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Research and Development

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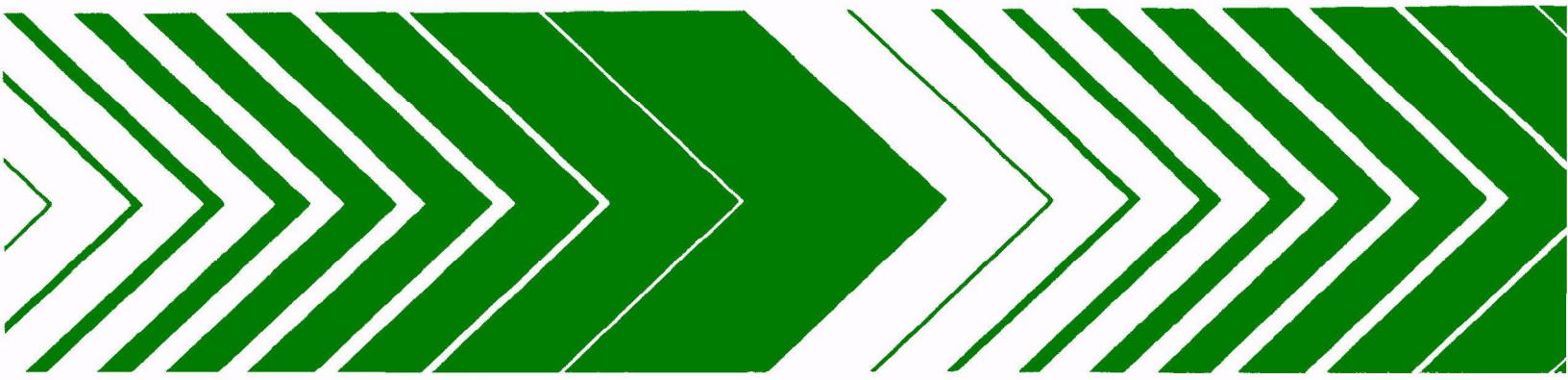
Effects of Selected Asbestos Fibers on Cellular and Molecular Parameters



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EFFECTS OF SELECTED ASBESTOS FIBERS ON CELLULAR AND MOLECULAR PARAMETERS

by

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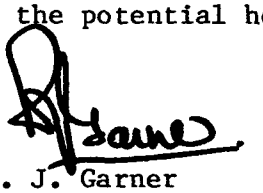
FOREWORD

The U. S. Environmental Protection Agency was created in response to increasing public concern about the dangers of pollution to the health and welfare of the American people and their environment. The complexities of environmental problems originate in the deep interdependent relationships between the various physical and biological segments of man's natural and social world. Solutions to these environmental problems require an integrated program of research and development using input from a number of disciplines.

The Health Effects Research Laboratory was established to provide sound health effects data in support of the regulatory activities of the EPA. Cultural mammalian cell-line studies provide biological endpoints which can be used to compare the potential health effects of various types of pollutants. A multidisciplinary approach is necessary to evaluate the effects on biological endpoints such as cytotoxicity, enhancement of virally directed cellular transformation, alteration of cell membrane composition, changes in cyclic nucleotide ratios, modification of chemical carcinogen metabolism, and association with genetic material.

The report that follows describes effects of varying time, type and dosage of asbestos fibers on the above biological endpoints in cultured mammalian cells. An understanding of the effects of asbestos on the individual cell is important in determining the potential health effects of asbestos in drinking water.



R. J. Garner
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PREFACE

Man and his biosphere must be protected from the adverse effects of asbestos acting by itself or in conjunction with other carcinogens. Efforts to protect the environment need appropriate biological endpoints. In regards to asbestos, the CBERG program of The Ohio State University contributes to this goal via a multidisciplinary approach involving studies of

- cytotoxicity
- enhancement of virally directed cellular transformation
- the alteration of cell membrane composition
- changes in cyclic nucleotide ratios
- modification of chemical carcinogen metabolism and association with DNA

The report that follows describes effects of varying both time and dosage of asbestos fibers on the above biological endpoints in cultured mammalian cells. Unlike chemical carcinogens, asbestos did not induce any DNA damage or decrease the rates of DNA replication.

Human cell failure to form colonies was 65 percent with wide ranges of all asbestos fibers. Syrian hamster cells in a different test system exhibited greater differential dose-dependent effects on cell death with different asbestos fibers. At low concentrations of selected forms of asbestos fibers there was differential enhancement of virally-directed cellular transformation. Differential changes in surface membrane sugar-containing lipid and proteins are reminiscent of such changes in transformed cells. Asbestos also induced elevations in the ratio cyclic AMP/cyclic GMP. This change in the ratio may be characteristic of the actions of a promoter of cell transformation rather than the actions of an agent which interacts with DNA to initiate transformation. This promoter concept is supported by the evidence that there is an enhanced benzo(a)pyrene association with cellular DNA after cell treatment with asbestos fibers.

Effects of Selected Asbestos
on Cellular and Molecular Parameters
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ABSTRACT

The purpose of this grant was to develop a cellular, biochemical and molecular basis to compare the effects of five asbestos materials, short chrysotile, intermediate chrysotile, crocidolite, tremolite and amosite.

The test systems employed were the normal human fibroblast cell strain Detroit 550 and Syrian hamster embryonic cultures. The effects on 1) deoxyribonucleic acid (DNA) damage, 2) DNA replication, 3) cytotoxicity, 4) virally directed cellular transformation, 5) cell membrane composition and 6) cyclic nucleotide concentrations were studied.

Results of these studies were expressed as percentages of controls for each of the variables measured. Neither induction of DNA per se nor replication of DNA was affected by treatment with phosphate buffered solution (PBS) washed asbestos. Cytotoxicity (65 percent) was exhibited in human cells at asbestos concentrations up to 10 µg/ml, but, in another system for measurement of this factor (Syrian hamster cells), a greater dose and fiber (chrysotile intermediate > chrysotile mixed > chrysotile short $\geq$ crocidolite > tremolite > amosite $\geq$ silica) dependence was observed. Virally induced cellular transformation frequency increase was in the order amosite > chrysotile intermediate > crocidolite.

Cell membrane monosialoganglioside (GM_1) is an index of a simpler cell surface glycolipid pattern. For GM_1 the order was crocidolite > chrysotile mixed > chrysotile intermediate > amosite. The reduction of molecular weight of glycoproteins also is a sign of simplification of the cell surface. The indicator of this process is the loss of a 85,000 molecular mass protein. The greatest loss is after crocidolite treatment. The order for this loss is crocidolite > chrysotile mixed > chrysotile intermediate > amosite. Cellular ratios of cyclic nucleotides increased toward controls in the order crocidolite > chrysotile intermediate > amosite > tremolite > silica. These results are consistent with asbestos acting as a promoter of carcinogenesis metabolism of benzo(a)pyrene.

This report was submitted in fulfillment of Grant No. R-804201 by The CBERG Group under the sponsorship of the U.S. Environmental Protection Agency covering the period April 15, 1976, to July 1, 1978, and completed as of August 15, 1978.

CONTENTS

FIGURES

TABLES

ABBREVIATIONS AND SYMBOLS

ABBREVIATIONS

AMP	-- adenosine 3′, 5, -cyclic monophosphate
BP	-- benzo(a)pyrene
BUdR	-- 5 bromo deoxyuridine
DABA	-- 3,5-diaminobenzoic acid
DEAE	-- diethyl acetate ethyl
DMSO	-- dimethylsulfoxide
DNA	-- deoxyribonucleic acid
DN-HC	-- DNA-hydrocarbon
dsb	-- double strand break
EDTA	-- ethylenedinitrilo-tetraacetic acid
EMEM	-- Earle's minimal essential medium
ESS	-- endonuclease sensitive site
FBS	-- fetal bovine serum
FFU	-- focus forming units
GD_{1a}	-- disialoganglioside
GL-4	-- globoside
GM_1	-- monosialoganglioside 1
GM_2	-- monosialoganglioside 2
GMP	-- guanosine 3′, 5′-cyclic monophosphate
GT_1	-- trisialoganglioside
^{3}H-TdR	-- tritiated thymidine
PBS	-- phosphate buffered saline
PAH	-- polycyclic aromatic hydrocarbon
Rf	-- ratio of compound migration to solvent front
RNA	-- ribonucleic acid
SDS	-- sodium dodecyl sulfate
SHE	-- Syrian hamster embryo

ssb	-- single strand break
ST FeSV	-- Snyder-Feline Sarcoma virus
TCA	-- trichloroacetic acid
TLC	-- thin-layer chromatography
UDS	-- unscheduled DNA synthesis
UV	-- ultraviolet

SYMBOLS

T_4	-- DNA identifier, T_4 virus
λ	-- DNA identifier, λ virus
ΦX-174	-- DNA identifier, ΦX-174 virus
CO_2	-- carbon dioxide
O_2	-- oxygen
mCi	-- milli Curie
pH	-- -log hydrogen ion concentration
mM	-- milliMolar

SECTION I

INTRODUCTION

OBJECTIVES

The primary objective of this grant was to develop a cellular, biochemical and molecular data base for the comparative effects of fibrous asbestos materials (short chrysotile, intermediate chrysotile, crocidolite, tremolite and amosite) which are being used in the animal feeding studies being conducted by the National Institute of Environmental Health Sciences. (1) Specifically, the purpose of this project was to evaluate the ability of these same materials to:

1) induce deoxyribonucleic acid (DNA) damage
2) modify DNA replication
3) induce cytotoxicity in vitro
4) modify virally directed cellular transformation
5) alter cell membrane composition
6) modify cyclic nucleotide concentration

SUMMARY OF RESULTS

Induction of DNA Damage

None of the asbestos materials used at any concentration studied induced either unscheduled DNA synthesis (as measured by autoradiography), endonuclease sensitive sites (utilizing S_1 endonuclease), single-strand breaks (as measured by sedimentation in alkaline sucrose) or double-strand breaks (as measured by sedimentation in neutral sucrose) when asbestos samples were washed prior to use in phosphate buffered saline and autoclaved to reduce any contamination by biological material. Nonwashed autoclaved samples produced a low level of unscheduled DNA synthesis in a dose independent fashion from .01-10 µg/ml. Although this finding is reproducible, the mechanism underlying it is unclear. Since this effect is induced only by short chrysotile and intermediate chrysotile and not by any of the other materials, and also since none of the fibers induced repaired regions sensitive to 313 nm light [5 bromo deoxyuridine (BUdR) - incorporated regions], strand breaks or endonuclease sensitive sites, it is assumed that the unscheduled DNA synthesis observed may have been the result of either alterations in the cellular membrane resulting in a greater exchange of hot and cold thymidine (TdR) or the leakage of cells into scheduled DNA synthesis in spite of the hydroxyurea blockage used to prevent this event.

Modification of DNA Replication

No retardation or stimulation of DNA replication (a recently developed in vitro assay for chemical carcinogens) was observed for any of the asbestos fibers studied between concentrations of 0.0001 and 10 µg/ml when fibers were washed and autoclaved prior to use. Nonwashed, autoclaved fibers, while cytotoxic to varying degrees depending upon fiber type (see following section), exhibit only a minor retardation of DNA replication for the short and intermediate chrysotile. This response may relate to a modest contamination of the asbestos which we have observed via elution washing and have identified as a possible hydrocarbon of unknown character.

Cytotoxicity

The cytotoxic potential of both washed and unwashed samples of all forms of asbestos studied was extremely constant in human cells. Even at "doses" as high as 10 µg/ml cytotoxicity following a 24-hour exposure using human fibroblast cell cultures yielded only a 65 percent reduction in colony formation. Thus, human fibroblasts are relatively resistant to the cytotoxic effects of asbestos and show no differential in cytotoxicity relative to the asbestos type used. On the other hand Syrian hamster embryo (SHE) cell cultures exhibit a cytotoxic curve dependent upon dose and asbestos type. The SHE cell system is composed of several cell types and cytotoxicity in this system can be measured only by measuring total cell number and correcting for any difference in growth rates. In this system, however, it appears that cytotoxicity is chrysotile intermediate > chrysotile mixed > chrysotile short > crocidolite $\geq$ tremolite > amosite > silica.

Virally Directed Cellular Transformation

All forms of asbestos in the unwashed but autoclaved series increased the frequency of virally directed transformation of human fibroblast cell cultures with Snyder-Theiler Feline Sarcoma virus (ST FeSV) at dose levels of .1 µg/ml or greater. The frequency of increase was similar for each form of asbestos tested, with amosite > chrysotile intermediate > crocidolite.

Cell Membrane Composition

Membrane composition is modified by the various forms of asbestos tested in a differential fashion. For example, after a 48-hour treatment with amosite at 10 µg/ml there was a significant increase in the labeling of monosialoganglioside (GM_1) and decreases in both disialoganglioside (GD_{1a}) and globoside GL-4. While chrysotile (intermediate) caused a similar change in GM_1, it caused a greater decrease in the gangliosides GD_{1a} and trisialoganglioside (GT_1) than did amosite. The neutral glycolipid patterns of cells treated with amosite or chrysotile (intermediate) showed no difference from one another. Crocidolite also induced similar changes in membrane structure as did chrysotile; however, the time to manifestation at the same dose was greater for the former than for the latter material. The asbestos fibers examined also appeared to have differential effects on the surface labeling of glycoproteins. The label patterns of untreated cells and amosite treated cells were identical, whereas those cells treated with chrysotile intermed-

iate and chrysotile mixed were similar to one another but different from the controls. The glycoprotein labeling pattern of crocidolite treated cells was remarkedly different from that obtained with either amosite or chrysotile.

Modification of Cyclic Nucleotide Ratios

The normal human fibroblasts were incubated for 48 hours with various types of asbestos and chemical carcinogens. At least three different concentrations were used with each type of asbestos. Cyclic adenosine 3′,5,-cyclic monophosphate (AMP) and cyclic guanosine 3′,5′-cyclic monophosphate (GMP) were determined by radioimmunoassay. The data are given only for that particular concentration of carcinogen which gives maximum response in altering the ratio of cyclic AMP to cyclic GMP. It is quite clear that the effects of asbestos are dependent upon the type of fiber used. Silica, which is a weak carcinogen, also has an effect on the cells that is similar to the effects of certain forms of asbestos. Asbestos increases the ratio of cyclic AMP to cyclic GMP by 26 to 65 percent. This increase is largely due to an increase in the concentration of cyclic AMP. (See Table 1)

TABLE 1. COMPARATIVE TABLE OF ASBESTOS TYPE VS. TEST AT 1.0 (*) OR 10.0 (+) µg/ml FOR A 24 HOUR EXPOSURE EXPRESSED AS A % OF CONTROL

Test	Crocidolite	Mixed Chrysotile	Intermediate Chrysotile	Short Chrysotile	Tremolite	Amosite	Silica
*DNA Damage (washed)							
a) UDS	-	-	-	-	-	-	
b) ESS (S_1)	-	-	-	-	-	-	
c) ssb	-	-	-	-	-	-	
d) dsb	-	-	-	-	-	-	
e) BUdR	-	-	-	-	-	-	
* DNA Damage (unwashed)							
a) UDS	135	155	140	170	120	125	
b) ESS(S_1)	-	-	-	-	-	-	
c) ssb	-	-	-	-	-	-	
d) dsb	-	-	-	-	-	-	
e) BUdR	-	-	-	-	-	-	
Cytotoxicity							
+ a) human	35		35			40	
+ b) hamster °	62	29	21	59	62	67	
+ Viral Transformation	190		210			230	
*cAMP/cGMP	154		143	111	127	111	

Test	Crocidolite	Mixed Chrysotile	Intermediate Chrysotile	Short Chrysotile	Tremolite	Amosite	Silica
+Glycolipids							
a) GM_1	190	150	143			163	
b) GM_2	112	168	135			99	
c) GD_{1a}	21.9	18.8	24.2			65.5	
d) GT_1	22	30.4	45.8			98.3	
e) Cerebroside	18.8	136	155			148	
f) GL-4	50.1	86.9	67.9			70.5	
+Glycoproteins							
a) MW=148000	99.6	67.1	62.6			74.3	
b) MW=85000	28.0	36.5	34.4			80.2	
c) MW=70000	33.5	48.6	78.0			70.2	
*Metabolism of B&P	98	70			84	98	99
*DN-BP association							
a) peak 3		-					
b) peak 4		750					
*Non DN-BP association							
a) peak 1		250					
b) peak 2		700					

[a]Cells were counted in trypan blue and thus cytotoxicity was determined by vital dye exclusion.
* 1.0 µg/ml asbestos concentration.
+10.0 µg/ml asbestos concentration.

SECTION 2

CONCLUSIONS

RELEVANCE OF PRESENT STUDIES TO CARCINOGENIC EFFECTS OF ASBESTOS

1. Asbestos in and of itself does not act as do most carcinogens by damaging cellular DNA.

2. Unwashed asbestos can and does contain trace levels of polyaromatic hydrocarbons which can produce DNA damage.

3. Compounds such as benzo(a)pyrene do associate with asbestos.

4. To varying degrees, depending on the form of asbestos used, asbestos does modify membrane structure in the same direction as does various chemical carcinogens.

5. Asbestos of all forms enhances virally directed transformation but the level of enhancement is relatively independent of the form of asbestos used.

6. Asbestos acts as would a promotor relative to the cyclic nucleotides.

7. There is an uptake of asbestos in normal human fibroblasts in culture.

8. Iron-containing asbestos leaches iron intracellularly but not extracellularly.

Thus it is now apparent that specific forms of asbestos such as chrysotile can: (a) strongly associate with polyaromatic hydrocarbons; (b) be taken up by fibroblasts as well as other cell types; (c) modify the metabolism of polycyclic aromatic hydrocarbons (PAH) yielding a metabolite that associates strongly with DNA; (d) alter membrane structure in the direction of a transformed cell; and (e) modify the cyclic nucleotides in the same manner as a classical promotor of carcinogenesis would be expected to do.

RELATIVE HAZARDS OF ONE FORM OF ASBESTOS VERSUS ANOTHER

It appears that crocidolite causes the most changes at the lowest dosage. Surprisingly, this asbestos fiber is the least cytotoxic. Perhaps the surviving cells are the most affected by the asbestos entering these cells. Amosite conversely was the most cytotoxic fiber, but had little effect based on the other biologic parameters.

SECTION 3

NARRATIVE

METHODS

General

Cell Culture--

Cultures are maintained in conventional CO_2 incubators at 37°C and routinely tested for pleuro-pneumonia-like organisms. Cells were grown in Earle's Minimal Essential Medium (EMEM) supplemented with 1 mM sodium pyruvate, 2 mM glutamine, 1 percent non-essential amino acids and 10 percent fetal bovine serum. Confluent monolayers were dispersed with trypsin plus methyl cellulose. The determination of the generation time and various cell cycle parameters was made by standard autoradiographic procedures (2 through 7).

