The Biochemistry of Epithelia

HYDROLYTIC ENZYMES OF HUMAN BRONCHIAL EPITHELIUM

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A quantitative study of human bronchial and tracheal epithelia from smokers and non-smokers has been undertaken in this Laboratory as part of an investigation into the role of the smoking habit in the aetiology of bronchiogenic carcinoma. Briefly, the plan of study was to measure various enzymes and substances in epithelia removed at autopsy from the trachea and bronchus of males over the age of 40 years and to correlate the findings with the clinical record and smoking habit with the view to the recognition of precancerous biochemical lesions which could be associated with smoking.

There is little information in the literature about the quantitative aspects of the enzyme activities of bronchial or tracheal epithelium and most of the qualitative information has been derived from histochemical studies. A study has therefore been made of certain hydrolytic activities of bronchial epithelium and, from consideration of the kinetics of the reactions and effects of inhibitors, it has been possible, in some cases, to characterize the enzymes responsible and to choose conditions of assay under which the hydrolysis of certain esters can be attributed to particular enzymes. Before routine assays of these enzymes could be undertaken, several important investigations were considered to be necessary to place the study on as unequivocal a basis as possible.

The epithelium of human trachea and bronchus is of the pseudostratified variety, in which four types of cell can be recognized: basal, intermediate, ciliated and goblet (Miller, 1932). In the normal epithelium of adult males these cells occur in the approximate ratio of 1.6 (basal):0.2 (intermediate):0.4 (goblet):1.0 (ciliated) (Spencer, 1958), but significant variations of this pattern occur (cf. Chang, 1957; Ide, Suntzeff & Cowdry, 1959) and basal-cell hyperplasia, transitional and squamous epithelia and carcinoma *in situ* are frequently seen. The occurrence of these atypical epithelia is greater in smokers than in non-smokers (Chang, 1957; Auerbach *et al.* 1957a; Auerbach *et al.* 1957b; Auerbach *et al.* 1956). Metaplastic changes also frequently occur in pneumonia and other chronic

pulmonary disease (Valentine, 1957; Ide *et al.* 1959) and in uraemia (Sanderud, 1956). The biochemical composition of epithelial samples might be expected to reflect these variations in cell population and a cytological basis for the expression of chemical entities was considered desirable for adequate interpretation of the results of the proposed routine analyses. Accordingly, a method of preparation of epithelial samples was devised (Spencer, 1958) in which the epithelial cells were removed from the specimen into suspension in 0.25 M-sucrose containing 0.01 M-ethylenediaminetetra-acetic acid. The cytological composition of such preparations could be readily and accurately assessed and the method was suitable for the routine collection of a large number of specimens. The amount of material obtained as a routine procedure consisted of 1.2 ml. of a cell suspension removed from 2 cm.² of bronchus or trachea and contained about $5-7 \times 10^6$ cells. Contamination with superficial mucus and connective tissue was negligible. Immediately after removal the samples were distributed among a number of small tubes in 10 or 40 μ l. portions and stored at -20° until required for assay (Spencer, 1958).

This method of obtaining epithelial samples possessed many advantages but at the same time introduced several factors of unknown importance. First, although the epithelial samples were removed from human trachea and bronchus and frozen within 6 hr. after death, post-mortem changes in enzyme activities may have occurred. Secondly, the effect of storage at -20° on the various enzymes required investigation. Finally, to preserve the integrity of the goblet cells and to help in the separation of cells for counting purposes 0.01 M-ethylenediaminetetra-acetic acid was incorporated into the medium used for the removal of the epithelium (Spencer, 1958), and the effect of the sequestering agent on the enzymes had to be assessed. This paper describes these various investigations.

EXPERIMENTAL

Substrates. Monopotassium *p*-nitrophenyl sulphate (NPS) was prepared by the method of Burkhardt & Lapworth (1926) and dipotassium 2-hydroxy-5-nitrophenyl

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sulphate (nitro catechol sulphate, NCS) was freed from impurities by recrystallization of the monopotassium salt (Dodgson & Spencer, 1956). Ribonucleic acid (RNA) (Schwarz) was dialysed against distilled water for 2 days, concentrated by freeze-drying, precipitated with ethanol and dried *in vacuo* over P_2O_5 . Disodium *p*-nitrophenyl phosphate (NPP), disodium adenosine triphosphate (ATP), adenosine 5-phosphoric acid (AMP) and phenolphthalein glucosiduronic acid were purchased from Sigma Chemical Co.

