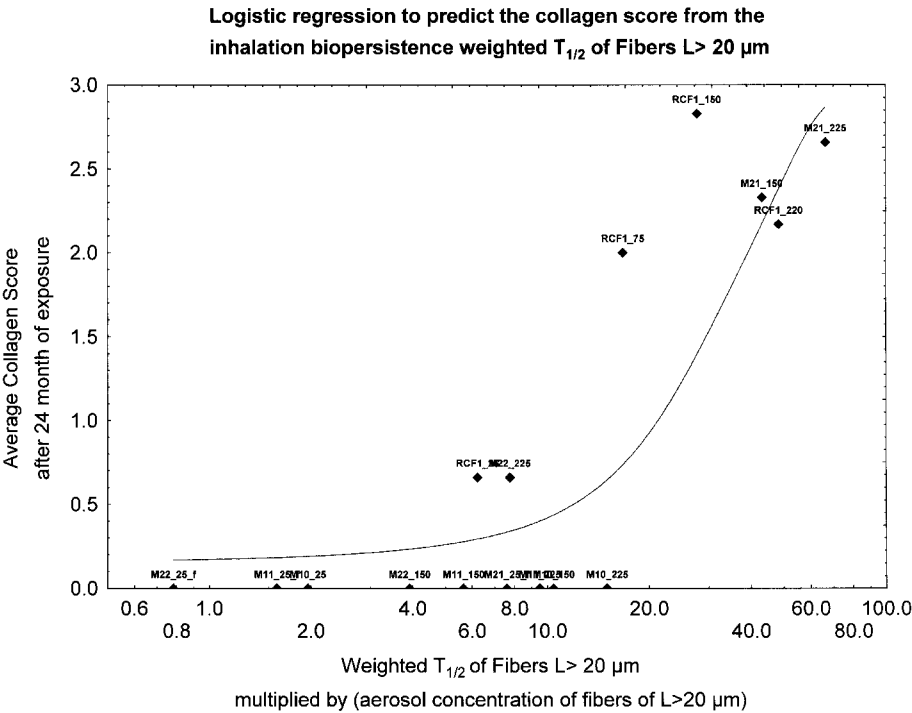


FIGURE 5. Plot of the logistic regression function showing the prediction of the collagen score at the broncho alveolar junction after 24 mo of exposure in the chronic inhalation studies from the weighted $T_{1/2}$ of the fibers with $L > 20 \mu\text{m}$ as determined in the inhalation biopersistence studies. The chi-square of 68.6 is highly significant (a p value of .0001).

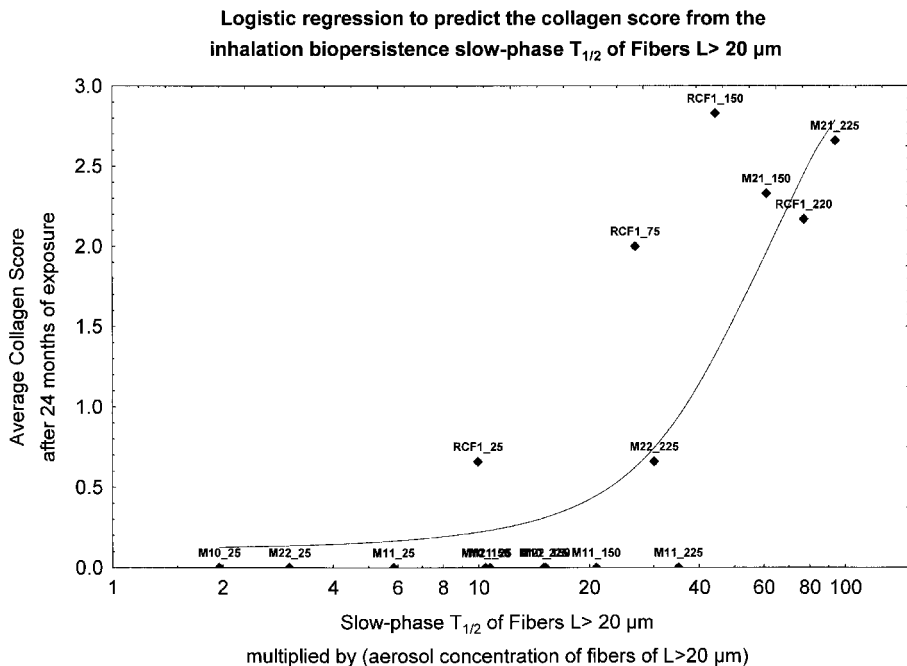


FIGURE 6. Plot of the logistic regression function showing the prediction of the collagen score at the broncho alveolar junction after 24 mo of exposure in the chronic inhalation studies from the “slow-phase” $T_{1/2}$ of the fibers with $L > 20 \mu\text{m}$ as determined in the inhalation biopersistence studies. The chi-square of 65.4 is highly significant (a p value of .0001).