DNA Damage

Unscheduled DNA Synthesis--

In this technique cells are incubated in the presence of radiolabel (generally ^{3}H-TdR) after exposure of the test cells to asbestos. The incorporation of radiolabel detected by autoradiography is a measure of excision repair. Details of this procedure and its limitations have been explained in detail elsewhere. Studies presented herein represent an average of 200 cells examined per point assayed. (2, 6, 7 through 11)

Strand-break Analysis--

Our method for determining DNA strand-breaks has been published in detail and for specific procedural aspects see 13 through 17. Generally, however, our procedure is a modification of the classical McGrath and Williams technique (18). Cells are layered onto a solution of 1 N NaCl, 0.01 M ethylenedinitrilo-tetraacetic acid (EDTA) on top of a 3.6 ml gradient of 5-20 percent sucrose containing 2 M NaCl, 0.01 EDTA, and 0.33 M NaOH in a 4.0 ml polyallomer centrifuge tube. After lysis for 60 minutes at 23°C, the denatured DNA is sedimented and fractions collected on paper strips. Our analysis for weight-average molecular weights is done by a computer program calibrated with single-stranded T_4, λ, and Φ X-174 DNA's.

BUdR Photolysis--

The method we use for BUdR photolysis was developed by Regan, Setlow and Ley (19) and has been reported by us in several separate papers including references 4, 5, 8, 17, and 20. Briefly, therefore, matched cell cultures are

labeled with either ^{3}H-TdR (experimental) or $^{32}PO_4$ (control), treated with test agents and permitted to repair in the presence of either 10^{-4} M BUdR or 10^{-4} M TdR for either 4 hours (a measure of the rate of repair) or 20 hours (a measure of extent of repair). Cells are then detached, adjusted for number of dpm/cell, mixed and exposed to high-intensity 313 nm light in the same cuvette, thereby breaking the BUdR-containing regions but not the TdR-containing regions of the DNA. The number of breaks detected by sedimentation in alkali permits one to calculate the number of repaired regions, while the fluence of 313 nm light (21) required to saturate all such sites permits a determination of the size of such repaired regions.

Endosites--

Essentially our endonuclease sensitive site assay is that of Wilkins and Hart (22), with the exception that S_1 rather than ultraviolet (UV)-endo is used, thus permitting recognition of single-stranded regions within the DNA.

DNA Replication

The number of cells performing DNA replication per unit time was determined by autoradiographic procedures described in previous pulications (3, 4, 5, 8, 17). In this procedure cells are exposed for a given length to the agent and ^{3}H-TdR added to the media for a prescribed length of time. After varying lengths of time, coverslips to which the cells are attached are removed and autoradiographs prepared. Those cells showing grains are assumed to have gone through a round of DNA replication.

Cytotoxicity

Human Fibroblast Cell Cultures--

The procedure we use for determining cytotoxicity via colony formation has been published in a paper by Blakeslee (25). In this procedure preconfluent monolayers are dispersed by trypsin and triturated to assure single cell suspension. Two hundred and fifty cells were plated in 35 mm diameter wells in EMEM supplemented with 10 percent fetal bovine serum, 1 mM sodium pyruvate, 2 mM glutamine, 1X nonessential amino acids (growth medium) and incubated with the cells for 24 hours. Six to eight wells were used for each test concentration. At the end of the incubation period, cells were washed and refed with growth medium. Cell cultures were incubated for 12 days, fixed in formalin or methanol, stained with Giemsa and clones containing fifty or more cells enumerated. Absolute and relative plating efficiencies were determined and survival curves calculated from the data.

Syrian Hamster Embryo (SHE) Cell Cultures--

SHE cultures are composed of a number of cell types, each with its own particular *in vitro* life span and cloning potential. It is used due to its capacity to be transformed *in vitro* and its low degree of spontaneous transformation (26, 27). Due to the fact that SHE cultures are composed of multiple cell types, standard cloning cannot be accurately performed on this system, and therefore cytotoxicity can only indirectly be measured by the change in cell number compared to a control culture (26, 27).

Modification of Virally Directed Transformation

Virus--

Briefly, 10 percent cell-free tumor homogenates from ST FeSV infected cats were prepared and stored in L-15 medium + 15 percent fetal bovine serum (FBS) at -70°C in 1 ml aliquots.

Infectivity Assay--

Cells were trypsinized and seeded onto either 16 mm (4×10^4 cells) or 35 mm (1×10^5 cells) diameter wells (Costar, Cambridge, MA) in 1.0 ml EMEM medium for the former and four ml for the latter and incubated 18 hours prior to treatment. Cells pre-treated with asbestos prior to virus infection were incubated with designated concentrations of asbestos for two, six or 24 hours, washed and treated with 0.2 ml (11 mm wells) or 1.0 ml (35 mm wells) of DEAE dextran (40 µg/ml) in serum-free EMEM. After twenty minutes, the cells were rinsed with EMEM + 5 percent FBS, infected at 0.05 ml per 16 mm wells or 0.2 ml per 35 mm wells with each of four twofold virus dilutions and allowed to absorb for two hours. Four wells were used per dilution of virus. The plates were rocked at 10 to 15 minute intervals to maintain an even distribution of inoculum and, after adsorption, the inoculum was removed and replaced with two ml or four ml of growth medium. Cells post-treated with asbestos after virus infection were incubated with designated concentrations of chemicals two, six or 24 hours after virus adsorption. The medium was removed from infected cells and the cells treated with medium containing only asbestos for 24 hours, washed and refed with growth medium. Cells were refed with fresh growth medium only on the sixth day after infection and subsequently fixed with buffered formalin and stained with Giemsa three to four days later. Foci appear as discrete areas consisting of round, hyperrefractile, enlarged fibroblast cells. These foci were counted at 25 to 40 times with a dissecting microscope.

Virus induced foci were counted in nontreated and asbestos treated wells. The mean number of focus forming units and standard deviation was determined for each treatment time and significance determined by Student's t-test.

Alterations in Cellular Membrane Composition

Surface Labeling--

For labeling in the presence of phosphate buffered saline (PBS) the media was decanted, cells washed three times with PBS, and the incubation volume adjusted to five ml with the same buffer (28,29). For labeling in the presence of media alone, minimal essential medium (MEM) was employed for washing and final incubation volume adjustment. For labeling with media containing 10 percent FBS, the incubation volume was adjusted to five ml. Galactose oxidase [Sigma Biochemical Type III (125 units/mg) or Grand Island Biological Co. (100 units/mg)] (200-250 µg) was added to give a final concentration of 25 units per dish, and the plates were incubated in a Precision Scientific Co. p^5 Model 2 oven under CO_2-O_2 atmosphere for three hours at 35°C. After incubation, the dish was washed twice with either PBS or MEM medium and the excess incubation mixture was aspirated. One (1) mCi of NaB^3H_4, with S.A. 9 Ci/mmol (New England Nuclear; stored in 0.01 N NaOH solution at -40° C) was added and allowed to stand with occasional shaking for 30 minutes at 37°C.

Finally, the cells were detached from the plates with a scraper having a wide rubber blade. The reaction mixtures obtained from the various plates were washed five times with PBS pH 7.4, and centrifuged at 1500 rpm for 10 minutes each time. The pelleted cells were suspended in 200 µl PBS, and aliquots were taken for the glycolipids and glycoproteins determinations. For glycoproteins the cells (10^6) were digested in PBS (100 µl) containing one percent sodium dodecyl sulfate (SDS) and five percent β-mercaptoethanol and heated in a water bath at 85°C for 10 minutes.

SDS-Polyacrylamide Gel Electrophoresis--

Electrophoresis was performed according to the method of Weber and Osborn (30). β-galactosidase, urease, albumin, peroxidase and lysosyme with molecular masses of 130,000, 83,000, 68,000, 44,050 and 13,930 respectively were used. The gels were sliced and each slice radioassayed.

Extraction of Syrian Hamster Cells Glycolipids (31)--

An aliquot (100 µl) of surface labeled cells (10^6) was homogenized with 1 ml of methanol for five minutes at room temperature, and chloroform (2 ml) was added so that the final ratio was 30 volumes of chloroform: methanol (2:1, v/v) to 1 volume of cells. The homogenate was left overnight at room temperature for efficient extraction, and then centrifuged at 1500 rpm for ten minutes and the precipitate washed twice with chloroform:methanol (1:1, v/v).

Isolation of Glycolipids (32)--

Following the extraction of lipid from the cells, polar glycolipids (gangliosides) were separated from neutral glycolipids by the procedure of Folch (33). After Folch partition the total upper layer was reduced in volume to one to two ml and dialyzed at 4°C against distilled water for 24 hours. The dialized upper phase was evaporated to dryness under N_2, and the residue extracted with a small volume of chloroform:methanol (2:1, v/v). This fraction represents a major part of the gangliosides and contains some neutral glycolipids in minor quantities. The analysis of gangliosides was performed by thin-layer chromatography (TLC) (Kontes/quantum, precoated TLC plates) and developed in a solvent system (Tetrahydrofuran:0.5 percent aqueous KCl, 7:1, v/v). Standard gangliosides were run separately in the same system at the same time. The standard gangliosides were detected with resorcinol reagent. Zones having the same R_f as the standards were scraped with a razor blade and counted for radioactivity. The lower phase, containing the neutral glycolipids, sulfatides, neutral lipid and phospholipid, was evaporated to dryness and the glycolipid fraction separated according to Laine et al (34). The isolated glycolipid fraction was separated into individual neutral glycolipids by TLC as described previously (35, 36).

