Phosphodiesterase II in Epithelial Cells from Guinea-Pig and Rat Small Intestine

By PETER R. FLANAGAN* and S. H. ZBARSKY
Department of Biochemistry, University of British Columbia,
Vancouver, B.C. V6T 1W5, Canada

(Received 4 March 1974)

Phosphodiesterase II activity was determined by using a synthetic substrate, the 2,4-dinitrophenyl ester of thymidine 3'-phosphate. The enzyme activity was determined in fractions obtained by differential centrifugation of homogenates of epithelial cells from the small intestinal mucosa of guinea pigs and rats. In guinea-pig preparations phosphodiesterase II occurred with highest specific activity in those fractions rich in succinate dehydrogenase and acid phosphatase. A lysosomal location for the guinea-pig enzyme was indicated by its structure-linked latency and by its association with particles that underwent a characteristic decrease in equilibrium density when Triton WR-1339 was injected into the animals. With rat preparations a much greater proportion of the phosphodiesterase II activity was found in the soluble fraction after ultracentrifugation. The rat enzyme exhibited a lower degree of latency and administration of Triton WR-1339 had no effect. The rat enzyme further differed from that of the guinea pig in other respects; it was more labile at 60°C, it exhibited a lower pH optimum and it had a higher molecular weight as determined by gel-filtration chromatography.

Phosphodiesterase II (oligonucleate 3'-nucleotidohydrolase, EC 3.1.4.18) catalyses the exolytic hydrolysis of nucleoside 3'-phosphates from the 5'-hydroxy termini of oligonucleotide fragments. Hynie & Zbarsky (1970) reported that approximately 60% of the total phosphodiesterase II activity in homogenates of mucosal scrapings from rat intestine was found in the supernatant fraction after ultracentrifugation. This observation, which has been repeated by Flanagan (1970), disagrees with findings of other workers (van Dyck & Wattiaux, 1968; Erecinska *et al.*, 1969) who have reported that phosphodiesterase II is a lysosomal enzyme. Although the observations of van Dyck & Wattiaux (1968) and Erecinska *et al.* (1969) were obtained with rat liver, one might expect a similar distribution in the small intestine according to the work of Hsu & Tappel (1964). These authors showed that six marker enzymes for lysosomes were similarly distributed among particulate fractions from liver and intestinal mucosa.

It seemed appropriate therefore to investigate more fully the subcellular location of phosphodiesterase II in intestinal mucosa. However, since mucosal scrapings can consist of a mixture of different cell types (Porteous & Clark, 1965), preparations of epithelial cells were used in the present work because these contain a relatively homogeneous population of cells

(Evans *et al.*, 1971). Experiments were carried out with intestinal preparations from the rat which contained large amounts of mucus and were somewhat difficult to fractionate by differential centrifugation (Clark & Porteous, 1965) and with preparations from the guinea-pig in which the mucus content was considerably lower (Hubscher *et al.*, 1965; Evans *et al.*, 1971).

The work reported here shows that phosphodiesterase II from guinea-pig intestinal epithelium is a lysosomal enzyme, but the location of the rat enzyme is less clear. Evidence is also presented that indicates that the phosphodiesterases from the two animals have different properties. A preliminary account of some of this work has been presented (Flanagan & Zbarsky, 1973).

Materials and Methods

Chemicals

Glucose 6-phosphate (disodium salt), 2-(*p*-iodophenyl) - 3 - (*p* - nitrophenyl) - 5 - phenyltetrazolium chloride and calf thymus DNA were obtained from Sigma Chemical Co., St. Louis, Mo., U.S.A. Yeast RNA was from Calbiochem, La Jolla, Calif., U.S.A. Disodium *p*-nitrophenyl phosphate and thymidine 3'-phosphate (bistriethylammonium salt) were purchased from Raylo Chemicals Ltd., Edmonton 82, Alta., Canada. Triton WR-1339 was obtained from the Ruger Chemical Co., Irvington-on-Hudson, N.Y., U.S.A., and bovine serum albumin (fraction V)

* Present address: The Hospital for Sick Children, Toronto, Ont., M5G 1X8 Canada.

was purchased from the Armour Pharmaceutical Co., Chicago, Ill., U.S.A. Protein molecular-weight markers were obtained from Pharmacia (Canada) Ltd., Dorval, P.Q., Canada. Other chemicals were the purest grade available. Diphenylamine and orcinol were recrystallized as described by Schneider (1957).

