Studies on the Fractionation of Mucosal Homogenates from the Small Intestine

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1. Homogenates of the mucosa of the small intestine of the guinea pig were separated by fractional sedimentation into seven different fractions. The enzymic properties of some of these subcellular fractions were compared with those obtained from the mucosa of the small intestine of the rabbit and cat. 2. The enzymic properties of the low-speed sediment (15 000g-min.) were investigated and it was shown that invertase and alkaline ribonuclease were predominantly located in this subcellular fraction, whereas alkaline phosphatase, aryl-amidase, acid phosphatase, acid ribonuclease and phosphoprotein phosphatase, though true constituents of this fraction, occurred to varying degrees in other subcellular structures also. 3. It was shown that the most probable source of the enzymic activities observed in the low-speed sediment was the brush border. Electron micrographs of the purified brush-border fraction indicated vesicles derived from the brush-border membrane. 4. A method is described for the fractionation of mucosal homogenates into a brush border-plus-nuclei fraction, a mitochondrial fraction, a microsomal fraction and a particle-free supernatant. The fractions were shown to be relatively pure, as indicated by the distribution of invertase, DNA, succinate dehydrogenase, glucose 6-phosphatase and 6-phosphogluconate dehydrogenase. 5. Most of the activity of four lysosomal enzymes present in the nuclei-free homogenate was sedimented at 375 000g-min., suggesting the occurrence of lysosomal particles in mucosal homogenates. 6. Further fractionation of the microsomal membranes into three fractions is described. The enzymic composition of the membrane fractions is given and discussed in relation to their structure as seen in electron micrographs.

The fractionation of homogenates of the mucosa of the small intestine has been described for preparations from the rat (Schmidt, Bessman & Thannhauser, 1957; Busch, Davis & Anderson, 1958; Triantaphyllopoulos & Tuba, 1959; Epstein & Shapiro, 1959; Senior & Isselbacher, 1962; Ailhaud, Samuel & Desnuelle, 1963; Robinson, 1963), the hamster (Brown & Johnston, 1964), the guinea pig (Glover & Green, 1957; Sherratt & Hübscher, 1963), the rabbit (Doell & Kretschmer, 1962; Carnie & Porteous, 1962*b*; Porteous & Clark, 1963), the cat (Hübscher, Clark, Webb & Sherratt, 1963), the dog (Baker, 1958), the pig (Buell & Reiser, 1959; Borgström & Dahlqvist, 1958), the calf (Morton, 1954) and man (Doell & Kretschmer, 1962). The suspending media described in these procedures vary in composition, and include salt solutions (Schmidt *et al.* 1957; Buell & Reiser, 1959), 0.25 M- or 0.3 M-sucrose (Busch *et al.* 1958; Baker, 1958;

Hübscher *et al.* 1963) and buffered mannitol solutions (Senior & Isselbacher, 1962).

It is difficult to assess the efficiency of separation of subcellular structures achieved by any of these methods since, with a few exceptions, no attempts were made to ascertain the purity of the fractions prepared. The most careful study so far undertaken is that of Porteous & Clark (1963), who examined the subcellular fractions prepared from rabbit small-intestinal mucosa by electron microscopy and by assaying a number of characteristic enzymes and cellular constituents.

The greatest difficulty encountered in the fractionation of mucosal homogenates is the heavy cross-contamination of subcellular fractions due to the presence of mucus. Busch *et al.* (1958) were probably the first to point out that the nuclear fraction comprises nuclei and a gelatinous material that may represent a relatively large volume of the

original homogenate. As a result, in many studies, extremely large volumes were employed in the preparation of the homogenate, or the nuclei-free homogenate was used as the starting material, disregarding all metabolic activities that may have been sedimented with the first low-speed centrifugation. Hübscher *et al.* (1963) reported the specific precipitation of the gelatinous material by the addition of a barium sulphate suspension to the homogenate.

Further difficulties arise because the cell of the intestinal mucosa has, in comparison with the liver cell, additional structural features such as the brush border and the terminal web. Miller & Crane (1961) described a method for the isolation of a brush-border fraction from the intestinal mucosa of the hamster by using a strongly hypo-osmotic medium, but no conclusions can be drawn about the sedimentation properties of this structure in iso-osmotic media with a higher density. Finally, the microsomal fraction obtained by conventional fractionation procedures is a mixture of several subcellular structures such as the smooth-surface membranes of the Golgi apparatus, the rough-surface membranes of the endoplasmic reticulum and possibly fragments of the cell wall.

The present investigation deals with the fractionation of the intestinal mucosa of the guinea pig and the intracellular distribution of a number of enzymes and other cell constituents. Several of these enzymes were reported to be of lysosomal origin in rat liver. A subfractionation of the microsomal fraction into three different components is also reported.

MATERIALS AND METHODS

Animals. Adult guinea pigs and rabbits of either sex and cats aged from 9 months to 2 years, also of either sex, were used. They were starved for approx. 16 hr. before the experiments.

Determination of RNA. RNA was determined by the method of Schneider (1957) by using the orcinol colour reaction for ribose. The concentration of RNA was calculated from a standard curve, which was prepared anew with each batch of determinations. The results were expressed in $\mu\text{g.}$ of RNA phosphorus and a hydrolysed yeast-RNA preparation served as standard.

Determination of DNA. Burton's (1956) modified diphenylamine reaction was employed, with the trichloroacetic acid extraction procedure described by Schneider (1957). A DNA preparation from thymus was used as standard.

Determination of protein. Protein was estimated by the method of Weichselbaum (1946) with slight modification. The protein was precipitated with trichloroacetic acid (final concn. 5%, w/v), and the precipitate was collected by centrifugation and then suspended in the biuret reagent by stirring with a glass rod. This procedure was included since sucrose as well as several ions were found to interfere. After incubation the solutions were extracted with 2 ml. of

water-saturated diethyl ether to remove turbidity due to lipid. Crystalline bovine serum albumin obtained from the Armour Pharmaceutical Co. Ltd. (Eastbourne, Sussex) was taken as the standard.