Methods

Reactions were carried out in tapered tubes, 50 mm. long, internal diam. 3 mm., or in round-bottomed tubes, 50 mm. long, internal diam. 4.5 mm. Tubes were capped with Parafilm and mixing was achieved by 'buzzing', i.e. holding against a flattened nail rotating at approx. 2000 rev./min. Solutions were delivered with Lang-Levy pipettes (Lowry, Roberts, Leiner, Wu & Farr, 1954a). Spectrophotometer readings were made in microcells of 1 cm. path length (Lowry & Bessy, 1946) in a Beckman DU spectrophotometer equipped with photomultipliers.

Cell suspensions. Epithelial-cell suspensions from human bronchii removed at autopsy within 6 hr. after death were made up in 0.25M-sucrose as previously described (Spencer, 1958) and 0.01M-ethylenediaminetetra-acetic acid (EDTA) was incorporated only in specified instances. All enzyme material was frozen and stored at -20° until required. Enzyme determinations were carried out in duplicate with duplicate controls in which epithelial-cell suspensions and buffer-substrate were incubated separately and then combined immediately before cooling to 0° and the addition of the agent was used to stop the enzyme action.

Non-specific phosphomonoesterases. The epithelial-cell suspension (10 μ l.), contained in a round-bottomed tube, was cooled to 0° and 40 μ l. of buffer containing NPP and sometimes $MgCl_2$ at the same temperature was added. After incubation at 38° for the requisite period the tubes were replaced in the bath at 0° and 200 μ l. of 0.2N-NaOH was added. The solutions were mixed by 'buzzing' and the amount of *p*-nitrophenol liberated was measured at 400 m μ (ϵ_{400} for *p*-nitrophenol, 18 200). In experiments at acid pH the buffer used was 0.2M-sodium acetate-acetic acid and incubation was for 30 min. At alkaline pH the buffer was 0.2M-2-amino-2-methylpropan-1-ol-0.2N-HCl and a 1 hr. incubation period was employed.

5'-Nucleotidase and adenosine triphosphatase. The orthophosphate split off from AMP or ATP was measured by the method of Lowry, Roberts, Wu, Hixon & Crawford (1954b). The epithelial-cell suspension (10 μ l.), contained in a tapered tube, was cooled at 0° and 10 μ l. of 0.1M-histidine hydrochloride-0.1N-NaOH buffer containing AMP or ATP and sometimes $MgCl_2$ was added. After mixing the contents, the tube was capped with Parafilm, incubated at 38° for 1 hr. and then cooled at 0° . Protein was precipitated with 25% (w/v) trichloroacetic acid (4 μ l.) and, after centrifuging at 0° , a 20 μ l. portion of the clear supernatant was mixed promptly and thoroughly with 200 μ l. of molybdate-ascorbic acid reagent. The absorption at 870 m μ was measured after 15-45 min. under the conditions suggested by Lowry *et al.* (1954a) and compared with orthophosphate standards prepared at the same time.

Ribonuclease. The method used was a scaled-down version of the Strauss (1956) modification of the Schneider-Hogeboom method. To 10 μ l. of epithelial-cell suspension in

a tapered tube at 0° was added 40 μ l. of 0.5M-acetate-borate-cacodylate buffer containing RNA. After incubation at 38° for 1 hr. the tubes were cooled to 0° and 20 μ l. of 20% (w/v) trichloroacetic acid containing 0.75% of uranyl acetate was added. The tubes were centrifuged, 10 μ l. of the clear supernatant was diluted with 80 μ l. of water and the absorption at 260 m μ measured. The ribonuclease activity was arbitrarily expressed in the extinction readings obtained in this manner.

β -Glucuronidase. To 10 μ l. of the epithelial-cell suspension in a tapered tube kept at 0° was added 10 μ l. of 0.2M-sodium acetate-acetic acid buffer containing phenolphthalein glucosiduronic acid. After incubation at 38° for 1 hr. the tube was cooled to 0° and 50 μ l. of glycine-NaCl- Na_2CO_3 mixture at pH 10.7 (Kerr & Levvy, 1951) was added. The tube was centrifuged and the absorption of the supernatant measured at 550 m μ (ϵ_{550} for phenolphthalein, 26 600).

Arylsulphatases A plus B and arylsulphatase C. To 40 μ l. of epithelial suspension in a tapered tube was added 40 μ l. of 0.2M-sodium acetate-acetic acid containing NCS (for arylsulphatases A plus B) or 40 μ l. of 0.2M-2-amino-2-hydroxymethylpropane-1,3-diol (tris)-0.2N-HCl containing NPS (for arylsulphatase C), both solutions being at 0° . After incubation at 38° for 1 hr. the tubes were cooled to 0° , 40 μ l. of N-NaOH was added and the tubes were centrifuged. The absorption at 515 m μ (NCS as substrate; ϵ_{515} for nitro catechol, 12 400) or at 400 m μ (NPS as substrate; ϵ_{400} for *p*-nitrophenol, 18 200) was measured.

