A Survey of Nine Common Laboratory Compounds as Causative Agents of Ciliostasis in Tracheal Explants from Mice

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A SURVEY OF NINE COMMON LABORATORY
COMPOUNDS AS CAUSATIVE AGENTS OF
CILIOSTASIS IN TRACHEAL EXPLANTS FROM MICE

by

Josie Marshall Fletcher

A Thesis
Submitted to the
Faculty of The Graduate College
in partial fulfillment
of the
Degree of Master of Science

Western Michigan University
Kalamazoo, Michigan
August 1978
ACKNOWLEDGMENTS

I wish to thank my employer, The Upjohn Company, for its generous policy which allowed me to use its fine research facility and equipment. I further thank Dr. Cecil McIntire for his help and constructive criticism from which I have benefited. To my faculty adviser, Dr. Darwin Buthala, goes my deep appreciation for his constant support and interest. I also express my gratitude to my husband, Frank P. Fletcher, for his continued encouragement. These, as well as others, have made the pursuit of this degree a rewarding experience.

Josie Marshall Fletcher
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Western Michigan University, M.S., 1978
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LITERATURE REVIEW

Ciliated Cells: Their Function

The ciliated cells of various forms of animal life are widely diversified as to function and serve an amazing variety of purposes. In the lower forms they vary in duty from feeding appendages to propulsion mechanisms, and in other life forms cilia serve as aids to digestion, respiration, reproduction, excretion, sensory reception and cleaning of surfaces (Sleigh, 1966). Since there are basically no structural differences in cilia from Protozoa to man (Ambrose and Easty, 1971), all are relevant to this investigation regardless of animal form or activity.

Ciliated cells are found in the bodies of mammals, in the eye, the ear, the respiratory tract, the oviducts of the female and the structurally identical flagella are found in the spermatozoa of the male. Differentiation of cilia and flagella are made upon the direction of stroke or beat (Sleigh, 1974). Cilia are seen to move with a waving motion, while flagella have a screw-like movement. There are such slight variations in movement in some intermediate organelles that the name "undulipodium" has been suggested to include both cilia and flagella (Shamagina, 1948), but has not been widely accepted.

In order to better understand the role of cilia, the structure of this organelle will be examined in greater detail. The advent of the high resolution electron microscope has made this possible. Since the
The purpose of all motile cilia is to propel cells through an aqueous environment or to propel that environment over a cell's surface (Warner, 1974), motion is the primary function for cilia. While there are variations (Warner, 1972), most cilia (and flagella) consist basically of a sheaf of filaments or microtubules whose pattern is a 9 + 2 arrangement, nine double structures surrounding a central pair of single elements. The total structure, an axoneme, is enclosed in a membrane extension of the cytoplasm. For this reason it is considered to be a protruding portion of the cell beneath (Satir, 1974). As a result of the work of many scientists (Satir, 1950; Hoffman-Berling, 1950; Satir and Child, 1960; Gibbons and Gibbons, 1962; Gibbons and Rowe, 1965; and Brokaw, 1966), it is now understood how the axoneme provides ciliary activity. This activity is not the product, as was formerly believed, of an appendage powered by contractile elements elsewhere in the cell. It is, instead, currently believed to be an intricate series of movements primarily dependent upon an energy supply which is located within the cilium. Satir (1974) has condensed and clarified the sequence of events in the following manner:

"Each tubular element is actually a microtubule...composed of small protein molecules called tubulins. There are at least two kinds of tubulin molecules in each microtubule. They are strung together...in protofilaments like columns to make up the cell of the microtubule. Each anemomal doublet is composed of two subfibers, A and B. Subfiber A is a complete microtubule with 13 protofilaments. Subfiber B is shorter in length and has fewer protofilaments—only ten or eleven—so that it sits along subfiber A like an intersecting, incomplete cylinder. The dynein (protein) molecules are spaced along subfiber A in two differently oriented rows; they form two sets of arms (inner and outer) that point toward subfiber B of the adjacent doublet and are long enough to bridge the space between the microtubules. If the ciliary microtubules could 'walk along' one another by means of their dynein arms one could envisage that, given the
right sequence of activity and appropriate shear resistance within the axoneme, bends would form and would propagate in such a way as to produce the movements characteristic of cilia. The hypothesis that this is the mechanics of ciliary motion is called the sliding microtubule hypothesis."

Also found in the ciliated cell is a basal body which lies at the origin of every cilium and has the same structure as a centrisome, a cylinder whose wall is formed by nine microtubules. The remainder of the ciliated cell may include the mitochondria, Golgi complex, cell nucleus and cytoplasm.

Ciliated cells and their components compose only part of the tracheal epithelium however. Dependent upon the life form there also may be found intermediate cells (Rhodin, 1966), mucous cells, brush cells (Pavelka, 1976), endocrine cells (Cuz, et al., 1975), basal cells and their various components. Thus, understanding the mechanism of ciliary activity, it is now possible to relate this activity to the impact of ciliary motion on pathogenicity. The presence or absence of cilia functioning in their accustomed places can make a vast difference in the well-being of the total organism, as will be reported subsequently.

A congenital condition of nonfunctioning cilia has been reported from several sources, (Camner, et al., 1975), (Eliasson, et al., 1977), (Guerrant, et al., 1978), and in every case the pathology is attributed to the same causative agent—abnormal dynein arms. Pathologic conditions which exist as a result of this condition include chronic airway infections, otitis media and macular degeneration. Congenital sterility frequently accompanies these. All are brought about by immotile cilia (Guerrant, et al., 1975).
Another factor in ciliary-associated pathology is thought to be concerned with number and distribution of cilia. It has been demonstrated by at least one study (Gabridge, et al., 1977) that attachment of pathogens (*M. pneumoniae*) in tracheal explants of the hamster was inversely related to the amount of ciliation present.

As has been noted, the responsibility of the cilia of the trachea is to move the mucus along the respiratory tract. If ciliostasis prevents this movement irritants may be retained and ultimately enter the epithelium (Kortin, et al., 1966). In the wake of many studies prompted by a statistical link between cigarette smoke and cancer, it has now been well established that the deposition of carcinogens of all kinds may occur following the sequence of events begun by ciliostasis, inhibition of mucus transport, retention of irritants, desquamation and penetration to the basal cells. It is here that regeneration provides an optimal opportunity for malignant transformation (Kortin, et al., 1966).