Determination of leucine aminopeptidase (aryl-amidase) (EC 3.4.1.1). The method of Goldbarg & Rutenberg (1958) was slightly modified. The assay system included, in a final volume of 2 ml., 50 mM-Sörensen phosphate buffer, pH 7.0, 0.53 mg. of L-leucyl- β -naphthylamide hydrochloride and the enzyme. The fractions of the intestinal mucosa had to be diluted and this was done with distilled water just before the assay. After incubation at 37° for 40 min., the reaction was stopped by adding 1 ml. of 10% (w/v) trichloroacetic acid. The colour was developed exactly as described by Goldbarg & Rutenberg (1958). Because of the different intracellular distribution of the enzyme hydrolysing L-leucylglycine (Robinson, 1963) and the increasing evidence for the occurrence of two leucine aminopeptidases in one tissue (Fleischer, Pankow & Warmka, 1964), the enzyme studied in the present investigation is referred to as 'aryl-amidase'.

The amount of β -naphthylamine liberated was estimated from a standard curve obtained by taking known amounts of β -naphthylamine through the second stage of the assay procedure in which β -naphthylamine is converted into a coloured azo derivative. Under the conditions used (i.e. a final volume of 5 ml.), 10 $\mu\text{g.}$ of β -naphthylamine gave an extinction of 0.48 at 560 $m\mu$ (1 cm. light-path).

Determination of succinate dehydrogenase (EC 1.3.99.1). The activity of this enzyme was determined as described by Pennington (1961) by using 2-(*p*-iodophenyl)-3-(*p*-nitrophenyl)-5-phenyltetrazolium chloride as the electron acceptor. A slight reduction of this dye occurred in the absence of succinate and this was allowed for in the determination.

Determination of β -glucuronidase (EC 3.2.1.31). The enzyme was estimated by measuring the hydrolysis of phenolphthalein glucuronide according to the method of Gianetto & de Duve (1955). After incubation for 30 min. at 37°, the reaction was stopped by the addition of 6 ml. of a glycine-NaOH-NaCl buffer, pH 10.7 (0.106 M-glycine-0.094 N-NaOH-0.106 M-NaCl). The reaction mixture was centrifuged in a bench centrifuge and the phenolphthalein content of the supernatant liquid determined colorimetrically by using $\epsilon_{550m\mu}^{1\text{cm.}}$ 2.26×10^4 (Talalay, Fishman & Huggins, 1946) for the calculation.

Determination of β -galactosidase (EC 3.2.1.23). The enzyme was assayed by an adaptation of the method of Seidman & Link (1950). The incubation mixture included, in a final volume of 2 ml., 0.65 M-sodium acetate buffer, pH 5.2, 12.5 mM-*o*-nitrophenyl galactoside and the enzyme. The mixture was incubated for 30 min. at 37° and the reaction stopped by the addition of 2 ml. of 0.4 M-glycine-NaOH buffer, pH 10.8. After centrifugation in a bench centrifuge the concentration of *o*-nitrophenol in the supernatant liquid was measured optically at 430 $m\mu$; the extinction coefficient of *o*-nitrophenol was found to be $\epsilon_{430m\mu}^{1\text{cm.}}$ 2.3×10^3 .

Determination of acid ribonuclease (EC 3.1.4.-). The ribonuclease was estimated by a slight modification of the method of de Duve, Pressman, Gianetto, Wattiaux & Appelmans (1955). The assay system consisted of 0.1 M-sodium acetate buffer, pH 5.0, and 3 mg. of purified sodium ribonuclease (see below). Enzyme and water were added to

give a final volume of 2 ml. After incubation for 30 min. at 37°, the reaction was stopped by adding 2 ml. of 2.5% (w/v) trichloroacetic acid containing 0.25% (w/v) of uranyl acetate. The mixture was left for 1 hr. at 0° and then centrifuged. The supernatant liquid was diluted with water before reading the extinction at 260 m μ . An assumed average extinction coefficient of $\frac{1}{\epsilon_{260\text{m}\mu}} 8.5 \times 10^3$ was used for the calculation of the amounts of product formed, as recommended by Stimson & Reuter (1945). The purified sodium ribonucleate used as substrate was prepared according to the method of Kunitz (1941) by dissolving yeast RNA in dilute NaOH followed by dialysis in the cold against 200 vol. of 0.1M-sodium acetate buffer, pH 5.0.

Determination of alkaline ribonuclease (EC 3.1.4.-). The assay system was that of P. Gresham & W. F. R. Pover (personal communication) and contained, in a final volume of 2 ml., 50 mM-veronal-HCl buffer, pH 8.6, 0.25 mM-CuSO₄, 2.5 mg. of ribonucleate and enzyme. The CuSO₄ was added to inhibit a ribonuclease with pH optimum 7.7 that is different from the ribonuclease assayed in this system and also different from the acid ribonuclease mentioned above (P. Gresham & W. F. R. Pover, personal communication). After incubation at 37° for 30 min., the reaction was stopped by adding 2 ml. of 2.5% (w/v) trichloroacetic acid containing 0.25% (w/v) of uranyl acetate. Estimation of liberated nucleotides followed, by the method described above.

Determination of phosphoprotein phosphatase (EC 3.1.3.16). The procedure of Paigen & Griffiths (1958) was employed except that the reaction was stopped by adding 2 ml. of 5% (w/v) trichloroacetic acid containing 12.5% (w/v) of charcoal (Norit A). The mixture was shaken and filtered, and inorganic phosphate was determined in the filtrate according to the method of King (1932).

Determination of alkaline phosphatase (EC 3.1.3.1), acid phosphatase (EC 3.1.3.2) and glucose 6-phosphatase (EC 3.1.3.9). The methods described by Hübscher & West (1965) were used.

