RESPIRATORY EPITHELIAL LESIONS PRODUCED BY BENZO(A)PYRENE OR AN AIR POLLUTION EXTRACT IN TRACHEOBRONCHIAL MUCOSA FROM PERINATAL HAMSTERS, DOGS AND MONKEYS AND FROM ADULT HUMAN SUBJECTS IN ORGAN CULTURE.

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Polluted air contains a variety of chemical carcinogens (1), the most commonly recognized of which is benzo(a)pyrene (BaP). Benzene-soluble materials from filters used to collect air-borne particles, and fractions of these extracts, not always of known composition, are carcinogenic (1,2) and may have biologic activity which is relevant to human respiratory carcinogenesis associated with air pollution. In order to learn whether the respiratory mucosa of man (or other primates) can be expected to be more or less susceptible than that of rodents or canines upon exposure to these materials, a test system is desirable that permits exposure of respiratory mucosa of several species to suspected materials under uniform conditions.

Tracheobronchial structures of suckling rats and hamsters and of fetal dogs and monkeys have been maintained for 2 to 3 weeks in organ culture. Late fetal or suckling (also referred to as "perinatal") animals were chosen in order to minimize environmental exposure to inhaled air and to make possible the use of whole respiratory structures (trachea, bronchi) which were small enough to be nourished by diffusion in the organ culture system. In addition, the rapid growth rate of cells of immature tissue was expected to permit more cell generations to occur during the limited time of maintenance of organ cultures. Thus, if more cell divisions could occur after initiation of a potential neoplasm, recognition of a neoplastic transformation might be more likely. The choice of these three orders of mammals was intended to include two orders (rodents and canines) in which experimental bronchogenic carcinoma has been produced by polycyclic environmental chemicals and one order (primates) in which BaP and AP have not been tested as a cause of bronchogenic carcinoma.

Polycyclic hydrocarbons known to be carcinogenic by various methods of testing in rodents have been added to organ culture media and have produced abnormal histologic states of respiratory epithelia of suckling rats (3,4,5,6). Among these are: (i) excessive height and crowding of columnar cells to form redundant epithelial folds; (ii) basal cell hyperplasia; (iii) replacement of differentiated columnar cells by one or more layers of undifferentiated pleomorphic cells or by stratified cells of a squamous epithelial type; and (iv) loss of all or most epithelial cells. Weakly carcinogenic and non-carcinogenic hydrocarbons have not produced these abnormal states (6). Whole benzene-soluble extracts of solids from filters used to collect air pollutants (AP) have been applied in this sytem, and one or more of the abnormal states described above have been induced. Effects of benzo(a)pyrene (BaP) and of AP are described in evaluating the biologic responsiveness of perinatal hamster, dog and monkey respiratory epithelia to a pure compound or an extract of material under consideration as having possible effects on human health.

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IBZN 17256

In addition to tests with perinatal animals as a source of tracheobronchial structures for culture, adult human subjects undergoing bronchial biopsy or lung resection have yielded some samples for testing. Control culture data for human mucosa are presented more fully elsewhere (7).

Materials and Methods

Addition of the whole benzene-soluble air pollution extract (AP) to culture media

A pool of crude whole benzene-soluble extracts of particulate material from air filters was provided through the courtesy of Dr. John Ludwig, Chief, Laboratory of Engineering and Physical Sciences, National Center for Air Pollution Control, Cincinnati, Ohio. The sample, designated "Miscellaneous Composite Vial #11964" was received as a brown glass vial of tarry material and was stored at 4°C. This material and the fractions obtained from it were described by Tabor and Smith (8).

Samples of AP were transferred to tared glass vials, weighed and exposed to a volume of dimethylsulfoxide (DMSO, Crown-Zellerbach Corporation) or acetone such that there was 180 mgm per ml of solvent. The vials were stoppered tightly and incubated at 37°C overnight. A clear supernate overlay a finely divided suspension which in turn was in contact with coarser particulate material. The entire sample was mixed well and 0.1 ml was transferred with a tuberculin syringe and 27 gauge needle to (i) 6.15 ml of chicken serum, with rapid mixing, and (ii) a pre-weighed glass vial. The serum preparation was stored at 4°C throughout an experiment and when added to medium it represented 25% of the final volume of medium.

The tared glass vial containing an acetone solution-suspension was exposed to a vacuum to exaporate acetone and re-weighed to determine the amount delivered. The amount of AP suspended or dissolved in 0.1 ml of DMSO was determined by the difference in weight of tared vials containing an aliquot of DMSO or of AP in DMSO.

The weights measured in 0.1 ml of solvent approximated the weight expected from the exposure of AP and solvent in a weight/volume ratio of 180 mg/ml. The estimates of concentration of AP in media based on direct weighings are, however, regarded as maximum possible values because of the presence of poorly suspended particles which could not be assumed to yield effectively dissolved or emulsified organic materials upon addition to serum. Final concentrations of samples in medium of about 700 ug/ml are therefore proposed as nominal values only.

Addition of control materials to culture media. BaP (Mann Research Laboratories) was dissolved in acetone or DMSO and 0.1 ml of the solution was rapidly mixed into 6.15 ml of chicken serum. This provided a stock sample which was stored at 4°C in the dark for the duration of the experiment. Media contained 25% of serum BaP stock suspension and were designated as containing the hydrocarbon at contractions indicated in the description of results. Actual concentrations were found to be lower than these nominal concentrations. When serum preparations of BaP were quantitated by absorption spectrophotometry, BaP was measured at 60 to 75% of the nominal concentration when nominal concentrations between 10 and 20 ug/ml were sought. This was associated with formation of a fine precipitate presumably of BaP and denatured serum proteins, but other sources of loss of apparently suspended and/or dissolved hydrocarbon, as adsorption on the vessel wall, also may have occurred. The difference between the expected and the measured among of BaP in serum was noted within 1 hour

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after mixing and was stable for 22 days, indicating no progressive decline in dissolved and/or suspended BaP.

Culture Media

1. Clot medium. Chicken embryos at 12 days of incubation age were minced and suspended in a volume of Tyrode's solution equal to the volume of mince to provide standard fresh embryo extract. Penicillin was included in the suspension at a concentration such that dilution of extract by 1:4 in making medium gave a final concentration of 200 units/ml. Roosters were starved for 2 days and then bled into siliconed glassware through a carotid artery to provide fresh sterile chicken plasma every 14 days or less. Commercial chicken serum (Microbiological Associates) was stored frozen until use and at 4°C during use. It was not heated to eliminate complement.

The above components were mixed in the center well of the Falcon plastic organ culture dish to form a clot having the following proportions: 50% plasma, 25% serum containing vehicle, AP or BaP and 25% embryo extract containing penicillin. Organ pieces on rayon mesh were rinsed in Tyrode's solution containing 2% chicken serum and 200 U/ml penicillin and moved from old to new clotted medium in fresh culture vessels every 2 or 3 days.

2. Liquid medium. A medium reported by Lasnitzki (9) was modified for use in parallel with the clotted medium and differed from it in that chicken plasma was omitted and the final medium consisted of 65% Medium 199, 25% chicken serum containing vehicle, AP or BaP and 10% embryo extract containing penicillin. Enough medium was placed in the center well of the organ culture dish to wet but not immerse organ pieces resting at the interface between medium and air on a rayon mesh supported by a stainless steel wire screen. Liquid medium was changed by replacing old medium in the same culture vessel.

3. Gassing. Tracheal and bronchial explants in covered culture dishes were gassed at 100 ml/min with humidified 97% O_2 - 3% CO_2 for ½hour daily in a black plastic box kept in a forced draft incubator at 37°C. All manipulations were performed in dark rooms lighted by incandescent bulbs delivering minimal energy at less than 4000 Å.

