

## INTRODUCTORY REMARKS

Evaluation of toxic damage to the respiratory tract, caused by inhalation of gases, fumes and dusts, is usually accomplished by pulmonary function tests and by morphologic examination of the diseased tissues. During the last few years, attention has begun to turn a third possibility to assess toxic lung damage: normal and pathological biochemistry of the lung. Biochemical tests have long proved their value in studying <sup>the</sup> ~~the~~ <sup>of</sup> hepatotoxicity and nephrotoxicity. It is conceivable that a similar approach will be useful in the evaluation of toxic lung damage.

The work described in this proposal wants to examine two questions: first, can we define any biochemical parameters which can be used as reliable indicators of lung damage produced by the inhalation of tobacco smoke. Second, once such parameters have been found, will they help to quantitate the biological effects produced by different tobaccos in the upper and low respiratory tract of laboratory animals. In other words, we plan to study whether undesirable effects of tobacco smoke can be detected and quantitated with a biochemical approach. At the same time, the tests to be developed should evaluate those biochemical processes in lung which may be underlying or be associated with human lung disease caused by excessive tobacco consumption.

Two regions of the respiratory tract will be analyzed: the tracheal epithelium and the lung parenchyma. Among tracheobronchial responses to insult in acute and/or chronic lung disease we find changes in mucus secretion, ciliary dysfunction, inflammation and neoplasia (Macklem and Kilburn, 1972). The alveolar

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response to insult shows essentially three patterns: alterations in a) the alveolar surface, b) in 5 defined pulmonary cell classes and c) in the interstitial spaces of lung parenchyma (Kilburn, 1972). We have attempted to select several biochemical parameters which conceivably represent tracheobronchial and alveolar response to chemical lung injury. They are: biosynthetic processes and signs of membrane damage in the cells of the tracheobronchial epithelium, the differential activation of enzymes implicated in the metabolic transformation of pulmonary carcinogens and the development of enzyme systems designed to strengthen lung tissue against oxidant damage.

1. Biosynthesis of macromolecules within the tracheal epithelium:

a) Evaluation of the biosynthesis of glycoproteins:

Glycoproteins are the principal non-diffusible components of the secretions that originate from the epithelial goblet cells and the submucosal tracheobronchial mucous glands. It has been shown recently that under pathological conditions, as for example Vitamin A deficiency, glycoprotein biosynthesis in tracheal mucosa is altered qualitatively and quantitatively (De Luca et al., 1968, 1971; Bonanni et al., 1973). It is conceivable that acute exposure to smoke will interfere with glycoprotein synthesis, too.

Canine tracheal explants (Ellis and Stahl, 1973) or tracheal strips from hamsters (Bonanni et al., 1973) incorporate radioactive labelled precursors (fucose, glucosamine, serine) into macromolecules during several hours of in vitro incubation. The techniques described will be used to measure the rate of glycoprotein synthesis and the rate of release of formed macromolecules into the medium. In a later stage an attempt will be made to establish whether qualitative

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differences can be found in the different species of glycoproteins formed during *in vitro* incubation.

b) Analysis of RNA synthesis in tracheobronchial epithelium:

Hamsters deficient in Vitamin A display an altered RNA metabolism in the tracheobronchial epithelium (Kaufman et al., 1972). Less RNA of low electrophoretic mobility is formed in the epithelial cells of tracheas from deficient hamsters. Evidence was obtained by incubating isolated tracheas with radioactive uridine. The newly formed RNA was analyzed with polycrylamide gel electrophoresis. The changes in RNA synthesis were fully reversible and disappeared upon treatment of the deficient animals with Vitamin A.

An analysis of the rate of RNA synthesis and of the RNA species formed might serve as another biochemical index of normal or disturbed cellular metabolism in the tracheal epithelium. Moreover, attempts will be made to develop methods which will allow to measure RNA and/or glycoprotein synthesis in small tracheal segments. This should allow to examine the effects of smoke in both the upper and lower regions of the tracheobronchial tree.

2. Isolation of and analysis for cell damage in tracheal epithelium:

Several techniques have now been described which allow to harvest a suspension of isolated epithelial cells from the trachea of small laboratory animals (Spencer, 1958; Smith et al., 1971; Nasr et al., 1971). Essentially, the tracheas are excised, opened and the mucosa is gently brushed off. It has been claimed that up to  $1.6 \times 10^6$  cells can be harvested from a rat trachea. It is planned to follow and, if necessary, to modify those techniques in order to

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obtain isolated epithelial cells. The composition and purity of the tracheal cell population will be evaluated by light and electron microscopy.

Once a satisfactory cell population can be isolated, several biochemical parameters such as oxygen uptake, glucose consumption, NADP/NADPH ratios and the activities of various enzymes could be studied. Such studies have been done to evaluate oxidant lung damage (Nasr et al., 1971). However, we plan to concentrate first upon one more particular aspect of cell damage: signs of lipid peroxidation in cellular membranes.