Determination of 6-phosphogluconate dehydrogenase (EC 1.1.1.44). The dehydrogenase activity was determined according to the method of Glock & McLean (1953) by following spectrophotometrically the rate of reduction of NADP at 340 m μ .

Determination of invertase (EC 3.2.1.26). Before assay the subcellular fractions were dialysed for approx. 16 hr. against 200–300 vol. of 10 mM-Sørensen phosphate buffer, pH 7.0. This was done to remove glucose, which was liberated from the preparations during the isolation of the fractions in 0.3M-sucrose. The assay system was based on that of Carnie & Porteous (1962a). The incubation mixture included, in a final volume of 1 ml., 0.04M-sodium phosphate buffer, pH 6.7, 0.1M-sucrose and the enzyme. The reaction was started by addition of enzyme and, after incubation at 37° for not more than 15 min., was terminated by immersing the tubes in boiling water for 3 min. The tubes were then cooled and 1 ml. of water was added to each, after which the tubes were centrifuged in a bench centrifuge. Portions of the supernatant liquid were withdrawn for the determination of glucose.

Glucose was determined by the glucose-oxidase method. Details of the method as well as the reagents were obtained from C. F. Boehringer und Soehne G.m.b.H., Mannheim, Germany. Since the commercial enzyme preparation contained invertase, it was heated for 10 min. at 60° in the

buffered solution before the addition of the chromogen. This procedure selectively inactivated invertase without affecting the glucose oxidase.

Determination of phosphatidate phosphatase (EC 3.1.3.4) and of acyl-CoA synthetase (EC 6.2.1.2). These enzymes were determined as described by Brindley & Hübscher (1965).

Preparation of homogenates of the mucosa of the small intestine. The whole of the small intestine of a guinea pig was removed immediately after death and irrigated with ice-cold sucrose, which was adjusted to pH 7.4 by adding a small amount of KHCO₃. The small intestine was placed on a cold glass plate and the mucosa collected by gentle scraping with a wooden spatula. The mucosal scrapings were suspended in about 20 ml. of 0.3M-sucrose, pH 7.4, and homogenized by six up-and-down strokes at 600 rev./min. with a Teflon-glass homogenizer (radius clearance 0.0055–0.0075 in.). Finally, the homogenate was filtered through a nylon cloth (St Martins Bolting Cloth, 9N obtained from Henry Simon Ltd., Cheadle Heath, Cheshire). The filtrate was made up to 50 ml. with 0.3M-sucrose, pH 7.4. The entire procedure was done at 4°.

The method of preparing homogenates from cat or rabbit small-intestinal mucosa was the same except that the volume of the filtered homogenate was approx. 120 ml.

Fractionation of mucosal homogenates. The centrifuges used for the preparation of subcellular fractions were the MSE High Speed 18 refrigerated centrifuge (with 8 × 50 ml. angle rotor; maximum radius 10.4 cm.) and the MSE Super Speed 40 refrigerated centrifuge (with either 8 × 50 ml. or 8 × 25 ml. angle rotors and maximum radii of 10.7 and 7.8 cm. respectively). The *g* values quoted are maximum R.C.F. values. The filtered homogenate obtained from one guinea pig was centrifuged at 15000*g*-min. (10 min. at 1500*g*). The sediment was suspended in 30 ml. of 0.3M-sucrose, pH 7.4, homogenized as described above but with only three up-and-down strokes and centrifuged at 15000*g*-min. This procedure was repeated three times and the final sediment is referred to as 'brush border-plus-nuclei fraction'.

The combined supernatants were centrifuged at 335000*g*-min. (15 min. at 22300*g*) and the sediment was resuspended in 30 ml. of 0.3M-sucrose, pH 7.4. The sediment was suspended by gentle homogenization and centrifuged at 335000*g*-min. This was repeated once, and the washed sediment is referred to as 'mitochondrial fraction'. The combined supernatant fluids were then centrifuged at 840000*g*-min. (60 min. at 140000*g*), giving a 'microsomal sediment' and a 'particle-free supernatant'.

All particulate subcellular fractions were suspended in a small volume of 0.3M-sucrose, pH 7.4, and stored at -8° or -20°, if not assayed on the same day.

The method used for the fractionation of homogenates of cat or rabbit small-intestinal mucosa was the same except that the volumes were increased correspondingly.

Subdivision of the microsomal fraction of guinea-pig and cat small-intestinal mucosa. The methods used were similar to those described by Dallner (1963) and Dallner, Orrenius & Bergstrand (1963) for preparations from rat liver. Slight modifications were made to suit the fractionation of preparations from the intestinal mucosa and the different type of centrifuge used. Two methods were used (Scheme 1), both relying on separation by means of a sucrose density gradient containing relatively high concentrations of metal ions.

Method I: separation in a sucrose density gradient containing MgCl_2 . The filtered homogenate obtained from one guinea-pig or one cat small intestine was centrifuged at 335000g-min. and the sediment, after resuspension and homogenization in 30 ml. of 0.3M-sucrose, was washed twice at the same centrifugal force. Then 1 ml. of M- MgCl_2 /100 ml. was added to the combined supernatants with continuous stirring. A small portion was taken and centrifuged at 840000g-min., the resulting sediment representing the 'total microsomal fraction'. Portions (6 ml.) of the remainder were layered over 12 ml. of 1.5M-sucrose-15 mM- MgCl_2 in 25 ml. centrifuge tubes. Centrifugation was performed in the MSE Super Speed centrifuge at 40000 rev./min. in an angle rotor, the tube angle with the rotor axis being 20° and the average radius 5.84 cm.