Sources of explants from perinatal animals.

Hamster. On 3 occasions pregnant females of the inbred MHA line (Lakeview Hamster Colony, Newfield, New Jersey) produced young which were used at 1 or 2 days of age to obtain whole tracheas. Each trachea resembled in diameter the medium sized bronchi which could be obtained and handled with reasonable ease upon dissection from lungs of fetal dogs and monkeys. Four to six tracheas were explanted for each period of incubation (8,11,15 days) for each experimental variable.

Canine (fetal beagle). Four fetuses taken 11 days and 5 fetuses taken 7 days before the estimated end of gestation (gestation time, 63 days), were used in 2 experiments. Beagles are not very mature even at birth. Ears open at 5 days and eyes at 8 days of age, and dogs walk at 2½ weeks of age. In newborn rodents the ears open at 2½ to 3½ days and the eyes at 14 to 17 days; sucklings can propel themselves to the mother at 9 to 11 days. Thus fetal dogs are probably less mature than the newborn rodents used in these studies.

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Simian Primates. One experiment using a single fetal M. rhesus and another using 2 fetal M. cynomolgus monkeys have been analysed. The fetal animals were at 130 to 140 days of gestational age (total gestation time, 160 to 163 days). Such fetuses are regarded as being reasonably mature since they are furred, [illegible] breathing attempts. Breathing was prevented at the time of surgical delivery by placing a tight ligature around the neck before opening the amniotic sac.

Preparation of dog and monkey tracheobronchial explants.

Dissection of the bronchial tree from the lung was rather easy in unaerated lung from late fetal animals because fine air bubbles surrounded by surfactant did not form as the tissue was manipulated. The clear field of vision permitted the lung tissue to be "combed" off bronchi down to the 5th order and facilitated separation of pulmonary vessels from bronchi.

The smallest bronchi were cut from the bronchial tree as 3 to 5 parallel airways in a group about 1.5 mm long and laid on rayon mesh as a single explant. This occurred most often with fetal dog bronchi because the less mature tissues of these animals, as compared with fetal monkeys, made it easier to separate small bronchi from lung. The histologic state of the epithelium of each bronchus was recorded (see below) and the large number of histologic states per explant of dog tracheo-bronchial epithelium is explained by the presence of many small bronchi per explant. Moderate sized bronchi (lumen diameter about 0.5 to 1 mm) were cut in 2 mm lengths as individual explants. Major bronchi and the trachea were opened and nearly flat plaques were cut parallel with the axis of the lumen and laid as individual explants on rayon, mucosa side up. Where tracheal cartilage was present the plaques were labeled to distinguish them from the membranous portion (posterior 1/3) of the trachea. Four to six explants of plaques and small bronchi were set up per treatment for each of 2 or 3 periods of time in culture.

Preparation of adult human bronchial mucosal explants.

Bronchial mucosal samples were obtained from persons undergoing biopsy or lung resection for chronic respiratory disease or lung cancer. Tissues used for culture were taken from apparently normal bronchi or zones of mucosa remote from inflammatory or neoplastic lesions. The report by T. V. O'Donnell and T. T. Crocker (7) describes preparation of explants. That report includes terms used in describing histologic states of epithelial differentiation before and during cultivation.

Histology and autoradiography.

Tritiated thymidine (^{3}HTdR) was added to each explant 30 minutes before fixation and the culture reincubated and gassed. Explants were rinsed 3 times in Tyrode's solution without serum and fixed in Bouin's solution for ½ hour.

After routine dehydration, acetone was used to dissolve the rayon acetate mesh before infiltration with paraffin. Serial sections were cut at 3 microns and were distributed on a series of slides so as to permit each slide to bear representative sections from 3 portions of the series. Some slides were stained with hematoxylin-eosin or colloidal iron-hematoxylin stains for histologic examination and others were used for autoradiography.

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Prior to autoradiography with NTB2 liquid emulsion, histologic sections were stained with Periodic Acid-Schiff's (PAS) stain or deparaffinized and air dried. After photographic development, hematoxylin or hematoxylin-eosin were used as counterstains through the film.

Microscopic analyses.

Descriptions of each of 4 to 6 tracheobronchial explants for each treatment at each period of time in culture were recorded on a form by slide number without knowledge of the experimental status of the tissue. Quantitation of the proportion of epithelial cells labeling with ^{3}HTdR was performed on autoradiographs. Some explants of dog bronchial tree contained several small bronchi; the epithelial state of each bronchus was recorded for that explant. Some explants bore epithelium in more than one state of differentiation. For example, a zone of columnar epithelium with basal hyperplasia might be adjacent to a zone of normal columnar epithelium in a single tracheo-bronchial plaque making up one explant. Each histologic state was recorded as an independent datum and labeling in autoradiographs over each epithelial zone was placed in one of the classes of labeling shown in Table 1. These factors led to the scoring of more epithelial states of differentiation and the associated labeling rates than could be accounted for by the total number of explants.

Results.

Respiratory structures exposed to whole benzene-soluble air pollution extract (AP) and benzo(a)pyrene (BaP).

Suckling hamster trachea: control cultures. Generally good maintenance of epithelia was observed for as long as 17 days on liquid or clotted acetone control media. (Figures 1-4) but occasionally a zone of undifferentiated epithelium or squamous metaplasia was noted and some devitalized explants occurred (Table 1). Data from both types of media are pooled in this review. Comparison of acetone and DMSO in control liquid media revealed that DMSO favored retention of a columnar cell population but no change in labeling as compared to acetone. While this suggests that DMSO had an effect slightly favoring differentiation, the results from use of both vehicles are pooled in this report.

Suckling hamster trachea: treated cultures. AP and BaP reduced the frequency of columnar differentiation and increased the frequency of basal hyperplasia (State 2, Table 1 and Figures 5 and 6) and of pleomorphic undifferentiated states (State 3, Table 1 and see, for example, Figures 7, 8). BaP and AP reduced the level of replication in columnar epithelium except when basal hyperplasia was present (Table 1). AP stimulated DNA synthesis in areas of basal hyperplasia more than BaP did. AP was toxic to hamster tracheas as indicated by increase in the percent of devitalized explants (Table 1). Squamous metaplasia occurred rarely with BaP or AP (State 4, Table 1 and Figures 9, 10) and was observed in one zone (1%) of 131 states recorded in 124 control explants.

Fetal dog tissue at the time of explanting. Respiratory epithelium in fetal dogs was not well differentiated. The columnar cells of trachea had rare cilia and PAS-positive but colloidal iron-negative goblets. Smaller airways were lined by columnar cells lacking mucous inclusions and cilia but

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filled with finely granular glycogen. Tritiated thymidine complexes with glycogen and labeling produced a pattern of autoradiographic grains over the cytoplasm generally or over the Golgi zone.

Fetal dog tissue: control cultures. Differentiation of columnar epithelial cells occurred during 8 to 17 days in control cultures. Ciliated cells and colloidal iron-positive mucous inclusions in goblet cells appeared in fetal tracheal epithelia, but the fetal dog bronchi did not acquire acid mucopolysaccharide-containing mucous inclusions indicating limits to the extent to which differentiation would be achieved in vitro. Coincident with evolution of differentiated states, the proportion of epithelial cells in DNA synthesis declined. The cells labeled with ^{3}HTdR at the time of planting fell in fetal dog trachea from 3.5 to 1.0%, and in fetal dog bronchi from 5 to 0.6%.