Thymidine 3'-(2,4-dinitrophenyl)phosphate was prepared by the method of von Tigerstrom & Smith (1969) with the following modifications: (1) at the end of the reaction period the reaction mixture was taken up in 150ml of ether and extracted with water (4×50ml); (2) triethylammonium bicarbonate was used to elute products from the DEAE-cellulose column since it could be removed easily and quickly from the eluted substances by flash evaporation; (3) no cation exchanger was added during the concentration of the desired product.

Because of the inherent instability of the diester in solution (von Tigerstrom & Smith, 1969) it slowly hydrolysed even when kept at 4°C and at pH 4–6 in the dark, and therefore at times it was necessary to repurify the compound on a DEAE-cellulose column. This is important since one of the products of hydrolysis, thymidine 3'-phosphate, is a competitive inhibitor of phosphodiesterase II (P. R. Flanagan & S. H. Zbarsky, unpublished work).

Animals

Rats (Wistar) weighing about 200 g each, and albino guinea pigs of the Hartley strain (300–350 g) were obtained from the animal unit of the University of British Columbia, and were starved for 24 h before death.

Methods

Preparation of epithelial cell suspensions. The suspensions of intestinal epithelial cells were prepared by the method of Connock & Pover (1970). About 2–3 g of cells (wet wt.) were obtained from each rat and 4–5 g from each guinea pig.

Fractionation of the cells. All operations were carried out at 2–5°C. The cells from one animal, suspended in 40 ml (about 8 vol.) of 0.3 M-sucrose–5 mM-sodium phosphate, pH 7, were homogenized in a Potter–Elvehjem homogenizer with ten complete strokes of the glass vessel against the Teflon pestle, which was rotating at 900 rev./min. The homogenate was centrifuged in a model PR-2 International centrifuge (rotor no. 269) for 10 min at 280 *g*_{av}, and the turbid supernatant carefully removed by using a wide-tipped Pasteur pipette and kept. The loosely packed sediment was resuspended in 40 ml of sucrose–phosphate medium and again homogenized with five strokes of the homogenizer. After centrifugation at 280 *g*_{av} for 10 min the supernatant was retained and the sediment washed twice by dispersion in 20 ml of sucrose–phosphate solution, gentle homogenization (three strokes of the vessel against the stationary

Teflon pestle) and centrifugation at 280 *g*_{av} for 10 min. The final supernatant was only slightly cloudy. The well-packed sediment was suspended in 40 ml of sucrose–phosphate buffer to give fraction (I). The supernatants from the four centrifugations were combined and a 1 ml sample was removed for analysis. Fractions (II)–(V) were obtained from this 'cytoplasmic' fraction by differential centrifugation. At each step the sediment was resuspended in 20 ml of sucrose–phosphate buffer and the supernatant re-centrifuged at the next highest speed. The 'cytoplasmic' fraction was centrifuged in the International centrifuge (rotor no. 269) for 10 min at 750 *g*_{av} to yield fraction (II). Fractions (III) and (IV) were obtained by using a Sorvall RC2-B centrifuge equipped with an SS-34 rotor (*r*_{av}, 8.3 cm); fraction (III) by centrifugation at 3850 *g*_{av} for 10 min and fraction (IV) by centrifugation at 7380 *g*_{av} for 20 min. The final centrifugation was carried out at 78 500 *g*_{av} for 60 min in a Spinco no. 30 rotor (*r*_{av}, 7.8 cm) to give fraction (V) (sediment) and fraction (VI) (supernatant).

In some experiments the number of centrifugations was decreased so that fractions (I)+(II) or fractions (III)+(IV) or fractions (III)+(IV)+(V) were isolated together.

Density-gradient centrifugation. In experiments where this procedure was used fractions (III)+(IV) were isolated together and resuspended in 0.3 M-sucrose–5 mM-sodium phosphate buffer, pH 7, as before. Portions (5 ml) of this combined fraction were gently layered on 30 ml linear sucrose gradients prepared from 20% (w/w) and 50% (w/w) sucrose solutions. The tubes were then centrifuged for 3 h at 95 000 *g*_{av} in a Spinco SW-27 rotor (*r*_{av}, 11.7 cm). After centrifugation, the bottom of each tube was pierced in a Beckman recovery system and the contents were removed from the top by slowly pumping 60% (w/w) sucrose through the hole. Ten 3.5 ml fractions were obtained from each gradient.

Analytical methods. Protein was determined by the method of Lowry *et al.* (1951) with bovine serum albumin as the standard. DNA and RNA were extracted and measured as described by Schneider (1957); the complete procedure was calibrated by applying it to known amounts of the nucleic acids. *P*_i was determined by the method of Ames (1966).