Centrifugation at 1680000g-min. (120 min. at 140000g) gave a pellet at the bottom of the tube and a turbid layer at the boundary of the two sucrose solutions. The upper layer and the turbid layer at the boundary were withdrawn and centrifuged together at 840000g-min., the resulting pellet being referred to as 'smooth-vesicle fraction'. The lower layer, which was usually not quite clear, was decanted, diluted with sufficient water to give a final concentration of 0.3M-sucrose and centrifuged at 840000g-min. The pellet was collected and combined with that obtained in the

initial density-gradient centrifugation, giving the 'rough-vesicle fraction'. All pellets were taken up in 0.1M-sucrose and, if not immediately assayed, stored at -20° .

Method II: separation in sucrose density gradients containing CsCl and CsCl plus MgCl_2 . The 335000g-min. supernatant was prepared as described for method I except that the intestinal mucosa of three guinea pigs was used. Then 1 ml. of 1.5M-CsCl was added/100 ml. of supernatant and a small portion was taken and centrifuged at 840000g-min. The sediment represented the 'total microsomal fraction'. The remainder was layered over 1.3M-sucrose-15 mM-CsCl by using 12 ml. of upper layer and 6 ml. of lower layer. Centrifugation at 1680000g-min. resulted in a pellet at the bottom of the tube ('pellet 1'), a slightly turbid lower layer ('fraction S-1') and a strongly turbid layer at the boundary plus an almost clear upper layer ('fraction S-2'). These were collected separately.

Fraction S-1 was diluted with water to give a final concentration of 0.3M-sucrose and centrifuged at 840000g-min. The sediment was collected and combined with pellet 1, giving the 'rough-vesicle fraction'. Then 1 ml. of M- MgCl_2 was added/100 ml. of fraction S-2, and 12 ml. portions were layered over 6 ml. of 1.15M-sucrose-10 mM- MgCl_2 . After centrifugation at 1680000g-min. the contents of the centrifuge tubes had the same appearance as in the initial

Method I: separation in a sucrose density gradient in the presence of MgCl_2

Method II: separation in a sucrose density gradient in the presence of CsCl and MgCl_2

Scheme 1. Diagrammatic representation of the methods employed for separating microsomal membranes of intestinal mucosa.

density-gradient centrifugation and were labelled 'pellet 2', 'fraction S-3' and 'fraction S-4' respectively.

Fraction S-3 was treated as described for fraction S-1 and the sediment was combined with pellet 2, giving the smooth-vesicle fraction I ('fraction SVFI'). Fraction S-4 was centrifuged at 840000g-min. and the resulting pellet, the smooth-vesicle fraction II ('fraction SVFII'), was collected.

Preparation of electron micrographs. The tissue samples were fixed in veronal buffer, pH 7.0, containing 1% of OsO_4 , dehydrated in alcohols and embedded in Araldite. Sections were cut with a Huxley microtome and examined in a Siemens Elmiskop I.

EXPERIMENTAL AND RESULTS

Preparation and properties of the low-speed sediment. Centrifugation of homogenates of the small-intestinal mucosa of the guinea pig at 15000g-

min. gave sediments containing 33–51% of the succinate-dehydrogenase activity and 34–55% of the glucose 6-phosphatase activity of the original homogenate, indicating a high degree of contamination with mitochondrial and microsomal particles. After resuspending the sediment in 30ml. of 0.3M-sucrose, pH 7.4, followed by homogenization and centrifugation at 15000g-min., a sediment was obtained that contained only 20–25 and 27–29% respectively of the succinate-dehydrogenase and glucose 6-phosphatase activities of the original homogenate. Three further washings of the low-speed sediment resulted in further decreases of the mitochondrial and microsomal contaminations (see Fig. 1). Also shown in Fig. 1 are the recoveries of DNA, invertase, alkaline phosphatase, acid phosphatase and β -glucuronidase. Whereas DNA and invertase were retained to a high degree in the low-speed sediment, only 62, 41 and 24% of the alkaline-phosphatase, acid-phosphatase and β -glucuronidase activities respectively were recovered. Because the enzymic activities of only the sediments were assayed the recovery of enzyme activity was not known and no conclusions could be drawn. However, in subsequent experiments it was observed that the percentage of enzymic activities retained in the low-speed sediment was fairly constant and in good agreement with the values quoted in Fig. 1 (see Table 1). Further, the total activity recovered in the low-speed sediment and supernatant fraction was, with the exception of acid phosphatase, near 100%. From these results one can conclude that there was no inactivation of enzymes during the repeated washing of the sediment fraction. Since the low-speed sediment was only slightly contaminated with mitochondrial and microsomal particles, as indicated by the low activities of succinate dehydrogenase and glucose 6-phosphatase, it seems justifiable to assume that enzymes with a higher specific activity in the sediment than in the original homogenate are true constituents of this fraction.

In view of their high recovery, alkaline ribonuclease and invertase may be regarded as being predominantly, if not exclusively, located in the low-speed sediment. Alkaline phosphatase, arylamidase, acid phosphatase, acid ribonuclease and phosphoprotein phosphatase are most likely to be located in more than one subcellular structure because their recovery in the low-speed sediment was found to lie between that of the mitochondrial and microsomal enzymes and that of alkaline ribonuclease and invertase. A partial solubilization of this group of enzymes from the low-speed sediment can be excluded as far as arylamidase, alkaline phosphatase and acid phosphatase are concerned. These three enzymes were found to be retained by the brush-border fraction of the low-

Fig. 1. Enzymic composition of low-speed sediments. A homogenate of the small-intestinal mucosa of the guinea pig was centrifuged at 15000g-min., and the sediment (1) obtained was suspended in 0.3M-sucrose and repeatedly washed at 15000g-min. to give sediments 2–5. x, Succinate dehydrogenase; Δ, glucose 6-phosphatase; ●, alkaline phosphatase; ○, acid phosphatase; ▲, β -glucuronidase; □, invertase; ■, DNA.

