Biochemical Studies of Isolated Hamster Tracheal Epithelium

by David G. Kaufman*

The epithelial lining of respiratory air passageways is a primary target tissue for toxicity and carcinogenesis in man and in animal models of human disease. The importance of this target tissue was the basis for development of methods to study its biochemistry, and with this information to distinguish the unique properties of this tissue from properties common to all cell types. Biochemical methods employed labeling of macromolecules in isolated hamster tracheas during brief (< 4 hr) incubation *in vitro*. Studies of RNA metabolism in isolated tracheas demonstrated a pattern of maturation of ribosomal RNA like that shown for other other cell types. Alterations in RNA metabolism were observed in isolated tracheas obtained from vitamin A-deficient hamsters and hamsters previously treated by intratracheal administration of benzo[a]pyrene (BP) plus ferric oxide (Fe_2O_3) *in vivo*. Studies with toyocamycin, actinomycin D, and α-amanitin, all inhibitors of RNA metabolism, were performed to characterize the class of RNA molecules with a decreased proportion of labeling in tracheas from vitamin A deficient hamsters. In another series of experiments, BP was shown to bind to DNA in epithelial cells of isolated tracheas. The quantity of BP binding was increased by prior intratracheal treatment of hamsters with BP plus Fe_2O_3 *in vivo*, this induced binding was inhibited by addition of 7,8-benzoflavone to the incubation medium. Increased BP binding was also observed in isolated tracheas from hamsters believed to be in states of increased susceptibility to respiratory carcinogenesis *in vivo*. The results show that biochemical studies are feasible with this tissue. Furthermore, a number of questions of importance with regard to this target epithelium are best studied directly in its constituent cells.

Introduction

That the respiratory tract is a primary site of interaction with the environment is a fact well known to many city-dwelling scientists and laymen alike. Environmental pollution is a fact of life that greets you at your front door as you open it to leave each day. But rather than being another one of the petty annoyances of modern life, respiratory exposure to carcinogens and other environmental contaminants, along with respiratory exposures faced in the home and at the workplace, may threaten our health and is responsible in part for the increasing toll from lung diseases including lung cancer.

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Rather than the whole body or the whole lung, the critical site of biological interaction with many of these environmental agents is the small mass of tissue constituting the respiratory passageways, and in particular, their epithelial lining cells. This is particularly evident in the case of lung cancer, since the majority of these tumors are presumed to arise from cells of this epithelium. This fact was the basis for the development of biochemical methods for study of a respiratory airway epithelium. The Syrian golden hamster was chosen as the tissue because a method for producing respiratory tract tumors resembling those of man had been developed with this species (1) and because of the near absence of respiratory infections in these animals. The tracheal epithelium was selected since it is most readily obtained and since is is a site at which a high in-

cidence of experimental tumors develops (1,2). This report describes studies related to nucleic acids of the tracheal epithelium from normal hamsters, hamsters treated with benzo[a]pyrene and hamsters made vitamin A-deficient. The aim is to show that a number of sophisticated biochemical studies are feasible with this important target tissue. These investigations are an example, rather than the limit, of biochemical studies of this tissue, since other studies have considered glycoprotein synthesis (3-8), carcinogen metabolism (9), and isoenzyme distributions (10) in the tracheobronchial epithelium.

Materials and Methods

Male random-bred Syrian golden hamsters (Mammalian Genetics and Animal Production, Division of Cancer Therapy, National Cancer Institute) were maintained in pairs on San-I-Cell (Paxton Processing Co.) and given water ad libitum. They were fed either Wayne Lab-Blox (Allied Mills, Inc.) or a vitamin A-deficient diet (General Biochemicals). Vitamin A-deficient hamsters and pair-fed controls were prepared as described previously (11). Benzo[a]pyrene (Aldrich Chemical Co.) was hand ground with an equal mixture of Fe_2O_3 dust (Type 3098; Charles Pfizer, Inc.) and a suspension of this mixture was instilled intratracheally into some hamsters by methods previously described (1).

Hamsters were anesthetized with sodium methohexital (Brevital sodium; Eli Lilly and Co.), exsanguinated via the abdominal aorta, and the respiratory tract from larynx to bifurcation was excised en bloc. The trachea was isolated from surrounding tissues, cut open longitudinally through the membranous portion and then washed in phosphate-buffered saline (PBS). Pairs of isolated tracheas were maintained in L-15 medium (GIBCO) at 37°C for up to 4 hr. Uridine-5-³H (20 μCi/ml; 26 Ci/mmole; New England Nuclear) was added directly to the medium where used; Benzo[a]pyrene-³H (20 μCi/ml; 25 Ci/mmole; Amersham-Searle) was first dissolved in DMSO then added to the medium (final concentration of DMSO was 0.04%) when it was used. Various inhibitors (as indicated later) were also added to the incubation medium in specific cases. At the end of timed incubations, the tracheas were washed with PBS. Epithelial cells were then scraped from the supportive structures of the trachea with a scalpel held perpendicular to the epithelial surface. The scrapings were washed from the trachea in 150 μl of 0.032M sucrose containing

$0.1mM$ Na_3EDTA and $0.1mM$ potassium phosphate, pH 6.8, and collected for further biochemical procedures.

For preparation of whole cell RNA, tracheal epithelial scrapings from two tracheas were pooled, 100 μl of 0.8% sodium dodecyl sulfate (SDS) was added, and the mixture homogenized at 0°C. Liquified anhydrous phenol (600 μl, 45-50°C) was added, and the mixture was further homogenized for 2 min at 0°C. The resultant emulsion was centrifuged at 10,000g to obtain phase separation, and the aqueous phase was saved. This aqueous phase was further concentrated with a second phenol treatment as described above (approximately 30 μl final volume).

Samples containing approximately 5 μg RNA were fractionated by electrophoresis on composite agarose (0.5%)-acrylamide (1.7%) gel slabs as previously described (12). Hamster liver cytoplasmic RNA labeled in vivo for 15 hr with uridine-2-¹⁴C was added to each experimental sample to provide internal markers to identify the 18S and 28S species. After electrophoresis, the gels were sliced into 1-mm pieces and the slices placed into scintillation counting vials with 0.1 ml water and 1.0 ml NCS solubilizer (Amersham-Searle). After 2 hr, 10 ml of tolune–PPO–POPOP scintillation counting solution was added to the vials; vials were allowed to stand overnight before counting. Counts per minute were corrected for spillover between channels, efficiency of counting, and background counts to give disintegrations per minute (DPM). The sum of DPM recovered from all slices of a gel was determined and the percentage of this total present in each slice was calculated. These percentages were plotted as a function of the gel slice number, which is equal to the migration distance in millimeters.

For purification of DNA, the epithelial scrapings were transferred to a small Dounce homgenizer, and the suspension was adjusted to 5mM Na_3EDTA and 2% SDS. The mixture was homogenized at 0°C and then treated at room temperature with an equal volume of water-saturated phenol. The aqueous phase was separated and saved. A second phenol treatment of the aqueous phase was performed, two volumes of ethanol were added to the final aqueous solution, and the mixture was stored at −20°C overnight. The resulting precipitate was sedimented by centrifugation at 10,000g for 2 min, and the supernatant was discarded. The precipitate was extracted with multiple aliquots of ether until there was no radioactivity present in the extracts, then resid-

ual solvent was evaporated in a stream of nitrogen. The precipitate was dissolved by homogenization in 0.1M Tris-HCl, pH 7.5, containing 0.15M NaCl in a Dounce homogenizer. The aqueous solution was then sequentially digested with pancreatic RNase (Worthington Biochemical Co.; final concentration 100 μg/ml, previously incubated at 90°C for 1 hr to inactivate DNase) at 37°C for 1 hr, and pronase (Calbiochem; final concentration 100 μg/ml) at 37°C for 1 hr. After enzymatic digestion, the samples were dialyzed at 4°C during a 3-day interval against multiple changes of 0.1M Tris-HCl, pH 7.5, containing 0.15M NaCl. After dialysis, the sample solutions were adjusted to 5M CsCl and 4.2mM Na$_3$EDTA and the samples were centrifuged at 35,000 rpm for 66 hr at 20°C in a Beckman SW 56 rotor. After centrifugation the CsCl gradients were fractionated into 0.2 ml portions while the absorbance of the effluent stream was continuously monitored at 254 nm. Radioactivity was determined for each fraction and the absorbance of peak fractions was determined at 260 and 280 nm.