Determination of Cyclic Nucleotide Levels

Incubation with Asbestos and Preparation of Cell-free Extracts--

Asbestos was suspended in the medium at the desired concentrations. Cells were incubated in the medium containing asbestos for various time periods (37, 38, 39). At the end of the incubation period, the medium was poured off, and the cells were washed twice with five ml phosphate-saline buffer (pH 7.4). Cells were harvested in two ml of five percent trichloroacetic acid (TCA) with the help of a rubber scraper. The cells in TCA were

sonicated for 10 seconds and centrifuged at 4°C at 3000 rpm for 20 minutes. The precipitate fraction was dissolved in 1 N NaOH and saved for measurement of protein, which was determined by the method of Lowry (40). The TCA from the supernatant fraction was removed by extracting three times with three volumes of water-saturated diethyl ether. The ether layer was aspirated and any traces of ethyl ether remaining after the last extraction removed by heating the aliquots for 10 minutes at 50°C. The pH of the extracts was adjusted to 6.2 by adding 0.1 volume of 1 M acetate buffer, pH 6.5.

Radioimmunoassay of Cyclic Nucleotides--

Succinylated cyclic nucleotides were coupled to keyhole limpet homocyanin as described by Steiner (41). This antigen was injected in rabbits to produce the antiserum to cyclic nucleotides. The antiserum obtained has very high specificity for cyclic nucleotides. Succinylated cyclic nucleotide-tryosyl methyl ester derivatives are iodinated with ^{125}I-labeled cyclic nucleotide derivative (5000 cpm) followed by 50 µl of 1:2000 dilution of antiserum (cyclic GMP) or a 1:4000 dilution of antiserum (cyclic AMP). The other details of this procedure are identical to those described by Steiner (42). This method permits the measurement of cyclic nucleotides in the femtomole range (37, 38, 39, 43, 44).

For each measurement, five plates of cells were harvested individually. The concentrations of cyclic nucleotides were measured in duplicate for each plate. The standard errors of the mean were calculated from 10 values.

Measurement of PAH Metabolism and Binding to Cellular DNA

Isolation of DNA--

DNA was isolated by either phenol extraction (45) or hydroxylapatite chromatography (26, 27, 46, 47).

Degradation of DNA--

DNA was degraded to deoxynucleosides by either enzymic or chemical means as appropriate: (i) the enzymic methods we employed were modified from those used by Dipple, et al (48). In this method purified DNA is converted to mononucleotides by incubation with bovine deoxyribonuclease I and snake venom phosphodiesterase (12 hours, pH 7.5, 37°C). Hydrolysis to nucleosides is accomplished by either wheat germ (pH 6) or bacterial (pH 8.5) phosphatases after pH adjustment. In our hands, this system gave quantitative hydrolysis of carcinogen bound DNA as judged by cellulose TLC; (ii) when necessary, DNA was hydrolyzed by incubation in 0.1 N HCl at 37°C (24 hours). Conversion to bases was accomplished by boiling nucleosides in 0.1 N HCl for 20 minutes (26, 27).

Analysis--

(i) Protein was quantitated by the Lowry method using either a BSA or lypholized microsomal standard curve; (ii) DNA was quantitated, as appropriate, by either UV spectotroscopy, diphenylamine reaction, fluorescene or total DNA phosphate content; (iii) the extent of RNA contamination of DNA samples was determined by the orcinal reaction (27).

Chromatography of Deoxynucleosides and Deoxynucleoside-hydrocarbon Products--

Separation of the normal deoxynucleosides from the deoxynucleoside products was accomplished by chromatography on Sephadex LH-20 employing a water-methanol gradient. Eluting fractions are monitored by ultraviolet spectroscopy continuously and, where appropriate, individual fractions are monitored for radioactivity or fluorescence. Several scintillation counting systems are presently employed in our laboratories and are used as appropriate. Double label fractions are converted to $^{14}CO_2$ and $^{3}H_2O$ by a Packard 306 sample oxidizer and analyzed radiometrically in separate vials or are analyzed directly employing a computer program (26,27).

Determination of DNA-Hydrocarbon Adducts--

These studies were performed at two concentrations of benzo(a)pyrene and four concentrations of each form of asbestos. The duration of benzo(a)pyrene exposure was held constant and the duration of asbestos exposure varied between zero and 48 hours at 12 to 24 hour intervals. The methodologies employed in these studies are as follows: confluent monolayers of Syrian hamster embryo cells and/or human fibroblast cells cultured as described previously were treated with selected asbestos fibers at the desired concentration (0.1 to 1 µg/ml). At various times subsequent to the addition of the asbestos, the media were inoculated with ^{3}H-benzo(a)pyrene (1-1.5 mCi/mmole) at final concentration of one to two mM. Twenty-four hours after the hydrocarbon addition the medium was decanted, the monolayers rinsed three times with PBS and the cells harvested in four ml of lysing solution (eight M urea, containing 1 percent SDS, 0.01 M EDTA, and 0.24 M sodium phosphate, pH 6.8). The DNA was then isolated by hydroxylapatite chromatography. The isolated DNA was dialyzed and its concentration determined by DABA fluorescent techniques. The total amount of bound benzo(a)pyrene was determined by liquid scintillation counting. The purified DNA was then hydrolyzed enzymatically and the nature of the benzo(a)pyrene-deoxynucleoside adducts determined with Sephadex LH-20 chromatography (26,27).

CONCENTRATION EFFECTS

DNA Damage and DNA Replication

Only one concentration of asbestos (10 µg/ml) was employed to elicit maximal response. Since little or no increase in DNA damage was observed at this high concentration, no additional studies of concentration effects were undertaken (49,50).

Cytotoxicity

For human Detroit 550 skin fibroblasts the dose response of plating efficiency for amosite asbestos ranged from 58 to 90 percent of control. A bimodal response was seen with greater survival in the middle range of amosite concentrations and lower survival in the upper and lower ranges (Table 2). Crocidolite had no effect at any concentration on cell plating efficiency (Table 3). Chrysotile asbestos was in general more cytotoxic at higher (0.1 - 10 µg/ml) doses of asbestos (Table 4)(51).

TABLE 2. DETROIT 550 CELL SURVIVAL AFTER TREATMENT WITH CROCIDOLITE ASBESTOS

	Concentration ug/ml							
	10^{1}	10^{0}	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	Control
No. clones per 250 cells (a)	32.3±3.5	36.3±1.9	33.0±3.1	35.5±2.9	32.0±3.3	33.8±3.9	30.16±4.3	33.2±3.3
Absolute plating efficiency (%) (b)	12.9	14.5	13.2	14.2	12.8	13.5	12.1	13.3
Relative plating efficiency (%) (c)	97	110	99	110	96	100	90	—

(a) Cells treated 24 hours, washed and refed with cloning medium

(b) APE- 250 cells were seeded in 35 mm wells, grown in MEM(E) ± 10% FBS plus NaPyr, NEAA, EAA, gluNH$_2$, 5% CO_2 humidified atmosphere for 12 days at 37°C

(c) RPE - compared to control cells grown under same conditions

Enhancement of Virally Directed Cellular Transformation

There was no dose-dependent effect of amosite asbestos fibers on ST FeSV transformation (Figure 1). No enhancement could be demonstrated at any dose employed. Only significant suppression could be demonstrated with both chrysotile and crocidolite asbestos, no dose response could be demonstrated (Figures 2 and 3).

Cell Membrane Composition

Due to the complexity and time-consuming character of the assays for cell membrane composition, only 10 µg/ml concentrations of asbestos were employed. As time permits dose responses at one and 0.01 µ/ml will be examined for selected fiber types (28,29).

Modification of Cyclic Nucleotide Ratios

The ratio of cyclic AMP to cyclic GMP at 24 hours was 130 percent of the ratio in the control at five µg/ml amosite concentrations (Figure 4). At 48 hours amosite concentrations of 0.05 to five µg cyclic AMP/cyclic GMP showed no differences (150-170 percent of control). Crocidolite, however, showed a paradoxical dose response consistent at 0.05, 0.5 and 5.0 µg/ml. With higher concentrations of asbestos, there was a reduction in the lesser cyclic AMP/cycle GMP ratio (Figure 5). Incubation with 0.01 to 10 µm/ml chrysotile (short) or chrysotile (intermediate) did not demonstrate that alteration in cyclic AMP/cyclic GMP varied directly with asbestos concentration (Figure 6). The cyclic nucleotide ratio, however, did

TABLE 3. DETROIT 550 CELL SURVIVAL AFTER TREATMENT WITH AMOSITE ASBESTOS

	Concentration µg/ml							
	10^{1}	10^{0}	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	Control
No. clones per 250 cells	23.8±4.3	24.8±1.8	28.2±4.9	30.7±3.5	30.5±3.3	26.3±4.2	20.0±5.7	34.0±1.0
Absolute plating efficiency (%)	9.5	9.9	11.3	12.3	12.2	10.5	8.0	13.6
Relative plating efficiency (%)	70*	72.9	82.9	90.3	89.7	77.4	58.8	—

* = (P)-0.05-0.001 determined by student t test.

