Incorporation and Rate of Loss of S^{35}-Methionine by Adult Rat Trachea in Organ Cultures and in Living Animals*

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ABSTRACT

Full-thickness pieces of adult rat trachea were supported on rayon on the surface of clotted medium in watch glasses. Differentiated epithelium was reduced in height during 25 days of cultivation because basal cells and some columnar cells migrated to cover exposed parts of the explants and because some differentiated cells died and were shed.

S^{35}-methionine was (*a*) placed on explants *in vitro* and (*b*) injected intraperitoneally in living rats. Cultured tissues and tissues of living rats were examined by autoradiography at 4 and 24 hours and 4, 7, and 11 days after labeling.

Although migratory undifferentiated epithelial cells appeared in cultured trachea, all living epithelial cells *in vitro* incorporated and subsequently lost S^{35}-methionine to the same extent as did epithelium of intact rats. The biologic half-life of methionine in rat tracheal epithelium *in vivo* and *in vitro* was about 5 days.

Hyperplasia of basal cells and squamous metaplasia of superficial cells of the human bronchus have been correlated with concurrent bronchiogenic carcinoma and a history of heavy cigarette smoking (1). In order to study the metabolic features accompanying hyperplasia and metaplasia and to induce these states experimentally in adult human tissue, an *in vitro* method is needed which retains the normal histologic pattern and functional activity of adult bronchial epithelium.

Mucous metaplasia of chick embryo skin grown in organ culture (3) was induced by treatment with excess vitamin A by Fell and Mellanby (2) and the incorporation of amino acids, sulphate, and nucleic acid precursors was studied by Fell and Pelc (4) employing autoradiographic methods. Hyperplasia and squamous metaplasia in prostatic epithelium of young adult mice has been produced with methylcholanthrene by Lasnitzki (7), and changes in DNA synthesis have been explored by autoradiography in this system (8). Lasnitzki has also demonstrated hyperplasia of bronchial epithelium in human fetal lung treated with 3,4-benzpyrene (9). The value of the organ culture method is evident, therefore, as applied to tissues so far studied. Moreover, the culture technique has been combined with application of carcinogens and with physiologic studies using isotopes and autoradiography. Methods for culture of *adult* tracheobronchial tissue, however, had not previously been explored.

This communication describes experiments designed to answer two questions:

(*a*) Do adult tracheobronchial tissues retain normal histologic features in organ culture of this type, and

(*b*) What functional similarity exists between the tissue maintained *in vitro* and the same tissue in the intact animal?

Rat trachea was studied because it is similar in diameter to the human bronchi of small to medium size which can be dissected free for use in projected experiments employing cultures of human tissue;

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animals were used to permit experiments in which high doses of radioisotopes could be given for parallel autoradiographic studies of epithelium in living animals and in cultured tissues.

Materials and Methods

Media for Cultivation.—Tissues were planted in watch glass cultures (3) with or without a rayon mesh (13). The nutrient consisted of equal parts of cock plasma and chick embryo extract from 11- to 13-day embryos. The fluid used for washing and diluting the embryo mince, designated hereafter as "diluting fluid," consisted of 70 per cent Gey's (5) balanced salt solution containing 200 units of penicillin, 200 μg. of streptomycin, and 0.05 per cent phenol red; 10 per cent of the amino acid and vitamin preparation described by Puck *et al.*, (12); and 20 per cent human serum (previously tested for non-toxicity to HeLa cells). One volume of minced embryo was diluted with 2 volumes of diluting fluid. This medium produced a "soft clot" within 2 minutes and had a pH of 7.6 to 8.0.

Preparation of Tissues for Planting.—Male Wistar rats, varying in weight from 130 to 160 gm., were used. For removal of tissue, each rat was anesthetized with sodium amytal and exsanguinated by cutting the abdominal aorta. Its trachea was exposed bloodlessly within one or two minutes of cessation of breathing, dissected free from adjacent organs, and removed from the thyroid cartilage to the main stem bronchi. The trachea was then cut with a scalpel blade into three longitudinal plates of tissue, each of which was then cut in 1.5 to 2 mm. lengths, yielding explants weighing about 2 mg. The pieces were transferred, mucosal side up, to small squares of rayon and laid, three to a watch glass, on the surface of clotted medium. Explantation to cultures was completed at room temperature within 1 to 2 hours of the animal's death.

Cultures were incubated at 36.5 to 37.5°C. and pieces were transferred to new media every 2 or 3 days. In experiments to test other methods of explantation and cultivation, rings or short tubular segments of the trachea were laid directly on the clot or on rayon.