sistence assay are correlated with the number of long fibers remaining in the lung at 24 mo in the chronic inhalation toxicity studies. The proportional odds model was used to determine if the clearance half-time values of the long fibers could also predict the collagen score in the chronic studies. Figures 5 and 6 and Tables 4 and 5 show that both the weighted $T_{1/2}$ and the slow-phase $T_{1/2}$ can predict the collagen scores at 24 mo in the chronic studies with similar R^2 as for the number of long fibers in the lung at 24 mo.

The analysis of maximum likelihood estimates shows that increasing clearance half-time of fibers increases the probability to have a high collagen score. The chi-square for the weighted $T_{1/2}$ of 68.6 is similar although slightly greater than that for the number of fibers (with a -2 LOG L difference of 64.9), while the chi-square for the slow-phase $T_{1/2}$ of 65.4 is similar but slightly less than that of the number of fibers. This indicates that the weighted and slow-phase clearance half-times of the fibers $L > 20 \mu\text{m}$ are equally as good predictors of the collagen score as the number of fibers with $L > 20 \mu\text{m}$ in the lung at 24 mo. The same can be concluded from the goodness of fit expressed by the R^2 .

Intratracheal Instillation Biopersistence As the Directive also includes the clearance half-time as determined using an intratracheal instillation (IT) biopersistence study as a criteria for exoneration of fibers as carcinogens, we also examined the ability of the IT biopersistence half-times to predict the collagen deposition at the broncho-alveolar junction in the chronic inhalation studies.

The proportional odds model was used to determine if the clearance half-time values of the long fibers could also predict the collagen score in the chronic studies. Figures 7 and 8 and Tables 6 and 7 show that both the $T_{1/2}$ of the WHO fibers and the $T_{1/2}$ of the fibers with $L > 20 \mu\text{m}$ can predict the collagen scores at 24 mo in the chronic studies. However, we did not have IT half-times for MMVF 10. Therefore, we had to omit MMVF 10 from the analysis in Figures 7 and 8. To make a direct comparison possible, we repeated the analysis of the inhalation data when MMVF 10 was excluded. For this reduced data set, we had the following results: The $T_{1/2}$ of the fibers with $L > 20 \mu\text{m}$ has a slightly greater R^2 (.75) as compared to that for the WHO fibers by IT (.61). The corresponding -2 LOG L differences (chi-squared) explained by the model are 65.9 and 52.9. The corresponding chi-squared values for the inhalation biopersistence weighted

TABLE 4. Logistic regression of collagen scores from inhalation weighted $T_{1/2}$ of long fibers

A. Model fitting information and testing global null hypothesis beta = 0							
Criterion		Intercept only	Intercept and covariates		Chi-square for covariates		
AIC		200.476	133.886		68.590 with 1 df (p = .0001)		
SC		204.01	138.598				
-2 LOG L		194.476	125.886				
B. Analysis of maximum likelihood estimates							
Variable	df	Parameter estimate	Standard error	Wald chi-square	Pr > chi-square	Standardized estimate	Odds ratio
INTERCP1	1	2.3095	0.3845	36.0778	.0001		
INTERCP2	1	2.7311	0.4223	41.8175	.0001		
INTERCP3	1	5.6295	0.8759	41.3075	.0001		
WEIGHTED $T_{1/2}$	1	-0.1149	0.0191	36.3128	.0001	-2.43036	.891
C. Association of predicted probabilities and observed responses							
Concordant = 82.90%				Somers D = 0.71			
Discordant = 11.90%				Gamma = 0.749			
Tied = 5.20%				Tau-a = 0.496			
(193 pairs)				c = 0.855			