Sucrose concentrations were measured by using an Abbe 60 refractometer. The densities of these solutions were then determined from tables in the Handbook of Chemistry and Physics (Chemical Rubber Company Press, 54th edn.).

Enzyme determinations. The assay for phosphodiesterase II was carried out in 0.1 M-sodium succinate, pH 6.1, 0.1% (v/v) Triton X-100 and 0.5 mM-thymidine 3'-(2,4-dinitrophenyl)phosphate with a suitable dilution of enzyme. The release of 2,4-dinitrophenol was determined in either of two ways: method (1)

was a continuous assay in which 1 ml of the reaction mixture was placed in a spectrophotometer cuvette and the increasing absorbance of the solution at 360nm was measured at 37°C in a Gilford model 2000 spectrophotometer. Method (2) was a fixed-time assay which was carried out in 0.3ml volume. The mixture was incubated for 20min at 37°C, the reaction stopped with 0.7 ml of 0.1 M-NaOH and the E_{360} of the solution measured. Controls contained the same components but the substrate was added after the NaOH. Whenever possible method (1) was used because of the ease with which initial reaction velocities could be determined. The release of 2,4-dinitrophenol was linear with time up to 30min and with protein concentration up to 150 μ g/ml. Non-enzymic hydrolysis of the substrate was negligible under these assay conditions.

Acid phosphatase (EC 3.1.3.2) activity was determined by measuring the fluoride-sensitive (see Hubscher & West, 1965) hydrolysis of *p*-nitrophenyl phosphate at pH5 and 37°C. Reaction mixtures (0.4ml) contained 50mM-sodium acetate buffer, pH5.0, 0.1% (v/v) Triton X-100, 4mM-EDTA, 5mM-*p*-nitrophenyl phosphate and enzyme. Control tubes contained, additionally, 2mM-KF. The reaction was initiated by the addition of substrate and stopped after 5–10min by adding 0.6ml of 0.1 M-NaOH. The absorbances of the solutions were measured at 400nm.

Succinate dehydrogenase (EC 1.3.99.1) activity was determined by the method of Pennington (1961), with the modifications described by Porteous & Clark (1965).

The assay for glucose 6-phosphatase (EC 3.1.3.9) was based on that of Hubscher & West (1965). The reaction mixture (0.3ml) contained 0.1M-sodium *N*-morpholinoethanesulphonate buffer, pH 6.5, 2mM-KF, 4mM-EDTA, 8mM-glucose 6-phosphate and enzyme (up to 100 μ g of protein). The reaction was started by the addition of enzyme and stopped after 15min by adding 0.7ml of the ascorbate-molybdate reagent described by Ames (1966). After further incubation for 20min at 45°C the E_{820} of the solution was measured. The control lacked glucose 6-phosphate.

The ϵ values for 2,4-dinitrophenol, *p*-nitrophenol and formazan under the experimental conditions used were 13900, 17600 (Flanagan, 1970) and 20100 (Pennington, 1961) litre·mol⁻¹·cm⁻¹ respectively.

A unit of enzyme activity was defined as the amount that produced 1 μ mol of product/min at 37°C.

Results

Differential centrifugation of epithelial-cell homogenates

Fractionation of cell preparations from guinea pig and rat gave the results presented in Table 1 which

Table 1. Subcellular distribution of components in homogenates of epithelial cells from guinea-pig and rat intestine