Results

Although the majority of our efforts have concerned nucleic acids of tracheal epithelium, our initial studies were designed to document the preservation of morphological and biochemical characteristics of the isolated tracheas. At the limits of our typical in vitro incubation, both histological and ultrastructural features of the tracheal explants were essentially normal. Linear incorporation of leucine into proteins during the period of in vitro maintenance indicated continuity of biochemical function. These results were reported previously (12,13). Autoradiographic studies demonstrated that grains from incorporated leucine-^{3}H were predominantly over mucus cells. Similar results have also been seen in long-term tracheal organ cultures from rats (M. J. Mass and B. P. Lane, personal communication). Intense labeling of cells with incorporated thymidine-^{3}H was infrequent in normal tracheas, but basal cells and nonciliated, nonbasal cells (sublumenal plus lumenal) were nearly equally represented among those cells labeled. Mechanical injury of the trachea in vivo (with a cannulation needle) prior to tracheal isolation increased the numbers of cells incorporating thymidine. Autoradiographic studies of tracheas incubated continuously with medium containing uridine-^{3}H initially (e.g., at 30 min) had grains predominantly over cell nuclei. With longer periods of incuba-

tions the distribution of grains between nucleus and cytoplasm become uniform (e.g., 3 hr). Accompanying studies demonstrated that precursors were also incorporated into the supportive structures of the trachea. Since supportive structures were discarded in studies of the epithelium, this incorporation did not influence the observed results.

Studies of RNA Metabolism in Isolated Hamster Tracheas

The first biochemical measure of RNA synthesis which we studied in the tracheal epithelium was the extent of incorporation of uridine-^{3}H into RNA as a function of time. After various periods of incubation in vitro, tracheal scrapings were homogenized and washed with 0.2M perchloric acid (PCA) to remove unincorporated radioactivity (12). The DNA and RNA in washed homogenates were then hydrolyzed with 0.6M PCA at 70°C, and radioactivity and DNA content (14) were quantitated. Uridine incorporation (normalized to DNA content) was approximately linear for up to 4 hr (Fig. 1). Evidence supporting the identity of the radioactivity as RNA were its lability to hydrolysis with 0.3M NaOH (37°C for 60 min) and the marked inhibition (91%) of incorporation caused by preincubation of tracheas in medium containing actinomycin D (5 μg/ml, 30 min). Inhibition of RNA synthesis has been seen in cultured rat tracheal rings incubated with a comparable concentration of actinomycin D (15).

Whole cell RNA was extracted from tracheal scrapings immediately after a 30 min pulse exposure to medium containing uridine-^{3}H and at 30, 90, and 150 min after transfer of tracheas to unlabeled medium. The RNA was separated electrophoretically on acrylamide-agarose gels, and the distribution of radioactivity in species larger than approximately 7S was determined for each of the observation times (Fig. 2). Uridine-^{14}C-labeled cytoplasmic RNA from hamster liver was mixed with each sample to provide an internal reference for identification of 28S and 18S species. At 30 min, label was principally in heterogenous high molecular weight RNA but with a distinct peak (at 27 mm migration) consistent in size with the 45S ribosomal precursor. By 60 min, small 28S and 18S peaks were present at the location of the ^{14}C- marker RNA. In addition to the persistent peak at 27 mm, a new peak was evident at 41 mm consistent in size with the 32S ribosomal precursor. By 120 and 180 min, sizeable 28S and 18S peaks were present. Estimates of the molecular weights of the presumed 45S and 32S
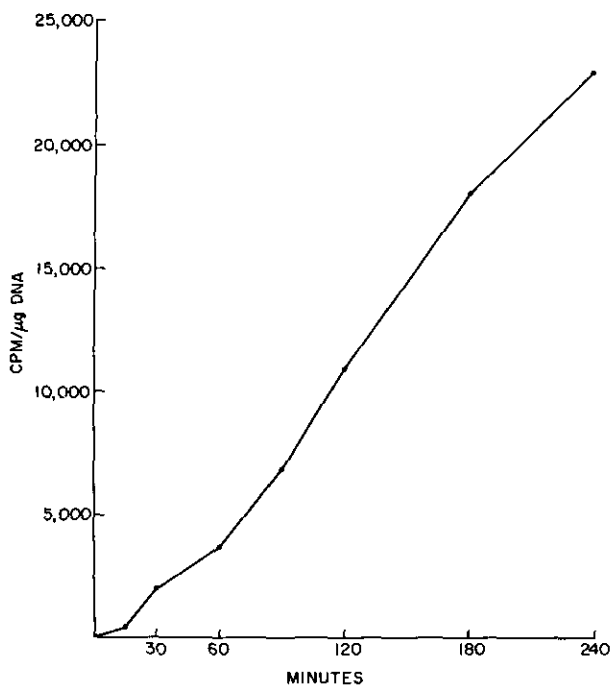
FIGURE 1. Incorporation of uridine as a function of time. Isolated tracheas were incubated *in vitro* for the indicated time intervals in medium containing 5μCi/ml of uridine-³H. Following incubation, the epithelium was scraped from supporting structures of the trachea and unincorporated radioactivity was removed by extraction with cold 0.2M PCA as described previously (*12*). RNA and DNA were hydrolyzed with 0.6M PCA at 70°C and both radioactivity and DNA content were quantitated in the hydrolyzate. CPM were normalized with respect to DNA content of the hydrolyzates and these ratios (i.e., specific activities) plotted as a function of time. The values indicated are average values for 4-8 animals.
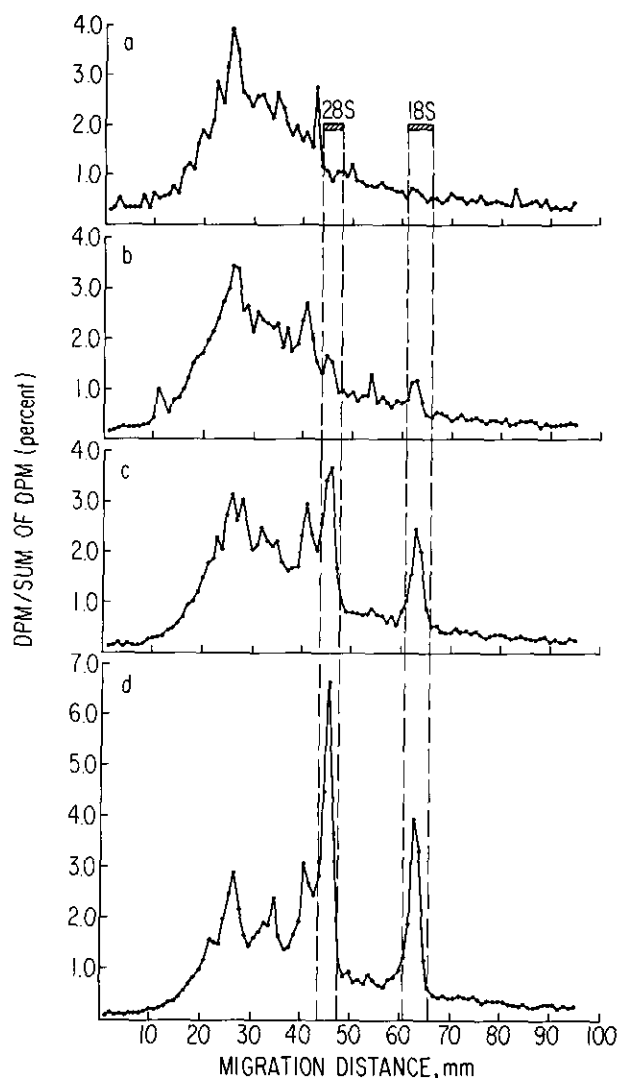