TABLE 4. DETROIT 550 CELL SURVIVAL AFTER TREATMENT WITH CHRYSOTILE ASBESTOS

	Concentration µg/ml							
	10^{1}	10^{0}	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	Control
No. clones per 250 cells	19.3±3.5	23 ±3.1	22.5±4.6	26.7±4.4	33.2±4.9	22.1±4.0	29.0±3.0	33.3±5.0
Absolute plating efficiency (%)	7.7	9.3	9.0	10.7	13.3	8.8	11.6	13.3
Relative plating efficiency (%)	58.0*	69.4	68.0	80.2	100	66.4	87.1	—

* =(P) 0.050-0.001 determined by student t test

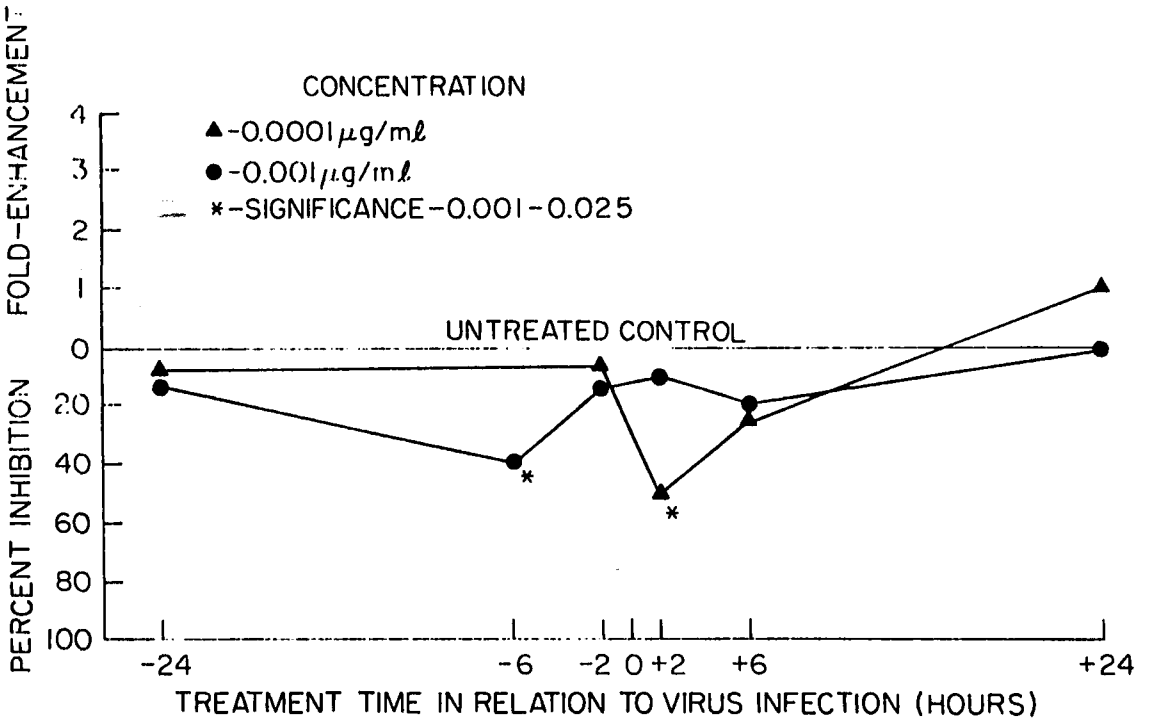

Figure 1. The effect of amosite asbestos on ST FeSV transformation of Detroit 550 cells.

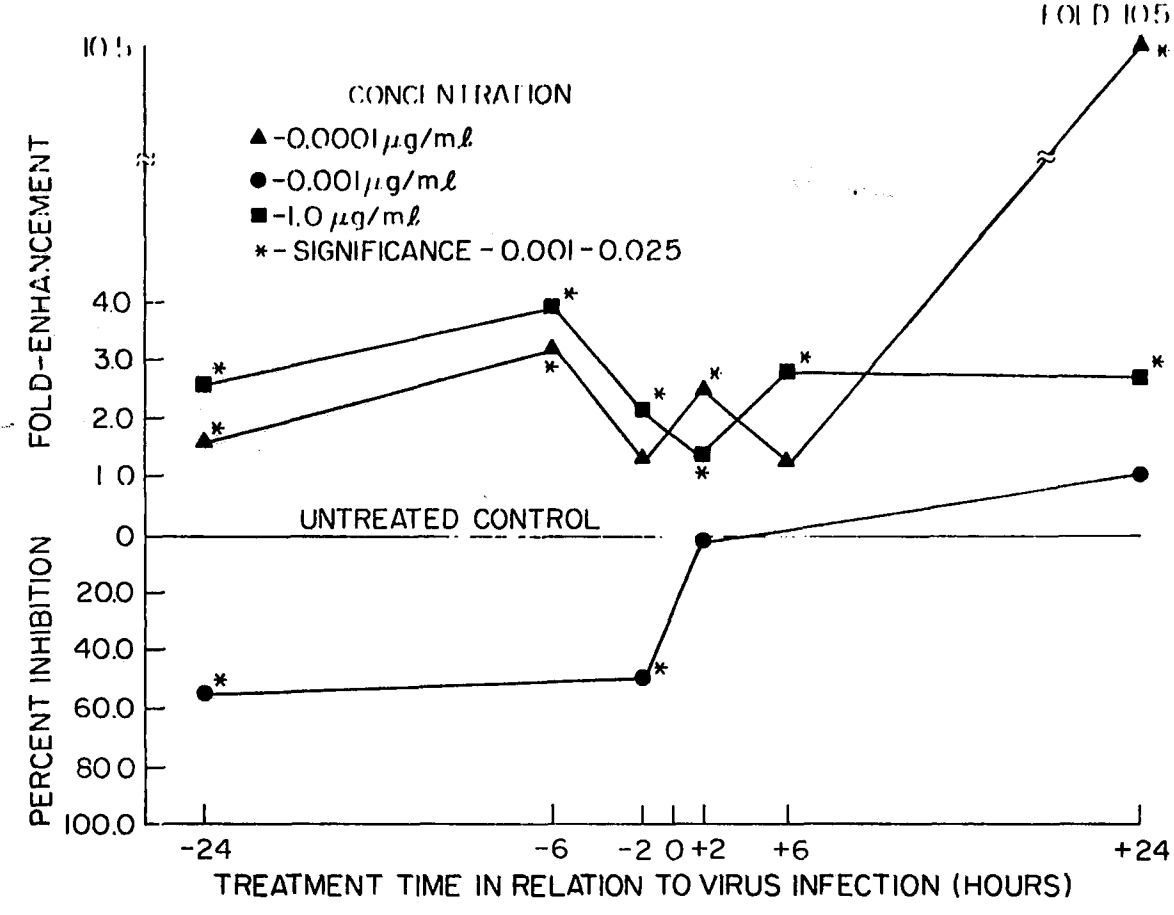

Figure 2. The effect of chrysotile asbestos on ST FeSV tranformation of Detroit 550 cells.

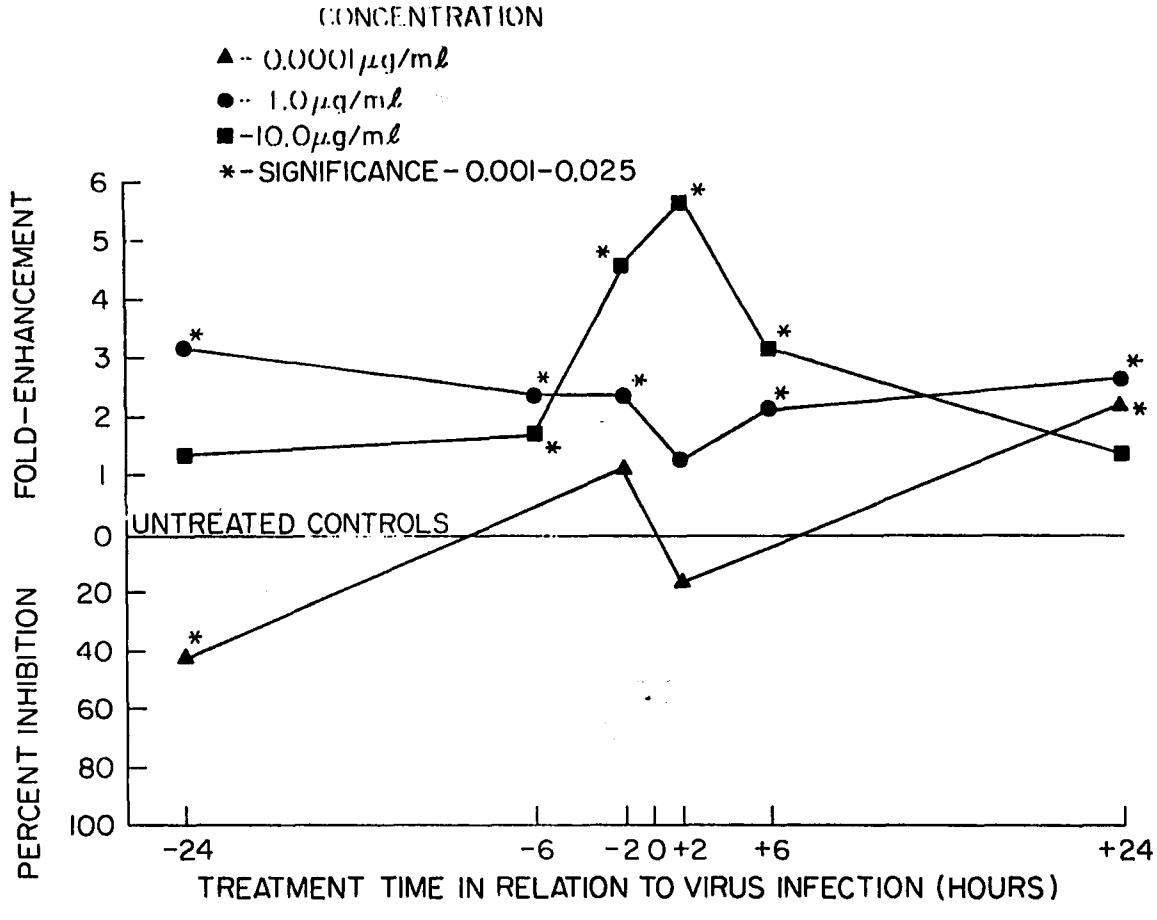

Figure 3. The effect of crocidolite asbestos on ST FeSV transformation of Detroit 550 cells.

exhibit a dose response to tremolite. This finding is the opposite of that found with cells incubated with the known chemical carcinogens, benzo(a)-pyrene and nitrosodimethyl amine (Figures 7 and 8)(39, 43, 44).