Nitrogen. The method of Nygar & Glick (1954) was adapted for use with 10 μ l. portions of epithelial-cell suspensions in 7 mm. $\times$ 70 mm. tapered tubes. After the addition of 40 μ l. of 1.25N-NaOH and mixing, the tube was allowed to stand at room temperature for 1 hr. before the addition of 125 μ l. of the colour reagent. The contents of the tube were mixed and then centrifuged. To 100 μ l. of the clear supernatant was added 1 ml. of 0.1N-NaOH and the absorption at 580 m μ measured (E_d). A blank determination (E_c) in which 10 μ l. of water was substituted for the tissue suspension was also carried out. To calculate the protein-nitrogen content of the tissue suspension the $E_c - E_d$ value was multiplied by a factor obtained by correlation with the micro-Kjeldahl nitrogen determination on trichloroacetic acid precipitates of representative larger epithelial samples.

RESULTS

Non-specific phosphomonoesterases. Maximum activity towards *p*-nitrophenyl phosphate was shown at pH 4.3 in 0.2M-acetate buffer at a substrate concentration of 0.01M, and at pH 10.3 and 0.006M-substrate in 0.2M-2-amino-2-methyl-1-propanol-HCl buffer (Fig. 1a). The shape of the pH curve on the acid side of neutrality was unaffected by 5 mM- Mg^{2+} ions (cf. Lowry *et al.* 1954b). The activity at pH 4.3 did not alter in the presence of 5 mM- Mg^{2+} ions or 0.5% formaldehyde (cf. Abul-Fadl & King, 1948), but was completely inhibited by 0.01M-fluoride and 80% inhibited by 0.01M-potassium sodium tartrate (cf. Abul-Fadl & King, 1949), and would therefore appear to be due to a phosphomonoesterase II (cf. Roche, 1950) similar

to prostatic acid phosphatase. The activity at pH 10.3 was Mg^{2+} ion-dependent and a concentration of 3 mM- MgCl_2 in the incubation mixture was required for full activity. This activity is that of a phosphomonoesterase I or alkaline phosphatase (cf. Roche, 1950). The optimum pH of alkaline phosphatase from various species is usually quoted as being between 9.2 and 9.6 when β -glycerophosphate is the substrate, but with NPP as substrate the optimum pH appears to be more alkaline; for example, the optimum pH for rabbit brain is 10.0 (Lowry *et al.* 1954b) and for human serum 10.0–10.1 (Bessy, Lowry & Brock, 1946).

5'-Nucleotidase. In the presence of 5 mM- Mg^{2+} ions and in 0.1M-histidine hydrochloride-NaOH buffer, bronchial epithelium shows a low activity

towards 0.01M-adenylic acid, which is fairly constant over the pH range 3.5–10.5 (Fig. 1c). In the absence of Mg^{2+} ions maximum liberation of orthophosphate is observed below pH 6.5 and is negligible above pH 8.0. The activity at pH 4.5 is totally inhibited by 0.01M-fluoride, 85% inhibited by 0.01M-tartrate and unaffected by 0.5% formaldehyde and 5 mM-EDTA. These facts are consistent with the explanation that the specific 5'-nucleotidase activity of the epithelium, which would be expected to have optimum activity around pH 7.8 (cf. Reis, 1951), is low and is largely masked in the assay *in vitro* by the non-specific acid and alkaline phosphatases (cf. Fig. 1).

Adenosine triphosphatase. In the presence of 5 mM- Mg^{2+} ions and in 0.1M-histidine hydrochloride-NaOH buffer, bronchial epithelium shows maximum activity for the liberation of orthophosphate from 2.5 mM-ATP at pH 9.6 with an inflexion at pH 7.5 (Fig. 1b). In the absence of added Mg^{2+} ions the activity above pH 7.5 drops considerably and maximum activity is actually obtained at pH 7.5 (Fig. 1). When 5 mM-EDTA is present the activity at high pH drops markedly and a single peak at pH 7.7 is seen. These facts could be explained on the assumption that the activity at high pH is due to the non-specific alkaline phosphatase and that the activity at pH 7.7 in the presence of EDTA is due to a specific ATPase. However, the action of epithelial-cell suspensions on ATP for 3 hr. at pH 7.7 led to the release of more than 1 mole of orthophosphate/mole of ATP and, under the same conditions, 27% and 10% of of the orthophosphate of 0.01M-adenosine diphosphate (ADP) and AMP respectively was liberated. The complexity of the breakdown of ATP by crude tissue extracts is well recognized (cf. Novikoff, Hecht, Podber & Ryan, 1952) and it seems probable that the release of orthophosphate from ATP by human bronchial epithelial suspensions at pH 7.7 is the combined action of several enzymes.