FIGURE 2. Electrophoretic distribution of newly synthesized, high molecular weight tracheal RNA as a function of time. Hamster tracheas were incubated *in vitro* for 30 min in medium with 20μCi/ml of uridine-³H to pulse label RNA. Whole-cell tracheal RNA was prepared for the epithelium (*a*) immediately after this 30 min pulse, and after further incubation in unlabeled medium for an additional times of (*b*) 30 min, (*c*) 90 min, or (*d*) 150 min. Hamster liver cytoplasmic RNA labeled with uridine-¹⁴C was added to each sample of tracheal RNA to provide an internal reference for the identification of 18S and 28S RNA species and electrophoresis on acrylamide-agarose composite gels was conducted for 2.5 hr. Gels were cut after electrophoresis, and RNA larger than approximately 7S were included in the analysis. Radioactivity for each gel slice was determined as indicated in the text. Data are presented as the proportion of the total uridine-³H radioactivity present in each gel slice plotted with respect to migration distance.

RNA species were made from electrophoretic mobility by the methods of Peacock and Dingman (*16,17*). Assuming molecular weights of 6-7×10⁵ and 1.5-1.9 × 10⁶ daltons for the 18S and 28S species, respectively, the presumed 45S and 32S species would correspond to molecular weights of 5.0 × 10⁶ and 2.3 × 10⁶ daltons, respectively. Thus a pattern of ribosomal RNA maturation can be seen in the tracheal epithelium which is comparable to that in other types of cells (*18-21*).

Subsequent studies of RNA metabolism concerned issues related to carcinogenesis *in vivo* in the respiratory tract of Syrian golden hamsters. First studied was the effect of prior intratracheal treatment with BP and Fe₂O₃ *in vivo* on RNA metabolism in isolated tracheas *in vitro*. Hamsters received one instillation of either 5 mg BP plus 5 mg Fe₂O₃ or 5 mg Fe₂O₃ alone (*1*) and were killed 48 hr later. Tracheas were isolated

and incubated with uridine-³H for various times. The electrophoretic pattern of RNA extracted

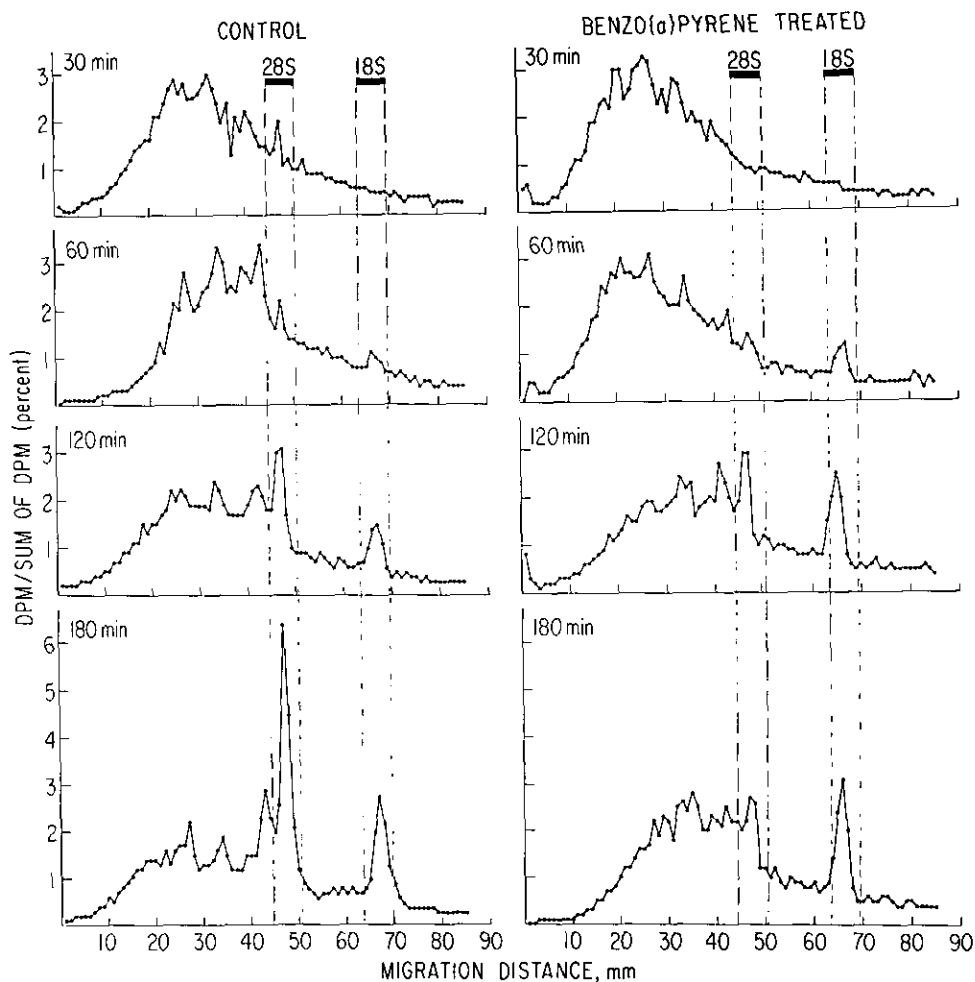CONTROL — BENZO(a)PYRENE TREATED

FIGURE 3. Effect of intratracheal instillation of benzo[a]pyrene *in vivo* on electrophoretic distribution of high molecular weight RNA synthesized *in vitro*. Hamsters received one intratracheal administration of either 5 mg BP plus 5 mg Fe$_2$O$_3$ (benzo[a]pyrene-treated) or 5 mg of Fe$_2$O$_3$ alone (control) 48 hr prior to sacrifice *(1)*. Labeling of RNA *in vitro*, gel electrophoresis and analysis of results were as described in the legend to Fig. 2. Distribution *(a)* after 30 min uridine-^{3}H pulse and after additional incubation in unlabeled medium for *(b)* 30 min, *(c)* 90 min, or *(d)* 150 min.

from the tracheas of hamsters treated with Fe$_2$O$_3$ alone and BP plus Fe$_2$O$_3$ are illustrated in Figure 3. No marked alterations of RNA larger than 28S were evident at 30 min or later. By the time that peaks of 28S and 18S RNA were distinct (60 min and later), however, a consistent alteration in the ratio of 28S to 18S RNA species was evident when tracheal RNA from BP plus Fe$_2$O$_3$-treated hamsters was compared to that of their controls (Fe$_2$O$_3$ alone). Current information suggests that 28S and 18S RNA are formed in equimolar proportions from a 45S precursor rbosomal RNA *(22)*. Based on their equimolar proportions and the estimated molecular weights, the ratios of uridine-^{3}H radioactivity in 28S and 18S RNA should be greater than 2.0 (assuming uridine con-

tent to be proportional to molecular weight). This situation is approximated in tracheas of either normal (Fig. 2) or Fe$_2$O$_3$-treated hamsters (Fig. 3). Small deviations from the calculated proportions are presumably a consequence of heterogenous RNA, probably of nuclear origin, which is present throughout the gel. The marked alteration in proportion of 28S and 18S species in tracheas from BP plus Fe$_2$O$_3$-treated hamsters (Table 1) is not a consequence of an alteration in heterogeneous RNA, but the method of analysis does not allow for a clear determination of whether there is a decrease in the quantity or rate of synthesis of 28S RNA, an increase for 18S RNA, or both. This result appears similar to those observed in other cell populations stim-