Biological membranes (cell membranes, membranes surrounding intracellular organelles such as mitochondria and lysosomes and the membrane system forming the endoplasmic reticulum) contain a wide variety of unsaturated lipid material. Such unsaturated fatty acids can undergo oxidative deterioration in the presence of a free-radical initiator and oxygen. Lipid peroxidation in vivo has been recognized as the molecular damage underlying a variety of adverse and degenerative tissue alterations. Available experimental evidence suggests that lipid peroxidation plays a considerable role in lung damage produced by oxygen, ozone and nitrogen dioxide (Thomas et al., 1968; Goldstein et al., 1969; Menzel, 1970).

Tappel and his associates have established that products of lipid peroxidation can react in turn with the amino groups of proteins, amino acids and phosphatidyl ethanolamine to form fluorescent compounds in small yield (Tappel, 1973). Fluorescence spectroscopy allows to detect and to measure these compounds. Methods have been described which should allow to determine and to measure

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quantitatively the occurrence of in vivo lipid peroxidation in biological samples (Fletcher et al., 1973). It is planned to examine whether these fluorometric methods, which have the advantage of great sensitivity, can successfully be adapted for an evaluation of lipid peroxidation in isolated tracheal epithelial cells and their organelles (mitochondria, lysosomes).

Lipid peroxidation can also be detected in biological material with the thiobarbituric acid test. The reaction is concerned with the formation of a colored product when malonaldehyde is heated in the presence of thiobarbituric acid. The method has been discussed by Slater (1972) in detail and used by Chow and Tappel (1972) to measure lipid peroxidation in ozone damaged lungs.

It is planned to concentrate first upon the development of a reliable assay which will allow to detect membrane damage and lipid peroxidation in small samples. It then should become possible to analyze cells from different heights of the tracheobronchial tree. An attempt will be made to correlate the extent of lipid peroxidation with the number of cells analyzed in any given sample. This can be done by analysis for DNA or cell count.

Should we be successful in developing a satisfactory technique and in obtaining positive results, then we would extend the technique to an analysis of other pulmonary cell types, especially type 2 pneumocytes. These cells can be isolated in a remarkably pure preparation with the method described by Kikkawa and Yoneda (1974). The adaptation of their methods to our needs would again require extensive verification of the fractionation and isolation procedure with light and electron microscopy.

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3. Studies on the metabolic transformation of polycyclic hydrocarbons by lung microsomes:

Polycyclic hydrocarbons (benzo(a)pyrene, 3-methylcholanthrene, dimethylbenzanthracene) are relatively inert compounds. However, certain enzyme systems are capable to activate them into highly reactive epoxides; these epoxides are then either converted to the corresponding dihydrodiols, or they rearrange into corresponding phenols or are converted into glutathione conjugates. Rat lung preparations are capable of carrying out all these reactions and, especially, do form the highly reactive K-region epoxides (Grover et al., 1974, Grover, 1974). It is these epoxides which most probably are responsible for the well-known carcinogenic and mutagenic action of polycyclic hydrocarbons.

The enzyme system responsible for the transformation of polycyclic hydrocarbons is known to be associated with the microsomal fraction of lung (and other) tissues. A variety of agents will stimulate the activity of those so-called microsomal enzymes and enhance transformation of a great many substrates. Cigarette smoke is one agent which stimulates the activity of microsomal enzymes in the lungs of animals (Welch et al., 1971). However, it has also been detected that different inducing agents will eventually induce qualitatively slightly different drug metabolizing enzymes systems (Lu et al., 1972). As a result, polycyclic hydrocarbons are metabolized at a different rate or even via different pathways (Rasmussen and Wang, 1974).

It is conceivable that different tobaccos will induce, in the lung of exposed animals, slightly different microsomal enzymes and that this will result

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in the formation of different metabolites of polycyclic hydrocarbons. This possibility will be explored as follows: aseptically prepared microsomal preparations from lungs will be incubated in the presence of selected polycyclic hydrocarbons and a defined bacterial strain. Enzymatic formation of highly reactive metabolites from the hydrocarbons during this incubation could lead to an interaction of these metabolites with bacterial nucleic acids. Microbiological methods will then allow to score the mutation rate in the exposed bacterial population. This system has already been demonstrated to differentiate between activation and inactivation of different carcinogens (Popper et al., 1973) and to detect that different enzyme inducers enhance the activity of microsomal enzymes which have different specialities towards benzo(a)pyrene (Rasmussen and Wang, 1974). It is possible that exposure to different blends of tobacco will also induce pulmonary enzymes with different specialities towards benzo(a)pyrene and other polycyclic hydrocarbons. The proposed test should help to gain information on this important question.

4. Search for the development of protective enzyme systems in lung parenchyma:

During the last few years a substantial number of work has been published which describes attempts to analyze damage to the alveolar region with a biochemical approach. Most of these studies have examined the acute effects of gases such as hyperbaric oxygen, ozone and nitrogen dioxide and of substances which produce specific lung damage after injection, such as paraquat, pyrrolizidine alkaloids and nickel carbonyl. So far, the techniques employed are inasmuch comparatively crude as practically all analysis have been done on total lung homogenates.

Nevertheless, several conclusions can be drawn from these studies.