Fetal dog tissue: treated cultures. There was a distinct tendency for emergency of basal hyperplasia and pleomorphic epithelial states in control cultures of fetal dog bronchi (Table 1). BaP did not reduce the overall proportion of differentiated epithelial states (States 1 and 2, Table 1) but did increase the frequency of basal hyperplasia with an associated increase in the proportions of labeled nuclei. The effect of AP was different from that of BaP; AP reduced the frequency of differentiated epithelial states, and increased the frequency of squamous metaplasia and pleomorphic epithelia associated with a moderate level of DNA synthesis (Table 1, States 3, 4). Figures 9 and 10 illustrate squamous metaplasia but were photographed from hamster tracheal sections.

Fetal monkey tissue at the time of explanting. The state of differentiation of tracheobronchial epithelium was not quite complete in these fetal simians. Not all surface cells bore cilia or mucous inclusions containing acid mucopolysaccharides and were not always arranged in a mature columnar state (Figure 11). The ratio of basal to differentiated cells was about 1 to 1 rather than the ratio 1 to 2 noted in adults.

Fetal monkey tissue: control cultures. Examination at intervals of 8 and 15 days of cultivation revealed progressive differentiation of columnar epithelia which were higher than in the planting controls. Pseudostratification occurred and basal cells increased in number, and the proportion of basal to columnar cells became similar to adult ratios in the monkey. Parts of some explants failed to acquire differentiated columnar epithelia and zones of cellular undifferentiated epithelia were present (State 3, Table 1). Cartilage, where present, was well maintained, as were smooth muscle cells and connective tissue elements from which fibroblasts grew into the rayon mesh (Figure 12).

Fetal monkey tissue: treated cultures. Fetal monkey bronchial explants had fewer instances of differentiated epithelium and basal hyperplasia emerged in some columnar epithelia which were retained upon treatment with AP and BaP (States 1 and 2, Table 1). Increased instances of pleomorphic undifferentiated states were produced, with relatively high levels of replicative activity (State 3, Table 1 and Figures 13 and 14). Squamous metaplasia was not observed in monkey tissue. BaP was toxic while AP was not, as shown by the percent of devitalized explants (State 5, Table 1).

Adult human bronchial mucosa.

Maintenance of spontaneous lesions and normal epithelia in control cultures.

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T. V. O'Donnell and T. T. Crocker (7) made a detailed analysis of control cultures of bronchial mucosa taken from persons with chronic respiratory disease or bronchogenic carcinoma. Since these tissue samples were initially [illegible] have been damaged during removal, extensive examination of control (untreated) explants was made to establish the range [illegible] states which might occur spontaneously. Histologic states that emerged while such samples of adult human bronchial mucosa were maintained in culture were than used to correct for the states that emerged during [illegible] pollutants and BaP.

The extensive control results (7) are condensed, without labeling data, in Table 2. The histologic states of the epithelium in control cultures are illustrated in Figures 11-16 (from reference 7). When a normal zone of mucosa was present in the bronchus dissected for explanting (Figure 15), it was well maintained for 15 days (Figure 16). If normal mucosa was damaged by handling, it lost differentiated epithelial cells but kept a single layer of regenerative epithelial cells in which labeling was high. This indicated that healthy adult human bronchial epithelia, both differentiated and regenerative, could be supported by this organ culture system.

A few samples of fresh bronchial mucosa which were labeled with ^{3}HTdR and fixed at the time of explanting contained zones of epithelium sufficiently dysplastic to raise the possibility that they represented pre-invasive carcinoma (Figures 17 and 18 and State 2a, Table 2). Labeling was high (17%) and labeled cells were found in all levels of this epithelium. After maintenance on control media some initially diseased mucosal samples retained zones of pleomorphic proliferative epithelium (Figures 19 and 20), demonstrating sustained replication of morphologically abnormal cells in vitro. These properties would seem appropriate to an incipient neoplasm and have therefore become the standard upon which to assess the significance of proliferative pleomorphic cells in epithelia of experimental animal tracheo-bronchial tissues in organ culture.

Effects of AP and BaP. Although the number of treated explants was small, toxicity of BaP and AP was indicated by the occurrence of 56% and 52% devitalized explants as compared with 27 devitalized among 133 (20%) of the pooled control explants (Table 2). In the control tissues which were directly related to AP-treated tissues only 4 of 24 control explants were devitalized while 12 of 25 AP-treated explants were devitalized. Clearer assessment of the effect of AP on human bronchial mucosa requires more tests on healthy tissue. Cytotoxicity was more common in adult human tissue than in perinatal animal tissue and stimulation of replication was less common.

Discussion.

This report reviews organ culture experiments with explants from 3 orders of mammals treated with a single chemical or a complex extract from polluted air. The more detailed analyses of the exposure of each animal species to the materials described here as well as to additional compounds will be presented separately. One function of this review has been to describe and illustrate evaluation of biologic activity of materials pertinent to air pollution in a relevant target tissue. Another object is to describe the potential for comparative studies in lower animals, which can be tested in vivo as well as in vitro and in higher animals and man in which controlled in vivo work is costly

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or ethically impossible but in which in vitro studies can be done.

Comparisons across species for AP and BaP. The data presented here may be examined to determine whether they give adequate evidence that tracheobronchial structures from members of several orders of mammals can be maintained in organ culture with predictable changes in the histologic and replicative states of their epithelia with and without exposure to test materials.

Comparison of control cultures can be aided, though possibly oversimplified, by combining the percentages of occurrence of differentiated columnar epithelial states (States 1, 2, Table 1) as an example of retention of differentiation, and by combining undifferentiated and squamous metaplastic states (States 3, 4, Table 1) as example of loss of differentiation. Increased replicative activity within each grouping can be epitomized by selecting the proportions of epithelial states having 4% or more of the epithelial cells in DNA synthesis. Data from Table 1 are abstracted in this way below for control cultures of animal tissues:

Histologic States (Table 1)	Percent of Control Cultures with Epithelia in Designated Histologic States		
	Hamster	Dog*	Monkey
1 + 2	78(17)**	73(14)	78(17)
3 + 4	15(50)	27(5)	22(40)
5	7(0)	0	0

* Dog tissue: only explants for which labeling data are available are included.

** Figures in parentheses give the percent of epithelial states with labeling of 4% or greater.

All orders had comparable retention of columnar differentiation and of DNA synthetic activity in columnar epithelia. The frequency with which undifferentiated states occurred was also not greatly different but labeling was low (5%) in undifferentiated dog epithelia. Devitalized explants were noted only in hamster tracheas on clot media. However, the frequency with which epithelia from control explants were found in states 3, 4 and 5 is important in interpreting the effect of test materials.

To evaluate the comparative effect of BaP in a similar way, a simplified tabulation of data from Table 1 is presented below:

Histologic States (Table 1)	Control and BaP-Treated Cultures					
	Hamster		Dog*		Monkey	
	Control	BaP	Control	BaP	Control	BaP
1 + 2	78(17)**	44(8)	73(14)	70(24)	78(17)	36(17)
3 + 4	15(50)	48(18)	27(5)	30(40)	22(40)	38(31)
5	7(0)	7(0)	0	0	0	26(0)

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* Dog tissue: only explants for which labeling data are available are included.

** Figures in parentheses give the percent of epithelial states with labeling of 4% or greater.

The distribution of histologic states for dog epithelia was not changed but lableling rose in both differentiated and undifferentiated states, indicating either stimulation of replication or release of inhibition of replication. In contrast to the effect of BaP on dog tissue, toxicity was marked in monkey tissue, and in both hamster and monkey tissues, DNA synthesis was reduced overall. (The latter statement excludes instances of highly proliferative, pleomorphic states which will be described separately in individual analyses by species).