Ribonuclease. The presence of alkaline and acid ribonucleases in rat liver has been reported (de Lamirande, Allard, da Costa & Cantero, 1954; Roth, 1953), but the pH curve obtained for the ribonuclease activity of human bronchial epithelium (Fig. 2), with a RNA concentration of 100 $\mu\text{g./50}\mu\text{l.}$ of incubation mixture, gave no indication that more than one enzyme was present. However, the substrate-concentration curve (Fig. 3) at the optimum pH, 6.3, showed a marked discontinuity, suggesting that two enzymes, with optimum substrate concentrations in the region of 40 and 600 $\mu\text{g.}$ of RNA/50 $\mu\text{l.}$ of incubation mixture, might be present. The pH curve was therefore repeated at these two substrate concentrations (Fig. 2). At the low RNA concentration a new peak at pH 5.7 appeared with an associated plateau

Fig. 1 (a). pH-activity curve for bronchial epithelium acting on NPP. Δ , In 0.2M-sodium acetate-acetic acid buffer and 0.01M-NPP; $\blacktriangle$, in 0.2M-2-amino-2-methyl-1-propanol-HCl buffer and 0.006M-NPP. (b) pH-activity curve of bronchial epithelium acting on 2.5 mM-ATP in 0.1M-histidine hydrochloride-NaOH buffer. $\square$, In the absence of Mg^{2+} ions; $\circ$, in the presence of 5 mM Mg^{2+} ions. (c) pH-activity curve for bronchial epithelium acting on 0.01M-adenylic acid in 0.1M-histidine-HCl buffer. $\circ$, In the absence of Mg^{2+} ions; $\square$, in the presence of 5 mM- Mg^{2+} ions.

around pH 7.3, whereas at the high RNA concentration only a single peak at pH 7.3 was observed. These two peaks indicate the presence in human bronchial epithelium of two ribonucleases possessing similar optimum substrate concentrations to the acid (optimum pH 5.8) and alkaline (optimum pH 8.2) ribonucleases of rat liver (de Lamirande *et al.* 1954).

β -Glucuronidase. Optimum activity towards phenolphthalein glucosiduronic acid in 0.1M-acetate buffer was observed at pH 5.2 and at a substrate concentration of 3 mM (Fig. 4). The inflexion in the pH curve at pH 4.5 is reminiscent of the multiple pH optima reported for ox spleen (Mills, Paul & Smith, 1953).

Arylsulphatases. All human tissues so far tested contain three different arylsulphatases, A, B and C (Dodgson, Spencer & Wynn, 1956), which can be differentiated by their relative substrate specificities, behaviour towards inhibitors, localization within the cell and ease of solubility (Spencer, 1956). Bronchial epithelium is no exception.

Fig. 2. pH-activity curves for bronchial epithelium acting on RNA in 0.5M-acetate-borate-cacodylate buffer; Δ , 100 μ g. of RNA/50 μ l.; $\bullet$, 50 μ g. of RNA/50 μ l.; $\blacksquare$, 600 μ g. of RNA/50 μ l. of incubation mixture.

Fig. 3. Substrate concentration-activity curve for bronchial epithelium acting on ribonucleic acid in 0.5M-acetate-borate-cacodylate buffer at pH 6.3.

Optimum activity towards NPS was observed at pH 7.5 in 0.2M-tris buffer at an optimum substrate concentration of 7 mM (Fig. 5), and this activity can be attributed to arylsulphatase C since the A and B enzymes have negligible activity towards this substrate under these conditions (Dodgson, Spencer & Thomas, 1955). With NCS as substrate in 0.2M-acetate buffer, peaks at pH 4.5 and 6.3 were observed (Fig. 5) which can be attributed to arylsulphatases A and B respectively (cf. Dodgson *et al.* 1956).

Confirmation of the presence of the three arylsulphatases was obtained by the method employed previously for other human tissues (Dodgson *et al.* 1956). An acetone-dried powder was prepared from 10 ml. of bronchial-epithelium suspension and separated into soluble and insoluble fractions as described previously (Dodgson *et al.* 1956). The original acetone-dried powder and the soluble and

Fig. 4. pH-activity curve for bronchial epithelium acting on 3 mM-phenolphthalein glucosiduronic acid in 0.1M-acetate buffer.