Kensler and Battista (1966) point out that any factor which interferes with the transport rate of the respiratory tract has potential for pathogenesis, even though the change in ciliary beat may be due to a change in the composition and physical properties of the mucus itself. This observation also is based upon the belief that environmental carcinogens, if not cleared, have the ability to penetrate to the epithelial cells which line the respiratory tree. These investigators have divided most of the agents which can be studied in conjunction with ciliary activity into three groups. They are endogenous regulating, general pollutants and those agents containing special
Based on studies by Satir (1974), it now appears that the endogenous regulating factors of greatest importance are ATP and dynein. ATP is believed to be the energy source for motility in cilia, while dynein is the name given to ATPase by its discoverers, Gibbons and Rowe (1965). The breakdown of ATP and conversion to ADP and phosphoric acid by dynein causes ciliary beat.

The second group, that of general pollutants, has been studied extensively in the last few years owing to an increasing concern over environmental contamination. Industry is held responsible in many cases, so industrial pollutants have been a popular area of experimental research. Chromates, which fall into this group, were found to cause ciliostasis in concentrations of 10 mg/ml within twenty minutes (Moss and Lane, 1975). This in vitro study of tracheal explants from rats was made with special interest toward workers in the chrome plating and chromate industries. In a study involving levels of $SO_2$ (Hirsch, Swenson and Wanner, 1974) based on amounts less than the concentrations found in some major metropolitan areas of this country during peak traffic and industrial pollution, an impairment of mucociliary activity was noted. Dalhamm (1961) found in an in vivo study in rats that ciliary response was directly related to the solubility ratio of compounds tested. The size of a carrier particle to which an irritant may become attached is a factor in determining how long said irritant is retained in the respiratory tract (Cresia, et al., 1976), with larger particles being eluted more slowly (Farrell and Davis, 1974). However, Henry and Kaufman (1973) have showed that increased retention
of benzo(a)pyrene, when bound tightly to large carbon particles, did not necessarily demonstrate greater tumorigenicity, but Kortin, et al., (1966), reported that irritants showing a nonspecific effect on the respiratory epithelium can and frequently do alter ciliary action and modify the properties of mucus.

In concurrence with studies reported by Harris, et al., (1971), Port, et al., (1973) found that multiple intertracheal instillation of ferric oxide in hamsters precipitated a loss of ciliated cells, leaving rough and wrinkled surfaces. In addition, these investigators determined that benzo(a)pyrene with ferric oxide caused squamous metaplasia and foci of normal protuberant cells.

An in vivo study conducted on the instillation of diethylnitrosamine into the tracheas of hamsters (Spit and Peron, 1975) showed resulting tumors to be devoid of ciliated cells, although ciliated cells were in a somewhat normal picture elsewhere in surrounding tissue, but low in incidence.

The third group includes negatively or positively charged ions which find their way into the respiratory tract and their effect upon ciliary activity both in vivo and in vitro. Satir (1975) showed that ionophore-mediated calcium caused ciliary arrest in mussel gills, surmising that the effect of the high Ca$^{2+}$ is upon the sliding microtubules of the axoneme. Krueger and Smith (1958) reported that positive and negative ions have marked effects on ciliary rates in the tracheas of mammals, both in vivo and in vitro, and further reported these patterns deviated when combined with commercially produced gases or cigarette smoke. An attempt to repeat this study with
confirming results by Kensler and Battista (1966) failed. However, these workers further stated that no experiments done until that time could be more than suggestive that the pollutants mentioned previously could, under ordinary circumstances of breathing or smoking, result in impaired ciliary transport. Jahn in 1962 established a relationship between ciliary activity and potassium and calcium ions. Thus the problem of ion antagonism is not new but does not appear to have been explored as extensively as some of the other causative agents in ciliostasis.
Tracheal Explant Controls

Literature surveyed on tracheal explant controls were of interest in that Palekar, et al., (1968) reported seeing in cultures of rat explants cells which degenerated initially and later differentiated into normal ciliated epithelium. Further, they observed "newly formed cuboidal cells...prominent newly formed cilia, then a regression with a loss of cilia by the third week." Dalhamn (1966) stated that actual growth in culture of ciliated cells has been accomplished many times and is related to control of Vitamin A content. On the other hand, Dirkson and Crocker (1968) found after eleven days in culture, structural features of explants from suckling rat tracheas did not deviate appreciably from normal morphology. Pavelka (1976) found that after six days in culture tracheal explants from mice did not show any appreciable changes, and utilizing hamster tracheal explants in culture for twenty weeks Mossman and Craighead (1975) found the explants were similar to cells in the intact animal.

The question arises, if indeed some of the explants did undergo changes, how would an investigator be able to monitor accurately explants under test circumstances in the face of such erratic "normal" behavior?
MATERIALS AND METHODS

Laboratory Equipment

Initial work was begun on this project in the fall of 1977. Some equipment problems had to be solved as the first step. It was not possible to obtain the exclusive use of a CO\textsubscript{2} incubator. Rather than share an incubator with researchers in other areas, thereby perhaps introducing a variable in conditions, it seemed wiser to make adaptations of the existing equipment.

A standard incubator with a hot water jacket was secured, along with a cylinder of 95\% CO\textsubscript{2}/5\% O\textsubscript{2}. A dessicator was adapted to serve as a CO\textsubscript{2} chamber by filing away the top and substituting a rubber stopper bored with one hole. This hole was fitted with a piece of glass tubing which ran to a point below the porcelain plate inside the dessicator. The top of the tubing made a 90\degree turn one inch above the stopper and terminated in a piece of rubber tubing which could be closed with a pinchcock. The standard aperture found on the dessicator was then utilized as an exhaust source for the spent atmosphere. An intermediate connection containing a millipore filter was inserted between the CO\textsubscript{2} tank and the specimen chamber to further ensure sterile conditions. It had been noted during pretest trials that plastic culture dishes were attacked by the fumes of some of the test compounds. Accordingly, sterile glass Petri dishes were used throughout. Wells were made to hold the test compounds by cutting off scintillation vials which had a diameter of 2.5 cm at a depth of 1.5 cm. All testing equipment was autoclaved at 15 lbs. pressure for twenty minutes before its use.
The Testing Compounds

1. Formaldehyde is used in the laboratory primarily as a fixative. It is a saturated solution of formaldehyde gas (40%) in water. The fixing fluid commonly used is formalin, a 4% dilution of formaldehyde. As a fixative it penetrates tissue well with a minimum of hardening and shrinkage.