The effects of AP are similarly abstracted and simplified from Table 1:

	Control and AP-Treated Cultures					
Histologic States (Table 1)	Hamster		Dog*		Monkey	
	Control	AP	Control	AP	Control	AP
1 + 2	78(17)**	58(27)	73(14)	49(14)	78(17)	54(64)
3 + 4	15(50)	23(15)	27(5)	51(26)	24(40)	46(66)
5	7(0)	19(0)	0	0	0	0

* Dog tissue: only explants for which labeling data are available are included.

** Figures in parentheses give the percent of epithelial states with labeling of 4% or greater.

Toxicity for hamster trachea, reduction of the proportion of differentiated states in dog tissues and increased proportions of epithelial cells in a higher replicative category without toxicity in monkey tissues are the major effects of this material, demonstrating that BaP, present in AP, does not account alone for biologic effects of AP.

This review demonstrates that tracheo-bronchial epithelia from perinatal animals of these 3 orders were altered by BaP and AP to a degree not attributable to cultivation alone, but that all 3 orders did not respond identically to control or test conditions of maintenance in organ culture. The complexity of AP components is too great to permit a discussion of mechanisms of action but the characteristics and metabolism of BaP are better known and allow at least preliminary discussion of the different responses of the 3 animal orders.

One trend in the response to BaP correlates with maturity of the animals whose tissues were explanted. The most mature were human, monkey and hamster and in these there was suppression of DNA synthesis or devitalization. Toxicity of BaP depends on intracellular enzymatic conversion to biologically active derivatives. The aryl hydroxylases responsible for this conversion are

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present in free-living animals and can be induced only at a fairly advanced
state of embryonic development or postnatally.

The microsomal enzyme system of which these hydroxylases are a ...
complex. Enzyme activity may be induced to varying degrees by many natural
and artificial substrates and enzyme action requires a number of co-factors.
The products of metabolism of any one substrate, as BaP, may vary from tissue
to tissue and the biologic activity of only a few products (as epoxides, mono-
hydroxy derivatives) has been proven or postulated. Some of the biologic
effects of BaP, as toxicity and carcinogenicity, may depend on the degree and
specificity of the BaP-hydroxylating activity which can be induced in this
group of microsomal enzymes, on the presence of cofactors or inhibitors and on
the enzymatic products formed. The greater toxicity of BaP for monkey than
for dog tracheo-bronchial epithelia may therefore reflect a genetic difference
between orders of mammals or the acquisition by the more mature animal of
more readily inducible microsomal enzyme systems which may, in turn, produce
BaP metabolites which are toxic in organ culture. The data from adult human
respiratory mucosal explants may contribute to either of the latter inter-
pretations, namely, that the toxicity noted in fetal primate epithelium is even
more evident in the adult (human) primate or that primates share a response
to BaP which is distinct from the response of canines.

Significance of Pathologic States of Respiratory Epithelia. Epithelia
composed of 3 or more layers of pleomorphic cells appeared occasionally in
association with increased labeling. Pleomorphic cells are predominant in
dysplastic, presumptively preneoplastic lesions of the human bronchus. The
presumptive human preneoplastic lesions have high proportions of labeled cells
both in the fresh state (Figures 17, 18) and after cultivation (Figures 19, 20).
Thus, when high labeling is present in a cellular pleomorphic lesion of an
animal respiratory epithelium in organ culture, the effect is interpreted as
morphologic transformation producing abnormal cells with the potential for
autonomous growth. This state could be regenerative, as in repair of injury
in vivo, but this is not likely in organ culture where injury usually leaves
a single layer of cells. For this reason such an epithelial state in an
organ culture is regarded here as potentially preneoplastic; the agent producing
it is regarded as potentially carcinogenic.

When pleomorphism plus low labeling is produced in treated organ cultures,
this state is admitted to represent morphologic alteration of epithelial cells
but since it is associated with suppression of DNA synthesis this state is
regarded as evidence of toxicity. The appearance and organization of cells in
an epithelium may not be adequate to define the neoplastic potential of a lesion
unless other indices, as replication, are available as well. In all attempts
to ascribe neoplastic potential to non-invasive lesions, the validity of the
interpretation depends ultimately on showing that similar lesions invade. This
finding has not yet been made for lesions produced in organ culture and inferences
as to the significance of epithelial lesions are understood to be subject to
this ultimate test.

The effects of BaP and AP were examined in this report under the assumption
that they would produce lesions compatible with the carcinogenic activity each
material has been shown to have under selected conditions of animal exposure.
Both BaP and AP have toxic as well as carcinogenic effects in animals, however,
hence their effects on respiratory tissue in organ culture are compatible with
their ranges of biologic activity in vivo.

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TABLE 1

COMPARATIVE EFFECTS OF BENZO(A)PYRENE AND AN AIR POLLUTION EXTRACT ON HAMSTER, DOG AND MONKEY TRACHEOBRONCHIAL EPITHELIUM IN ORGAN CULTURE

Histologic States of Epithelium	Control Acetone and DMSO			Benzo(a)pyrene 10-20 μg/ml			Whole benzene-soluble extract (AP)		
	Hamster	Dog	Monkey	Hamster	Dog	Monkey	Hamster	Dog	Monkey
1. Simple to pseudostratified differentiated columnar		8*			2*			4*	
0-0.9**	43	21	7	25	15	4	12	13	1
1.0-3.9	37	13	8	2	19	2	6	11	2
4.0-	16	2	3	0	6	1	2	3	3
Subtotal states	96	44	18	27	42	7	20	31	6
Percent of total states	74%	52%	78%	33%	36%	21%	35%	34.5%	23%
2. As above, with basal hyperplasia		5			6			5	
0-0.9	1	2	0	5	8	2	3	3	0
1.0-3.9	3	6	0	1	14	2	3	3	2
4.0-	1	5	0	3	12	1	7	2	6
Subtotal states	5	18	0	9	40	5	13	13	8
Percent of total states	4%	21%	0%	11%	34%	15%	23%	14.5%	31%
3. Pleomorphic undifferentiated		4			5			7	
0-0.9	6	10	2	26	10	5	10	12	2
1.0-3.9	4	8	1	7	5	4	1	11	2
4.0-	9	1	2	5	12	4	1	7	8
Subtotal states	19	23	5	38	32	13	12	37	12
Percent of total states	14.5%	27%	22%	46%	27%	38%			

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TABLE 1 (continued)

COMPARATIVE EFFECTS OF BENZO(A)PYRENE AND AIR POLLUTION EXTRACTS ON HAMSTER, DOG AND MONKEY TRACHEOBRONCHIAL EPITHELIUM IN ORGAN CULTURE

	[illegible]			Benzo(a)pyrene			Whole benzene-soluble extract (AP)		
	Acetone and DMSO			10-20 μg/ml					
Histologic States of Epithelium	Hamster	Dog	Monkey	Hamster	Dog	Monkey	Hamster	Dog	Monkey
4. Squamous metaplasia		0			2			5	
0-0.9	0	0	0	0	0	0	0	1	0
1.0-3.9	0	0	0	0	1	0	0	1	0
4.0-	1	0	0	2	0	0	1	2	0
Subtotal states	1	0	0	2	3	0	1	9	0
Percent of total states	1%	0%	0%	2%	3%	0%	2%	10%	0%
5. Devitalized	10	0	0	6	0	9	11	0	0
Percent of total states	7.5%	0	0%	7%	0%	26%	19%	0%	0%
Total number of States	131	85	23	82	117	34	57	90	26
Total number of Explants	124	39	15	57	43	25	49	38	13

*Not exposed to ^{3}HTdR.