Fig. 5. pH-activity curves for bronchial epithelium acting on NCS and NPS. $\bullet$, 0.02M-NCS in 0.2M-acetate buffer; $\blacksquare$, 7 mM-NPS in 0.2M-tris buffer.

insoluble components were assayed against 7 mM-NPS in 0.2M-tris buffer, pH 7.5, and 0.02M-NCS in 0.2M-acetate buffer, pH 6.0, i.e. the optimum conditions for arylsulphatase C and arylsulphatases A plus B respectively. Most of the NPS activity was found in the insoluble portion of the acetone-dried powder whereas most of the NCS activity was soluble (Table 1), indicating that both 'insoluble' (arylsulphatase C) and 'soluble' enzymes (arylsulphatases A or B or both) were present. The soluble material of the acetone-dried powder was concentrated by freeze-drying and subjected to paper electrophoresis on horizontally held Whatman no. 100 paper over 16 hr. at 200 v in the presence of 0.1M-acetate buffer, pH 5.0, and at an ambient temperature of 4°. The enzyme activity was located by incubating 1 cm. strips with 1 ml. of 0.02M-NCS in 0.2M-acetate at pH 5.6 for 1 hr. followed by the addition of 1 ml. of N-NaOH. Two bands of enzyme activity, indicated by the red colour of the liberated nitro catechol, were observed and were seen to possess the same electrophoretic mobilities as purified arylsulphatases A and B prepared from human liver by the method of Dodgson *et al.* (1956).

Effect of ethylenediaminetetra-acetic acid on enzyme activities. EDTA aids the total and differential counting of the cells by dissolving the inter-cellular cement, thus allowing easy separation of the cells, and also preserves the integrity of the goblet cells by changing the ionic state of the mucus in the theca (Spencer, 1958). The effect of EDTA on the various enzymes was studied by comparison of the activity of an epithelial-cell suspension in sucrose alone with the same suspension to which 0.01M-EDTA had been added. The value of Mg^{2+} ions as an EDTA-binding agent to offset any effect of the EDTA on the enzymes was assessed by incorporating a concentration of $MgCl_2$ equivalent to that of the EDTA into the cell suspension.

Arylsulphatase A + B, arylsulphatase C, β -glucuronidase, acid phosphatase and alkaline and acid ribonuclease activities were unaffected by the

EDTA but alkaline phosphatase was almost completely inhibited. This inhibition was only restored to 85 % of the original activity by the addition of the Mg^{2+} ions and further additions of Mg^{2+} ions did not increase the alkaline phosphatase activity. The arylsulphatase A + B activity was depressed 45 % by $MgCl_2$ but in the presence of the equivalent amount of EDTA this activity was restored.

Effect of time after death on enzyme activities. Although autopsy specimens for routine assay were to be confined to those from which the epithelial-cell samples could be removed and frozen at -20° within 6 hr. after death it was important to know whether post-mortem changes in enzyme activities had occurred during this period. Since human-autopsy specimens of bronchial epithelium could not be obtained sooner than 2 hr. after death and no suitable surgically removed specimen became available it was decided to use the trachea of rabbits for this investigation.

A rabbit was killed by injection of air and the trachea immediately exposed. A portion of the trachea about 1 cm. long was resected and the epithelium removed with isotonic sucrose containing 0.01M-EDTA, as previously described (Spencer, 1958). Samples were taken for cell counting and the remainder of the epithelial-cell suspension was cooled to 0° within 5 min. of the death of the animal and distributed in 10 and 40 μ l. portions among a number of small tubes, which were then frozen at -20° . The whole operation, from the death of the animal to the freezing of the last cell-suspension sample, lasted about 15 min. The rest of the trachea was allowed to remain in the animal at room temperature and further samples were removed and treated as above at intervals of 2, 4 and 6 hr. after death.

There was no significant difference in the ratio of different cell types in the four samples removed and the enzyme activities could be compared on the basis of the total number of cells present. The enzyme activities of the four samples were measured at the same time under the optimum conditions as

Table 1. *Fractionation of acetone-dried human bronchial-epithelium cells*

The acetone-dried powder was separated into soluble and insoluble fractions as previously described (Dodgson *et al.* 1956) and the activity of each fraction assayed against 7 mM-NPS in 0.2M-tris buffer, pH 7.5, and 0.02M-NCS in 0.2M-acetate buffer, pH 6.0, over 1 hr. at 37.5° . The activities of each fraction are related to 1 g. of the acetone-dried cells.

Activity (μ m-moles of substrate hydrolysed)	Suspension of whole acetone-dried powder		'Insoluble' fraction		'Soluble' fraction		Recovery ('insoluble' and 'soluble' fraction)	
	NPS	NCS	NPS	NCS	NPS	NCS	NPS	NCS
	8 200	30 400	7 800	5 400	400	26 500	8 200	31 900
Percentage of activity of whole powder	100	100	95.1	17.7	4.9	87.2	100.0	104.9
Ratio of activities	1:3.7		1:0.69		1:66.2		—	—

Table 2. *Optimum conditions for the assay of hydrolytic enzymes of human bronchial and tracheal epithelium*

No attempt has been made to assay arylsulphatases A and B separately (cf. Spencer, 1956) and the conditions chosen for A plus B activity are somewhat arbitrary.