Table 1. *Enzymic composition of a sediment obtained from guinea-pig intestinal mucosa after repeated low-speed centrifugation*

The original homogenate ('homogenate') was centrifuged at 15000g-min. The resulting sediment was collected and suspended in 0.3M-sucrose. It was again centrifuged at 15000g-min. This washing of the sediment was repeated three more times. The supernatants were combined ('supernatant') before assay. The values quoted are the averages of two preparations. Specific activities are expressed as μ moles of substrate converted/15min./mg. of protein, and total activities as μ moles of substrate converted/15min./total fraction; total DNA is expressed as μ g. of DNA phosphorus, and total protein as mg. of protein.

	Homogenate			Sediment		Supernatant	
	Specific activity	Total activity	Specific activity	% of total	Purification	Specific activity	% of total
DNA	—	17.3 $\pm$ 2.5	—	102.0 $\pm$ 2.0	—	—	< 0.5
Alkaline ribonuclease	0.174 $\pm$ 0.052	53.4 $\pm$ 7.8	0.884 $\pm$ 0.116	85.0 $\pm$ 5.0	5.1	0.048 $\pm$ 0.010	23.1 $\pm$ 6.0
Invertase	0.743 $\pm$ 0.062	224.5 $\pm$ 18	3.65 $\pm$ 0.52	82.1 $\pm$ 5.2	4.9	0.455 $\pm$ 0.047	10.3 $\pm$ 0.8
Alkaline phosphatase	4.81 $\pm$ 0.84	1435 $\pm$ 30	18.50 $\pm$ 3.65	65.8 $\pm$ 3.1	3.9	1.34 $\pm$ 0.22	24.1 $\pm$ 0.3
Aryl-amidase	1.38 $\pm$ 0.16	415.3 $\pm$ 18.3	5.18 $\pm$ 0.30	63.2 $\pm$ 3.3	3.8	0.59 $\pm$ 0.20	36.6 $\pm$ 5.1
Acid phosphatase	0.209 $\pm$ 0.013	62.4 $\pm$ 5.8	0.683 $\pm$ 0.104	54.9 $\pm$ 4.8	3.3	0.573 $\pm$ 0.067	232 $\pm$ 9.0
Acid ribonuclease	0.356 $\pm$ 0.092	109 $\pm$ 21.3	0.685 $\pm$ 0.210	32.3 $\pm$ 6.8	1.9	0.328 $\pm$ 0.075	77.5 $\pm$ 9.4
Phosphoprotein phosphatase	0.084 $\pm$ 0.008	25.3 $\pm$ 1.3	0.121 $\pm$ 0.015	24.0 $\pm$ 0.6	1.4	0.073 $\pm$ 0.009	73.1 $\pm$ 1.4
β -Glucuronidase	0.012 $\pm$ 0.001	3.5 $\pm$ 0.5	0.013 $\pm$ 0.001	18.5 $\pm$ 0.7	1.1	0.012 $\pm$ 0.001	86.3 $\pm$ 2.0
Glucose 6-phosphatase	0.716 $\pm$ 0.069	214.5 $\pm$ 14.5	0.461 $\pm$ 0.017	11.0 $\pm$ 1.3	—	0.753 $\pm$ 0.040	89.5 $\pm$ 3.0
β -Galactosidase	0.307 $\pm$ 0.030	95.6 $\pm$ 23.9	0.204 $\pm$ 0.022	10.7 $\pm$ 1.3	—	0.354 $\pm$ 0.037	94.7 $\pm$ 0.3
Succinate dehydrogenase	0.181 $\pm$ 0.005	56.2 $\pm$ 9.3	0.072 $\pm$ 0.004	6.5 $\pm$ 0.4	—	0.204 $\pm$ 0.005	94.7 $\pm$ 0.2
Protein	—	306.5 $\pm$ 47.5	—	16.5 $\pm$ 0.5	—	—	84.1 $\pm$ 0.5

speed sediment under less mild conditions than were used for the preparation of the low-speed sediment (see below).

The sum of the acid-phosphatase activities recovered in the low-speed sediment and supernatant fraction was 2.8 times that of the original homogenate. This may have been due to an activation of the enzyme in the supernatant fraction during preparation. Gianetto & de Duve (1955) reported on the activation of lysosomal enzymes from rat liver by homogenization in a Waring Blendor. Although treatment in a Waring Blendor was not used in the preparation of the supernatant fraction such a treatment of a mucosal homogenate from the small intestine of the guinea pig brought about an activation of some acid hydrolases (see Table 2). If acid ribonuclease, acid phosphatase and β -glucuronidase, which were found to be significantly activated, were contained in lysosomal particles as defined by de Duve (1963) for liver, they should be washed out from the low-speed sediment at similar rates. However, this was not the case (see Fig. 1 and Table 1). From this it follows that in the intestinal mucosa of the guinea pig some acid hydrolases are located in more than one subcellular structure. One of the subcellular structures containing acid hydrolases sedimented from mucosal homogenates at low centrifugal forces. These forces are far too low for the sedimentation of lysosomal particles as isolated from liver homogenates (de Duve *et al.* 1955).

The main components of the low-speed sediment were likely to be nuclei and brush border. Electron microscopy and DNA analysis showed that this was in fact so (see also Plate 1). However, with respect to the enzymic composition of the low-speed sediment, further fractionation was required to establish the exact site of the enzymic activities. A low-speed sediment was washed four times in 0.3M-sucrose. The washed sediment was then suspended in 0.03M-sucrose and treated in a Waring Blendor for 2min. After centrifugation at 500000g-min., a sediment and a supernatant fraction were obtained, the analyses of which are summarized in Table 3. Most of the DNA was recovered in the supernatant fraction, whereas approximately three-quarters of the enzymic activity of aryl-amidase, alkaline ribonuclease, alkaline phosphatase, invertase and acid phosphatase was in the sediment fraction.