**Percent of epithelial cells labeled after 30 minutes incubation with ^{3}HTdR before fixation.

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LEGENDS FOR FIGURES

Figures 1 - 10 are photomicrographs of suckling hamster trachea, Figures 11 - 14 are photomicrographs of fetal <u>M. cynomalgus</u> and Figures 15 - 20 are photomicrographs of adult human respiratory mucosa. Figures 1, 5, 7, 9 and 11 - 16 are of sections stained with colloidal iron-hematoxylin. Acid mucopolysaccharide in cartilage matrix and in goblet cells appear grey to black. Figures 2 - 4, 6, 8, 10 are autoradiographs that were stained by the Periodic Acid - Schiff method, dipped in NTB2 photographic emulsion and counterstained with hematoxylin after development. Figures 17 - 20 are autoradiographs which were dipped in NTB2 photographic emulsion and stained after development with hematoxylin - eosin. These photomicrographs illustrate the various histologic states referred to in Tables 1 and 2.

<u>Suckling hamster tracheas.</u>

Figure 1. Trachea at time of explanting. The pseudostratified columnar epithelium (State 1, Table 1, and State 1a, Table 2) is well differentiated with cilia and goblet cells. Note intense mucopolysaccharide staining in cartilage and cellularity of connective tissue. X400.

Figure 2. As for Figure 1. The labeling rate in this epithelium is 4.5%. X1000.

Figure 3. Acetone control, 11 day culture, liquid medium. This columnar epithelium (State 1, Table 1, and State 1a, Table 2) is well differentiated. Its appearance is much the same as it is at the time of explanting. X400.

Figure 4. As for Figure 3. The labeling rate of the epithelium has declined with time in culture and is now 2.9%. X1000.

Figure 5. Air pollution extract 11 day culture, liquid medium. This highly cellular pseudostratified columnar epithelium exhibits basal cell hyperplasia (State 2, Table 1, and State 1c, Table 2). There is a reduction of cartilage cellularity and mucopolysaccharide staining intensity. X400.

Figure 6. As for Figure 5. The irregularity of basal cell size and shape, and the increase in number is more clearly seen. The labeling rate is 2.6% in this epithelium. X1000.

Figure 7. Air pollution extract 15 day culture, liquid medium. In this undifferentiated metaplastic epithelium the cells are irregular in size, shape, orientation and staining intensity. This epithelial state is termed pleomorphic undifferentiated (State 3, Table 1, and State 3a, Table 2). There is also some vacuolization of cytoplasm and irregularity in nucleolar staining. X400.

Figure 8. As for Figure 7. The pleomorphism of the cells is more apparent. The labeling rate is 2.2% in this epithelium. X1000.

Figure 9. Air pollution extract, 8 day culture, clotted medium. In this squamous metaplastic epithelium (State 4, Table 1, and State 2b, Table 2) the basal cells are vertically oriented, with flattening of the cells occurring as the luminal surface is approached. There is irregularity of cell size, shape, and orientation, and an indication of intracellular bridges. X400.

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Figure 10. As for Figure 9. The orientation of cells is more apparent, and variation in staining intensity of nuclei is seen. The labeling rate is 7.0%. X1000.

Fetal Monkey Trachea.

Figure 11. Trachea at the time of explanting. The columnar epithelium is not well differentiated, perhaps because of the age of the fetus, but note intense mucopolysaccharide staining in cartilage and cellularity of connective tissues. X400.

Fetal Monkey Bronchi, Clotted Medium

Figure 12. Dimethylsulfoxide (DMSO) control, 8 day culture. This pseudostratified columnar epithelium has ciliate as well as goblet cell differentiation. X400.

Figure 13. Benzo(a)pyrene (BaP), 15 ug/ml, in acetone, 8 day culture. In this undifferentiated epithelium the cells are irregular in size, shape, orientation and staining intensity. In the lower right corner is a portion of cartilage in which there is loss of cellularity as well as mucopolysaccharide staining. X400.

Figure 14. Air pollution extract in DMSO, 8 day culture. This undifferentiated epithelium is similar to that produced by BaP (Figure 13). Note the destruction of connective tissue. X400.

Adult human bronchial mucosa.

Figure 15. Normal respiratory mucosa at the time of explanting. This columnar epithelium (State 1, Table 1, and State 1a, Table 2) has a high proportion of goblet cells in which mucous inclusions appear black. The labeling rate is 0.6% and labeled cells are present only in the basal cell layer. X400.

Figure 16. Normal respiratory mucosa cultured for 15 days on control clot medium. This columnar epithelium demonstrates preservation of the state of differentiation observed in Figure 15. The labeling rate was reduced to 0.0%. X400.

Figures 17 and 18. Abnormal respiratory mucosa at time of explanting. In the right portion of Figure 13 (X170), differentiated columnar epithelium is present with a labeling rate of 1.6%. In the left portion and in Figure 18 (X400) a dysplastic epithelium, possibly carcinoma in situ (State 2a, Table 2), has a labeling rate of 17.1% with labeled cells present at all levels in the epithelium.

Figures 19 and 20. Abnormal respiratory mucosa cultured for 11 days on clot medium with BaP or AP. In Figure 19 columnar epithelium with basal cell hyperplasia on the left (labeling rate 10.5%) joins a cellular dysplastic zone regarded as possible carcinoma in situ (State 2a, Table 2) in the right half of the figure. X160. In Figure 20, pleomorphic cells are seen to make up this dysplastic lesion, and DNA synthesis is occurring in 26.6% of cells at all levels in the epithelium. X400.

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TABLE 2

EFFECTS OF BaP AND AIR POLLUTION EXTRACTS
ON HUMAN BRONCHIAL MUCOSA IN ORGAN CULTURE

Histologic States of Epithelium	Pooled Control Data	BaP	AP	Controls Specific to AP Experiments
1. Pseudostratified differentiated columnar				
a. well preserved	22	1	1	3
b. technically not well preserved	26	2	1	1
c. with basal hyperplasia	15	2	2	1
Subtotal	63	5	4	5
Percent of total	46%	14%	14%	21%
2. Initial state abnormal				
a. localized dysplasia. Possible carcinoma *in situ*	6	0	0	0
b. squamous metaplasia	3	0	1	1
Subtotal	9	0	1	1
Percent of total	7%	0%	4%	4%
3. Initial state unknown				
a. now stratified undifferentiated, with or without squamous changes	21	5	9	10
b now regenerative	17	6	2	4
c. now devitalized	27(20)*	20(56)*	12(52)*	4(17)*
Subtotal	65	31	23	18
Percent of total	47%	86%	82%	75%
Total number of States	137	36	28	24
Total number of Explants	133	32	25	

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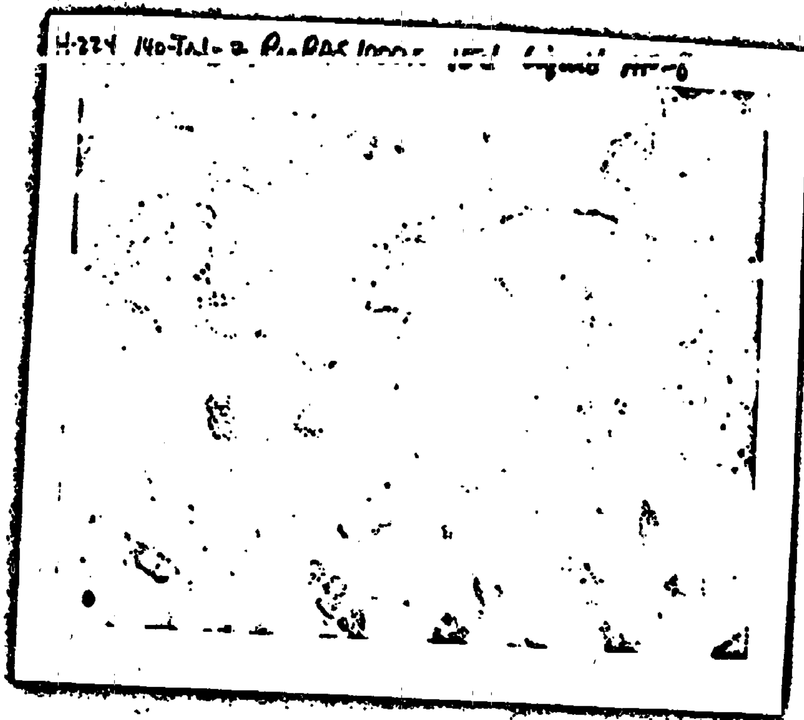
*Figures in parentheses indicate the percent of devitalized epithelia relative to the total number of epithelial states.