TABLE 5. Logistic regression of collagen scores from inhalation “slow-phase” $T_{1/2}$ of long fibers

A. Model fitting information and testing global null hypothesis beta = 0							
Criterion		Intercept only	Intercept and covariates	Chi-square for covariates			
AIC		200.476	137.083				
SC		204.01	141.795				
-2 LOG L		194.476	129.083	65.393 with 1 df (p = .0001)			
B. Analysis of maximum likelihood estimates							
Variable	df	Parameter estimate	Standard error	Wald chi-square	Pr > chi-square	Standardized estimate	Odds ratio
INTERCP1	1	2.7008	0.4432	37.1369	.0001		
INTERCP2	1	3.0964	0.4764	42.2383	.0001		
INTERCP3	1	5.7193	0.8473	45.5634	.0001		
SLOW-PHASE T _{1/2}	1	-0.0764	0.0123	38.7351	.0001	-2.2892	.926
C. Association of predicted probabilities and observed responses							
Concordant = 81.3%				Somers D = 0.679			
Discordant = 13.5%				Gamma = 0.716			
Tied = 5.2%				Tau-a = 0.475			
(193 pairs)				c = 0.839			

Note. Goodness of fit for individual rats: $R^2 = .634$.

and slow-phase $T_{1/2}$ of the fibers with $L > 20 \mu\text{m}$ in the reduced data set were 61.3 and 51.6, respectively.

DISCUSSION

This analysis has shown the clearance half-times as determined by either the inhalation biopersistence study (Bernstein & Riego-Sintes, 1999) or the intratracheal instillation biopersistence study (Bernstein & Riego-Sintes, 1999) to be good predictors of the collagen deposition at the broncho-alveolar junction in the chronic inhalation studies. All predictions considered appear to give rather similar goodness of fit.

As mentioned earlier, the synthetic mineral fibers tested in these chronic inhalation studies did not—with one exception, RCF 1—produce a statistically significant level of tumors as compared to the air controls. The RCF 1 fiber had an elevated collagen score of 2.2. In humans, pulmonary carcinomas have been reported to be very rare in asbestos workers unless asbestosis is also present (Browne, 1982; Kipen et al., 1987). In animals, Kushner (1987) has suggested that pleural fibrosis may be a necessary

precursor to mesothelioma. Davis and Cowie (1990) have reviewed animal inhalation studies with a variety of fiber types and have found that high levels of pulmonary fibrosis were always associated with high levels of pulmonary tumors.

When examining the collagen score as a function of either the number of long fibers in the lung (Figure 2), there appears to be a cutoff value or threshold at approximately 500,000 fibers with $L > 20 \mu\text{m}$ in the lung. Of all the animals with fiber burden less than 500,000 fibers with $L > 20 \mu\text{m}$ per lung, 42 animals had a collagen score of 0; 4 animals a collagen score of 1; and 2 animals a collagen score of 2 (in the RCF 1 low-dose and the MMVF 22 high-dose groups).

It therefore appears that a minimum persistence of long fibers is necessary before even very early changes start to appear in the lung. As presented in Figures 3 and 4, the persistence of the long fibers is directly related to the biopersistence of these fibers.

In Note Q of the EC Directive 97/69/EEC, it is stated that the classification as a carcinogen need not apply if it can be shown that the substance fulfills one of the following conditions:

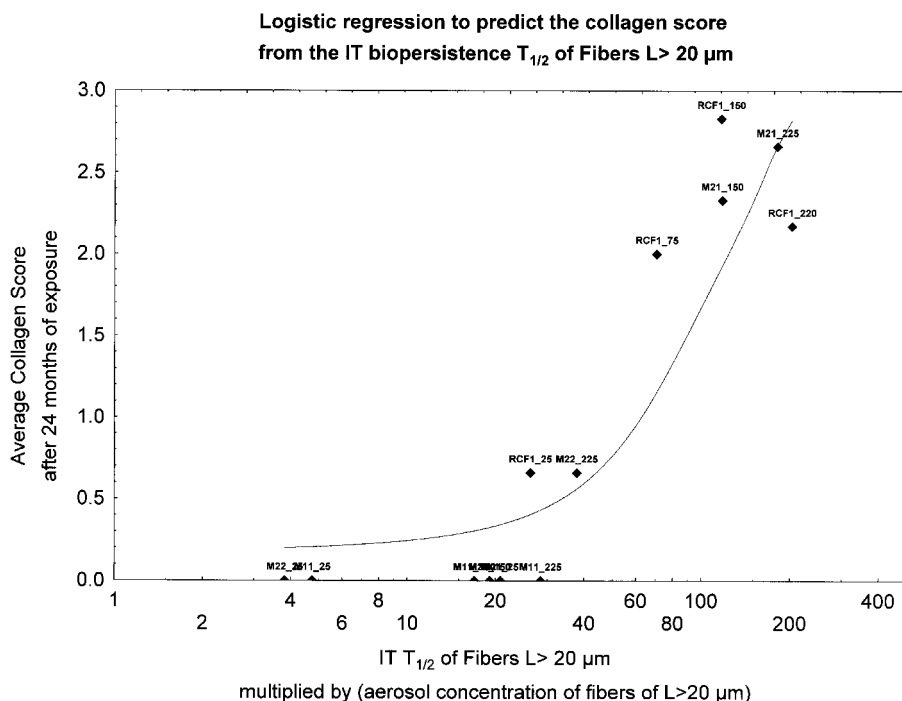


FIGURE 7. Plot of the logistic regression function showing the prediction of the collagen score at the broncho alveolar junction after 24 mo of exposure in the chronic inhalation studies from the $T_{1/2}$ of the fibers with $L > 20 \mu\text{m}$ as determined in the intratracheal instillation biopersistence studies. The chi-square of 65.9 is highly significant (a p value of .0001).

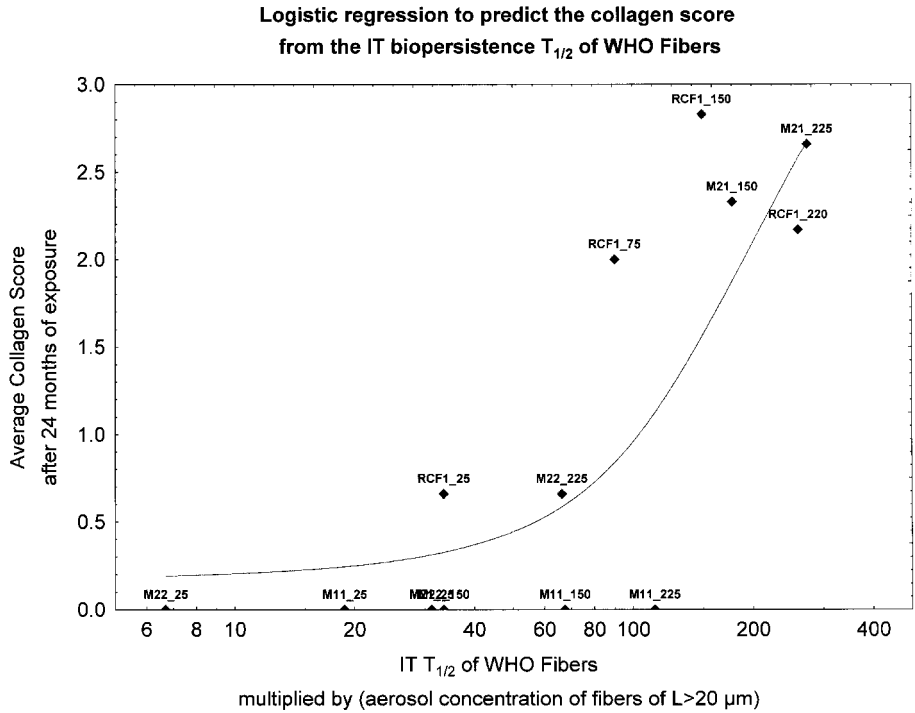


FIGURE 8. Plot of the logistic regression function showing the prediction of the collagen score at the broncho alveolar junction after 24 mo of exposure in the chronic inhalation studies from the $T_{1/2}$ of the WHO fibers as determined in the intratracheal instillation biopersistence studies. The chi-square of 52.9 is highly significant (a p value of .0001).