Electron micrographs of the washed low-speed sediment showed mainly intact brush-border structures, nuclei, mucus and some mitochondrial contamination together with a few rough-surface vesicles (see Plate 1). The diameter of the microvilli varied a great deal and there were signs of formation of vesicles at the tip of some microvilli. Only very few intact brush-border structures could be seen

EXPLANATION OF PLATE I

Low-speed sediment of a homogenate of guinea-pig small-intestinal mucosa, washed four times. The enzymic composition of this fraction is given in Table 1. In (a) (magnification $\times 9000$), brush borders, parts of the lateral cell membranes, smooth-surface vesicles and some mucus can be seen. In (b) (magnification $\times 36000$), the varying diameters of the microvilli are shown in more detail than can be seen in (a).

EXPLANATION OF PLATE 2

Purified brush-border fraction prepared from guinea-pig small-intestinal mucosa. The enzymic composition of this fraction is summarized in Table 3 (sediment I). Both (a) (magnification $\times 18000$) and (b) (magnification $\times 36000$) show the mainly vesicular nature of the membranes obtained. In some instances, the membranes of the vesicles are still continuous with part of a microvillus, indicating their origin.

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(a)

(b)

(c)

EXPLANATION OF PLATE 3

Membrane fractions prepared from the microsomal fraction of cat small-intestinal mucosa. The enzymic composition of these fractions is shown in Table 8. For the preparation of the electron micrographs, the fractions were treated as indicated in the Materials and Methods section and additionally stained with uranyl acetate after cutting. The fractions represented are: (a) (magnification $\times 54000$) rough-vesicle fraction; (b) (magnification $\times 54000$) fraction SVFI; (c) (magnification $\times 54000$) fraction SVFII.

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Table 2. *Activation of some acid hydrolases of guinea-pig intestinal mucosa*

Scrapings of the intestinal mucosa were homogenized in 0.3 M-sucrose in a Potter-Elvehjem homogenizer (fresh homogenate) or were further homogenized for 4 min. in a Waring Blendor (Blendor-treated homogenate). For comparison, the change in activity of succinate dehydrogenase is included. Activities are expressed as μ moles of substrate converted/15 min./ml.

Animal	Enzyme tested	Activity		Activation
		Fresh homogenate	Blendor-treated homogenate	
Cat	β -Galactosidase	0.465	0.602	1.3
Cat	β -Glucuronidase	0.058	0.086	1.5
Guinea pig	Phosphoprotein phosphatase	1.86	1.98	1.1
Guinea pig	β -Galactosidase	0.780	0.849	1.1
Guinea pig	β -Glucuronidase	0.041	0.076	1.9
Guinea pig	Acid phosphatase	2.87	5.75	2.0
Guinea pig	Acid ribonuclease	0.185	0.564	3.0
Guinea pig	Succinate dehydrogenase	0.667	0.631	0.9

Table 3. *Enzymic composition of a purified brush-border fraction from guinea-pig intestinal mucosa*

The purified brush-border fraction (sediment I) was obtained by preparing a washed low-speed sediment as described in the legend of Table 1. The low-speed sediment was then treated as indicated in the text, giving, after centrifugation at 500000g-min., a sediment (sediment I) and a supernatant (supernatant I). Specific activities are expressed as μ moles of substrate converted/15 min./mg. of protein, and total activities as μ moles of substrate converted/15 min.; total DNA is expressed as μ g. of DNA phosphorus, and total protein as mg. of protein. The values in parentheses indicate the ranges.

	No. of preparations tested	Low-speed sediment		Sediment I		Supernatant I	
		Specific activity	Total activity	Specific activity	% of total	Specific activity	% of total
Protein	3	—	35.0 (31.4-41.9)	—	34.8 (31.2-39.3)	—	58.4 (55.2-60.1)
DNA	3	—	8.2 (7.2-9.1)	—	6.5 (4.9-7.7)	—	91.6 (86.8-96.3)
Alkaline phosphatase	3	14.16 (7.32-23.10)	489 (227-734)	30.81 (16.87-39.40)	79.8 (67.7-91.1)	4.48 (4.02-5.84)	21.8 (18.7-27.1)
Aryl-amidase	3	5.29 (4.3-6.0)	186 (167-201)	11.85 (9.9-12.2)	83.0 (80.2-88.1)	1.81 (1.46-2.0)	21.5 (19.0-25.2)
Alkaline ribonuclease	2	0.736 (0.688-0.784)	25.4 (22.3-28.5)	1.51 (1.24-1.78)	67.1 (61.1-73.1)	0.213 (0.117-0.309)	17.3 (7.9-26.7)
Acid phosphatase	2	0.533 (0.505-0.561)	16.0 (15.9-16.1)	1.12 (1.05-1.19)	75.4 (66.7-82.1)	0.214 (0.166-0.262)	21.5 (18.7-24.2)
Invertase	1	1.44	45.6	3.25	89.5	0.44	17.7

in the purified brush-border fraction (see Plate 2; for the enzymic composition of this fraction see sediment 1, Table 3). Instead, numerous vesicles were seen arranged in groups and in some instances still of such a shape as to suggest that they were derived from microvilli. The membrane dimensions of the vesicles were similar to those of the brush-border membrane and significantly different from those of the microsomal vesicles (cf. Finean, 1965; Wilson, 1962).

The results obtained by enzyme assay and by electron microscopy suggest therefore that the enzymic activities observed in the original low-speed sediment were mainly, if not exclusively, due to the brush border.

Fractionation of mucosal homogenates. The combined supernatant liquids obtained from repeated washing of the low-speed sediment were further fractionated into mitochondrial, microsomal and particle-free supernatant fractions (see the

Table 4. *Enzymic composition of subcellular fractions from guinea-pig intestinal mucosa*

The values quoted are averages of two fractionations, except for invertase, which was assayed in only one fractionation procedure. Specific activities are expressed as μ moles of substrate converted/15 min./mg. of protein. The values in parentheses refer to the total activity of the homogenate expressed as μ moles of substrate converted/15 min. Fractions were prepared as described in the Materials and Methods section.