Enzyme	Substrate	Buffer	pH	Concn. in incubation mixture of		Time of incubation (hr.)
				MgCl ₂ (mM)	Substrate (M)	
Acid phosphatase	NPP	0.2M-Sodium acetate-acetic acid	4.3	0	0.01	0.5
Alkaline phosphatase	NPP	0.2M-2-Amino-2-methyl-1-propanol-HCl	10.3	3	0.006	1.0
Acid ribonuclease	RNA	0.5M-Acetate-borate-cacodylate	5.7	0	0.8*	1.0
Alkaline ribonuclease	RNA	0.5M-Acetate-borate-cacodylate	7.3	0	12.0*	1.0
β -Glucuronidase	Phenolphthalein glucosiduronide	0.2M-Sodium acetate-acetic acid	5.2	0	0.003	1.0
Arylsulphatase A + B	NCS	0.2M-Sodium acetate-acetic acid	6.0	0	0.02	1.0
Arylsulphatase C	NPS	0.2M-Tris-HCl	7.5	0	0.007	1.0

* mg./ml.

Table 3. *Activity of hydrolytic enzymes in human bronchial epithelium*

Activities were measured under the conditions outlined in Table 2 and are expressed as μ m-moles of substrate hydrolysed/mg. of protein nitrogen except for acid and alkaline ribonucleases, where the activity is expressed in arbitrary log I_0/I values (see text)/mg. of protein nitrogen. Figures are the averages for six samples and the ranges are quoted in parentheses.

Enzyme	Activity
Acid phosphatase	14 200 (8 230-22 300)
Alkaline phosphatase	3 820 (1 040-6 910)
Acid ribonuclease	92 (47-210)
Alkaline ribonuclease	365 (205-576)
β -Glucuronidase	239 (188-288)
Arylsulphatase A + B	238 (140-322)
Arylsulphatase C	50 (24-82)

frozen. When measured under the optimum conditions (Table 2), the activity of acid and alkaline phosphatases and ribonucleases, β -glucuronidase, arylsulphatases A plus B and arylsulphatase C decreased by less than 5% during 6 months' storage at -20° . It was advisable, however, to measure activities as soon after thawing the samples as possible, or to keep thawed samples at 0° , since most of the enzymes lost activity after standing for 0.5 hr. at room temperature. Protein-nitrogen values also dropped under the same conditions.

The enzyme activities of the bronchial epithelia from six male cadavers selected at random have been measured (Table 3).

DISCUSSION

detailed in Table 2. Less than 5% diminution in the activity of any of the enzymes or in protein nitrogen of rabbit tracheal epithelium was observed in the samples which were removed up to 6 hr. after death. The conditions employed in this experiment were comparable with those experienced by human-autopsy material, which was usually obtainable within 2-6 hr. *post mortem*, and it seems reasonable to assume that the human material behaves similarly to that of rabbits.

Effect of storage at -20° on enzyme activities. Immediately after removal of the epithelium from the autopsy specimen, the epithelial suspensions in sucrose plus EDTA were frozen at -20° in tubes capped with Parafilm until required. The effect of storage on the various enzymes was investigated by measuring the activities of samples from the same autopsy specimen after time intervals of approximately one month and including one sample which was thawed and assayed immediately after being

The present study has been mainly concerned with the validity of the assay of certain enzymes in human bronchial and tracheal epithelium removed at autopsy. The choice of enzymes to be studied in routine examinations, in attempting to demonstrate pre-cancerous lesions in bronchii associated with smoking, has been restricted to those enzymes which are known from the literature to be present in raised or lower amounts in various carcinomas as compared with their respective tissue of origin (Greenstein, 1954). Within this framework, the small amount of epithelial tissue available, the technique of collection and the method of storage (Spencer, 1958) have imposed further limitations. Thus the suitability of any particular enzyme for the proposed study depends on the availability of a convenient micro-assay method, the ease of reversing any effect of the added EDTA and the preservation of activity for long periods at -20° . A series of hydrolytic enzymes were chosen for

initial investigation and, of these, acid and alkaline phosphatase, acid and alkaline ribonuclease, β -glucuronidase, arylsulphatase A plus B and arylsulphatase C appeared to fulfil the criteria demanded, and conditions for their assay were established (Table 2). Specific adenosine triphosphatase and 5'-nucleotidase could not be distinguished from other non-specific enzymes attacking ATP and AMP respectively and, although both the specific enzymes have been said to alter in carcinoma, no attempt has been made to consider them further in the present study.