1. A short-term biopersistence test by inhalation has shown that the fibers longer than 20 μm have a weighted half-life less than 10 days, or
2. A short-term biopersistence test by intratracheal instillation has shown that the fibers longer than 20 μm have a weighted half-life less than 40 days, or
3. An appropriate intraperitoneal test has shown no evidence of excess carcinogenicity, or
4. Absence of relevant pathogenicity or neoplastic changes in a suitable long-term inhalation test.

From Figure 3, the weighted $T_{1/2}$ of 10 days for the fibers with $L > 20 \mu\text{m}$ (normalized to the highest chronic exposure concentration) corresponds to approximately 260,000 fibers with $L > 20 \mu\text{m}$ remaining in the lung. As is seen in Figure 5, this is within the plateau region of the collagen score. In the initial report to the ECB there was an indication that the slow phase of the $T_{1/2}$ for fibers with $L > 20 \mu\text{m}$ provided an improved fit. However, when applying the more appropriate statistical methods used herein there is no difference between the weighted $T_{1/2}$ and the slow-phase $T_{1/2}$ as is illustrated in Figures 5 and 6 and Tables 4 and 5.

The directive also provides for the use of the IT $T_{1/2}$ for fibers with $L > 20 \mu\text{m}$ for exoneration if the $T_{1/2}$ is less than 40 days. As is illustrated in Figure 7, this value lies just at the transition region. When considering the IT $T_{1/2}$ for WHO fibers, the 40-day value is in the middle of the collagen plateau region (Figure 8). It is difficult to assess the importance of this difference, especially as most of the IT studies that were included in this analysis were not fully screened for agglomeration which has been shown to influence the results (Bernstein et al., 1999).

The biopersistence of the long fibers in the lung serves to modulate the effects seen in the lung by simply reducing the dose of these fibers in the lung. Compared to durable natural fibers, the mineral wools are from 20 to a 1000 times more soluble. [In vitro dissolution rate (K_{dis}) for asbestos $< 1 \text{ ng/cm}^2\text{-h}$, and for mineral wools from $20 \text{ ng/cm}^2\text{-h}$ to several hundred (Zoitos et al., 1997).] From this analysis there is a dose to the lung of long fibers below which even the earliest indications of pathological change do not occur. The clearance half-time values set in the directive for exoneration are within this range.

In a recent publication, Marsh et al. (2001) reported on the epidemiological results following examination of the historical cohort of U.S. man-made vitreous fiber workers through 1992, and reported that there was no significant elevation of respiratory system cancer. The exposures that took

TABLE 6. Logistic regression of collagen scores from IT $T_{1/2}$ of long fibers

A. Model fitting information and testing global null hypothesis beta = 0							
Criterion		Intercept only	Intercept and covariates		Chi-square for covariates		
AIC		181.125	117.213		65.912 with 1 df (p = .0001)		
SC		184.258	121.391				
-2 LOG L		175.125	109.213				
B. Analysis of maximum likelihood estimates							
Variable	df	Parameter estimate	Standard error	Wald chi-square	Pr > chi-square	Standardized estimate	Odds ratio
INTERCP1	1	2.1183	0.4146	26.1068	.0001		
INTERCP2	1	2.6699	0.4656	32.8784	.0001		
INTERCP3	1	6.0308	0.9888	37.2008	.0001		
T _{1/2}	1	-0.0373	0.00615	36.7653	.0001	-2.67139	.963
C. Association of predicted probabilities and observed responses							
Concordant = 86.4%				Somers D = 0.792			
Discordant = 7.1%				Gamma = 0.847			
Tied = 6.5%				Tau-a = 0.581			
(193 pairs)				c = 0.896			

TABLE 7. Logistic regression of collagen scores from IT $T_{1/2}$ of WHO fibers

A. Model fitting information and testing global null hypothesis beta = 0							
Criterion		Intercept only	Intercept and covariates		Chi-square for covariates		
AIC		181.125	130.179		52.946 with 1 df (p = .0001)		
SC		184.258	134.357				
-2 LOG L		175.125	122.179				
B. Analysis of maximum likelihood estimates							
Variable	df	Parameter estimate	Standard error	Wald chi-square	Pr > chi-square	Standardized estimate	Odds ratio
INTERCP1	1	2.2422	0.4508	24.7436	.0001		
INTERCP2	1	2.6612	0.4826	30.4091	.0001		
INTERCP3	1	5.3087	0.8384	40.0929	.0001		
T _{1/2}	1	-0.0227	0.00389	34.176	.0001	-2.11966	.978
C. Association of predicted probabilities and observed responses							
Concordant = 80.5%				Somers D = 0.688			
Discordant = 11.7%				Gamma = 0.746			
Tied = 7.8%				Tau-a = 0.505			
(193 pairs)				c = 0.844			