	Protein		DNA		Invertase		Alkaline phosphatase		Succinate dehydrogenase		Glucose 6-phosphatase		6-Phosphogluconate dehydrogenase	
	mg./fraction	% of total	μ g. of DNAP	% of total	Specific activity	% of total	Specific activity	% of total	Specific activity	% of total	Specific activity	% of total	Specific activity	% of total
Homogenate	261.5 ± 76.5	100	15.9 ± 3.8	100	0.93 (248)	100	4.35 ± 0.46	100 (1120 ± 38)	0.204 ± 0.044	100 (51.7 ± 3.8)	0.682 ± 0.150	100 (184 $\pm$ 65)	0.207 ± 0.058	100 (53.8 ± 23.5)
Brush-border + nuclei fraction	44.8 ± 8.9	17.2 $\pm$ 1.0	13.6 ± 2.7	86.5 $\pm$ 3.6	4.05	73.3	16.58 ± 1.42	65.0 $\pm$ 5.2	0.113 ± 0.029	9.8 $\pm$ 0.3	0.300 ± 0.113	7.3 $\pm$ 1.6	—	Absent
Mitochondrial fraction	38.9 ± 4.2	14.9 $\pm$ 0.5	0.6 ± 0.3	4.5 $\pm$ 2.9	0.73	11.3	4.23 ± 0.65	14.6 $\pm$ 1.0	1.133 ± 0.223	84.1 $\pm$ 0.4	0.638 ± 0.227	13.6 $\pm$ 1.3	—	Absent
Microsomal fraction	47.6 ± 0.9	18.2 $\pm$ 2.2	0.05 ± 0.05	0.3 $\pm$ 0.3	0.44	8.5	3.99 ± 0.62	16.8 $\pm$ 3.1	0.017 ± 0.016	1.6 $\pm$ 0.7	2.968 ± 1.812	76.1 $\pm$ 4.3	0.044 ± 0.037	3.8 $\pm$ 3.2
Particle-free supernatant	129.0 ± 26	49.4 $\pm$ 3.1	0.4 ± 0.4	2.1 $\pm$ 2.1	—	Absent	0.09 ± 0.07	1.0 $\pm$ 1.0	0.001 ± 0.001	0.3 $\pm$ 0.3	—	Absent	0.451 ± 0.088	109.7 ± 3.8
Recovery (%)	...	99.7	—	93.4	—	93.1	—	97.4	—	95.8	—	97.0	—	113.5

Table 5. *Enzymic composition of subcellular fractions from rabbit intestinal mucosa*

Specific activities are expressed as μ moles of substrate converted/15 min./mg. of protein. The values in parentheses refer to the total activity of the homogenate expressed as μ moles of substrate converted/15 min. Fractions were prepared as described in the Materials and Methods section.

	Protein		DNA		Invertase		Aryl-amidase		Succinate dehydrogenase		Glucose 6-phosphatase		6-Phosphogluconate dehydrogenase	
	mg./fraction	% of total	μ g. of DNAP	% of total	Specific activity	% of total	Specific activity	% of total	Specific activity	% of total	Specific activity	% of total	Specific activity	% of total
Homogenate	497	100	14.6	100	0.93 (463)	100	1.42 (710)	100	0.154 (76.3)	100	0.122 (60.6)	100	0.395 (191)	100
Brush-border + nuclei fraction	63	12.7	13.2	90.5	4.51	61.4	5.15	45.7	0.063	5.2	0.115	12	—	Absent
Mitochondrial fraction	70	14.1	0.2	1.4	1.05	15.9	2.45	24.2	0.910	83.4	0.165	19	—	Absent
Microsomal fraction	71	14.3	0.2	1.4	1.25	19.1	1.64	16.4	0.256	2.4	0.638	74.7	0.057	2.1
Particle-free supernatant	300	60.3	1.5	10.3	0.07	4.8	0.15	6.3	—	Absent	—	Absent	0.580	91.1
Recovery (%)	...	101.4	—	103.6	—	101.2	—	92.7	—	91.0	—	95.7	—	93.2

Table 6. *Acid hydrolases in subcellular fractions from the mucosa of guinea-pig small intestine*

Specific activities are expressed as μ moles of substrate converted/15 min./mg. of protein, and total activity as μ moles of substrate converted/15 min./total fraction. Fractions were prepared as described in the Materials and Methods section.														
Protein		Succinate dehydrogenase		Glucose 6-phosphatase		Acid phosphatase		Acid ribonuclease		β -Glucuronidase		β -Galactosidase		
		Total mg.	Specific activity	Total activity	Specific activity	Total activity	Specific activity	Total activity	Specific activity	Total activity	Specific activity	Total activity	Specific activity	
Mitochondrial fraction	5.2	69.5	1.36	93.8	0.42	29.1	0.69	48.1	0.45	31.3	0.052	3.56	0.209	14.5
Microsomal fraction	5.8	93.5	0.01	0.8	2.06	192.0	0.17	16.3	0.08	7.3	0.012	1.10	0.015	1.4
Particle-free supernatant	1.0	170.0	—	<1	—	<1	0.16	27.3	0.07	11.9	0.013	2.21	0.052	8.8

Table 7. *Composition of microsomal subfractions of intestinal mucosa prepared by method I (see Scheme 1)*

Specific activities are expressed as $m\mu$ moles of glucose 6-phosphate hydrolysed or phosphatidate hydrolysed or hydroxamate formed/hr./mg. of protein. RNA is expressed as μ g. of RNA phosphorus/mg. of protein.																									
Guinea pig																									
Protein						Glucose 6-phosphatase		RNA		Protein		Glucose 6-phosphatase		RNA		Acyl-CoA synthetase		Phosphatidate phosphatase							
mg./fraction		% of total		Specific activity		% of total		P/mg. of protein		% of total		mg./fraction		% of total		Specific activity		% of total		Specific activity		% of total			
99.0		100		16700		100		10.4		100		208		100		507		100		19.7		100			
Total microsomal fraction		61.5		62		21600		81		17.8		106		165		79		550		86		22.2		89	
Rough-vesicle fraction		33.4		34		6100		12		3.7		12		42		20		0		0		7.2		7	
Smooth-vesicle fraction		...		96		—		93		—		118		—		99		—		86		—		96	
Recovery (%)		...		...		...		...		...		...		...		...		...		...		...		...	