The resistance of certain enzymes to post-mortem changes in brain has already been adequately established. An examination of rat brain left at room temperature for 50 hr. *post mortem* showed that one group of enzymes, including acid and alkaline phosphatase, showed little or no fall in activity during this period, whereas a second group, which included adenosine triphosphatase and 5'-nucleotidase, dropped markedly (Naidoo & Pratt, 1954). In a similar study over 6 hr. *post mortem*, Smith, Robins, Eydt & Daesch (1957) found that acid and alkaline phosphatase, lactic, malic and glutamic dehydrogenases and hexokinase showed changes that averaged only $\pm 7.6\%$, but phosphofructokinase, in contrast, showed a decrease in activity of approximately 70%. The present work adds acid and alkaline ribonuclease, β -glucuronidase and the arylsulphatases to the list of enzymes which show relatively little change in activity after death although, as Smith *et al.* (1957) point out, the results may not be applicable to other sites where there are more active autolytic processes. Indeed, since the tracheo-bronchial epithelium is exposed to the atmosphere and consequently highly contaminated with bacteria it is perhaps unexpected to find the enzymes showing the same post-mortem stability as that found in brain. However, the tracheo-bronchial tract is normally lined with a viscous mucus layer which presumably protects the epithelium for at least a short while after death and it may be noted that cilia of excised ox trachea may continue beating for as long as 36 hr. at room temperature without any special attention apart from placing the specimen in a plastic bag (Hilding, 1956). Several of the enzymes measured, for example β -glucuronidase and arylsulphatases A + B, are normally present in granules surrounded by semipermeable membranes, and a post-mortem increase in activity might have been expected, attributable to a release of the enzymes from the granules during progressive autolysis (cf. Smith *et al.* 1957). This was not observed, probably because each epithelial-cell sample was frozen and thawed before assay, a treatment which is known to rupture granule membranes.

The epithelium lining the human tracheo-

bronchial tract possesses two main activities, the secretion of mucus and the transport of the mucus by ciliary action, and it might therefore be expected that the epithelium is also active in a biochemical sense. As far as hydrolytic enzymes are concerned this supposition would seem to be true, for the activity of the enzymes measured (Table 3) compares favourably with that found in some of the more active organs of the body. For example, the arylsulphatase A and B activity of human bronchial epithelium is approximately one-third of that of liver, equal to that of spleen and twice that of brain (cf. Dodgson *et al.* 1956) when measured on a protein basis. The high acid phosphatase activity (Table 3) is also worthy of note. Other points of interest which have emerged are the confirmation of the ubiquity of arylsulphatases A, B and C in the human tissues so far tested and the presence of acid and alkaline ribonucleases which, although known in mouse and rat (de Lamirande, 1956), have not hitherto been reported in human tissue.

SUMMARY

1. The hydrolysis of *p*-nitrophenyl phosphate, adenosine triphosphate, adenylic acid, ribonucleic acid, phenolphthalein glucosiduronide, *p*-nitrophenyl sulphate and nitrocatechol sulphate by human bronchial and tracheal epithelia has been examined.

2. The presence of acid and alkaline phosphatase, acid and alkaline ribonuclease, β -glucuronidase and arylsulphatases A, B and C has been recognized and conditions have been established for their assay. Specific adenosine triphosphatase and 5'-nucleotidase could not be distinguished from other enzymes which hydrolysed the same substrates.

3. In rabbit trachea no appreciable change in activities was observed when the trachea was left in the cadaver at room temperature for periods of up to 6 hr. after death. The activity of the enzymes survived at least 6 months' storage of the human-autopsy material at -20° .

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REFERENCES

- Abul-Fadl, M. A. M. & King, E. J. (1948). *J. clin. Path.* **1**, 80.
- Abul-Fadl, M. A. M. & King, E. J. (1949). *Biochem. J.* **45**, 51.
- Auerbach, O., Gere, J. B., Forman, J. B., Petrick, T. G., Smolin, H. J., Meusam, G. E., Kassouny, D. Y. & Stout, A. P. (1957a). *New Engl. J. Med.* **256**, 97.