2. Gluteraldehyde is widely used as a fixative in electron microscopy. It has ability which is superior to that of all other fixatives in the cross linkage of proteins (Hyatt, 1970). Its rate of penetration is relatively slow compared to that of formalin. In order to effect complete penetration of tissue and uniform fixation by gluteraldehyde, tissue to be fixed usually is reduced to 1 mm. cubes.

3. Osmium is also a slowly-penetrating fixative. It has the added advantage of acting as an electron stain and is frequently used as a post-fixative after gluteraldehyde. Osmium stabilizes proteins in tissue by transforming them into clear gels, preserving structural features (Hayat, 1970).

4. Ethyl alcohol also has certain fixation qualities, coagulating protein by denaturing it. Ethyl alcohol is not generally used as a fixative because it causes shrinkage and hardening. Rather in tissue work, it is used to dehydrate after fixation has taken place. In addition to the previously mentioned qualities, ethyl alcohol has widespread use in the laboratory, ranging from those of a diluent to those of a disinfectant.

5. Methyl alcohol has specified laboratory uses and also is used
widely in industry in thinners, resins and perfumes. It is used externally in hospitals for its astringent and disinfectant qualities. Its toxicity is mostly irreversible if taken internally.

6. Ether is used in the laboratory in certain extraction procedures and for other specific techniques, but its widespread use has been as an anesthetic. In recent years many other anesthetics have been developed, somewhat lessening the popularity of ether. It is extremely volatile in nature and is not used widely in industry.

7. Chloroform is a potent anesthetic but has undesirable side effects (liver damage, ventricular fibrillation), and readily diffuses across the placental membrane. (Martindale, 1977). It is a fat solvent and is useful in the laboratory because of this property. Chloroform is used in industry in spite of the aforementioned biological hazards. Industrial uses include that of a solvent for resins, oils, rubber and as a preservative and a flavoring agent.

8. Acetone is used in the tissue laboratory in dehydrating and staining techniques. It has other laboratory uses based on its drying qualities and low boiling point. Acetone has been used commercially as a solvent for glue, as nail polish remover, and as setting fluid for casting limbs. Acetone is toxic through inhalation and by absorption. Severe acute poisoning has occurred as a result of absorption (Martindale, 1977), as well as anticipated toxicity from inhalation.

9. Toluene, a product of coal tar, is used as a reagent in the laboratory and in industry is used in the manufacture of benzoic acid, dyes, explosives and as a solvent. Toluene may be contaminated with benzene, which is a carcinogen. Inhalation of toluene causes intoxica-

Physical properties of the preceding agents may be found in Table 1.
<table>
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<td><strong>Table of Testing Agents and Properties</strong></td>
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<table>
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<tr>
<th>Sol. in 100 parts</th>
<th>Water</th>
<th>Alc.</th>
<th>Ether</th>
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<tr>
<td>Methyl alcohol (wood alc.) ( \text{CH}_3\text{OH} )</td>
<td>s.alk.</td>
<td>s.</td>
<td>s.</td>
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<tr>
<td>Ethyl alcohol (ethanor) ( \text{CH}_3\text{CH}_2\text{OH} )</td>
<td>oo</td>
<td>chl.</td>
<td>oo</td>
</tr>
<tr>
<td>Acetone (propanone) ( \text{CH}_3\text{CO-CH}_3 )</td>
<td>oo</td>
<td>oo</td>
<td>oo</td>
</tr>
<tr>
<td>Ether ((di)ethyl ether) ( \text{(C}_2\text{H}_5)_2\text{O} )</td>
<td>7.5 (^{20^oC})</td>
<td>oo</td>
<td>chl.</td>
</tr>
<tr>
<td>Chloroform (trichloromethane) ( \text{CHCl}_3 )</td>
<td>....</td>
<td>....</td>
<td>....</td>
</tr>
<tr>
<td>Toluene (methyl benzene) ( \text{CH}_3\text{C}_6\text{H}_5 )</td>
<td>i;s.act.</td>
<td>abs.</td>
<td>oo</td>
</tr>
<tr>
<td>Formaldehyde (methanal) ( \text{H-C:0} )</td>
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<td>vs.</td>
<td>vs.</td>
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<tr>
<td>Gluteraldehyde ( \text{CHO(CH}_3)_2\text{CHO} )</td>
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<td>....</td>
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<tr>
<td>Osmium tetroxide ( \text{OsO}_4 )</td>
<td>vs.</td>
<td>vs.</td>
<td>i.</td>
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**Abbreviations:**

abs., absolute  
act., acetone  
al., ethyl alcohol  
alk., alkali  
chl., chloroform  
i., insoluble  
s., soluble  
v., very
Organ Cultures

**Control Specimens**

Explants were prepared from the excised tracheas of 25 gram adult white mice of both sexes. Each mouse was anesthetized by an intraperitoneal injection of 0.1 ml. sodium pentabarbital (Dibutol Diamond). The trachea was removed from a point just below the larynx to the bifurcation into the bronchi. It was placed immediately in sterile Eagle's Essential Medium with 10% fetal calf serum and mycostatin (150 ppm solution) 15 ml./1.5 liters, which had been warmed to 37°C. Here, in as aseptic a manner as possible, all excess bits of tissue and muscle were removed and the organ was opened along the anatomic fault. The trachea was washed through several fresh changes of media, placed on a teflon square under a dissection scope and as rapidly as possible separated into c-shaped rings with a Börd-Parker scalpel. These rings were placed in a dish of fresh, sterile 37°C. media which was to serve as a pool for successive explants. When tracheal rings from six mice had been accumulated in this manner, they were separated into Petri dishes of six to eight rings each. These groups were maintained in 95% CO₂/5% O₂ at 37°C for three weeks. Media was replenished every second day and explants were checked at this time for ciliary activity by use of an inverted microscope (Nikon). Representative explants were removed at 5, 10, 15 and 18 day intervals and processed as stated below.