Modification of Chemical Carcinogen Metabolism and Association with DNA

This study was initiated at the beginning of the last grant period, so it is too early to include such data.

TIME EFFECTS

DNA Damage and DNA Replication

Since neither extent of DNA damage nor amount of DNA replication was altered after treatment of cells with PBS-leached asbestos, no time studies were attempted in this area.

Cytotoxicity

Not determined at this time.

Enhancement of Virally Induced Cellular Transformation

Amosite asbestos fibers inhibited ST FeSV directed transformation if the asbestos was introduced six hours prior to or two hours after viral infection. At all other times transformation frequency did not differ from control values (See Figure 1). Chrysotile fibers enhanced virally of 0.001 µg/ml) at all times of asbestos fiber exposure relative to viral infection (See Figure 2). Crocidolite, in general, showed enhancement of virally directed transformation at all times of asbestos incubation relative to viral infection times (See Figure 3). At 10 µg/ml the enhancement was most notable when the ST FeSV infection occurred either simultaneously with or within two hours of the asbestos induction (See Figure 3)(51).

Alteration of Cell Membrane Composition

The alteration of the cell membrane glycolipids (Table 5) and glycoproteins (Figures 9 to 11) by chrysotile asbestos was tested at two, 24, 48 and 72 hours of incubation (28,29). At two hours there was little change in the glycolipids or glycoproteins but at 24, 48, and 72 hours the ganglioside composition in the cell membrane progressively lost sialic acid moieties. This loss is reflected in a shift from GD_{1A} and GT_1 to GM_1 and GM_2, simpler gangliosides. Surface glycoproteins after two hours of asbestos incubation were unchanged. At 24 and 48 hours there was a loss of total glycoprotein as well as a reduction in the higher molecular mass proteins. At 72 hours there was a rebound in the total surface glycomolecular mass constituent (Figure 11).

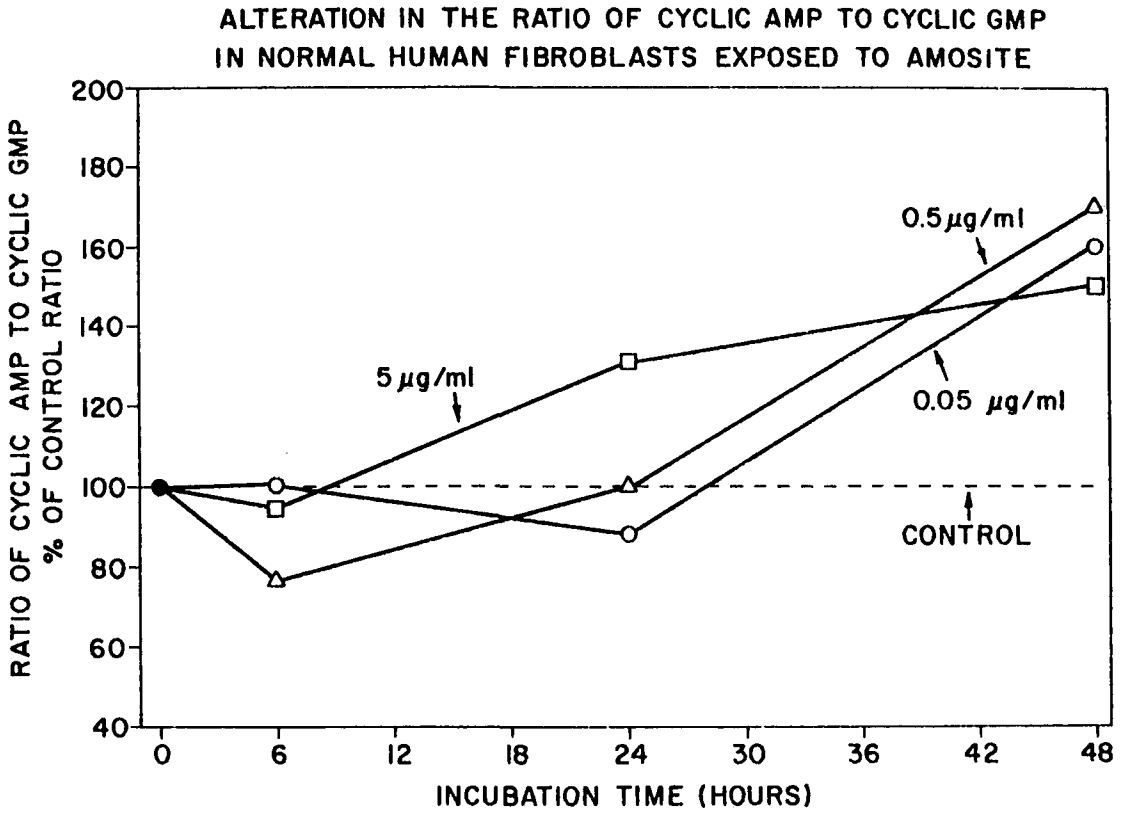

Figure 4. The effect of various concentrations of amosite on the ratio of cyclic AMP to cyclic GMP in normal human fibroblasts. The ratio of cyclic AMP to cyclic GMP in control cells is expressed as 100% and the ratio in the asbestos-treated cells is expressed as a percent (%) of control value. ------, control; o——o, 0.05 µg/ml, Δ——Δ, 0.5 µg/ml; □——□, 5.0 µg/ml.

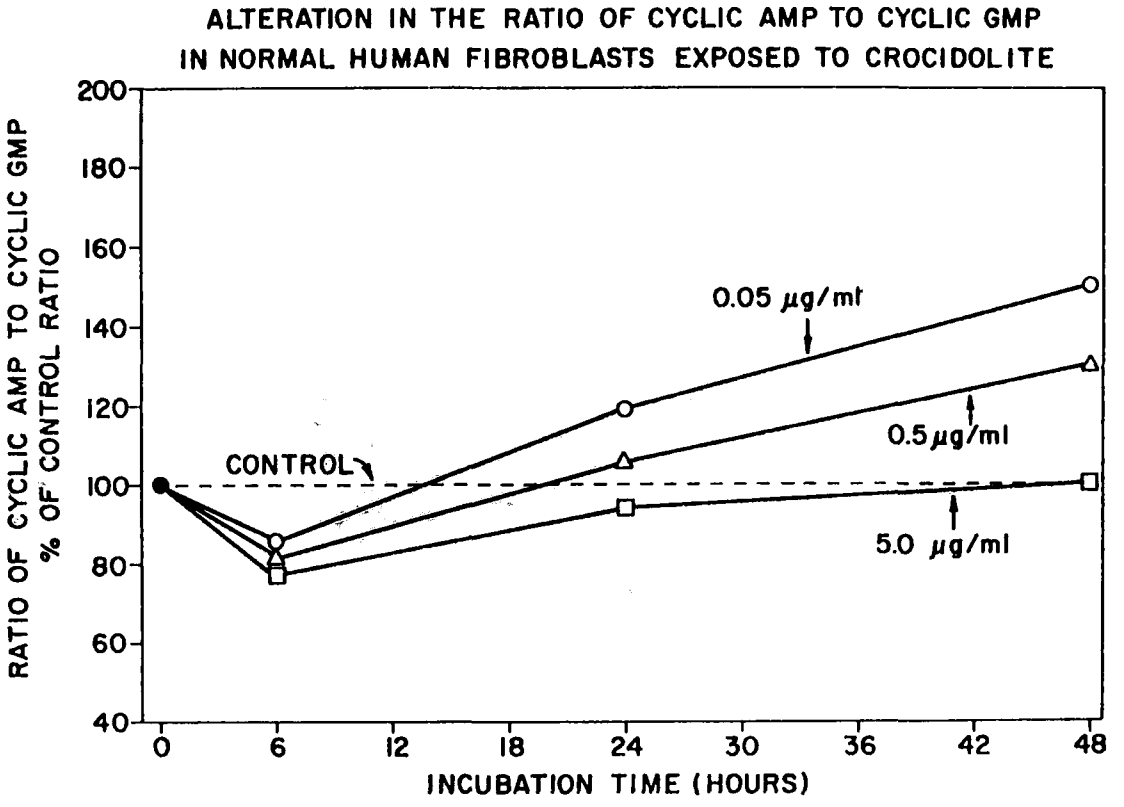

Figure 5. The effect of various concentrations of crocidolite on the ratio of cyclic AMP to cyclic GMP in normal human fibroblasts. The ratio of cyclic AMP to cyclic GMP in the control cells incubated without asbestos is taken as 100%. The ratio of cyclic AMP to cyclic GMP in the asbestos-treated cells is expressed as a percent (%) of control value. ------, control; o——o, 0.05 µg/ml; Δ——Δ, 0.5 µg/ml; □——□, 5.0 µg/ml.