In vivo and in vitro Application of Radioisotopes.—Protein metabolism is a reasonable indicator of the state of living tissue and for this reasons labeling with S^{35}-methionine was undertaken. Experiments were designed to compare the initial incorporation and the subsequent loss of methionine (*a*) in tracheal explants labeled in culture (C series), and (*b*) in tissues of the living animal labeled *in vivo* (V series).

One rat trachea was planted as the C series of organ cultures. One day later each explant received one drop of diluting fluid containing S^{35}-DL-methionine in a concentration of 200 μc./ml. After 30 seconds the fluid around each explant was pipetted off and the cultures were returned to the incubator. Three explants each were fixed at intervals of 4 and 24 hours, and at the latter time all remaining explants were washed and subcultured in the usual way. Three explants each were fixed at 4 and at 7 days after labeling.

At approximately the same time as the C series of cultures were labeled, five rats received 1 or 2 μc./gm. of S^{35}-DL-methionine and were killed at 4 and 24 hours, and at 4, 7, and 11 days after labeling.

Cultured tissues were fixed in acetic acid–alcohol (30:70) for 15 minutes, followed by formol saline for 45 minutes. Fresh animal tissues which had not been cultured were fixed for 1 hour in acetic acid–alcohol followed by formol saline for 24 hours. Tissues were embedded in paraffin, sectioned serially at 5 μ, and autoradiographs prepared by the stripping film technique (11) and stained with hematoxylin and eosin. The remaining slides were stained with hematoxylin and eosin or by the periodic acid–Schiff (PAS) method. In all, 27 tracheal pieces labeled in culture and 13 rats were used in the methionine studies. Six tracheas, yielding 40 explants of various types, were studied in relation to response of this adult tissue to cultivation in organ culture by the watch glass method.

RESULTS

Histology and Cytology.—The trachea of the rat is lined by a pseudostratified epithelium consisting of closely packed columnar cells, about one-half of which are ciliated and one-half goblet cells. On the basement membrane is a population of reserve or basal cells (Fig. 1 *a*). Glandular elements are sparse. During the first 4 to 6 days of cultivation, mucus is discharged from the surface of explants, and during the first 2 or 3 days this material is moved to one end of the explant, indicating oriented ciliary beating. Disorganized beating may be detected by movement of carbon particles up to the fifth day.

When the trachea is cut to provide flattened plates for explantation, the epithelium stretches, and at the cut edges is reduced to a single layer of columnar or cuboidal cells. After 2 days of cultivation most of the epithelium loses the original pseudostratified aspect and becomes a simple columnar structure overlying basal cells (Fig. 2 *b*). By 8 days the columnar cells become cuboidal (Fig. 3 *a*), but occasional patches of columnar cells are retained for as long as 25 days. Cilia are not well preserved by the fixative but appear to be retained by columnar cells and by some cuboidal cells. Goblet cells remain PAS-positive and persist in the original proportions but become flatter and have progressively less mucoid material. Death and sloughing of columnar cells occurs in variable degree during

the 1st week of culture and accounts for some thinning of the epithelium.

Migration of epithelial cells appears by 24 hours; cuboidal or flattened cells extending around the edge of a 48-hour explant are shown in Fig. 2 *a*. By 4 days all the exterior of the explant which is not attached to rayon has been covered by a thin layer of flattened cells. Among these migrating cells former goblet cells filled with PAS-positive granules are present in 4-day cultures, but by 5 days none of the migrating cells contain mucoid material. By 9 days the exterior of the explant is covered by a double layer of cells, which often extend richly into the rayon mesh. Practically all migrating cells have nuclei which resemble those of basal cells of the original epithelium.

The mitotic rate of normal tracheal epithelium of the rat is very low (6). After the first 2 days of cultivation, mitoses appear in appreciable number among the migrating cells and among basal cells near the point from which migration is proceeding.

Distribution of S^{35}-Methionine in Epithelium.—Peak incorporation of methionine in intact rats was observed 4 hours after labeling. S^{35}-methionine was uniformly distributed throughout the entire height of the epithelium and was also present to some degree in the scant mucus layer on the surface. A continuous decline in density of tracer was noted after 4 hours (Table I and Text-fig. 1). The appearances of autoradiographs at 4 hours and 11 days after administration of S^{35}-methionine are shown in Figs. 1 *b* and 1 *c*.