place in this cohort occurred prior to the implementation of any biopersistence requirements by any regulatory authority. The median exposure was 0.4 fibers/cm³ across all plants with plant median values ranging from 0.001 to 0.167 fibers/cm³. The highest mean cumulative dose was 23.5 fibers/cm³-mo (Marsh et al., 2001, Table 4). The effect of using less biopersistent fibers would be to reduce the effective dose even further.

CONCLUSIONS

This analysis indicates that all the indicators of biopersistence considered are equally good predictors of the early long-term change that occurs in the lung in response to more durable fibers. This change, the collagen deposition at the broncho-alveolar junction, is a precursor of interstitial fibrosis, which has been shown to be associated with tumor response in fiber-exposed animals. The clearance half-times set in Directive 97/69 are within the baseline for this parameter.

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APPENDIX

Fiber	Target dose	Number of fibers, $L > 20 \mu\text{m}$	AERL20	CHV COLL	WT12_20	LG_T_20	IT_TWHO	IT_WHO_D	IT_TL20	IT_20_DW
RCF1	25	138,000	13	0	55	88	296	33	233	26
RCF1	25	138,000	13	0	55	88	296	33	233	26
RCF1	25	138,000	13	0	55	88	296	33	233	26
M21	25	163,000	13	0	67	95	276	31	184	21
M21	25	163,000	13	0	67	95	276	31	184	21
M21	25	163,000	13	0	67	95	276	31	184	21
M21	25	163,000	13	0	67	95	276	31	184	21
M21	25	163,000	13	0	67	95	276	31	184	21
M21	25	163,000	13	0	67	95	276	31	184	21
M21	25	163,000	13	0	67	95	276	31	184	21
M11	225	482,000	84	0	13	48	155	113	39	28
M11	225	482,000	84	0	13	48	155	113	39	28
M11	225	482,000	84	0	13	48	155	113	39	28
M11	225	482,000	84	0	13	48	155	113	39	28
M11	225	482,000	84	0	13	48	155	113	39	28
M11	150	230,000	50	0	13	48	155	67	39	17
M11	150	230,000	50	0	13	48	155	67	39	17
M11	150	230,000	50	0	13	48	155	67	39	17
M11	150	230,000	50	0	13	48	155	67	39	17
M11	150	230,000	50	0	13	48	155	67	39	17
M11	150	230,000	50	0	13	48	155	67	39	17
M11	25	77,000	14	0	13	48	155	19	39	5
M11	25	77,000	14	0	13	48	155	19	39	5
M11	25	77,000	14	0	13	48	155	19	39	5
M11	25	77,000	14	0	13	48	155	19	39	5
M11	25	77,000	14	0	13	48	155	19	39	5
M11	25	77,000	14	0	13	48	155	19	39	5
M22	225	155,000	99	0	9	35	77	66	44	38
M22	225	155,000	99	0	9	35	77	66	44	38
M22	225	155,000	99	0	9	35	77	66	44	38

(Table continues on next page)