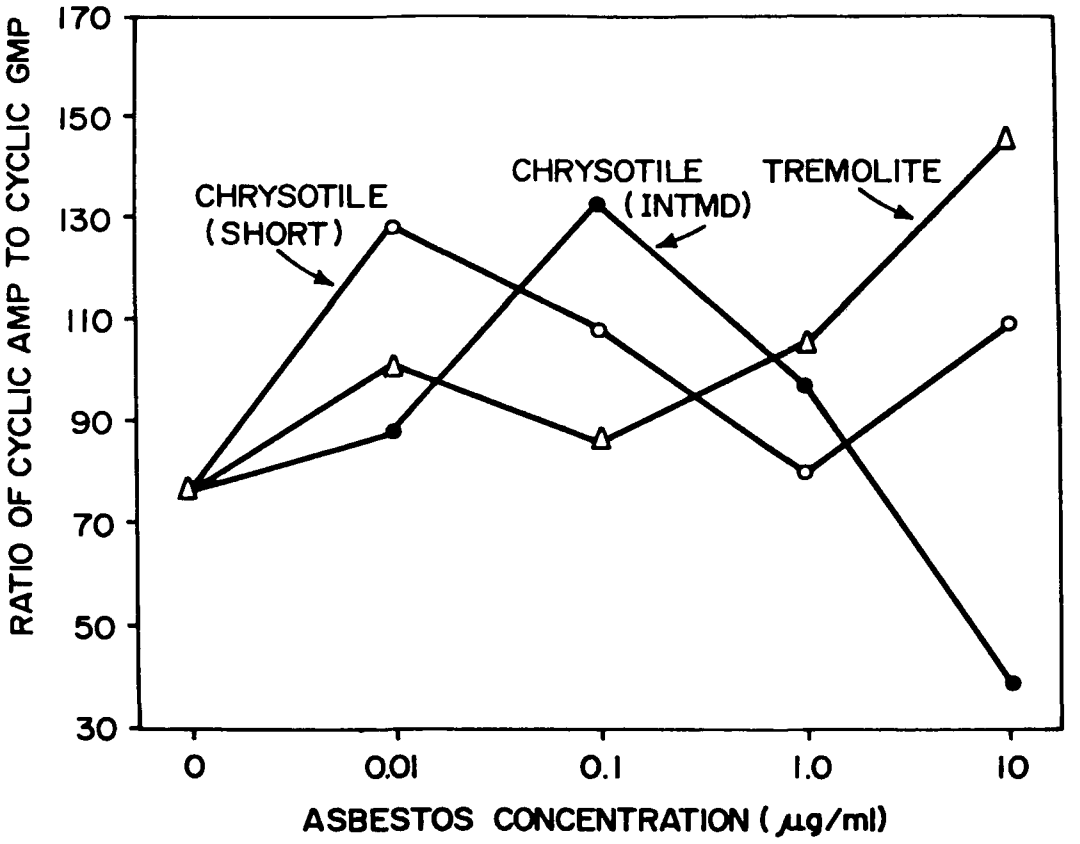

Figure 6. The effect of various types of asbestos on the ratio of cyclic AMP to cyclic GMP in normal human fibroblasts. o——o, chrysotile short type; ●——●, chrysotile intermediate; Δ——Δ, tremolite.

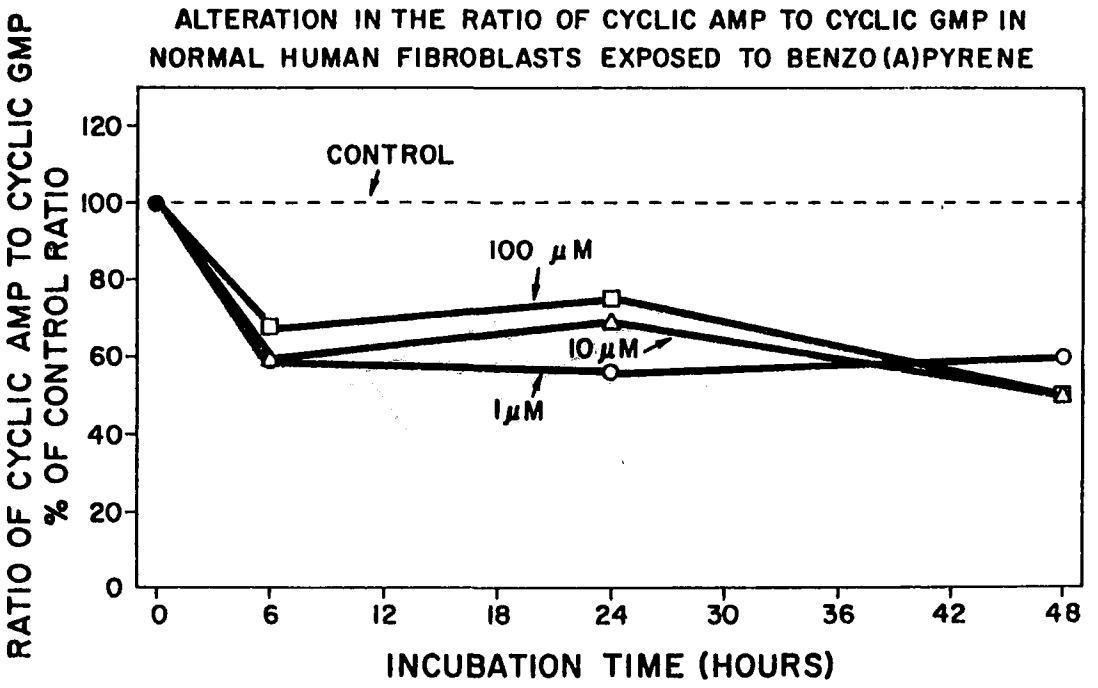

Figure 7. The effect of benzo(a)pyrene on the ratio of cyclic AMP to cyclic GMP in normal human fibroblasts. Benzo(a)pyrene (BP) was dissolved in dimethylsulfoxide (DMSO). The ratio of cyclic AMP to cyclic GMP is expressed as 100% of the control value. ------, control DMSO only; o——o, 1 μM BP; Δ——Δ, 10 μM BP; □——□, 100 μM BP.

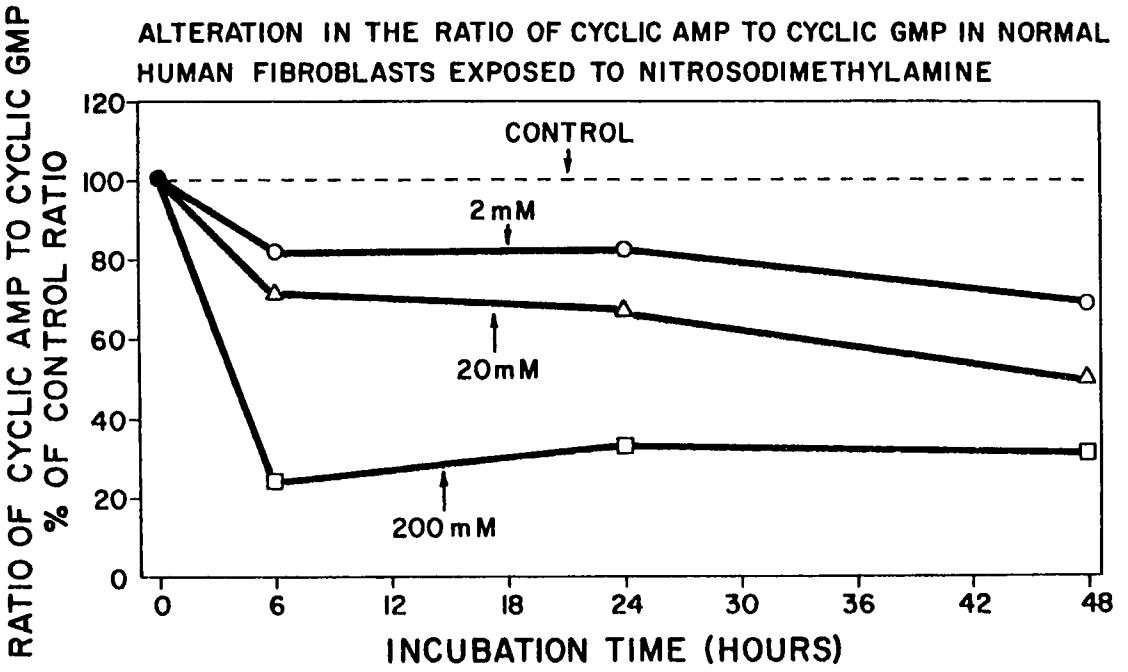

Figure 8. The effect of nitrosodimethylamine on the ratio of cyclic AMP to cyclic GMP in normal human fibroblasts. The ratio of cyclic AMP to cyclic GMP in the control cells is expressed as 100%. The ratio of cyclic AMP to cyclic GMP in nitrosodimethylamine-treated cells is expressed as a percent (%) of control value. ------, control; o·····o, 2 mM; Δ——Δ, 20 mM; □——□, 200 mM nitrosodimethylamine.