Table 1. Effect of intratracheal instillation of BP plus Fe_2O_3 or Fe_2O_3 alone on ratio of 28S to 18S RNA.[a]

Incubation time, min	Ratio 28S/18S RNA species[b]	
	BP plus Fe_2O_3	Fe_2O_3 Alone
60	1.34	1.87
120	1.25	1.86
180	1.10	2.00

[a] *In vivo* treatment; intratracheal administrations of 5 mg BP plus 5 mg Fe_2O_3 or 5 mg Fe_2O_3 alone (*1*) were performed 48 hr prior to sacrifice.

[b] The ratio of 28S to 18S RNA was calculated from the mean of the five highest points of each peak to give an adequate estimate of the area under the curve.

ulated to divide (*23*). Lymphocytes transformed by phytohemagglutinin are the most thoroughly studied of these populations, and in studies with comparable short labeling intervals they show a marked increase in the quantity of 18S RNA as compared to 28S RNA in response to the mitogen (*20*). This alteration in tracheal RNA metabolism may reflect the stimulation of cell division of tracheal epithelium in response to the BP treatment but not to Fe_2O_3 alone, which is observed morphologically as a modest hyperplasia during this acute phase after treatment (*24*).

The observation (*25*) of an inhibitory effect of vitamin A treatment on experimental respiratory carcinogenesis *in vivo* was the basis for study of the effect of the *in vivo* vitamin A status of hamsters on the metabolism of RNA in isolated tracheas. As previously reported (*26*), an alteration of RNA metabolism was observed in the case of vitamin A deficiency. In this study, isolated tracheas from vitamin A-deficient hamsters and their pair-fed controls were labeled with uridine-^{3}H, and RNA was isolated and separated electrophoretically as usual. In the case of vitamin A deficiency, there was a consistantly smaller proportion of RNA among the high molecular weight, slowly migrating species (Fig. 4; Table 2). This abnormality was reversed following administration of vitamin A *in vivo* (*26*). Subsequent studies of RNA metabolism were designed to determine the class of RNA species which were effected in the case of vitamin A deficiency. Specific inhibitors of RNA metabolism were employed to determine whether the decrease in large RNA species related to the heterogenous nuclear RNA or the ribosomal precursor species.

Low concentrations of toyocamycin, an analog of adenosine, selectively inhibited the synthesis of 18S and 28S ribosomal RNA and caused the ac-
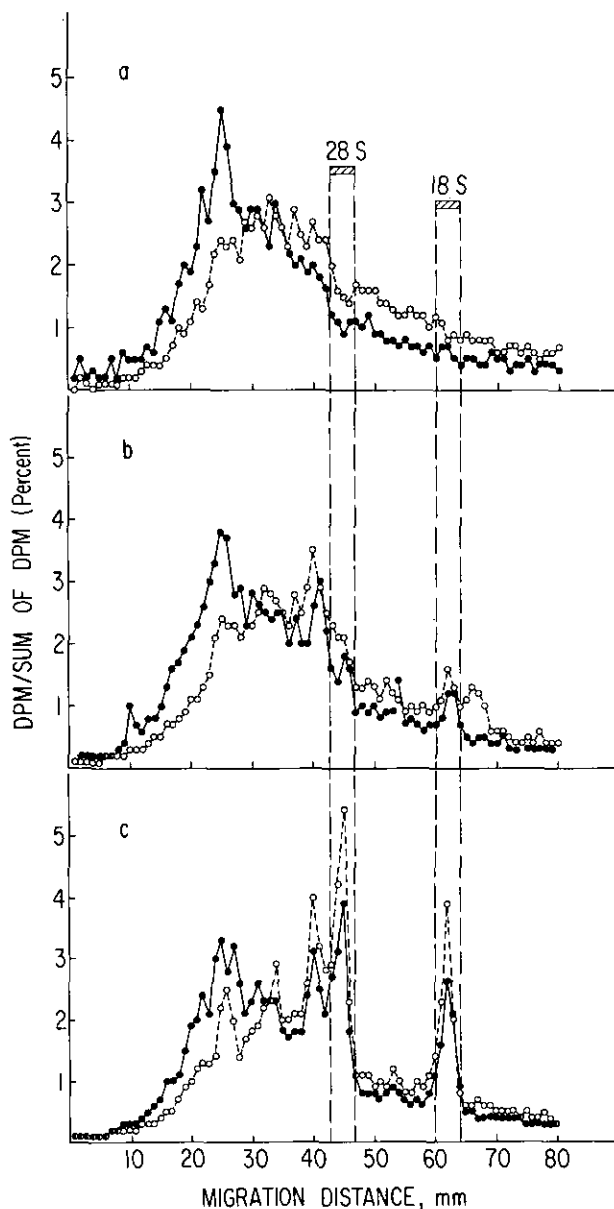

FIGURE 4. Electrophoretic distribution of high molecular weight RNA synthesized *in vitro* in tracheal epithelium from normal and vitamin A-deficient hamsters. Tracheas were isolated from vitamin A-deficient hamsters and their pairfed controls. Labeling of RNA *in vitro*, gel electrophoresis and analysis of results were as described in the legend to Fig. 2. Distribution (a) after 30 min pulse and after additional incubation for (b) 30 min or (c) 90 min of RNA from tracheas of (O) vitamin A-deficient hamsters and (●) pair-fed, vitamin A-normal hamsters. From Kaufman (*26*) with permission of the American Association for the Advancement of Science.

cumulation of the 45S ribosomal RNA precursor in L-cells (*27,28*) and Novikoff hepatoma cells (*21*). Toyocamycin had the same effect in isolated

tracheas (Fig. 5a). The region of the gel containing the 45S ribosomal RNA precursor (delimited by the vertical lines) was calculated from the observed mobility of the 18S and 28S RNA reference markers. There was a clear increase in size of the peak at this location when toyocamycin was used. Continuous labeling (i.e., tracheas incubated with uridine-^{3}H for the entire incubation period rather than only the first 30 min) was found to accentuate the alteration in slowly

Table 2. Electrophoretic pattern of RNA species from tracheal epithelium of vitamin A-deficient and normal hamsters.