Distribution of methionine in cultured material resembled that in the intact rat. Tissues labeled in culture incorporated more tracer in 24 hours than in 4 hours, presumably because labeled material remained available for longer than in the intact rat. When tissues were washed and placed on fresh, unlabeled medium, loss occurred at a rate similar to that observed in the epithelium of intact rats (Table I and Text-fig. 1). The biological half-life of S^{35}-methionine was about 5 days from the peak values in each series of tissue. The autoradiographs at 24 hours and at 7 days after labeling of cultures with S^{35}-methionine are shown in Figs. 2 *c* and 3 *b*.

Migrating epithelial cells retained label in the same way as cells remaining in their original location on the explant.

Distribution of S^{35}-Methionine in Non-Epithelial Tissues.—In both living animals and tissues labeled in culture, radioactive material is found in the cellular zones of cartilage. Labeling is most intense where new cartilage is being formed and there is prolonged retention with slow diffusion of tracer from the cellular areas into the surrounding matrix accompanied by a slow decline in labeling over the entire mass of cartilage. Connective tissues are sparsely labeled compared to epithelium, though the expanded cytoplasm of fibroblasts usually contains some radioactive material.

TABLE I

Mean Grains per Standard Field Counted over Epithelium of Rat Tracheas Labeled in Vivo or as Organ Cultures with S^{35}-Methionine

Time after adding label*	Mean grains/Standard field	
	Animals	Cultures
4 hrs.	80 ± 14 (100 per cent)‡	131 ± 22 (51 per cent)‡
24 hrs.	69 ± 23 (86 per cent)	256 ± 23 (100 per cent)
4 days	42 ± 10 (52 per cent)	155 ± 27 (61 per cent)
7 days	32 ± 5 (40 per cent)	100 ± 17 (39 per cent)
11 days	15 ± 5 (18 per cent)	

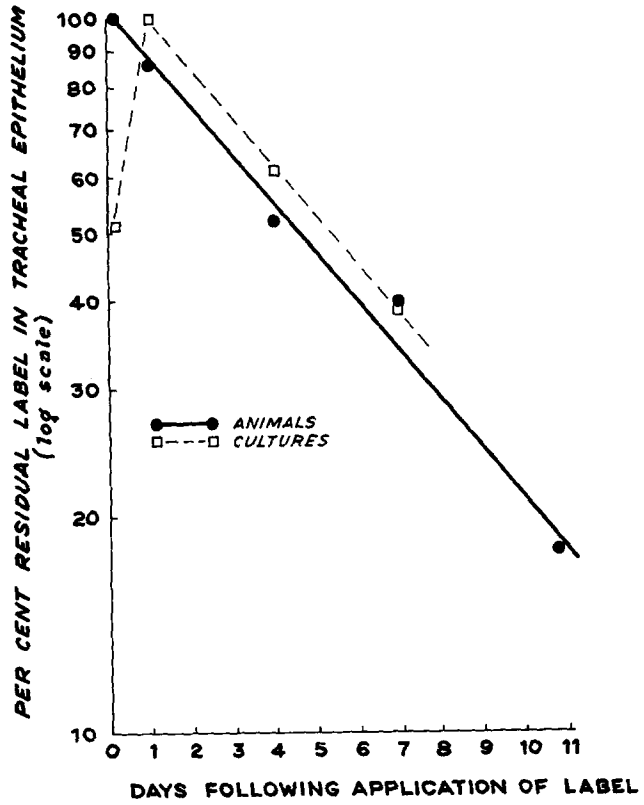
* The times refer to (*a*) the period between intraperitoneal injection of S^{35}-methionine in living animals and fixation of animal tissues, and to (*b*) the period between application of label on 1-day old cultures and fixation of cultured tissue, except that cultures exposed to label for 24 hours but not fixed were washed and subcultured on label-free media prior to fixation at 4 and 7 days.

‡ Values in parentheses express the mean grain count as a percentage of the highest count reached in each series. The standard errors were computed from the variance of counts of autoradiographs.

DISCUSSION

In full thickness explants from adult rats the epithelium progressively loses its original differentiated structure in organ cultures of the watch glass type. The original epithelium thins out because (*a*) basal cells, and some columnar cells, migrate and proliferate to cover the exposed surfaces of the explant, and (*b*) some differentiated cells die.

The watch glass culture seems to promote an epithelial reaction resembling that stage in early



TEXT-FIG. 1. Graphic presentation of data from Table I, demonstrating decline in numbers of grains per standard area counted in autoradiographs over tracheal epithelium in living rats (●——●) and in organ culture (□-----□) at intervals after exposure to a single administration of S^{35}-methionine. Actual grain counts were transformed to per cent values, giving the highest counts in each series the value 100.

wound healing during which migration and proliferation predominate (14). This stage in wound healing has been correlated by Needham (10) with low oxygen tension, products of cell breakdown, and a predominantly glycolytic metabolism. All these conditions may exist within relatively large explants in watch glass cultures which are not given added oxygen and which contain embryo extract.