TABLE 5. RELATIVE DISTRIBUTION OF SURFACE LABELED GLYCOLIPIDS OF SYRIAN HAMSTER EMBRYONIC CELLS

Compounds	Percentage labeling*				
	Untreated cells	Chrysotile asbestos treated cells			
		2 hr	24 hr	48 hr	72 hr
Polar glycolipids					
Monosialoganglioside (G_{M1})	24.9 ± 2.33	25.2 ± 2.05	26.0 ± 2.47	37.5 ± 1.62	48.1 ± 2.05
Monosialoganglioside (G_{M2})	30.3 ± 3.74	29.8 ± 1.76	48.1 ± 2.89	51.2 ± 0.91	43.1 ± 1.20
Disialoganglioside (G_{D1a})	20.7 ± 1.97	20.2 ± 2.96	6.9 ± 1.89	3.9 ± 1.12	4.4 ± 1.68
Trisialoganglioside (G_{T1})	24.0 ± 3.39	24.7 ± 3.25	18.8 ± 1.48	7.3 ± 1.86	4.2 ± 0.89
Neutral glycolipids					
"Glucocerebroside"	32.8 ± 1.69	30.5 ± 1.20	21.2 ± 1.76	26.4 ± 0.56	29.6 ± 1.06
Cerebroside	31.1 ± 1.56	36.0 ± 1.20	35.4 ± 4.24	42.4 ± 1.69	56.5 ± 1.62
Globoside GL-4	35.9 ± 0.42	33.4 ± 2.40	43.3 ± 2.47	31.2 ± 2.26	13.8 ± 0.56

* Based on TLC comparison with known glycolipid standards
Mean ± standard deviation, n = 2

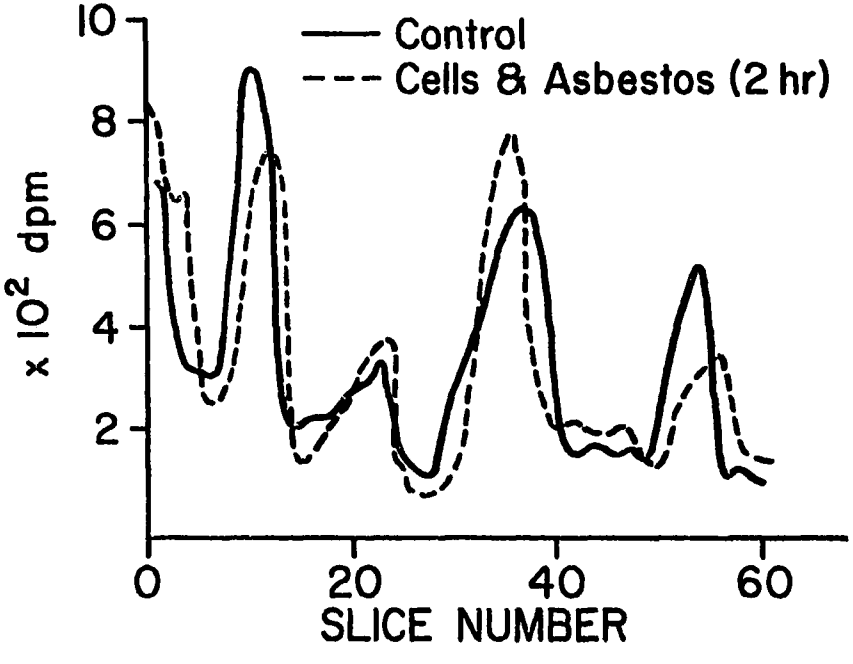

Figure 9. Control and 2 hr electropheretic distributions of cell surface ^{3}H-glycoproteins.

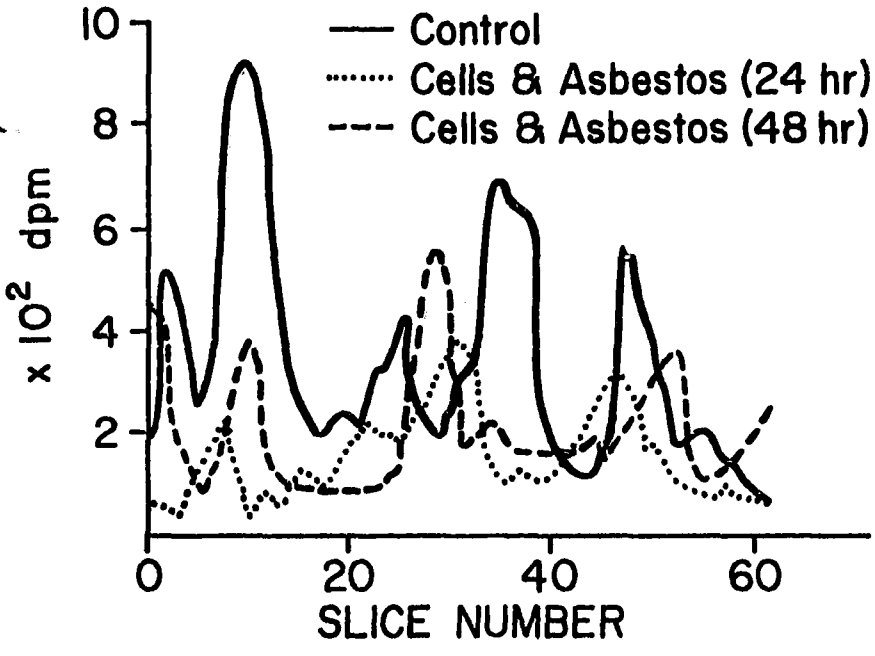

Figure 10. Control, 24 & 48 hr electropheretic distribution of cell surface ^{3}H-glycoproteins.

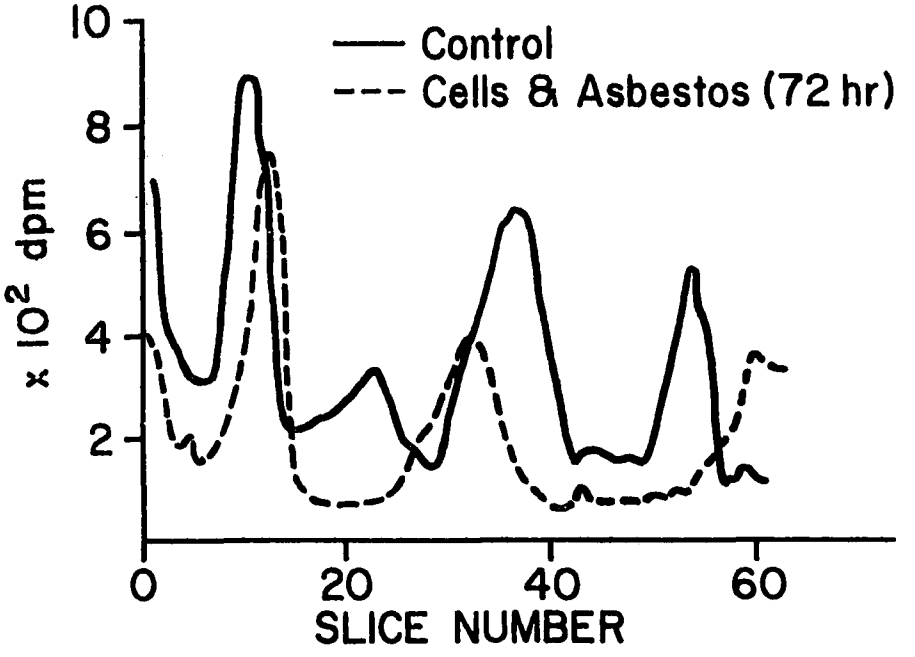

Figure 11. Control and 72 hr electropheretic distributions of cell surface ^{3}H-glycoproteins.

Changes in Cyclic Nucleotide Ratios

Brief (six hours) exposures of Detroit 550 human fibroblasts to either amosite or crocidolite asbestos decreased the cyclic AMP/cyclic GMP below the control value (See Figures 4 and 5). At 24 hours the ratio for both amosite and crocidolite fibers bracketed the control ratio. At 48 hours the ratio was almost universally greater than that of the control (39, 43, 44).

Modification of Chemical Carcinogen Metabolism and Association with DNA

No time studies of alteration of this factor are presented here.

SECTION 4

NEGATIVE OR NEGLIGIBLE RESULTS OF STUDIES WITH ASBESTOS

All asbestos fiber types at the highest concentration employed had little or no effect on induction of DNA damage based on unscheduled DNA synthesis if the samples were washed. Unwashed fibers caused small amounts of DNA damage. Crocidolite caused no cytotoxicity at fiber concentrations of 10^{-5} - 10^{1} µg/ml.

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16. ABSTRACT

Embryonic cultures were used to compare the effects of five asbestos materials on a cellular, biochemical and molecular basis. Chrysotile was found to be the most cytotoxic followed by crocidolite, tremolite, amosite, and silica. Results of tests involving cellular ratios of cyclic nucleotides were consistent with asbestos acting as a promoter of carcinogenesis. Washed asbestos fibers had little or no effect on induction of DNA damage based on unscheduled DNA synthesis tests.

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