Incubation time, min	Migration 30 mm or less, % of DPM*	
	Vitamin A normal	Vitamin A deficient
30	46.3	28.1
60	43.3	27.5
120	36.4	23.3

* The reference point of 30 mm migration has no inherent significance but operationally it permits a numerical comparison of distribution of radioactive RNA in normal and vitamin A-deficient animals (Fig. 4).
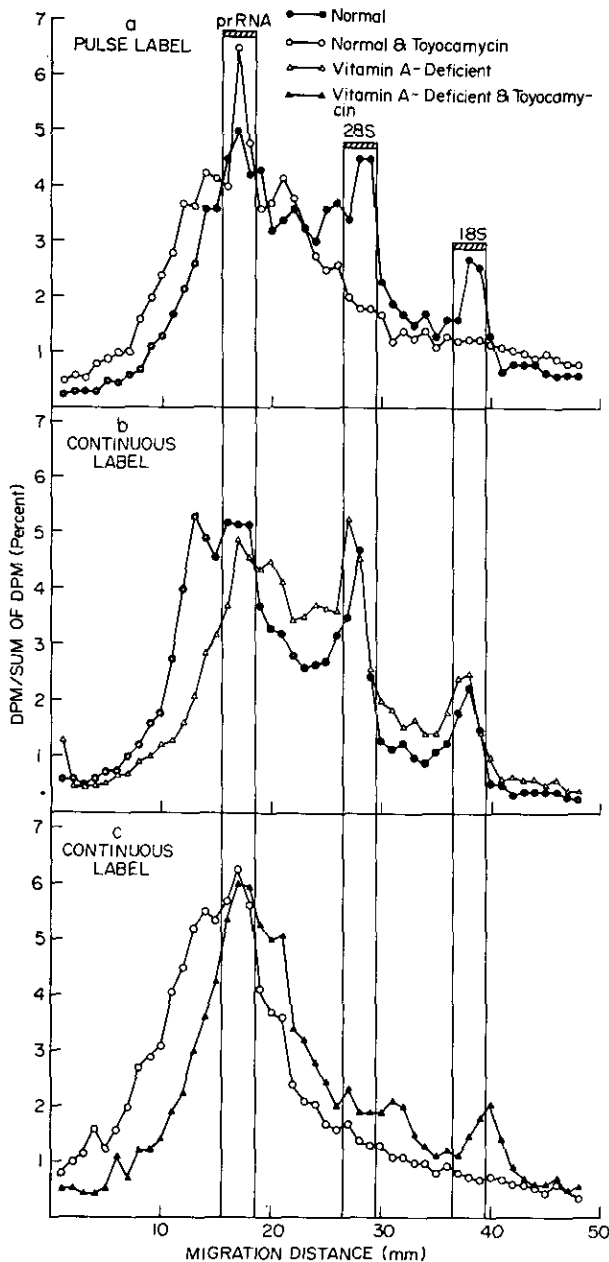
migrating RNA species in vitamin A deficiency (Fig. 5b) as compared to pulse labeling (Fig. 4). In subsequent experiments, continuous labeling was used to evaluate the effects of inhibitors. The broad peak with 12-18 mm migration is biphasic with continuous labeling. It appears that the more slowly migrating portion of this peak (left half) is the specific region which is decreased in the case of vitamin A deficiency (Fig. 5b). Treatment of tracheas with toyocamycin in vitro did not affect the alteration of RNA pattern in tracheas of vitamin A-deficient hamsters as compared to controls (Fig. 5c). In both cases, however, radioactive RNA accumulated in the region of the biphasic peak presumed to contain the 45S ribosomal RNA precursor (right half).

Further evidence that the right half of the biphasic peak contains the ribosomal precursor comes from an inhibition study using actinomycin D. Synthesis of 18S and 28S RNA was appreciably inhibited in tracheas treated with 0.2

FIGURE 5. Effect of toyocamycin on synthesis of high molecular weight RNA in tracheal epithelium from normal and vitamin A-deficient hamsters. Tracheas were obtained from untreated hamsters, vitamin A-deficient hamsters, or pair-fed vitamin A-normal controls. In cases were toyocamycin was used, tracheas were preincubated in medium containing 1 μg/ml toyocamycin for 15 min prior to labeling, and toyocamycin at this concentration was also present throughout the subsequent 180 min of incubation. (a) Tracheas were from normal hamsters; labeling with uridine-^{3}H was for 30 min followed by an additional 150 min incubation without uridine-^{3}H: (●) normal tracheas; (o) normal tracheas incubated with toyocamycin. (b) Tracheas were incubated continuously for 180 min in medium containing uridine-^{3}H, but with toyocamycin: (△) vitamin A-deficient; (●) pair-fed controls. (c) Tracheas were incubated continuously in medium containing uridine-^{3}H for 180 min and incubated with toyocamycin: (▲) vitamin A-deficient hamsters; (o) pair-fed control hamsters. The region denoted by prRNA is the calculated location of the 45S ribosomal RNA precursor based upon the migration of 18S and 28S RNA species. Note that in this and subsequent cases electrophoresis was conducted for 1.5 hr, and only 48 slices were analyzed for each gel.

μg/ml actinomycin D and continuously labeled with uridine-^{3}H (Fig. 6). At 0.1 μg/ml there was considerably less inhibition and lower doses (e.g., 0.01 or 0.001 μg/ml) did not cause selective inhibition of ribosomal RNA synthesis. Actinomycin D, unlike toyocamycin, prevents the formation of the 45S ribosomal RNA precursor as well as 28S and 18S RNA (29). Consistent with this previous observation, there was a decreased proportion of labeled tracheal RNA in the portion of the gel calculated to contain the 45S precursor in the experiments where actinomycin D was used. The greater inhibition of labeling of 28S RNA than 18S RNA obseved in this instance has also been found in phytohemagglutinin-stimulated lymphocytes (30).
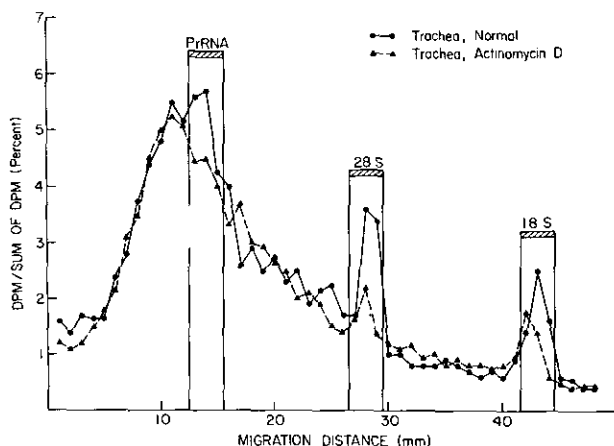

FIGURE 6. Effect of actinomycin D on synthesis of high molecular weight RNA in tracheal epithelium. Tracheas from normal hamsters were continuously labeled with uridine-^{3}H for 180 min. In the case where actinomycin D was used, tracheas were incubated in medium containing 0.2 μg/ml for 15 min prior to labeling and throughout the labeling period. (●) normal tracheas; (▲) tracheas treated with actinomycin D. The region denoted as prRNA was calculated from the observed mobility of the uridine-^{14}C labeled 28S and 18S RNA markers.

In contrast to the preferential inhibition of nucleolar (ribosomal) RNA synthesis by low doses of toyocamycin or actinomycin D, α-amanitin has been shown to have much greater effect on nucleoplasmic RNA synthesis (31,32). This results from a direct interaction with the nucleoplasmic RNA polymerase which is responsible for the synthesis of the DNA-like heterogenous nuclear RNA. The electrophoretic pattern of RNA synthesized in isolated tracheas in the presence of α-amanitin had a slightly more prominent 45S region than in untreated controls (Fig. 7). This prominence could be the result of a slightly decreased proportion of synthesis of
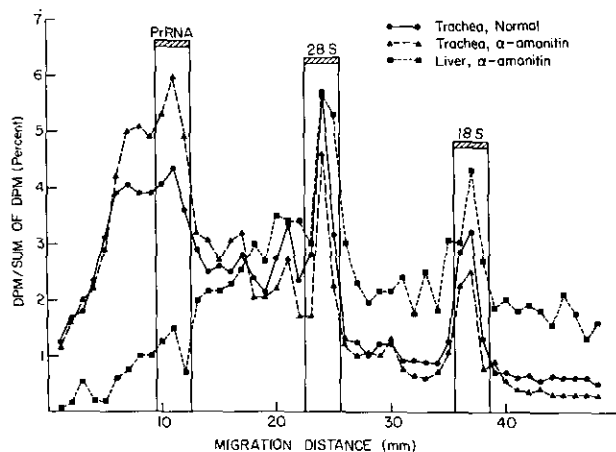