Component	Absolute values	Recovery (%)	Recovered component in each fraction (%)					
			(I)	(II)	(III)	(IV)	(V)	(VI)
Guinea pig								
Protein (4)	450 $\pm$ 57	102.0 $\pm$ 6.7	21.3 $\pm$ 3.9	9.8 $\pm$ 2.1	9.4 $\pm$ 1.0	9.1 $\pm$ 1.9	11.1 $\pm$ 0.2	39.4 $\pm$ 0.6
Phosphodiesterase II (6)	3.76 $\pm$ 0.78	95.9 $\pm$ 8.1	10.0 $\pm$ 3.0	15.3 $\pm$ 2.3	24.8 $\pm$ 1.0	18.0 $\pm$ 2.7	11.9 $\pm$ 1.1	20.0 $\pm$ 2.1
Acid phosphatase (5)	7.06 $\pm$ 1.20	101.5 $\pm$ 3.9	16.6 $\pm$ 7.7	8.7 $\pm$ 2.6	25.2 $\pm$ 3.7	23.2 $\pm$ 3.2	17.2 $\pm$ 1.5	9.1 $\pm$ 3.2
DNA (4)	37.4 $\pm$ 7.8	92.3 $\pm$ 10.3	51.3 $\pm$ 9.4	34.8 $\pm$ 7.8	9.1 $\pm$ 2.8	2.0 $\pm$ 0.9	1.6 $\pm$ 0.9	1.1 $\pm$ 2.3
RNA (5)	38.3 $\pm$ 8.3	94.3 $\pm$ 8.5	20.0 $\pm$ 6.7	8.6 $\pm$ 1.9	6.9 $\pm$ 1.9	8.8 $\pm$ 1.0	30.8 $\pm$ 2.8	24.9 $\pm$ 4.1
Succinate dehydrogenase (5)	6.85 $\pm$ 2.31	88.0 $\pm$ 10.1	15.9 $\pm$ 10.2	5.7 $\pm$ 1.4	42.2 $\pm$ 9.4	28.2 $\pm$ 1.2	4.2 $\pm$ 1.0	3.8 $\pm$ 3.3
Glucose 6-phosphatase (4)	9.31 $\pm$ 3.06	87.1 $\pm$ 4.1	27.6 $\pm$ 13.8	6.6 $\pm$ 2.1	9.4 $\pm$ 4.1	19.7 $\pm$ 6.3	29.0 $\pm$ 3.9	7.7 $\pm$ 4.7
Rat								
Protein (5)	273 $\pm$ 58	100.9 $\pm$ 2.7	34.2 $\pm$ 2.4	6.0 $\pm$ 0.7	7.0 $\pm$ 0.6	5.2 $\pm$ 0.4	10.4 $\pm$ 1.82	37.2 $\pm$ 1.3
Phosphodiesterase II (4)	1.93 $\pm$ 0.75	90.6 $\pm$ 3.5	16.4 $\pm$ 2.5	10.1 $\pm$ 2.4	17.5 $\pm$ 4.1	9.2 $\pm$ 1.6	7.9 $\pm$ 1.7	39.0 $\pm$ 5.7
Acid phosphatase (5)	2.63 $\pm$ 0.67	100.4 $\pm$ 9.0	35.1 $\pm$ 6.4	6.0 $\pm$ 2.0	18.1 $\pm$ 5.6	13.5 $\pm$ 2.0	15.6 $\pm$ 3.5	11.6 $\pm$ 3.1

Fig. 1. Localization of phosphodiesterase and other components in subcellular fractions obtained by differential centrifugation of intestinal epithelial-cell homogenates

The ordinates indicate the relative specific activities (or amounts) of the components calculated for each fraction as percentage of component/percentage of protein by using the data in Table 1. Fractions (I)–(VI) are depicted from left to right according to their protein contents (abscissa). (a), (e) Phosphodiesterase; (b), (f) acid phosphatase; (c) DNA; (d) succinate dehydrogenase; (g) glucose 6-phosphatase; (h) RNA; (a)–(d), (g) and (h) are results for guinea pig, (e) and (f) for the rat.

shows the distribution patterns of phosphodiesterase II and a number of markers for subcellular components: DNA for nuclei, succinate dehydrogenase for mitochondria, acid phosphatase for lysosomes, RNA for ribosomal particles and glucose 6-phosphatase for endoplasmic reticulum. The relative specific activities or amounts of these components are shown in Fig. 1. Preliminary examination by light microscopy revealed that greater amounts of a mucus-like amorphous material were present in fraction (I) of the rat than that from the guinea pig. This observation could explain the differences found

in the relative amounts of protein recovered in fractions (I)–(V) from both animals (Table 1), since the mucus could trap some of the slower-sedimenting particles in the low-speed sediment (fraction I). The large amount of mucus in rat intestinal preparations has been shown to impede fractionation of the tissue (Robinson, 1963; Clark & Porteous, 1965). Microscopic examination also showed that besides numerous nuclei, fraction (I) contained many unbroken cells whereas fraction (II) was mostly nuclear.

Since a slightly better separation of the particulate fractions was achieved with the guinea pig, a more

extensive biochemical characterization of these was carried out (Table 1 and Fig. 1). Considerable cross-contamination amongst the fractions was apparent especially in the 'mitochondrial' fractions (III) and (IV), which contained the highest specific activities of both succinate dehydrogenase and acid phosphatase (Fig. 1). The results of other workers (Porteous & Clark, 1965; Hubscher *et al.*, 1965; Wrigglesworth & Pover, 1966) also indicate that mitochondria and lysosomes from intestinal preparations tend to sediment at similar centrifugal forces. In any event we were unable to separate particles containing succinate dehydrogenase from those containing acid phosphatase by differential centrifugation.