**Explants for testing**

These were prepared identically to the controls as described above.
but were held for only 12 hours. At the end of the 12-hour stabilizing period, explants to be used in testing were examined for ciliary activity. Having ascertained this feature, four ml. of each of the compounds selected were placed into a well 2.5 cm in diameter and 1.5 cm deep. This was placed inside the Petri dish containing the explants and held at 37°C except when being monitored at intervals for ciliary activity. When ciliostasis was complete these explants were processed as described for scanning electron microscopy.

**Fixation and dehydration**

Explants were fixed for a period of two hours in 2% gluteraldehyde in phosphate buffer. They were washed in phosphate buffer and post-fixed for 1-1/2 hours in Osmium tetroxide which had been diluted to 2% in distilled water and further diluted 1:1 in Millonig's double-strength buffer (in ice). The explants were washed in distilled water briefly and dehydrated through a series of graded alcohols (Table 2). At this point the explants were divided as follows:

**Preparation for scanning electron microscopy**

Specimens to be examined by SEM were critical point dried with liquid CO₂ (100%) at a pressure of 100 lbs. and a temperature of 31°C. (Table 3). Having undergone removal of all moisture, the explants were then mounted with the aid of double-faced tape and silver paste on standard SEM stubs and sputter coated with gold in the following manner: stubs with the mounted explants were placed in the Denton Vacuum Evaporator. In the presence of a vacuum into which Argon gas had been released, a charge was applied to an electrode containing a thin gold sheet. The ionization of the gold in the Argon atmosphere caused a
Table 2

Processing of Specimens for Electron Microscopy

<table>
<thead>
<tr>
<th>Step</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Glutaraldehyde Fixation 1-1/2 hrs., 4°C</td>
</tr>
<tr>
<td>2</td>
<td>Phosphate Buffer Wash</td>
</tr>
<tr>
<td>3</td>
<td>Osmium Fixation (in ice) 1-1/2 hrs.</td>
</tr>
<tr>
<td>4</td>
<td>Distilled H₂O Wash</td>
</tr>
<tr>
<td>5</td>
<td>50% EtOH 5-15 min.</td>
</tr>
<tr>
<td>6</td>
<td>75% EtOH 5-15 min.</td>
</tr>
<tr>
<td>7</td>
<td>85% EtOH 5-15 min.</td>
</tr>
<tr>
<td>8</td>
<td>95% EtOH 5-15 min.</td>
</tr>
<tr>
<td>9</td>
<td>100% EtOH 10 min.</td>
</tr>
<tr>
<td>10</td>
<td>100% EtOH 10 min.</td>
</tr>
</tbody>
</table>

Further Processing for SEM (See Table)  Further Processing for TEM (See Table)
Further Processing for SEM

100% EtOH

Critical Point Drying (Pelco Instrument)

The essence of operation is as follows:

a) Cool the instrument to about 8°C. adiabatically by running gas in the Gas In valve and out the Vent valve. (Gas In valve should be open about 3/4 turn and the Vent valve about 1-1/2 turns.)

b) Fill the chamber by bringing in gas through the Gas In valve (about 3/4 turn) and venting through the Vent valve (about 1/4 turn).

c) Flush the material by opening the Vent valve further (up to about 1 full turn open) until gas is heard exhausting, but with a full chamber level being maintained.

d) After flushing, close both valves and activate the Auto. Heat switch. Heating the chamber also builds up pressure.

e) After critical temperature (31°C.) and critical pressure (1070 lbs.) have been exceeded, by opening the Vent valve slightly, bleed off all the gas.

f) Remove the material, mount, coat (if desired), and examine in a microscope.

The purpose of critical point drying is to minimize problems encountered in "air drying", such as surface tension effects of distortion and wrinkling.

The fluids remaining in the tissue are exchanged for a transitional fluid, CO₂, which is then, by means of increased pressure and elevated temperature, converted to a gaseous state which is then "bled off" leaving the specimen dried and intact at atmospheric pressure.
shower of gold over the specimens below, forming a coat which ultimately served as an outline for the features of the specimen and also as a ground for the electron beam. The metal coating prevents the accumulation of localized charging. The specimens were then examined with a Cambridge 150 scanning electron microscope and subsequent photographs were recorded on Polaroid P/N film, type 55.

Preparation for transmission electron microscope

The control explants to be examined by TEM were removed from the 100% alcohol, placed in several changes of propylene oxide and then into a mixture of propylene oxide and epon resin (Table 4). They were left overnight in this mixture in order for the propylene to evaporate and for the infiltration of the epon resin to occur. The next morning the explants were dessicated under vacuum in fresh epon resin and embedded in tissue molds. They were placed in a 60°C drying oven for 72 hours while polymerization was completed. Thin sections were cut from the polymerized embedments using a Sorvall Mt-2 microtome with a diamond knife. The sections were cut at the silver interference level (80 nm). They were picked up on 200 mesh copper grids and stained with uranyl acetate and lead citrate (Reynolds, 1963). The ultrastructure of the control specimens was examined using a Phillips 201 Transmission Electron Microscope and photographs were recorded using Kodak Electron film #4463.
<table>
<thead>
<tr>
<th>Step</th>
<th>Solution</th>
<th>Time</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>100% EtOH</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>Propylene Oxide</td>
<td>10 min.</td>
</tr>
<tr>
<td>3</td>
<td>Propylene Oxide</td>
<td>10 min.</td>
</tr>
<tr>
<td>4</td>
<td>Propylene Oxide, Epon Resin</td>
<td>1:1, 1-1/2 hrs.</td>
</tr>
<tr>
<td>5</td>
<td>Propylene Oxide, Epon Resin</td>
<td>1:2, 2 hrs.</td>
</tr>
<tr>
<td>6</td>
<td>Propylene Oxide, Epon Resin</td>
<td>1:3, Overnight on rotary drum</td>
</tr>
<tr>
<td>7</td>
<td>Fresh Epon Resin</td>
<td>1 hr. (min.) vacuum dessicator</td>
</tr>
<tr>
<td>8</td>
<td>Embed with fresh Epon Resin</td>
<td></td>
</tr>
<tr>
<td>9</td>
<td>Drying Oven 60°C</td>
<td>72 hrs.</td>
</tr>
</tbody>
</table>
Solutions