FIGURE 7. Effect of α-amanitin on synthesis of high molecular weight RNA in hamster trachea and liver. Liver was obtained from a hamster 2 hr after an intraperitoneal administration of 300 μg/kg of α-amanitin in 0.9% NaCl solution. Liver was minced finely and incubated in medium containing uridine-^{3}H for 180 min. After incubation the fine pieces of liver were collected and processed identically to tracheal epithelium. Tracheas were from normal hamsters and they were incubated for 180 min in medium containing uridine-^{3}H. Tracheas treated with α-amanitin were incubated with it at a concentration of 0.2 μg/ml during the 180 min labeling period only: (●) normal trachea; (▲) α-amanitin-treated trachea; (■) liver from a hamster treated in vivo with α-amanitin.

species larger than 45S. These large RNA species were most affected by α-amanitin in an experiment in which hamsters were treated in vivo and uridine-^{3}H labeling of liver followed in vitro (Fig. 7). In contrast to this marked reduction of large RNA, normal liver RNA synthesized under comparable conditions has a greater proportion of label in species 45S and larger than does trachea (see Fig. 8). This result suggests that an alteration of metabolism of heterogenous nuclear RNA could produce an electrophoretic pattern which would have a deficit of very large (>45S) RNA as its most obvious effect.

The preceding results suggest that the alteration in RNA metabolism observed in tracheas of vitamin A-deficient hamsters concerns the heterogenous nuclear RNA. Although a complete understanding of this class of RNA has not emerged yet, it is clear that it contains RNA related to messenger RNA (33-36). The proportion and presumably the types of heterogenous RNA are related to the state of tissue differentiation. The greatest differences between electrophoretic patterns of RNA synthesized in trachea, liver, and esophagus in vitro were in species 45S and larger (Fig.8). The differentiation of the tracheal epithelium of a vitamin A-deficient hamster is morphologically changed from that of the normal trachea.
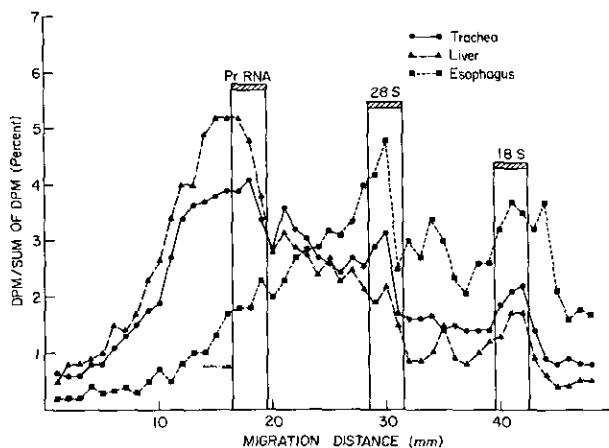
FIGURE 8. Electrophoretic distribution of newly synthesized high molecular weight RNA from hamster trachea, liver, and esophagus. Tissues were obtained from normal hamsters and all were incubating for 180 min in medium with uridine-³H. Tracheas were prepared and labeled as described previously. Liver from a normal hamster was minced, labeled, and analyzed as described in the legend of Fig. 7. The esophagus of a normal hamster was washed extensively with phosphate buffered saline then cut open. It was incubated with medium containing uridine-³H as a flat sheet comparable to an isolated trachea. Following incubation the epithelium was scraped from the surface of the esophagus and the scrapings processed like tracheal epithelium: (●) trachea; (▲) liver; (■) esophagus.

In the deficient state there are focal areas of squamous metaplasia (11) as well as basal cell hyperplasia, and a decreased proportion of ciliated cells (37). There are fewer goblet cells, although nonciliated lumenal cells are very numerous. The most likly conclusion is that the change in the electrophoretic pattern of RNA in vitamin A-deficiency is the consequence of the alteration of differentiation. The reduced proportion of the more differentiated ciliated and goblet cells, the increase in the numbers of basal cells and simple nonciliated cells (in intermediate and lumenal locations), and focal squamous metaplasia might explain this change.

Studies of Benzo[a]pyrene Binding to DNA in Isolated Hamster Tracheas

Studies concerning binding of BP to DNA in isolated tracheas form a second major area of our biochemical studies of tracheobronchial epithelium. These observations have been published recently (38-40), and the following is limited to an abbreviated reiteration of these results.

The motivation for these studies was also to learn more about factors related to the induction of respiratory tract tumors in vivo in life-time carcinogenesis studies with Syrian golden hamsters. Initial experiments were designed to demonstrate purification and banding of DNA from tracheas, and to determine whether binding of carcinogens during in vitro incubation reached detectable levels. Tracheas were obtained from normal hamsters and from hamsters which received an intratracheal administration of BP plus Fe_2O_3 or Fe_2O_3 alone, 48 hr prior to sacrifice. The tracheas were incubated in vitro in medium containing BP-³H and following incubation the epithelium was removed for further study. Unincorporated BP-³H was carefully extracted from the partially purified nucleic acids. RNA and proteins were enzymatically digested, leaving a DNA preparation which banded as a sharp peak in CsCl gradients with low background absorbance at 254 nm (see examples in Fig. 9). Radioactivity was determined for all gradient fractions, and ultraviolet absorption was carefully quantitated for peak fractions. A greater quantity of radioactivity banded coincidently with the DNA peak from the tracheas of hamsters previously treated with BP plus Fe_2O_3 in vivo than in untreated hamsters. In contrast, an intratracheal dose of Fe_2O_3 alone did not induce greater binding of BP-³H. These results are presented quantitatively in Table 3. Here, the specific activity of binding is determined as the quantity of radioactivity from bound BP-³H (DPM) normalized to the DNA content (μg DNA) of the peak fractions.

The next experiments were to demonstrate that the increased BP binding in tracheas of hamsters treated with BP plus Fe_2O_3 in vivo was the result of activation of BP in vitro rather than a nonspecific phenomenon. Tracheas were obtained from animals treated with BP plus Fe_2O_3 48 hr earlier. Tracheas incubated at 0°C or with 7,8-benzoflavone (BF), an inhibitor of BP metabolism (41), added to the medium were compared to tracheas incubated under standard conditions. The results of these studies (Table 3) indicate that both BF and 0°C incubation inhibit binding of BP-³H to DNA in isolated tracheas. These results suggest that the observed BP binding of isolated tracheas is the summation of two processes. Binding is low in tracheas of normal hamster or in tracheas treated with BF, but somewhat higher than that observed after incubation at 0°C. Prior treatment with BP plus Fe_2O_3 in vivo induces a much higher level of binding in isolated tracheas, and this induced binding is reduced to normal levels by addition of BF to the incubation medium.