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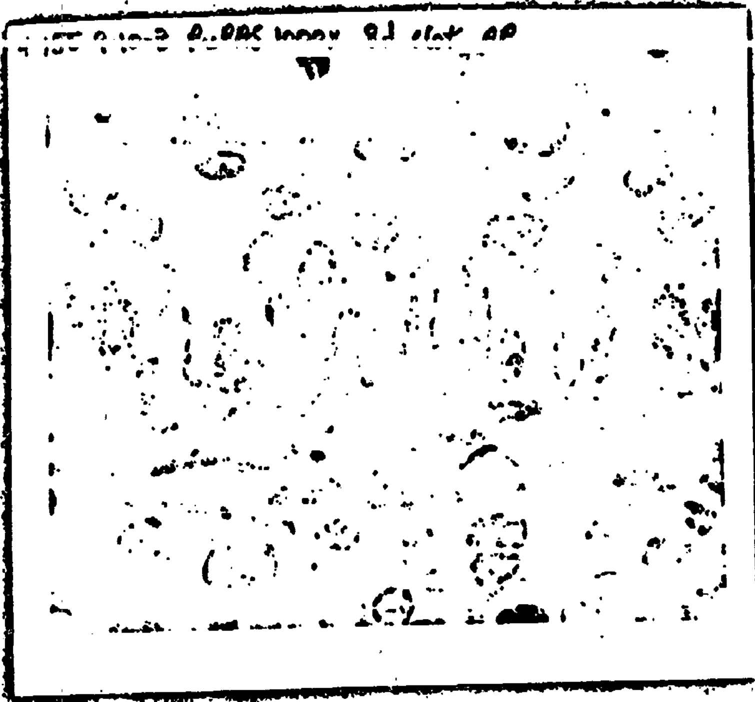

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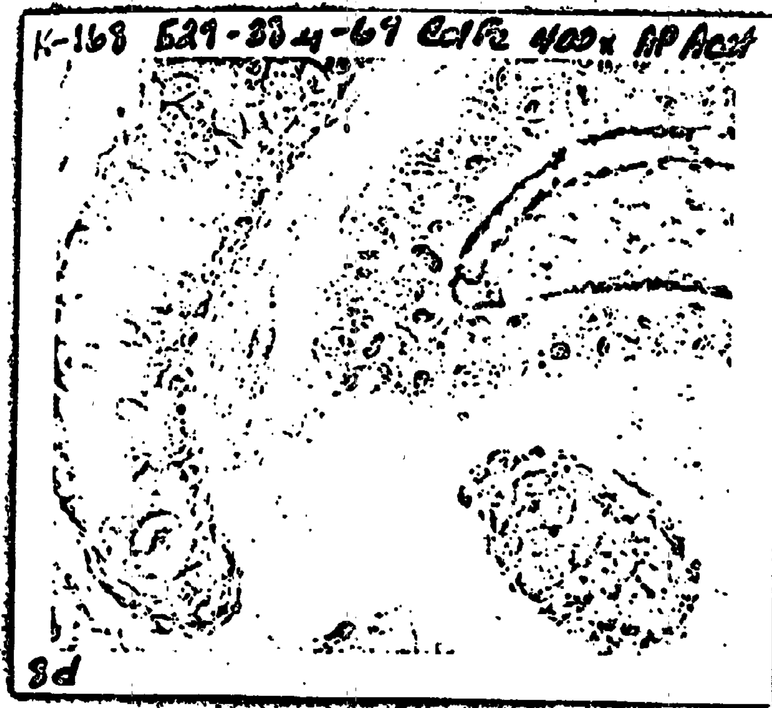

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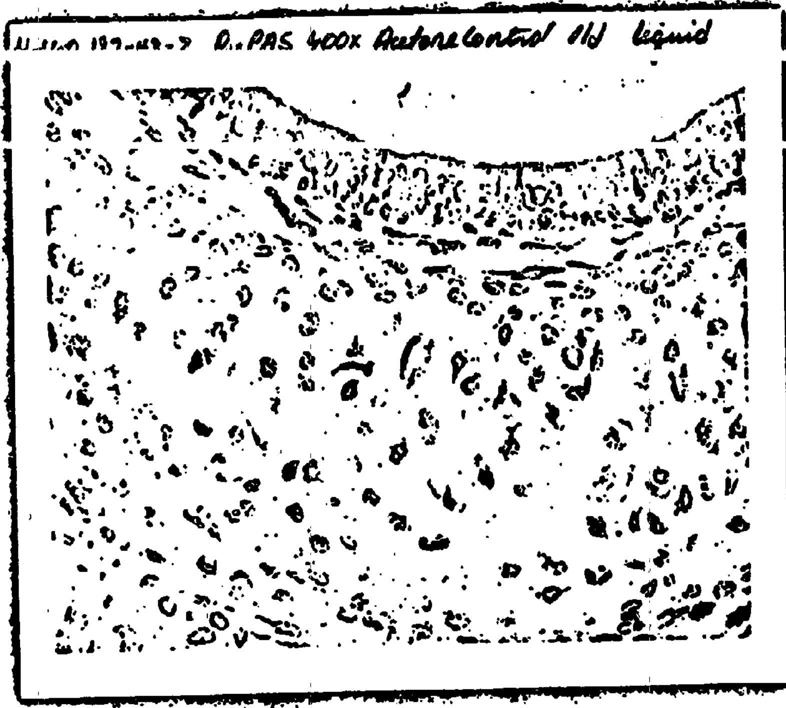

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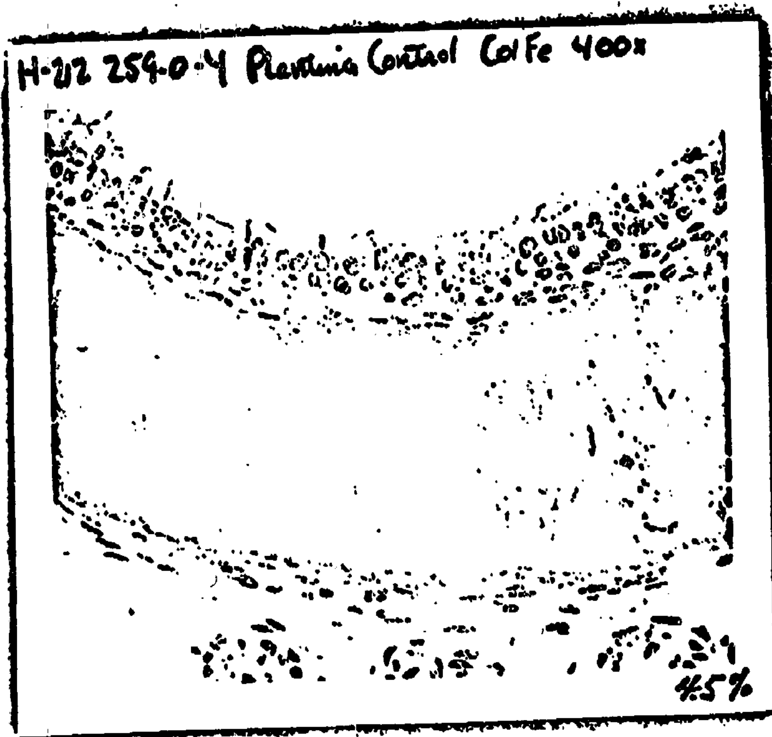