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First it has to be realized that so far no single biochemical deficiency has been found which could be associated with damage to the alveolar zone. Direct biochemical signs of cell damage or of cell death have yet to be defined for lung tissue.

On the other hand, more and more evidence accumulates which suggests that exposure of lung to a wide variety of toxic chemicals produces a biochemical pattern which can be labelled as "reparative-adaptive response". Essentially, as was recently summarized by Cross (1974), lung tissue damaged by oxidants is repaired soon by a proliferation of type 2 epithelial cells. This process not only repairs tissue lesions but might actually render the lung more resistant against chemical injury.

Several biochemical responses have been associated with this reparative-adaptive response (Chow and Tappel, 1973; Mustafa et al., 1973; Tierney et al., 1973; DeLucia et al., 1972). It is planned to examine the following ones among them:

a) Glucose-6-phosphate dehydrogenase (G6PDH)

The activity of G6PDH has been found to increase substantially in lung tissue after low-dose exposure to oxidants. The increased activity probably reflects an increased activity of the hexose-monophosphate shunt. This could result in an increased production of nucleotides (needed for nucleic acid synthesis in tissue repair) or an increased formation of NADPH (required for oxidoreductive synthesis).

b) 6-Phosphogluconate dehydrogenase

This is another enzyme in the pentose shunt reaction sequence and might

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behave like G6PDH.

c) Glutathione peroxidase

Glutathione peroxidase is another enzyme which increases in oxidant-damaged rodent lung.

d) Superoxide dismutase (SOD)

An increase in the activity of SOD has been found to accompany the development of oxygen tolerance in rats (Crapo and Tierney, 1974). SOD is an enzyme which dismutates superoxide anion, a highly reactive free radical produced by the one electron reduction of oxygen, to less toxic forms. The role of SOD in pulmonary toxicology only now begins to be appreciated (Saltzman and Fridovitch, 1973).

e) Thymidine kinase

This enzyme is involved in the biosynthesis of DNA. In resting tissue, practically no thymidine kinase activity is found. However, if any cell type is stimulated into proliferation, thymidine kinase activities can increase substantially over basal values. Thymidine kinase would serve as a convenient biochemical marker of pulmonary cell proliferation.

It has to be emphasized again that all eventual changes observed in the activities of these enzymes only would indicate that an abnormal process is going on in lung tissue. Most probably pneumonia would alter substantially the activity of any of the mentioned enzymes. (It has to be added that this was not the case in any of the studies mentioned earlier). Any changes in the pattern of the "re-

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parative-adaptive response" would have to be verified by a histopathological evaluation of the affected tissue in order to exclude trivial explanations of biochemical alterations. On the other hand, the enzyme assays do not represent major-technical difficulties, and several enzymes could be measured on material derived from one or even half a lung. The suggested experiments could serve as a convenient, rapid and easily to quantify screen for damage to the alveolar zone.

5. Over-all experimental approach:

The technique of exposure to tobacco smoke will be described elsewhere in this proposal. Animals to be used are guinea pigs. Preliminary work will establish whether all the suggested biochemical parameters can be found in guinea-pig lungs in reasonably high activities so that precise and meaningful measurements can be made. For example, it is well known that the lungs of certain species (e.g. rabbits) contain high activities of drug metabolizing enzymes, whereas in rat lungs the same enzymes are almost not detectable. Studies on the biochemistry of guinea-pig lungs are few and baselines for most of the proposed assays will have to be established first. It is also planned to use model substances such as certain inducers of microsomal enzymes or other agents known to affect lung biochemistry in order to find out how susceptible our parameters will be to alterations induced in lung metabolism.

In a second step it will be examined how the different parameters will change (if at all) in animals exposed acutely to the smoke given off by a standard cigarette. This will be done with time-response and dose-response studies. For example, if we want to study how smoking affects biosynthetic processes in

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the tracheal mucosa, animals will be exposed to the smoke of 5 cigarettes. Three 6, 12 and 24 hours later, 2 - 3 animals will be killed and it will be examined whether acute smoke exposure affects the rate of synthesis and release of glycoproteins from tracheal mucosa. Hopefully, a time-point will be found which provides a maximal response. Next, a dose-response study will be done at this particular time-point in order to examine whether the biochemical response is dose-dependent. Four to 5 doses will be applied, with 3 - 4 animals per dose. If a satisfactory dose-response can be established, then it is possible to evaluate the effects of other brands of tobacco under the same, rigorously standardized conditions of combustion and inhalation. The resulting dose-response curves may serve as guidelines in the evaluation.

Not all projects can be carried out simultaneously. It is felt that project No 2 (studies on lipid peroxidation) and project No 3 (studies on the transformation of polycyclic hydrocarbons) have the greatest potential to provide useful information. However, both projects will require some special methodology and high priority will have to be assigned to the development of this particular aspects. The two other projects are considered to be the secondary ones (biosynthesis of macromolecules within the tracheal mucosa and search for the development of protective enzyme systems). Considerably less technical difficulties are anticipated in these studies. On the other hand, these two projects will require a substantial number of animal exposures in order to allow enough data which can be subject to a statistically meaningful analysis.

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