- Auerbach, O., Gere, J. B., Pawlowski, T. M., Muehsam, G. E., Smolin, H. J. & Stout, A. P. (1957b). *J. thorac. Surg.* **34**, 298.
- Auerbach, O., Petrick, T. G., Stout, A. P., Statsinger, A. L., Muehsam, G. E., Forman, J. B. & Gere, J. B. (1956). *Cancer*, **9**, 76.
- Bessy, O. A., Lowry, O. H. & Brock, M. J. (1946). *J. biol. Chem.* **164**, 321.
- Burkhardt, G. N. & Lapworth, A. (1926). *J. chem. Soc.* p. 684.
- Chang, S. C. (1957). *Cancer*, **10**, 1246.
- de Lamirande, G. (1956). *Trans. Roy. Soc. Can.* **50**, 48.
- de Lamirande, G., Allard, C., da Costa, H. C. & Cantero, A. (1954). *Science*, **119**, 351.
- Dodgson, K. S. & Spencer, B. (1956). *Biochim. biophys. Acta*, **21**, 175.
- Dodgson, K. S., Spencer, B. & Thomas, J. (1955). *Biochem. J.* **59**, 29.
- Dodgson, K. S., Spencer, B. & Wynn, C. H. (1956). *Biochem. J.* **62**, 500.
- Greenstein, J. P. (1954). *Biochemistry of Cancer*. New York: Academic Press.
- Hilding, A. C. (1956). *Ann. Oto-laryng.* **65**, 736.
- Ide, G., Suntzeff, V. & Cowdry, E. V. (1959). *Cancer* (in the Press).
- Kerr, L. M. H. & Levvy, G. A. (1951). *Biochem. J.* **48**, 209.
- Lowry, O. H. & Bessy, O. A. (1946). *J. biol. Chem.* **163**, 633.
- Lowry, O. H., Roberts, N. R., Leiner, K. Y., Wu, M. L. & Farr, A. L. (1954a). *J. biol. Chem.* **207**, 1.
- Lowry, O. H., Roberts, N. R., Wu, M. L., Hixon, W. S. & Crawford, E. J. (1954b). *J. biol. Chem.* **207**, 19.
- Miller, W. S. (1932). In *Special Cytology*, p. 133. Ed. by Cowdry, E. V. New York: Hoeber.
- Mills, G. T., Paul, J. & Smith, E. E. B. (1953). *Biochem. J.* **53**, 232.
- Naidoo, D. & Pratt, O. E. (1954). *Enzymologia*, **17**, 1.
- Novikoff, A. B., Hecht, L., Podber, E. & Ryan, J. (1952). *J. biol. Chem.* **194**, 153.
- Nyayar, S. N. & Glick, D. (1954). *J. Histochem. Cytochem.* **2**, 282.
- Reis, J. L. (1951). *Biochem. J.* **48**, 548.
- Roche, J. (1950). In *The Enzymes*, vol. 1, pt. 1, p. 473. Ed. by Summer, J. B. & Myrback, K. New York: Academic Press Inc.
- Roth, J. S. (1953). *Biol. Bull., Wood's Hole*, **105**, 359.
- Sanderud, K. (1956). *Brit. J. Cancer*, **10**, 226.
- Smith, D. E., Robins, E., Eydt, K. M. & Daesch, G. E. (1957). *Lab. Invest.* **6**, 447.
- Spencer, B. (1956). In *Colloque sur la Biochimie du Soufre*, p. 135. Paris: Editions du Centre National de la Recherche Scientifique.
- Spencer, B. (1958). *J. Histochem. Cytochem.* **6**, 105.
- Strauss, W. (1956). *J. biophys. biochem. Cytol.* **2**, 513.
- Valentine, E. H. (1957). *Cancer*, **10**, 272.

A Method for the Assay of Insulin by Paper Chromatography

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Methods for the assay of insulin *in vitro* have appeared at intervals in the literature. Among the most familiar are the fibril-precipitation method of Waugh, Thompson & Weimer (1950) and the more recent version of the same method (Grodsky, 1958). The former method was the subject of an investigation by Foster, Macdonald & Smart (1951), who, although able to obtain reliable results in assays on freshly prepared solutions of insulin, protamine insulin and commercial concentrates, reported failure with solutions of low-potency insulin (11 units/mg.), with globin insulin and with a 5½-year-old solution of insulin.

Numerous chromatographic methods for the separation of insulin from associated impurities may be found in the literature. Porter (1953) separated insulin from crude preparations by using a silane-treated kieselguhr column. Porath & Li (1954) used a charcoal column successfully and Dickinson (1956) a column of calcium phosphate and kieselguhr.

Other workers have achieved satisfactory separation of insulin from other proteins by paper

chromatography; Robinson & Fehr (1952) were able to estimate the percentage of insulin in protamine zinc insulin, and Grodsky & Tarver (1956) separated insulin from liver proteins and pancreatic extracts. Light & Simpson (1956) achieved separation from similar mixtures.

The high cost of biological assays of insulin has always suggested that a more economical process would be of great value, and it appeared worthwhile investigating the possibility of adapting a chromatographic method to fulfil this need.

There is abundant evidence in the literature for the heterogeneity of insulin samples, and it seemed reasonable to assume that a suitable solvent system might be capable of separating biologically active insulin from the impurities with which it is associated. If this could be done, there should be a relationship between the amount of biologically active insulin separated from a given amount of sample and the biological activity of the sample on a weight basis.

Light & Simpson (1956) found the biological activity of insulin to be associated with a specific