1. Phosphate Buffer (0.1N, pH 7.4)

   Monobasic sodium phosphate 2.760 gm/100 ml \( H_2O \) dist. \( H_2O \)
   Diabasic sodium phosphate 14.196 gm/500 ml \( H_2O \) dist. \( H_2O \)

   The above are stock solutions

   19 ml stock solution monobasic sodium phosphate
   81 ml stock solution diabasic sodium phosphate
   100 ml dist. \( H_2O \)

   Calibrate buffer to pH 7.4 with pH meter

2. Millonig's Buffer (double strength)

   6.752 gm monobasic sodium phosphate
   2.160 gm dextrose
   19.278 ml sodium hydroxide, 2N

   Quantity sufficient to 200 ml with dist. \( H_2O \)

3. Uranyl Acetate

   2% aqueous solution (stock)

   Dilute to .2% for stain

4. Reynold's Lead Stain

   Chemicals:

   Lead nitrate (Malin)
   Sodium citrate
   Sodium hydroxide "Acculate" 1N (anachemia)
   \( H_2O \), double distilled, \( CO_2 \) free
Preparation:

(1) Boil and cool 700 ml. distilled H\textsubscript{2}O

(2) Prepare 2N NaOH

(3) Into a chemically clean 50 ml. volumetric flask
   a. Weigh:
      lead nitrate 1.33 gm.
      sodium citrate 1.76 gm.
   b. Swirl to mix dry chemicals and distribute evenly over bottom of flask.
   c. Add approximately 10 ml. \textsubscript{H}2O, swirl quickly to avoid caking.
   d. Add H\textsubscript{2}O to approximately 30 ml.
   e. Stopper and shake flask vigorously by hand for 30 minutes.
   f. Add 4 ml freshly prepared 2N NaOH.
      Swirl to clear ppt.
   g. Quantity sufficient to make 50 ml with water and mix.

5. Media
   Eagle's Minimum Essential Medium (Hanks' base)
   10\% fetal calf serum
   Mycostatin suspension 150/ppm (Grand Island Bio. Co., Grand Island, New York)

6. Osmium Tetroxide (Polysciences)
   1 gm. dissolved in 50 ml. dist. H\textsubscript{2}O (stock)
   Store in stoppered bottle in plastic bag at 4°C.
   Make fresh day of use equal parts of osmium and Millonig's buffer (keep stoppered).

7. Glutaraldehyde (Ladd) 70\%
   Dilute to 2\% in phosphate buffer, store at 4°C.
Epon Resin
(Ladd Research Industries)

The weight per epoxide equivalent was determined based on the WPE number given on the epon 812. A general formula for calculating the amount of anhydride used follows:

\[
\text{weight of anhydride} = \frac{\text{wt. of resin}}{\text{WPE}} \times \frac{\text{anhydride equivalent}}{\text{anhydride to resin}} \times \frac{\text{ratio of anhydride to resin}}{1}
\]

In the equation:

1. WPE = weight of epoxy resin containing one equivalent weight of epoxide.
2. Anhydride equivalent = molecular weight of anhydride for dibasic anhydrides such as DDSA or NMA.
3. Ratio = ratio of equivalent of anhydride to equivalent of epoxy resin, i.e., 0.7:1.

Assuming a W.P.E. of 160

Wt. of DDSA = \(\frac{80}{160} \times 266 \times 0.7 = 93.1 \text{ gms.}\)

Wt. of NMA = \(\frac{100}{160} \times 178 \times 0.7 = 77.87 \text{ gms.}\)

Mixture A
93 gms. DDSA in 8 oz. bottle
80 gms. epon 812
Cap and shake well

Mixture B
18 gms. NMA in 8 oz. bottle
100 gms. epon 812
Cap and shake well

Use in ratio 3A:7B with .14 mL DMP (accelerator) for each 10 mL complete resin. Stir mechanically and deaerate in vacuum dessicator.
THE EXPERIMENT

An Attempt to Use Toluene in Solution for Testing

The elusive significance of the ciliated cells of the tracheal epithelium has been surveyed from many aspects— as indicators of cytotoxicity of an invading particle or gas, as a clearing agent for a part of the respiratory tract, as a critical function in a sequel of events whose absence could herald pathogenesis and as an organ to be evaluated as a segment of the total respiratory tract are but a few of these.

The original intent of this experiment was to measure the dose level at which toluene caused ciliostasis in mouse tracheal explants which were growing in culture medium. Toluene is defined as being only slightly soluble in water, but soluble in acetone and ethyl alcohol. Since benzene studies, according to available literature, had been made utilizing acetone as the intermediate vehicle, and since acetone is soluble in both water and toluene, acetone seemed to be the vehicle of choice. Log 10 dilutions of acetone and toluene into Eagle's medium were made with the first tube containing 1:1 toluene/acetone. It was determined by these dilutions that the smallest amount of acetone required to keep toluene in even suspension in 10 ml. media was a 1:1 dilution. When greater amounts of toluene/acetone were used, the acetone became miscible in the media and the toluene rose to the surface in concentrated bubbles. These could be solublized briefly by agitating on a vortex mixer, but the toluene soon returned to its former state.
Therefore, the explants were set up using a 1:1 dilution of acetone/toluene in 10 ml media, and log 10 dilutions of the 1:1 dilution were made. It appeared after two log 10 runs and one run in which multiples of 20 were used that ciliostasis could be established at 1:200. Duplicate cultures were made and dilutions using acetone alone were set up as controls. In the resulting tests, the acetone was found to be capable of ciliostasis in dilutions comparably as high as with toluene. Since it appeared impossible to utilize toluene in the amounts necessary for acute toxicity studies without some intermediate solvent, an attempt was made to use either ethyl alcohol or Tween 80. Too much alcohol was required to hold the toluene in solution to be practical. Upon adding toluene to Tween 80 in any of a vast number of dilutions a precipitate was always observed. At this point new parameters were sought which will be described subsequently.