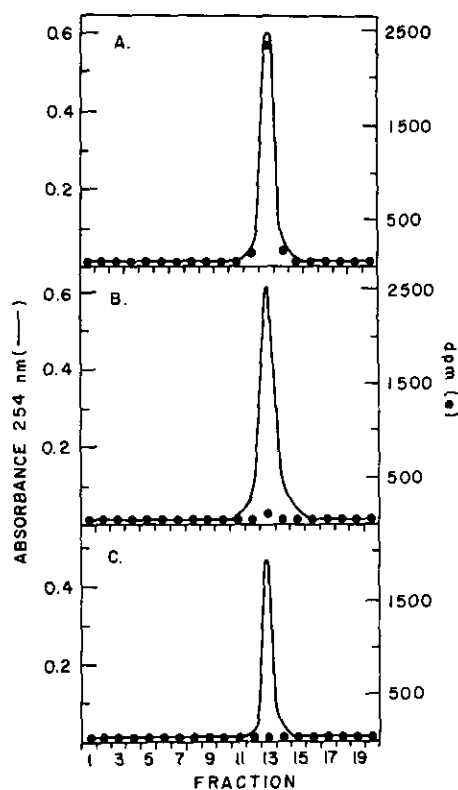Since initial studies demonstrated that BP binding to tracheal DNA was detectable and the

FIGURE 9. Effects of conditions of incubation in vitro on binding of BP-³H to DNA of tracheas from vitamin A-deficient hamsters. Tracheas were removed from vitamin A-deficient hamsters and incubated in medium containing (a) BP-³H at 37°C (b) benzoflavone (BF) added to the incubation medium; (c) incubation was at 0°C. The continuous line is the tracing of the ultraviolet absorption of the gradient as it was displaced through a flow cell monitored at 254 nm.

result of both basal and inducible components, the objective of these studies became the evaluation of these properties for their correlation with susceptibility to carcinogenesis in vivo. It was hoped that, in addition to evaluating genetic metabolic capacity, which previous reports related to susceptibility in man (42) and mouse (43), these properties could evaluate other biological properties in the target organ which serve as cofactors in determining susceptibility. Vitamin A deficiency was one such biologic factor of interest since marginal vitamin A status has been shown to increase the incidence of aflatoxin-induced colonic carcinomas in rats (44). Vitamin A-deficient and pair-fed, vitamin A-normal hamsters were prepared as described previously and the binding of BP-³H to DNA in isolated tracheas determined following incubation in vitro. The BP binding level in the tracheas from vitamin A-deficient hamsters was nearly four times greater than in the vitamin A-normal controls. This elevated

Table 3. Effects of in vivo treatment and invitro incubation conditions on specific activities of binding of BP-³H to hamster tracheal DNA.

Incubation conditions	Binding, DPM (BP-³H)/µg DNA		
	In vivo Pretreatment with BP plus Fe₂O₃	In vivo Pretreatment with Fe₂O₃	Untreated
BP-³H, 37°C	12.1	2.4	2.7
BP-³H, BF, 37°C	2.4	2.4	2.6
BP-³H, 0°C	0.8	1.1	1.3

binding level was also inhibited by incubation with BF in the medium or by incubation at a temperature of 0°C (Fig.9). Addition of 5-15 µg/ml of retinyl acetate to the medium in which tracheas from vitamin A-deficient hamsters were incubated caused substantial inhibition of BP binding, but less than that caused by BF. In other studies two inbred strains of hamsters were selected for their demonstrated susceptibility to subcutaneous and gastrointestinal carcinogenesis (45). Levels of binding of BP in vitro in both inbred strains were about twice those of random bred hamsters following treatment with BP plus Fe₂O₃ in vivo. In one additional experiment, levels of BP binding were evaluated in random bred hamsters of 4, 8, and 12 weeks of age. There was substantially greater binding in the tracheas from the two younger groups. The significance of these observations will not be clear until we know the relationship of respiratory tumor incidence to vitamin A-deficiency, inbred hamster strain, and age at the time of carcinogen treatment. For the moment, however, the observation of high levels of binding in biologic states which appear likely to be states of increased susceptibility suggests that this type of binding assay may hold promise of distinguishing both genetic and acquired susceptibility factors which predispose to the development of lung cancer in the hamster model.

Conclusion

These results illustrate that biochemical studies on a respiratory epithelium, from hamster trachea in this case, can reach substantial sophistication. Methods now exist for a variety of biochemical investigations on this tissue, and the accomplished studies, hopefully, will give encouragement to further exploration of the biochemical properties of this target tissue.

RNA metabolism in the epithelium of isolated tracheas from normal hamsters shares a number of characteristics previously reported for other tissues and cell types. Alterations in the electrophoretic distribution of newly synthesized RNA were observed for two abnormal states *in vivo*: the acute phase following intratracheal treatment with BP plus Fe_2O_3, and vitamin A deficiency. Efforts to distinguish the class of RNA decreased in vitamin A deficiency have provided information on the effects of three inhibitors of RNA metabolism which illustrate a variety of acute states of biochemical toxicity in this target tissue. Comparable studies of the effects of vitamin A deficiency on RNA metabolism might not have been possible in many other tissues, or tissue culture cells, whose differentiation is not vitamin A dependent. In the case of BP binding studies, it was useful to learn that BP is metabolized in the tracheal epithelium and that it binds to DNA. This could have been anticipated, however, from the fact that instillation of BP into the respiratory tract can cause tracheal tumors. Only by direct studies of this epithelium, however, could we learn of the capacity of this tissue to undergo induction of a mechanism which increases BP binding. Furthermore, the evaluation of other biological states *in vivo* (e.g., vitamin A deficiency, genetic strain) for their effects on BP binding at this target site could only be studied in a respiratory epithelium. It is clear, therefore, that certain kinds of questions can only be studied, or studied best, in the specific target tissue. This belief forms the basis for initial studies of human respiratory epithelium *(46)* in an effort to identify specific biochemical characteristics of this human target tissue.

The ultimate importance of biochemical studies thus goes beyond comparison with other tissues. The human population shows a diversity of inherited and acquired characteristics, and this may explain why at a given level of injurious exposure, clinical cancer or emphysema, etc. develops in some individuals and not in others. It is to be hoped that biochemical and physiological studies of the respiratory tract in experimental animals can offer some insight into the genetic properties and acquired conditions which make one individual susceptible and another refractory to a given exposure. By specifically protecting the susceptible, perhaps we can more finely adjust the scales of societal benefit versus individual risks to maximize the advantages for all.

REFERENCES

1. Saffiotti, U., Cefis, F., and Kolb, L. H. A method for the experimental induction of bronchogenic carcinoma. Cancer Res. 28: 104 (1968).
2. Saffiotti, U. Morphology of respiratory tumors induced in Syrian golden hamsters. In: Morphology of Experimental Respiratory Carcinogenesis. P. Nettesheim, M. G. Hanna, Jr., and J. W. Deatherage, Jr., Eds., U.S. Atomic Energy Commission, Division of Technical Information. Oak Ridge, Tennessee, 1970.
3. Yeager, H., Jr., Massaro, G., and Massaro, D. Glycoprotein synthesis by the trachea. Amer. Rev. Resp. Dis. 103: 188 (1971).
4. Chakrin, L. W., et al. Synthesis and secretion of macromolecules by canine trachea. Amer. Rev. Resp. Dis. 105: 368 (1972).
5. Chakrin, L. W., et al. Effect of cholinergic stimulation on the release of macromolecules by canine trachea in vitro. Amer. Rev. Resp. Dis. 108: 69 (1973).
6. Ellis, D. B., and Stahl. G. H. Biosynthesis of respiratory-tract mucin. Incorporation of radioactive precursors into glycoproteins by canine tracheal explants in vitro. Biochem. J. 136: 837 (1973).
7. Stahl, G. H., and Ellis, D. B. Biosynthesis of respiratory-tract mucin. A comparison of canine epithelial goblet-cell and submucosal-gland secretions. Biochem. J. 136: 845 (1973).
8. Bonanni, F., and DeLuca, L. Vitamin A-dependent fucose-glycopeptide from rat tracheal epithelium. Biochim. Biophys. Acta 343: 632 (1974).
9. Pal, K., Grover, P. L., and Sims, P. The metabolism of carcinogenic polycyclic hydrocarbons by tissues of the respiratory tract. Biochem. Soc. Trans. 3: 174 (1975).
10. Turner, D. M., Davis, B. R., and Crabb, H. L. Alteration in the LDH isoenzymes of rat bronchial mucosa during chemical carcinogenesis. Enzyme 14: 166 (1973).
11. Harris, C. C., et al. Histogenesis of squamous metaplasia in the hamster tracheal epithelium caused by vitamin A deficiency or benzo[a]pyrene-ferric oxide. J. Nat. Cancer Inst. 48: 743 (1972).
12. Kaufman, D. G., et al. Coordinated biochemical and morphologic examination of hamster tracheal epithelium. J. Natl. Cancer Inst. 49: 783 (1972).
13. Kaufman, D. G., et al. Short term culture studies on tracheal epithelium. Proc. Am. Assoc. Cancer Res. 12: 10 (1971).
14. Burton, K. Study of the conditions and mechanism of the diphenylamine reaction for the colorimetric estimation of deoxyribonucleic acid. Biochem. J. 62: 315 (1956).
15. Donnelly, G. M. RNA synthesis in ciliated cells of the trachea. J. Cell Biol. 63: 88a (1974).
16. Peacock, A. C., and Dingman, C. W. Molecular weight estimation and separation of ribonucleic acid by electrophoresis in agarose-acrylamide composite gels. Biochemistry 7: 668 (1968).