The distribution of guinea-pig phosphodiesterase II (Fig. 1) was similar to the patterns shown by the markers for mitochondria and lysosomes. A similarity of the distributions of phosphodiesterase and acid phosphatase between the rat and guinea pig was also observed (Fig. 1). However, there were important differences. For instance, in the guinea pig 60% of the phosphodiesterase and acid phosphatase activities were recovered in fractions (II)–(IV) (Tables 1 and 2), whereas in the rat less than 40% of these enzymes was present in these fractions (Table 1). Further, the final supernatant from the rat tissue contained a much higher proportion of the total phosphodiesterase than the corresponding fraction from the guinea pig (Table 1). Since the rat preparations were more viscous because of their higher mucus content, it was thought that the larger shearing forces that develop during homogenization might lead to a greater disruption of the subcellular particles. However, when the viscosity of guinea-pig homogenates was artificially increased to approximately that of rat preparations by the addition of a suitable amount of Ficoll [Pharmacia (Canada) Ltd.], there was no difference in the distributions of acid phosphatase and phosphodiesterase II (Table 2).

Activation of acid phosphatase and phosphodiesterase II

If phosphodiesterase II is a lysosomal enzyme in the intestinal epithelium it would be expected to exhibit the 'structure-linked latency' common to other acid hydrolases (de Duve, 1965). When the activities of phosphodiesterase and acid phosphatase were measured under conditions where the access of substrate to enzyme was unrestricted by a limiting membrane, a 2.5–3-fold increase in phosphodiesterase and a 2-fold increase in acid phosphatase over their 'free' amounts was observed in mitochondrial-lysosomal fractions from the guinea pig (Table 3). The degree of activation of these enzymes was not as great in the rat particulate fractions, nor was the pattern similar to that obtained from the guinea pig.

Effect of Triton WR-1339 on the equilibrium density of particulate phosphodiesterase II

Probably the most conclusive evidence on the association of a component with lysosomes involves use of the non-ionic detergent Triton WR-1339. This compound, when injected into experimental animals, is taken up by lysosomes thereby causing a selective decrease in their equilibrium density in sucrose gradients (Wattiaux *et al.*, 1963). If an enzyme is associated with these granules its position in the gradient will shift accordingly. Fig. 2 shows a typical experiment with a guinea pig. In a gradient prepared with material from a control animal succinate dehydrogenase activity was at a density of 1.178 whereas the acid phosphatase and phosphodiesterase activities exhibited broad peaks with median densities close to 1.20. With a similar fraction prepared from a guinea pig injected with Triton WR-1339, the succinate dehydrogenase activity was again found at a density close to 1.18 whereas the acid phosphatase and phosphodiesterase activities were at densities of 1.168 and 1.152 respectively. This shift of guinea-pig phosphodiesterase II activity to a lower-density region of the sucrose gradient provides strong evidence of a lysosomal location. In similar experiments performed with fractions from rats no shift in either phosphodiesterase II or acid phosphatase could be detected. Hence firm conclusions about the location of the rat enzyme were not possible.

Some properties of guinea-pig and rat phosphodiesterase II

In view of the differences noted in the subcellular distribution of these enzymes, some biochemical properties of each were compared. These studies were done on sonicated extracts of fraction (III).

Influence of pH. The effect of pH on the hydrolysis of thymidine 3'-(2,4-dinitrophenyl)phosphate by extracts of fraction (III) from guinea pig and rat is shown in Fig. 3. In the absence of SO_4^{2-} , there was considerable hydrolysis of the substrate at low pH values, especially in the case of the guinea pig. This was probably due to DNAase* II in these preparations since it was not seen in the presence of SO_4^{2-} , an inhibitor of DNAase II (Rauenbusch & Altman, 1960). Further experiments (P. R. Flanagan & S. H. Zbarsky, unpublished work) showed that DNAase II activity was much more extensive at pH values 2.5–4 in guinea-pig preparations than in fractions prepared from rats thus accounting for the difference between the animals seen at low pH values in the absence of SO_4^{2-} (Fig. 3).

The phosphodiesterase activities of guinea-pig and rat preparations exhibited very similar profiles but the maximum activities were found at pH 6.2–6.3 for

* Abbreviation: DNAase, deoxyribonuclease.

Table 2. *Distribution of phosphodiesterase, acid phosphatase and protein in homogenates of guinea-pig intestinal epithelial cells prepared in media of different viscosity*

A less protracted version of the normal procedure was used in which only three fractions were obtained; the first corresponded to fractions (I)+(II), the second to fractions (III)+(IV)+(V) and the third to fraction (VI) of Table 1. In Expt. (a) the homogenizing medium was 0.3 M-sucrose-5 mM-sodium phosphate, pH 7; in Expt. (b) it contained, additionally, 3% (w/v) Ficoll. In each case the mean values ($\pm$ S.D.) for four experiments are shown.