New Parameters

Since it appeared that toluene could not be successfully combined with another compound for the original testing agent, it was decided to allow the toluene to vaporize within the Petri dish, designating its solubility ratio as the greatest amount of gas which could be present and soluble at that temperature. This was done as will be later described with eight other commonly used laboratory compounds. The complete list of test compounds is as follows:

- toluene
- acetone
- ethyl alcohol
- methyl alcohol
- ether
- chloroform
- formaldehyde
- gluteraldehyde
- osmium tetroxide

Pertinent data concerning these compounds may be found in Table 1.

When guidelines were drawn up for this investigation, the format
appeared to be of an environmental nature. Pursuing this approach, it became evident that compounds in a gaseous state were more allied to an environmental condition than those found in a liquid state. Only in isolated instances would the ciliated cells of the trachea be in contact with any of the test compounds in liquid forms. There are existing statistics which have been documented from accidents and suicides or attempted suicides dealing with such situations and effects.

On the other hand, the plight of big-city dwellers who are forced to live out their lives under a cloud of noxious fumes from many sources is becoming increasingly common, and it is to a relevant minuscule of this problem that this effort is addressed.

Compounds selected as testing agents for this study were but a few of those which usually are found on the shelves of a biological laboratory. It was felt that a study such as this would be of particular interest to those who encounter these and similar compounds on a daily basis, whether it be in the field of scientific education, research, health or industry. Some of the compounds reach many people disguised as consumer products. In spite of conscientious attempts by manufacturers to label products adequately, potential hazards to the public and to environmental health seem to be a constant problem. One such problem of the last decade is glue-sniffing by young builders of toy models. At first an inadvertent by-product of a hobby, the practice mushroomed until manufacturers were forced to abandon the hydrocarbon-based glue for a less toxic substitute.

In industry a potential for toxicity might be encountered based on a time/quantity relationship, similar to that found in a laboratory.
In the leather industry benzene is used in the tanning process. A worker for a period of years in such a factory could be subjected to extreme amounts of toxicity based upon occupational exposure. Frequently many more years elapse between termination of a job in an industry under study and the onset of a catastrophic disease in a former employee, which may or may not be job related. For this reason a time lapse factor must be taken into consideration along with other data (Ott, et al., 1978). It is becoming increasingly difficult for investigators to follow the subjects of such a study as the population becomes more mobile, but as chemicals are used more freely in an expanding industry, such research becomes more important to public health and even survival than ever before.
RESULTS

Controls

The control explants, when viewed by the scanning electron microscope, were seen to be composed of a surface covered by a network of cells, both ciliated and nonciliated. The ciliated cells occurred in patches for the most part and the cilia were uniform in size and overall appearance. The nonciliated cells had many microvilli protruding from their surfaces and cellular junctions were clearly defined.

Explants from the same control group were viewed by the transmission electron microscope. The familiar 9 + 2 configuration was in evidence in the cross sections of axonemes and the remaining ultrastructure of the cell appeared to conform to standard observations (Pavelka, et al., 1976). (Fig. 2)
Figure 2

TEM photomicrograph of the tracheal epithelium of a mouse showing a cross section of the tracheal epithelium. Cilia may be seen with their axonemes in the upper segments and basal bodies below. Microvilli are interspersed between the groups of cilia. Mitochondria may be seen in numbers below the basal bodies. 30,000 x.
Figure 3

TEM photomicrograph of cilia in cross section showing 9 + 2 arrangement of microtubules and double walled cell membrane. 60,000 x.

Figure 4

TEM photomicrograph of basal bodies with their microtubules. Microvilli may also be seen. By contrast the microvilli have no visible internal structure. 45,000 x.
Figure 5

SEM photomicrograph of the tracheal epithelium showing ciliated cells, nonciliated cells and cell junctions. 3,800 x.

Figure 6

SEM photomicrograph of ten-day untreated cultures demonstrating visibly intact cilia. Microvilli are apparent in the background. 29,000 x.
Figure 7

SEM photomicrograph showing cilia from an untreated five-day culture. 9,500 x.

Figure 8

SEM microphotograph from a five-day untreated culture showing patterns of both ciliated and nonciliated cells. 3,800 x.
Figure 9

Cells in this SEM photomicrograph from a five-day culture appear rounded and the cilia seem sturdy and well defined. 7,250 x.

Figure 10

The cilia and surrounding cells in this SEM photomicrograph from a five-day culture appear intact and healthy. 2,900 x.
Ciliary activity was established as being present in all cultures. The cultures had been allowed to stabilize for 72 hours prior to testing. Wells containing the compounds to be tested were placed in the Petri plates, covered Petri plates were returned to covered jars, gassed with the CO₂/O₂ mixture and returned to the 37°C incubator. Initially, the explants were monitored every ten minutes in order to evaluate roughly the ciliostatic qualities of the compound being observed. When tentative parameters had been established, all tests were run again and repeated a third time under conditions identical to the original. Results obtained in terms of time may be seen in Table 5.

Ciliostasis is defined in this case as the event in which all of the cilia on the luminal surface no longer display ciliary activity detectable by light microscopy.