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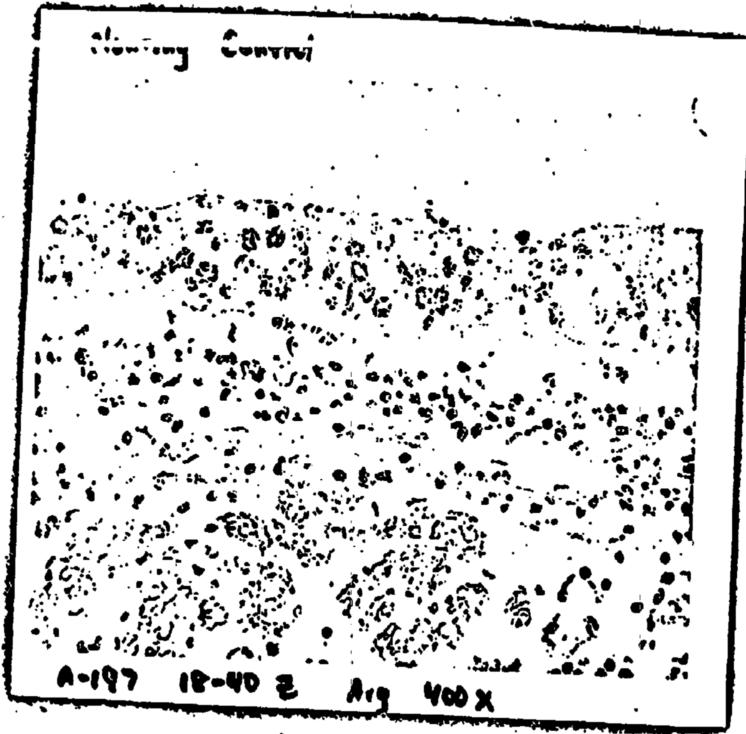

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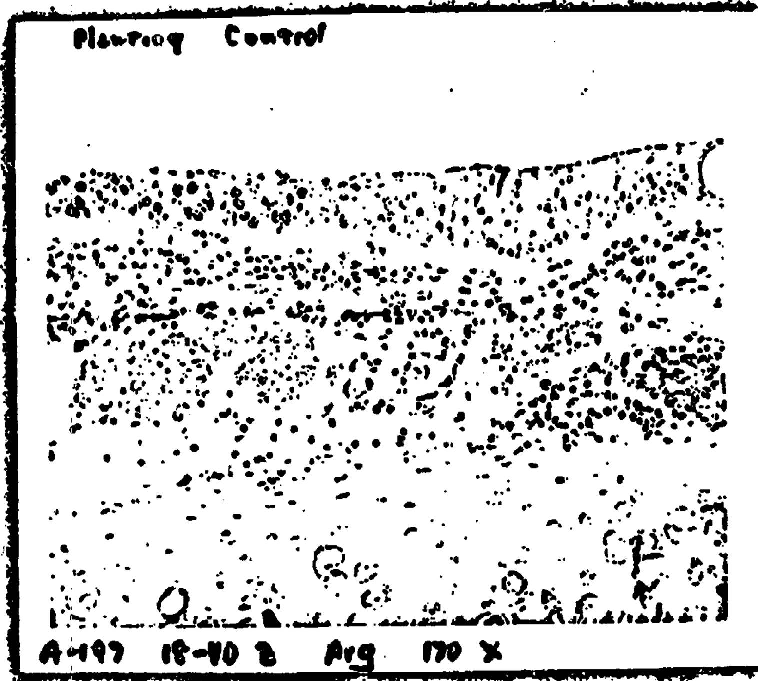

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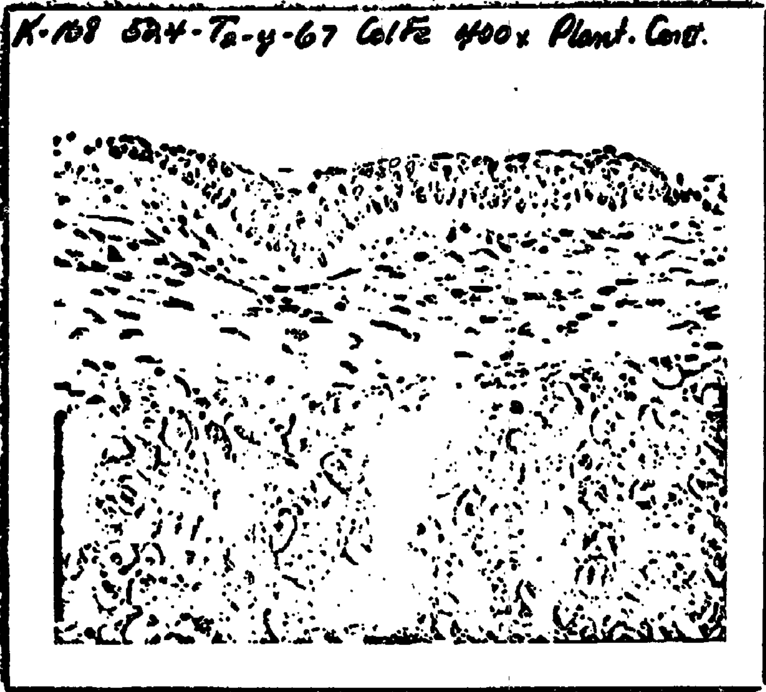

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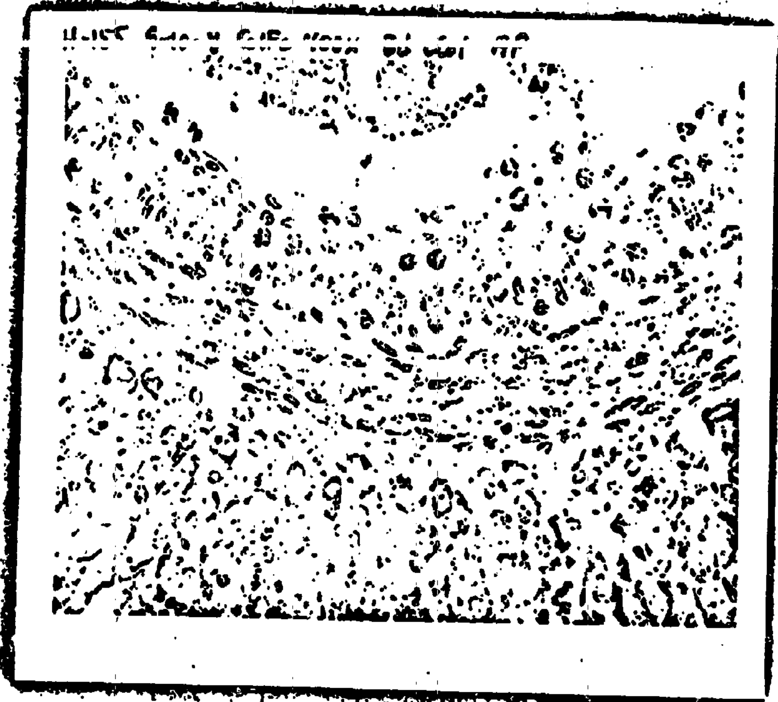