Component	Recovered component in each fraction (%)		
	(I)+(II)	(III)+(IV)+(V)	(VI)
Expt. (a)			
Protein	25.4 $\pm$ 7.6	32.5 $\pm$ 5.3	42.0 $\pm$ 2.1
Phosphodiesterase II	22.6 $\pm$ 7.6	57.6 $\pm$ 6.6	19.9 $\pm$ 3.0
Acid phosphatase	27.3 $\pm$ 4.7	59.9 $\pm$ 3.9	12.7 $\pm$ 1.4
Expt. (b)			
Protein	28.0 $\pm$ 7.3	25.9 $\pm$ 6.5	46.2 $\pm$ 2.7
Phosphodiesterase II	20.6 $\pm$ 6.1	59.8 $\pm$ 4.7	19.7 $\pm$ 1.9
Acid phosphatase	26.6 $\pm$ 10.6	59.5 $\pm$ 9.9	13.9 $\pm$ 3.2

Table 3. *Degree of activation of phosphodiesterase and acid phosphatase by Triton X-100 in particulate fractions of intestinal epithelial cells*

The degree of activation was obtained by dividing the total activity of a fraction by its 'free' activity. The latter was determined in the presence of 0.3 M-sucrose without added detergent. The means ($\pm$ S.D.) for the numbers of experiments shown in parentheses are given.

Fraction	Degree of activation			
	Guinea pig		Rat	
	Phosphodiesterase (5)	Acid phosphatase (4)	Phosphodiesterase (5)	Acid phosphatase (4)
(I)	1.97 $\pm$ 0.88	1.73 $\pm$ 0.32	1.01 $\pm$ 0.83	0.96 $\pm$ 0.16
(II)	2.09 $\pm$ 0.15	1.33 $\pm$ 0.15	1.30 $\pm$ 0.47	1.13 $\pm$ 0.29
(III)	2.50 $\pm$ 0.21	2.00 $\pm$ 0.29	1.57 $\pm$ 0.39	1.41 $\pm$ 0.31
(IV)	3.05 $\pm$ 0.58	2.06 $\pm$ 0.14	1.94 $\pm$ 0.59	1.21 $\pm$ 0.23
(V)	1.64 $\pm$ 0.28	1.36 $\pm$ 0.17	1.81 $\pm$ 0.87	1.03 $\pm$ 0.09

the rat and pH 6.6-6.8 for the guinea pig (Fig. 3). This small difference was also observed when other fractions from both animals were tested. Despite these differences it was possible to assay the maximum activity of both enzymes at pH 6.1 in sodium succinate buffer (see the Materials and Methods section) because guinea-pig intestinal phosphodiesterase II exhibited a relatively flat activity profile at pH values between 6.0 and 6.6 in that buffer.

Heat stability. The phosphodiesterase activities of guinea-pig and rat preparations differed markedly in their thermal sensitivities. Heating at 60°C for 12 min decreased guinea-pig activity to 20-28% of its original value, whereas only 0-2% of the activity of rat preparations remained after heating for half that time under these conditions.

Molecular weight. The two phosphodiesters had different molecular weights by gel-filtration chromatography on Sephadex G-150 columns. The rat enzyme had a molecular weight of 170000 and that

from guinea pig 125000. The standard proteins used were aldolase, *Escherichia coli* alkaline phosphatase, ovalbumin and chymotrypsinogen. Menon & Smith (1970) have reported that phosphodiesterase II from salmon testis has a molecular weight of 100000. It should be noted that the experiments described here were carried out at high ionic strength since the guinea-pig phosphodiesterase (but not the rat enzyme) tends to aggregate at *I* values less than 1.

Discussion

The experiments of van Dyck & Wattiaux (1968) and Erecinska *et al.* (1969) with rat liver provide strong evidence that phosphodiesterase II is lysosomal. Others, however, have reported the presence of the enzyme in other cell fractions in a variety of tissues, e.g. in the mitochondria of hog kidney (Razzell, 1961), in the soluble fraction of rat pancreas homogenates (van Venrooij & Poort, 1970) and in the nuclear frac-

Fig. 2. Sucrose-density-gradient centrifugation of fractions (III)+(IV) from guinea-pig epithelial-cell homogenates

The data on the right (B) were obtained from an animal that received a single intraperitoneal injection of Triton WR-1339 (1.3 mg/g body wt.) dissolved in 0.9% NaCl, 4 days before death, whereas the information on the left (A) came from a control animal that received a similar sized injection of 0.9% NaCl alone. Relative concentration values were calculated by dividing the enzyme concentration in a particular fraction (C) by the initial concentration (C_i) it would have had if it had been distributed homogeneously throughout the whole gradient. The distances from the top of the gradient (abscissa) were expressed as a percentage of the volume of the gradient; no correction was made for the sphericity of the tube bottom. The numbers in parentheses in each histogram represent the percentage recovery of activity in each case. (a) Succinate dehydrogenase; (b) acid phosphatase; (c) phosphodiesterase. For other details see the Materials and Methods section.