When viewed by the scanning electron microscope, the explants which had been subjected to the vapors of the designated compounds displayed widespread and severe damage which will be discussed below:

**Formaldehyde (Figures 11, 12):**

Cilia treated with formaldehyde vapors show evidence of irreversible destruction. The cilia are roped together with apparent loss of individuality and regard to direction. Microvilli seem to be well preserved. Cellular junctions are in evidence. Fixation by formaldehyde vapors takes place fairly rapidly (Table 6) leaving most structures recognizable at SEM magnifications.
<table>
<thead>
<tr>
<th>Compounds</th>
<th>Start</th>
<th>Ciliostasis</th>
<th>Time to Produce Ciliostasis</th>
</tr>
</thead>
<tbody>
<tr>
<td>EtOH</td>
<td>1. 1:45</td>
<td>3:30</td>
<td>1 hr. 45 min.</td>
</tr>
<tr>
<td></td>
<td>2. 11:45</td>
<td>12:49</td>
<td>1 hr. 4 min.</td>
</tr>
<tr>
<td>Meth. Alc.</td>
<td>1. 12:45</td>
<td>1:45</td>
<td>1 hr.</td>
</tr>
<tr>
<td></td>
<td>2. 1:15</td>
<td>2:00</td>
<td>45 min.</td>
</tr>
<tr>
<td>Toluene</td>
<td>1. 1:55</td>
<td>2:30</td>
<td>35 min.</td>
</tr>
<tr>
<td></td>
<td>2. 10:21</td>
<td>11:00</td>
<td>39 min.</td>
</tr>
<tr>
<td>Gluteraldehyde</td>
<td>1. 12:10</td>
<td>1:20</td>
<td>1 hr. 10 min.</td>
</tr>
<tr>
<td></td>
<td>2. 1:40</td>
<td>2:29</td>
<td>49 min.</td>
</tr>
<tr>
<td>Formaldehyde</td>
<td>1. 12:45</td>
<td>12:55</td>
<td>10 min.</td>
</tr>
<tr>
<td></td>
<td>2. 10:35</td>
<td>10:50</td>
<td>15 min.</td>
</tr>
<tr>
<td>Acetone</td>
<td>1. 12:16</td>
<td>1:10</td>
<td>54 min.</td>
</tr>
<tr>
<td></td>
<td>2. 2:08</td>
<td>3:15</td>
<td>1 hr. 7 min.</td>
</tr>
<tr>
<td>Chloroform</td>
<td>1. 12:45</td>
<td>12:50</td>
<td>5 min.</td>
</tr>
<tr>
<td></td>
<td>2. 12:25</td>
<td>12:34</td>
<td>9 min.</td>
</tr>
<tr>
<td>Ether</td>
<td>1. 2:20</td>
<td>3:40</td>
<td>1 hr.</td>
</tr>
<tr>
<td></td>
<td>2. 1:15</td>
<td>2:30</td>
<td>1 hr. 15 min.</td>
</tr>
<tr>
<td>Osmium</td>
<td>1. 3:37</td>
<td>3:40</td>
<td>3 min.</td>
</tr>
<tr>
<td></td>
<td>2. 2:33</td>
<td>2:35</td>
<td>2 min.</td>
</tr>
</tbody>
</table>
Table 6

TIMES OF CILIOSTASIS IN TWO SERIES OF TESTS

<table>
<thead>
<tr>
<th>Substance</th>
<th>Ethyl Alcohol</th>
<th>Ether</th>
<th>Gluteraldehyde</th>
<th>Acetone</th>
<th>Methyl Alcohol</th>
<th>Toluene</th>
<th>Formaldehyde</th>
<th>Chloroform</th>
<th>Osmium</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

TIME IN MINUTES

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Figure 11

Tracheal explants treated with vapors of formaldehyde. 14,500 x.

Figure 12

Tracheal explants treated with vapors of formaldehyde. 7,250 x.
Gluteraldehyde (Figure 13):

While the background structures of the tissue fixed by the vapors of gluteraldehyde appear to be intact with clear lines of demarcation separating the cells and microvilli, the cilia themselves appear to have lost their identity as separate structures. This method of invoking ciliostasis was one of the slowest of the compounds tested. Perhaps the length of time involved enabled greater damage to occur.

Osmium (Figure 14):

By contrast osmium vapors caused complete ciliostasis in only minutes, and the affected cilia appear to have retained their structural integrity, although they seem somewhat softened. Microvilli are apparent and well defined.

Ethyl alcohol (Figure 15):

This was also one of the more lengthy of the vapors tested in effecting ciliostasis (Table 6). Cilia do not seem to demonstrate as much structural damage as would be anticipated if time were the only factor. The treated cilia, while roped together, still may be seen as individual units. Microvilli appear intact, as do cell surfaces.

Methyl alcohol (Figure 16):

Vapors of this agent appear to produce ciliostasis more rapidly than those of EtOH (Table 6), and structural qualities in the treated cilia are as good or better. Other features are comparable as well.
Figure 13

Tracheal explants treated with vapors of gluteraldehyde.

2,900 x.

Figure 14

Tracheal explants treated with vapors of osmium tetroxide.

14,500 x.
Figure 15

Tracheal explants treated with vapors of EtOH. 14,500 x.

Figure 16

Tracheal explants treated with vapors of MetOH. 14,500 x.
Ether (Figure 17):

These vapors had a much slower ciliostatic effect than had been anticipated by this investigator. In view of its anesthetic qualities and of its volatility, the time factor for ciliostasis seems lengthy. The great destruction evident is also surprising. Cilia are hardly recognizable as such and have a seared or melted appearance. Background structures appear equally damaged.

Chloroform (Figure 18):

Ciliostasis occurred rapidly as a result of exposure to these vapors and left cilia firm and upright. The appearance is favorably comparable to that of control cilia (Figures 5-10). Cell junctions are clearly defined and microvilli are well preserved. Even the ciliary synchronization is evident, as if all ciliostasis occurred simultaneously to all structures.

Acetone (Figure 19):

These vapors, although not appearing as destructive as those of ether, seem to be so toxic that there is loss of most structural integrity by the cilia and a general softening or coating obscures detail.

Toluene (Figure 20):

Vapors are more ciliostatic based upon time than those of acetone, but the preservation of the cilia and of the background structures is much better. The rapidity of ciliostasis by toluene vapors is surprising considering the insolubility of toluene in media.

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Figure 17

Tracheal explants treated with vapors of ether. 7,250 x.

Figure 18

Tracheal explants treated with vapors of chloroform. 7,250 x.
Figure 19

Tracheal explants treated with vapors of acetone. 7,250 x.

Figure 20

Tracheal explants treated with vapors of toluene. 29,000 x.
DISCUSSION

From these experiments it is difficult to establish a cause/effect relationship. Although time must be a critical factor in toxicity, it is obviously not the only influence. Solubility in fluid (i.e., media) of the testing compounds also must be taken into consideration as this would, no doubt, be an influencing factor in ciliostasis. The stability of the vapors involved and the availability of unoccupied sites on molecules might be a determining factor in the concentration of the vapors in the environment containing the tracheal explants.