There is no difference in protein metabolism, as measured by methionine incorporation and turnover, between tracheal tissues *in situ* and in culture. Further, methionine incorporation and retention *in vitro* were similar in non-migrating and migrating (presumably less differentiated) epithelial cells. *In vivo* also, no difference was found between the differentiated (columnar) and less differentiated (basal) cells of the epithelium. Protein metabolism, as shown by methionine utilization, therefore, does not reflect the state of differentiation of the epithelial cells in these experiments. If the present cultures produced no more extreme "de-differentiation" than occurs normally in wound healing, then methionine utilization would seem to be constant over the range of differentiation expected under presumed physiologic circumstances. Methionine apparently enters relatively stable cellular components since the half-life of approximately 5 days is too long to suggest incorporation in secretions.

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REFERENCES

1. Auerbach, O., Gere, J. B., Forman, J. B., Petrick, T. G., Smolin, H. J., Muehsam, G. E., Kassouny,

D. Y., and Stout, A. P., Changes in the bronchial epithelium in relation to smoking and cancer of the lung: a report of progress, *New England J. Med.*, 1957, **256,** 97.

2. Fell, H. B., and Mellanby, E., Metaplasia produced in cultures of chick ectoderm by high vitamin A, *J. Physiol.*, 1953, **119,** 470.
3. Fell, H. B., and Robison, R., Growth, development and phosphatase activity of embryonic avian femora and limb-buds cultivated *in vitro*, *Biochem. J.*, 1929, **23,** 767.
4. Fell, H. B., and Pelc, S. R., The effect of excess vitamin A on the uptake of labeled compounds by embryonic skin in tissue culture, *Exp. Cell Research*, in press.
5. Gey, G. O., *in* Methods of Tissue Culture, (R. C. Parker, editor), New York, Paul B. Hoeber, Inc., 2nd edition, 1950, 81.
6. Leblond, C. P., and Walker, B. E., Renewal of cell populations, *Physiol. Rev.*, 1956, **36,** 255.
7. Lasnitzski, I., Influence of hypervitaminosis on effect of 20-methylcholanthrene on mouse prostate glands grown *in vitro*, *Brit. J. Cancer*, 1955, **9,** 434.
8. Lasnitzki, I., and Pelc, S. R., Effect of 20-methylcholanthrene on DNA synthesis in mouse prostates grown *in vitro*, *Exp. Cell Research*, 1957, **13,** 140.
9. Lasnitzki, I., Discussion, Proceedings Decennial Review Conference on Tissue Culture, Woodstock, Vermont, September 1957, *J. Nat. Cancer Inst.*, 1957, **19,** 777.
10. Needham, A. E., Regeneration and Wound Healing, Methuen's Monographs on Biological Subjects, London, Methuen and Co., Ltd., 1952.
11. Pelc, S. R., The stripping-film technique of autoradiography, *Internat. J. Appl. Rad. and Isotopes*, 1956, **1,** 172.
12. Puck, T. T., Cieciura, S. J., and Fisher, H. W., Clonal growth *in vitro* of human cells with fibroblastic morphology. Comparison of growth and genetic characteristics of single epithelioid and fibroblast-like cells from a variety of human organs, *J. Exp. Med.*, 1957, **106,** 145.
13. Shaffer, B. M., The culture of organs from the embryonic chick on cellulose-acetate fabric, *Exp. Cell Research*, 1956, **11,** 244.
14. Wilhelm, D. L., Regeneration of tracheal epithelium, *J. Path. and Bact.*, 1953, **65,** 543.