Fiber	Target dose	Number of fibers, $L > 20\text{ }\mu\text{m}$	AERL20	CHV COLL	WT12_20	LG_T_20	IT_TWHO	IT_WHO_D	IT_TL20	IT_20_DW
M22	150	85,000	50	0	9	35	77	33	44	19
M22	150	85,000	50	0	9	35	77	33	44	19
M22	150	85,000	50	0	9	35	77	33	44	19
M22	10	85,000	50	0	9	35	77	33	44	19
M22	150	85,000	50	0	9	35	77	33	44	19
M22	150	85,000	50	0	9	35	77	33	44	19
M22	25	30,800	10	0	9	35	77	7	44	4
M22	25	30,800	10	0	9	35	77	7	44	4
M22	25	30,800	10	0	9	35	77	7	44	4
M22	25	30,800	10	0	9	35	77	7	44	4
M22	25	30,800	10	0	9	35	77	7	44	4
RCF1	25	138,000	13	1	55	88	296	33	233	26
RCF1	25	138,000	13	1	55	88	296	33	233	26
M22	225	155,000	99	1	9	35	77	66	44	38
M22	225	155,000	99	1	9	35	77	66	44	38
RCF1	220	1,293,000	101	2	55	88	296	260	233	205
RCF1	220	1,293,000	101	2	55	88	296	260	233	205
RCF1	220	1,293,000	101	2	55	88	296	260	233	205
RCF1	220	1,293,000	101	2	55	88	296	260	233	205
RCF1	220	1,293,000	101	2	55	88	296	260	233	205
RCF1	150	758,000	58	2	55	88	296	149	233	118
RCF1	75	589,000	35	2	55	88	296	90	233	71
RCF1	75	589,000	35	2	55	88	296	90	233	71
RCF1	75	589,000	35	2	55	88	296	90	233	71
RCF1	75	589,000	35	2	55	88	296	90	233	71
RCF1	75	589,000	35	2	55	88	296	90	233	71
RCF1	75	589,000	35	2	55	88	296	90	233	71
RCF1	25	138,000	13	2	55	88	296	33	233	26
M21	225	1,096,000	114	2	67	95	276	274	184	182
M21	225	1,096,000	114	2	67	95	276	274	184	182
M21	150	637,000	74	2	67	95	276	178	184	118

M21	150	637,000	74	2	67	95	276	178	184	118
M21	150	637,000	74	2	67	95	276	178	184	118
M21	150	637,000	74	2	67	95	276	178	184	118
M22	225	155,000	99	2	9	35	77	66	44	38
RCF1	220	1,293,000	101	3	55	88	296	260	233	205
RCF1	150	758,000	58	3	55	88	296	149	233	118
RCF1	150	758,000	58	3	55	88	296	149	233	118
RCF1	150	758,000	58	3	55	88	296	149	233	118
RCF1	150	758,000	58	3	55	88	296	149	233	118
RCF1	150	758,000	58	3	55	88	296	149	233	118
M21	225	1,096,000	114	3	67	95	276	274	184	182
M21	225	1,096,000	114	3	67	95	276	274	184	182
M21	225	1,096,000	114	3	67	95	276	274	184	182
M21	150	637,000	74	3	67	95	276	178	184	118
M21	150	637,000	74	3	67	95	276	178	184	118

Note. Subsequent detailed confirmation of the data that were used for the inhalation analyses in the European Commission document ECB/TW/15(97) revealed some errors in the original data set. These are explained as follows. In some of the summary publications from which the results were derived, the collagen scores were not clearly presented. At the time the EC report was prepared, the original pathology files were not available and have only been provided recently.

1. For RCF 1, the Mast publication (REF) on this study presented a Chevalier fibrosis score that was interpreted as the collagen deposition at the bronchiolar junctions. In the EC report ECB/TW/15(97) these values were used in the analyses. Upon receiving the pathology report from Dr. Chevalier (the pathologist who read these studies), it was found that Mast reported interstitial fibrosis scores, not the collagen deposition at the bronchiolar junctions scores. In the revised data tables included herein, the collagen deposition at the bronchiolar junctions scores are now listed for RCF 1.
2. For MMVF 10 and 11, the publications did not present the Chevalier scores. Tables were provided that included a summary table from the pathology report, which again were interpreted as the collagen deposition at the bronchiolar junctions scores and used in the EC report ECB/TW/15(97). However, upon receiving the pathology report from Dr. Chevalier the original summary table was confirmed to be pleural collagen scores, not the collagen deposition at the bronchiolar junction scores. In the revised data tables included herein, the collagen deposition at the bronchiolar junctions scores as derived from the full pathology report are now listed for MMVF 10 and 11. Dr. Chevalier explained that the reason that the summary table for the collagen deposition at the bronchiolar junctions scores was not included in the pathology report was that all the values had a score of zero. The confusion apparently arose as in all other of the pathology reports from Dr. Chevalier for the other fibers the summary table for collagen deposition at the bronchiolar junctions scores was presented.
3. For RCF 1 and MMVF 21, the clearance half-times were inverted in the original EC report ECB/TW/15(97). This was a typographical transcription error that was not noticed until after the EC analysis was reviewed and completed. This is now corrected in the data tables herein.