An attempt was made to observe reversability in the two testing compounds which have had histories of use as anesthetics, ether and chloroform. It seemed reasonable to speculate that no lasting damage or fixation had occurred. Wells containing the testing agents were removed and tops were left off culture plates for one-half hour to allow any vapors to escape. At the end of this time the explants were again observed. No ciliary activity could be seen. The reason for lack of recovery was obvious in the case of the ether-vapor treated explants. Based on the subsequent micrographs, recovery would not have been feasible in view of the damage incurred, as was discussed previously. The specific reason for lack of recovery of the explants from chloroform vapors is not known.

Although the actual sequence of events can only be surmised, it seems reasonable to suggest that the missing cilia, through loss of integrity, broke away from the tracheal epithelium and disintegrated. Rodin (1966) postulated that cilia cannot be regenerated by the same

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cell from which they were lost. Mass and Lane (1975) were of the opinion that ciliostasis, as a rule, indicated ensuing cell death. Based on these and other premises which have been advanced as a result of scientific investigation, some of which are covered in this paper, it is not difficult to believe that ciliostasis frequently heralds an ominous cascade of events.

There have been recent studies reporting successful regeneration of eukaryote cilia in Tetrahymena (Wolfe, 1973; Renard, 1974; and Quinones and Renard, 1975) after having first deciliated the cells by the addition of calcium ions to an acid medium.

However, in the case of the explants treated in the foregoing study, the extensive damage noted would surely preclude recovery.

It is not possible to study all the pertinent factors in such an event with a single technique and yield a comprehensive assessment. The SEM enables one to survey the topography of an entire explant with specific attention to designated areas. The TEM permits reconstruction by the viewer of the pseudostratified epithelium of the trachea and of its histologically complex population and relationships. By utilizing both instruments it is possible to form a mental picture of the complete structure under study.

The trachea epithelium is referred to as being "pseudostratified," for it has the appearance of a layered arrangement. In reality it is a single layer, but all its cells are not visible simultaneously from every direction. As an example, the basal cells are not seen from the top surface; there the goblet cells are much better seen in their entirety from a longitudinal section.
For this reason, if time permits, it is highly desirable to utilize several techniques in studying even a single feature, such as the ciliated cells, in order to enable one to visualize the sequence of events accurately in cellular function.

In this study the transmission electron microscope was used only briefly to verify normal cytological structure of the control explants. Since the ciliated cells themselves are best seen from a topographical standpoint, as has been indicated, the scanning scope was utilized extensively for this purpose. Unfortunately scanning electron microscopes are greatly limited in resolution as compared to the transmission electron microscope. In addition, the gold coatings on the specimens by its very nature would obscure some of the fine detail if it were resolvable.

It is the feeling of this investigator that a more in-depth study in this area utilizing additional techniques and instrumentation would be useful and informative.

Various degrees of toxicity could be selected from the established base line and the fate of the cellular ultrastructure could be monitored more closely.

Also it is suggested that a chronic toxicology study should be conducted in order to determine what events lead up to lethal toxicity. Since it has been established by other studies that cilia are not totally dependent upon membrane integrity nor upon that of the basal bodies (Satir, 1974), one can only speculate upon the modes of action when ciliostasis and cell death occur.
There is some question concerning the value of working with organs in culture as opposed to those in their natural environment, i.e., the body of the subject. Pollack (1977) sees the cell culture as the "system with which to resolve the outstanding paradox of biology--normal development."

Lane, et al., (1976) regards the organ culture as a means of retaining complete control of the environment, the model and a method of continuing monitoring. At the same time there is no risk of immune or inflammatory response. Proetz, et al., (1962) observed that sources compromising a proper function were, at the same time, capable of inducing disease.

Implications of a study such as the one presented here may be of intrinsic value based upon the information obtained as it relates to the designated compounds alone, but it is also conceivable that even greater value may lie in these results in combination with those of others.

If each of the chemicals determined to be ciliostatic in this study could be regarded as an indicator or potential for pathogenesis based upon these findings, a set of factors obtained here could be utilized as a baseline in a more extensive study involving products containing any one or a combination of the aforementioned chemicals.

An illustration of the value of established baselines in studies involving respiratory illnesses may be seen in the subsequent examples.

In one such study which concerned respiratory illnesses occurring as a result of allergic or immunologic reaction giving rise to asthma or chronic bronchitis in an individual exposed to epoxy resin, workers...
were found to be sensitive to trimellitic anhydride (TMA), a curing agent for epoxy resin (Zeiss, et al., 1977). In other studies involving epoxy resin, respiratory illnesses alone have been attributed to ethylene amines (Dernehl, 1951), phthalic acid anhydride (Malten and Zielhuis, 1964) and triethylene tetramine (Fawcett, et al., 1977).

In the plastics industry toluene diisocyanate has been identified for the last decade as an agent responsible for the provocation of asthma and/or bronchitis (Sweet, 1968; Paisley, 1969; and Pepys, et al., 1972). It may be recalled that toluene alone was one of the compounds under observation for ciliostatic properties in the preceding study.

From the information obtained above it may be speculated that it is not sufficient to say that a particular resin or plastic produces respiratory distress, but rather it is essential to know which ingredient of the product under consideration is the causative agent of the resulting illness. Substitutions may then be effected to eliminate this toxicity.

Another useful outgrowth of this and other related studies may be as a contribution to a handbook or encyclopedia listing manufactured products having chemical components and their biological hazards. A journal such as this might publish biological hazards encountered by workers in the aircraft industry who use a resin laminate in their work. This information would in turn be of benefit to the laboratory worker who preserves the ultrastructure of tissue in polymerized epoxy resin.

Well-defined boundaries obtained from a study of any magnitude may be useful when incorporated into another study for the purpose of
systematically eliminating unknown substances.

Therefore, to an investigator embarking upon the hypothetical but expansive task of cataloguing the pollutants of the atmosphere of a city, an almost endless task could be envisioned. If, however, he had available documentation of all previously determined biological hazards of this nature, his work would not appear so formidable. In this way also, small but carefully performed studies find a meaningful place in scientific achievement.
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