Fig. 3. Influence of pH on the activity of rat and guinea-pig phosphodiesterase II

By using method (2) the enzymic hydrolysis of thymidine 3'-(2,4-dinitrophenyl)phosphate (0.43 mM) was measured in mixtures composed of 25 mM-sodium succinate-25 mM-sodium phosphate-25 mM-Tris-HCl buffer adjusted to different pH values. The assays were done in the absence ($\square$, $\circ$) or presence ($\blacksquare$, $\bullet$) of 125 mM- $(NH_4)_2SO_4$ at 25°C and the pH of each reaction mixture was measured before stopping the reaction. A sonicated extract of fraction (III) from each animal was used. $\square$, $\blacksquare$, Rat enzyme (58 μ g of protein); $\circ$, $\bullet$, guinea-pig enzyme (89 μ g of protein).

tion of salmon testis preparations (Menon & Smith, 1970). In the present paper we also report a broad distribution of the enzyme in subcellular fractions of intestinal epithelial cells. However, the highest specific activity of the enzyme was always found in those fractions which were enriched with lysosomes. In addition, the guinea-pig enzyme followed a shift in lysosomal density when the animals were injected with the non-ionic detergent Triton WR-1339, a finding considered conclusive evidence of a lysosomal location (see Beaufay, 1972). However, the location of the rat enzyme is less obvious from our results. Although the specific activity was again high in the mitochondrial-lysosomal fraction, a large portion (40%) of the total activity appeared in the soluble fraction of the homogenate. This distribution was similar to that reported for rat mucosal scrapings (Flanagan, 1970; Hynie & Zbarsky, 1970) although less soluble activity was found in the present case.

A difference between the latency of guinea-pig and rat acid hydrolases was also noted, in agreement with the results of other investigators. Hubscher *et al.* (1965) observed a modest latency effect for a number of acid hydrolases in guinea-pig intestine whereas lack of such an effect has been reported for these enzymes in intestinal preparations from the rabbit (Porteous & Clark, 1965) and the rat (Gresham & Pover, 1967). In this connexion it may be significant that we were unable to demonstrate in the rat the

shift in density of particulate acid phosphatase and phosphodiesterase II on Triton WR-1339 administration which we observed with the guinea pig. Since lysosomes have been repeatedly seen in the epithelial cells of rat intestine (Behnke & Moe, 1964; Moe *et al.*, 1965; Trier, 1968), it is puzzling that acid hydrolases from these cells did not display the behaviour expected for lysosomal enzymes. Possibly this indicates that the granules in this tissue were more easily damaged by the manipulations used to prepare the tissue fractions than those from guinea-pig epithelium, since it is recognized that lysosomes from different sources vary greatly in their fragility (Turnbull & Neil, 1969; Beaufay, 1972; Reid, 1972).

Hsu & Tappel (1964) have reported that intestinal epithelium, in common with kidney (Straus, 1956), contains a more heterogeneous population of lysosomes than does liver. We have observed that the particulate distribution of acid phosphatase and phosphodiesterase in intestinal epithelium were not identical; granules containing phosphodiesterase II appeared to sediment slightly faster than those containing acid phosphatase. Hubscher *et al.* (1965) have also noted that some particles containing acid hydrolases sedimented from mucosal homogenates at low centrifugal forces. Perhaps the situation in the intestine is somewhat analogous to that in the liver where acid phosphatase and acid DNAase are contained in different populations of lysosomes (Beaufay *et al.*, 1964).

Finally, as de Duve *et al.* (1955) have pointed out the 'free' activity of enzymes can become redistributed among the fractions as a result of adsorption and other phenomena during fractionation studies. However, we do not feel that such artifacts were important in the present study since in experiments to test the possibility of adsorption only fraction (I) adsorbed any phosphodiesterase II activity from a soluble fraction of epithelial cells.

The phosphodiesterases of guinea-pig and rat intestine differed in other properties also. The rat enzyme had a higher molecular weight, was more labile at 60°C and exhibited a lower pH optimum. One explanation for these observed differences is that the activities studied arise from two different proteins. Another possibility which must be considered is that the differences are due to the presence of contaminating substances in the relatively crude preparations used in these experiments.