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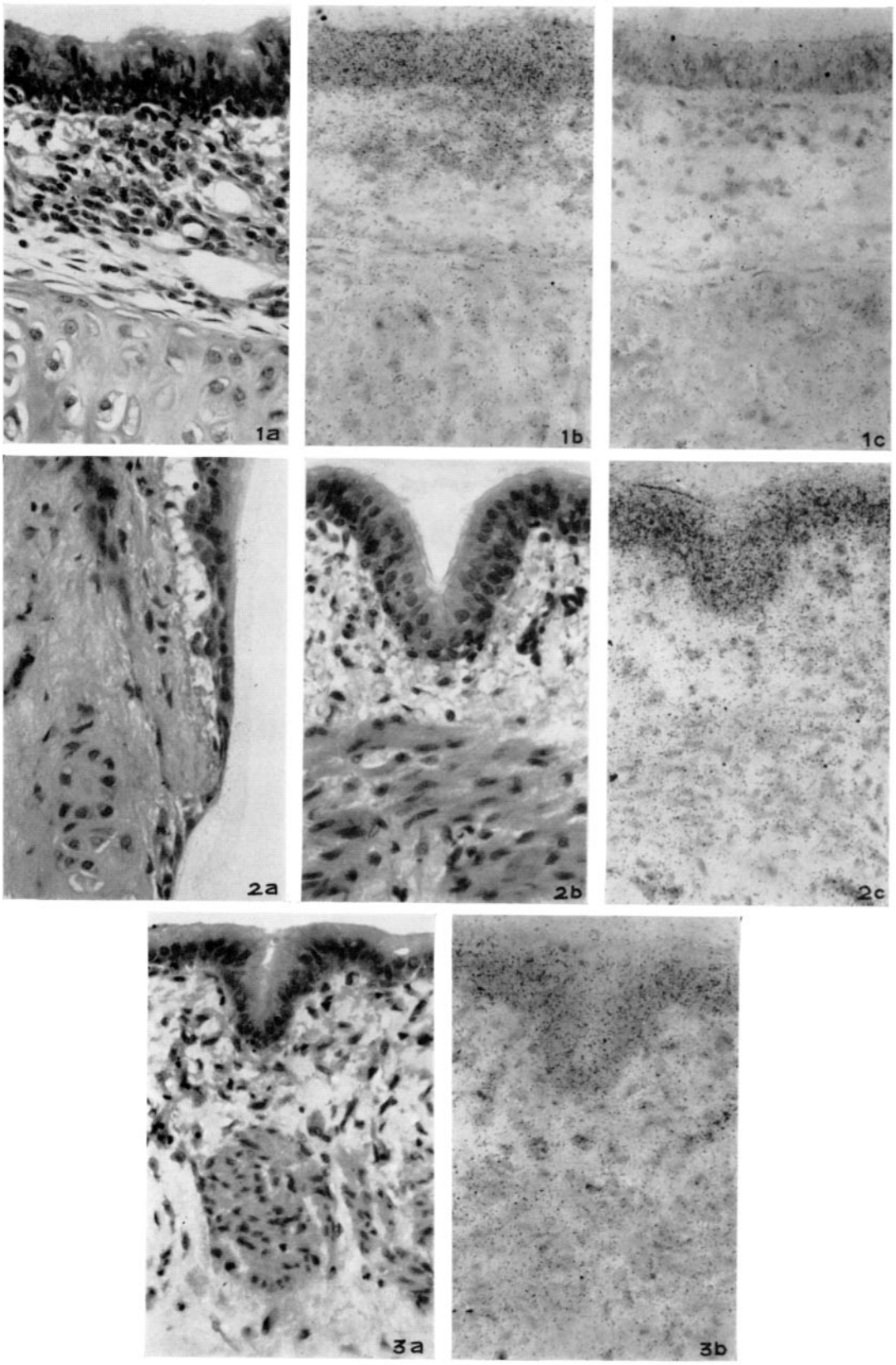
FIGS. 1 *a*, *b*, *c*. Trachea from adult rat autopsied 4 hours (1 *a*, *b*) or 11 days (1 *c*) after intraperitoneal inoculation with 1 μc per gram of S^{35}-methionine. Standard histologic preparation, 1 *a*; autoradiographs (ARG's), 1 *b* and 1 *c*, exposed 10 days.

FIGS. 2 *a*, *b*, *c*. Adult rat trachea fixed after 2 days maintenance as an organ culture. During 24 hours preceding fixation this tissue was in contact with S^{35}-methionine. Fig. 2 *a* demonstrates the transition between intact epithelium (upper right) and migrating epithelial cells (lower right) as the migrating cells move past the cut edge of the original epithelial sheet and cover adjacent exposed tissue. Figs. 2 *b* and 2 *c* are taken from the center of the epithelial sheet; 2 *c* is an ARG exposed 10 days.

FIGS. 3 *a*, *b*. Adult rat trachea maintained as an organ culture for 8 days. After the first day of cultivation, the explant was exposed to S^{35}-methionine for 24 hours, then washed, and transferred to fresh, non-labeled medium; similar transfers were made at 2- or 3-day intervals thereafter. Fig. 3 *b* is an ARG exposed 10 days. All magnifications are $\times$ 326.



(Crocker and Pelc: Loss of S^{35}-methionine in organ cultures)