17. Dingman, C. W. The relationship between the molecular weight of polyribonucleotides and their electrophoretic mobility in agarose-acrylamide gels. In: Proceedings of the First European Biophysical Congress. E. Broda, A. Locker, and H. Springer-Lederer, Eds., Wiener Medizinischen Akademe Verlag, Vienna, 1971.
18. McConkey, E. H., and Hopkins, J. W. Molecular weights of some HeLa ribosomal RNA's. J. Mol. Biol. 39: 545 (1969).
19. Green, M. R., Bunting, S. L., and Peacock, A. C. Changes in labeling pattern of ribonucleic acid from mammary tissue as a result of hormone treatment. Biochemistry 10: 2366 (1971).
20. Cooper, H. L. Control of synthesis and wastage of ribosomal RNA in lymphocytes. Nature 227: 1105 (1970).
21. Weiss, J. W., and Pitot, H. C. Inhibition of ribosomal RNA maturation in Novikoff hepatoma cells by toyocamycin, tubercidin and 6-thioguanosine. Cancer Res. 34: 581 (1974).
22. Attardi, G., and Amaldi, F. Structure and synthesis of ribosomal RNA. Ann. Rev. Biochem. 39: 183 (1970).
23. Cooper, H. L. Biochemical alterations accompanying initiation of growth in resting cells. In: The Cell Cycle and Cancer. R. Baserga, Ed., Marcel Dekker, Inc., New York, 1971.
24. Harris, C. C., et al. Acute ultrastructural effects of benzo[a]pyrene and ferric oxide on the hamster tracheobronchial epithelium. Cancer Res. 31: 1977 (1971).
25. Saffiotti, U., et al. Experimental cancer of the lung. Inhibition by vitamin A of the induction of tracheobronchial squamous metaplasia and squamous cell tumors. Cancer 20: 857 (1967).
26. Kaufman, D. G., et al. RNA metabolism in tracheal epithelium: alteration in hamsters deficient in vitamin A. Science 177: 1105 (1972).
27. Travitian, A., Uretsky, S. C., and Acs, G. Selective inhibition of ribosomal RNA synthesis in mammalian cells. Biochim. Biophys. Acta 157: 33 (1968).
28. Tavitian, A., Uretsky, S. C., and Acs, G. The effect of toyocamycin in cellular RNA synthesis. Biochim. Biophys. Acta 179: 50 (1969).
29. Perry, R. P. The cellular sites of synthesis of ribosomal and 4S RNA. Proc. Nat. Acad. Sci. (U.S.) 48: 2179 (1962).
30. Kay, J. E., and Cooper, H. L. Differential inhibition of 28S and 18S ribosomal RNA synthesis by actinomycin. Biochem. Biophys. Res. Commun. 35: 526 (1969).
31. Reeder, R. H., and Roeder, R. G. Ribosomal RNA synthesis in isolated nuclei. J. Mol. Biol. 67: 2861 (1971).
32. Zylber, E. A., and Penman, S. Products of RNA polymerases in HeLa cell nuclei. Proc. Nat. Acad. Sci. (U.S.) 68: 2861 (1971).
33. Darnell, J. E., et al. Studies on the relationship of mRNA to heterogenous nuclear RNA in mammalian cells. Cold Spring Harbor Symp. Quant. Biol. 35: 555 (1970).
34. Williamson, R. Drewienkiewicz, C. E., and Paul, J. Globin messenger sequences in high molecular weight RNA from embryonic mouse liver. Nature New Biol. 241: 66 (1973).
35. Torelli, U., and Torelli, G. Poly(A)-containing molecules in heterogenous nuclear RNA of normal PHA-stimulated lymphocytes and acute leukaemia blast cells. Nature New Bio. 244: 134 (1973).
36. Stevens, R. H., and Williamson, A. R. Isolation of nuclear pre-mRNA which codes for immunoglobulin heavy chains. Nature New Biol. 245: 101 (1973).
37. Boren, H. G., et al. Cell populations in the hamster tracheal epithelium in relation to vitamin A status. Internat. J. Vit. Nutr. Res. 44: 382 (1974).
38. Kaufman, D. G., et al. Binding of ^{3}H-labeled benzo[a]-pyrene to DNA in hamster tracheal epithelial cells. Cancer Res. 33: 2837 (1973).
39. Genta, V. M., et al. Vitamin A deficiency enhances binding of benzo[a]pyrene to tracheal epithelial DNA. Nature 247: 48 (1974).
40. Kaufman, D. G., Genta, V. M., and Harris, C. C. Studies on carcinogen binding in vitro in isolated hamster tracheas. In: Experimental Lung Cancer. Carcinogensis and Bioassays. E. Karbe and J. F. Park, Eds., Springer-Verlag, Heidelberg, 1974.
41. Wiebel, F. J., et al. Aryl hydrocarbon (benzo[a]pyrene) hydroxylase in microsomes from rat tissues: differential inhibition and stimulation by benzoflavones and organic solvents. Arch. Biochem. Biophys. 144: 78 (1971).
42. Kellerman, G., Shaw, C. R., and Luyten-Kellerman, M. Aryl hydrocarbon hydroxylase inducibility and bronchogenic carcinoma. New Engl. J. Med. 289: 934 (1973).
43. Kouri, R. E., Salerno, R. A., and Whitmire, C. E. Relationship between aryl hydrocarbon hydroxylase inducibility and sensitivity to chemically induced subcutaneous sarcomas in various strains of mice. J. Natl. Cancer Inst. 50: 363 (1973).
44. Newberne, P. M., and Rogers, A. E. Rat colon carcinomas associated with aflatoxin and marginal vitamin A. J. Natl. Cancer Inst. 50: 439 (1973).
45. Homburger, F., et al. Inherited susceptibility of inbred strains of Syrian hamsters to induction of subcutaneous sarcomas and mammary and gastrointestinal carcinomas by subcutaneous and gastric administration of polynuclear hydrocarbons. Cancer Res. 32: 360 (1972).
46. Harris, C. C., et al. Carcinogenic polynuclear hydrocarbons bind to macromolecules in cultured human bronchi. Nature 252: 68 (1974).