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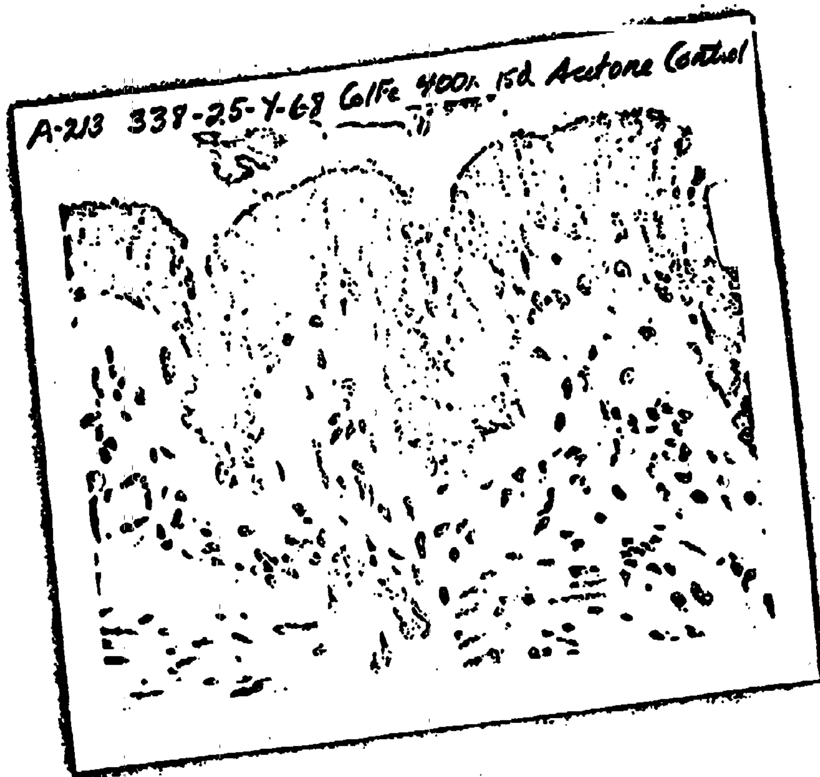

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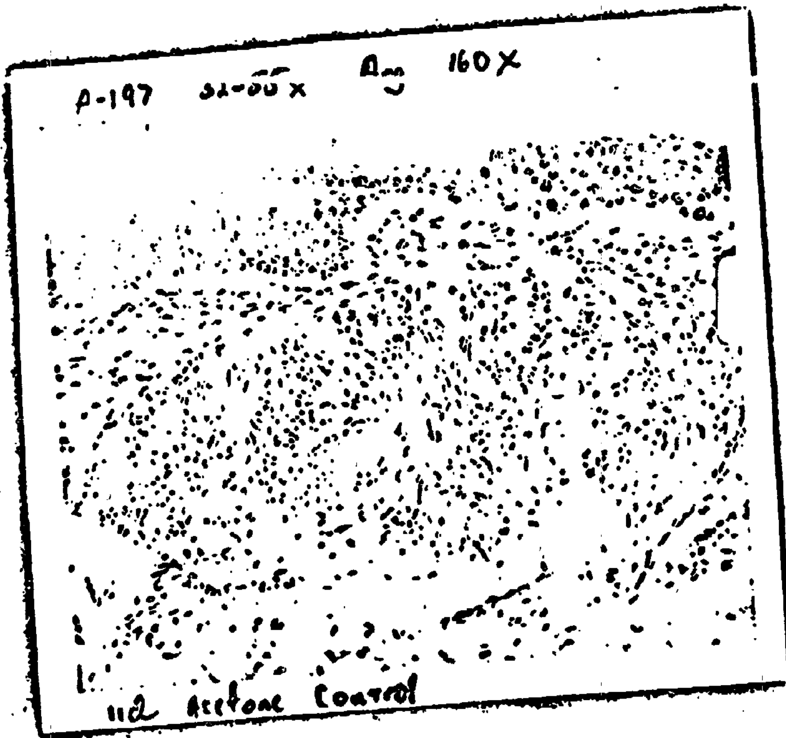

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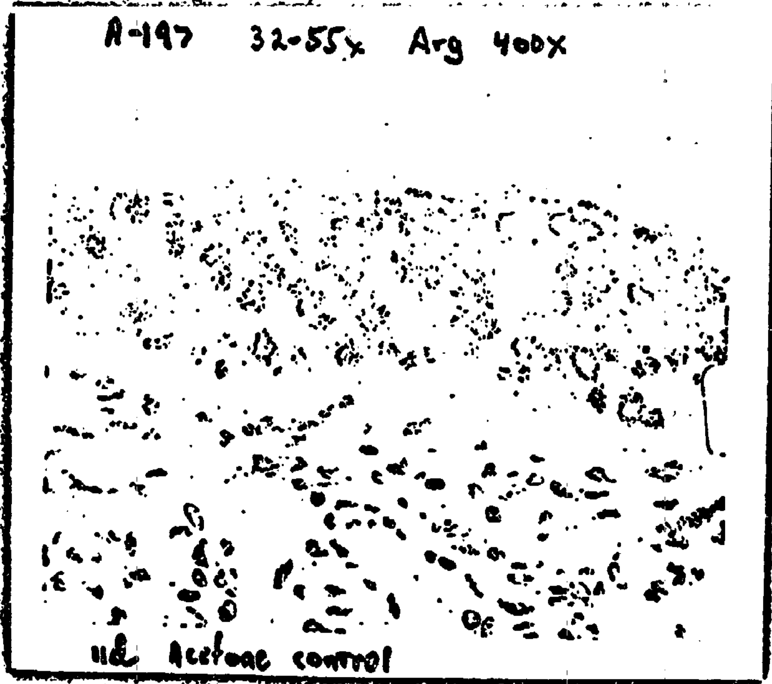

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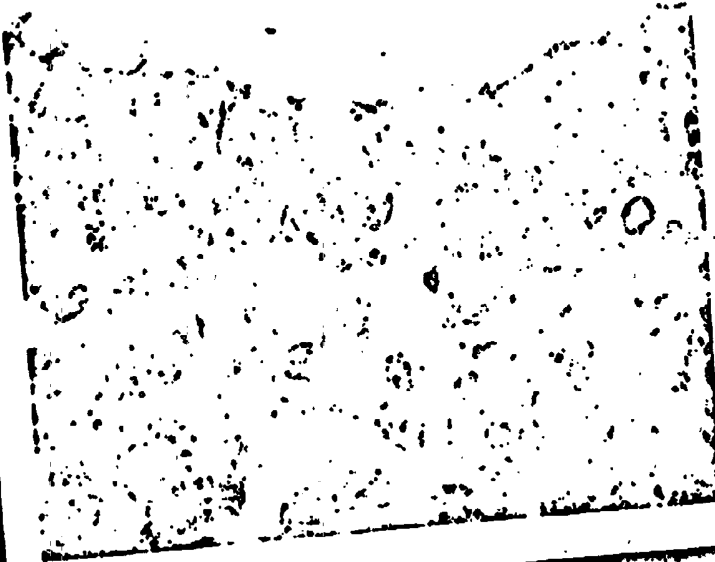

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