This work was supported by the Medical Research Council of Canada. P. R. F. was the recipient of a Medical Research Council Studentship and a University of British Columbia Graduate Fellowship.

References

- Ames, B. N. (1966) *Methods Enzymol.* **8**, 115-118

- Beaufay, H. (1972) in *Lysosomes, a Laboratory Handbook* (Dingle, J. T., ed.), pp. 1-45, North-Holland Publishing Co., London and Amsterdam
- Beaufay, H., Jacques, P., Baudhuin, P., Sellinger, O. Z., Berthet, J. & de Duve, C. (1964) *Biochem. J.* **92**, 184-205
- Behnke, O. & Moe, H. (1964) *J. Cell Biol.* **22**, 633-652
- Clark, B. & Porteous, J. W. (1965) *Biochem. J.* **96**, 539-551
- Connock, M. & Pover, W. (1970) *Histochem. J.* **2**, 371-380
- de Duve, C. (1965) *Harvey Lect.* **59**, 49-87
- de Duve, C., Pressman, B. C., Gianetto, R., Wattiaux, R. & Appelmans, F. (1955) *Biochem. J.* **60**, 604-617
- Erecinska, M., Sierakowska, H. & Shugar, D. (1969) *Eur. J. Biochem.* **11**, 465-471
- Evans, E. M., Wrigglesworth, J. M., Burdett, K. & Pover, W. F. R. (1971) *J. Cell Biol.* **51**, 452-464
- Flanagan, P. R. (1970) M.Sc. Thesis, University of British Columbia
- Flanagan, P. R. & Zbarsky, S. H. (1973) *Proc. Can. Fed. Biol. Soc.* **16**, 314
- Gresham, P. A. & Pover, W. F. R. (1967) *Int. J. Radiation Biol.* **13**, 275-278
- Hsu, L. & Tappel, A. L. (1964) *J. Cell Biol.* **23**, 233-240
- Hubscher, G. & West, G. R. (1965) *Nature (London)* **205**, 799-800
- Hubscher, G., West, G. R. & Brindley, D. N. (1965) *Biochem. J.* **97**, 629-642
- Hynie, I. & Zbarsky, S. H. (1970) *Can. J. Biochem.* **48**, 1141-1150
- Lowry, O. H., Rosebrough, N. J., Farr, A. L. & Randall, R. J. (1951) *J. Biol. Chem.* **193**, 265-275
- Menon, K. M. J. & Smith, M. (1970) *Biochemistry* **9**, 1584-1592
- Moe, H., Rostgaard, J. & Behnke, O. (1965) *J. Ultrastruct. Res.* **12**, 396-403
- Pennington, R. J. (1961) *Biochem. J.* **80**, 649-654
- Porteous, J. W. & Clark, B. (1965) *Biochem. J.* **96**, 159-171
- Rauenbusch, E. C. & Altman, K. L. (1960) *Proc. Soc. Exp. Biol. Med.* **104**, 385-388
- Razzell, W. E. (1961) *J. Biol. Chem.* **236**, 3028-3030
- Reid, E. (1972) in *Subcellular Components, Preparation and Fractionation* (Birnie, G. D., ed.), 2nd edn., pp. 93-118, Butterworths, London
- Robinson, G. B. (1963) *Biochem. J.* **88**, 162-168
- Schneider, W. C. (1957) *Methods Enzymol.* **3**, 680-684
- Straus, W. (1956) *J. Biophys. Biochem. Cytol.* **2**, 513-521
- Trier, J. S. (1968) in *The Handbook of Physiology* (Code, C. F., ed.), section 6, vol. 3, pp. 1125-1175, American Physiological Society, Washington, D.C.
- Turnbull, J. M. & Neil, M. W. (1969) *Biochem. J.* **111**, 503-507
- van Dyck, J. M. & Wattiaux, R. (1968) *Eur. J. Biochem.* **7**, 15-20
- van Venrooij, W. J. W. & Poort, C. (1970) *Eur. J. Biochem.* **13**, 391-397
- von Tigerstrom, R. G. & Smith, M. (1969) *Biochemistry* **8**, 3067-3070
- Wattiaux, R., Wibo, M. & Baudhuin, P. (1963) in *Ciba Symp.: Lysosomes* (de Reuck, A. V. S. & Cameron, M. P., eds.), pp. 176-200, J. and A. Churchill Ltd., London
- Wrigglesworth, J. M. & Pover, W. F. R. (1966) *Life Sci.* **5**, 1365-1371