Specific activities are expressed in the same values as those in Table 7.																
Guinea pig										Cat						
Protein		Glucose 6-phosphatase		RNA		Protein		Glucose 6-phosphatase		RNA		Acyl-CoA synthetase		Phosphatidate phosphatase		
mg./fraction	% of total	Specific activity	% of total	µg. of RNA	P/mg. of protein	% of total	mg./fraction	% of total	Specific activity	% of total	µg. of RNA	P/mg. of protein	% of total	Specific activity	% of total	
Total microsomal fraction	204	100	21500	100	10.8	100	226	100	1065	100	17.4	100	2410	100	1100	100
Rough-vesicle fraction	90.8	45	26700	55	19.8	81	108	48	1205	54	27.2	75	3540	70	1040	45
Fraction SVFI	61.5	30	14300	20	10.1	28	41.3	18	371	6	17.1	18	3060	23	1005	17
Fraction SVFII	14.9	7	5700	2	3.8	3	43	19	326	6	74	8	990	8	1780	31
Recovery (%)	...	82	—	77	—	112	—	85	—	66	—	101	—	101	—	93

membranes were those of Dallner (1963) and Dallner *et al.* (1963), who had developed these procedures for the subfractionation of microsomal fractions from rat liver. As shown below, similar results to those of Dallner (1963) were obtained with microsomal fractions from the mucosa of the small intestine of the guinea pig and cat.

With method I, two fractions were obtained. One fraction contained most of the RNA and glucose 6-phosphatase activity of the original microsomal fraction, whereas the second fraction contained very little RNA and glucose 6-phosphatase and had correspondingly lower specific activities (see Table 7). There appeared to be good agreement between the composition with respect to these enzymes of the subfractions derived from guinea-pig and cat small-intestinal mucosa.

With method II, the membrane fraction sedimenting as rough-vesicle fraction in method I was further divided into a rough-vesicle fraction and a smooth-vesicle fraction (SVFI). Fraction SVFII corresponded to part of the smooth-vesicle fraction of method I. The results of analyses of these fractions are summarized in Table 8. The composition of the various fractions prepared from guinea-pig intestine was similar to that of cat intestine, the rough-vesicle fraction containing most of the RNA, smaller amounts being in fraction SVFI and negligible amounts in fraction SVFII. The distribution of glucose 6-phosphatase is difficult to compare since the recovery of this enzyme was rather low in the preparations obtained from the cat. The recovery of protein in the various fractions was similar in both sets of preparations except that fraction SVFII from the guinea pig contained only 7% of the protein of the original microsomal preparation, whereas 19% was recovered in this fraction when prepared from the cat. It is obvious that the determination of RNA and of glucose 6-phosphatase is not sufficient to characterize the three subfractions prepared by using method II. Additional marker enzymes were therefore sought and the distribution of phosphatidate phosphatase and of acyl-CoA synthetase in the fractions from the cat indicates further differences in the composition of the membranes. Phosphatidate phosphatase was mainly recovered in the rough-vesicle fraction and fraction SVFII, the latter fraction having the highest specific activity. In contrast, acyl-CoA synthetase was mainly in the rough-vesicle fraction and fraction SVFI, the former having the highest specific activity.

Electron micrographs of the fractions prepared from the cat showed certain structural differences (see Plate 3). The rough-vesicle fraction contained, in addition to rough-surface vesicles, numerous free ribosomes. Fraction SVFI showed predominantly smooth-surface vesicles but also some rough-

surface vesicles, an observation that is in agreement with the distribution of RNA in these fractions. The structures observed in fraction SVFII were quite different in that there were long stranded membranes and vesicles with a larger diameter.

DISCUSSION

The present work was undertaken to study systematically the isolation of subcellular structures from homogenates of the mucosa of the small intestine. Electron microscopy and the assay of typical marker enzymes were employed for the identification of the subcellular structures isolated. By these means it was possible to obtain some insight into the intracellular distribution of a number of enzymes. Thus the brush-border fraction contained a number of acid hydrolases (acid phosphatase, acid ribonuclease, phospho-protein phosphatase), a neutral hydrolase (aryl-amidase) and alkaline hydrolases (alkaline ribonuclease, alkaline phosphatase). Of these, only invertase and alkaline ribonuclease seemed to be exclusively in the brush border, the other enzymes being located partly in the brush border and partly in other subcellular structures. The almost complete retention of invertase and alkaline ribonuclease in the low-speed sediment, and the partial removal of alkaline phosphatase and aryl-amidase from this fraction during repeated washing, could also be due to leakage of the latter two enzymes from the subcellular structures of the low-speed sediment. However, this is unlikely for two reasons. First, if alkaline phosphatase and aryl-amidase had leaked out from the subcellular structures of the low-speed sediment during its preparation one would have expected to recover corresponding proportions of these enzymes in the particle-free supernatant. This was not the case (see Tables 4 and 5). Further, treatment of the low-speed sediment in strongly hypo-osmotic solution did not result in significant differences in the distribution of these four enzymes in the resulting sediment and supernatant fractions (see Table 3).

The evidence presented for the localization of the enzymic activities of the low-speed sediment in the brush border rather than in the nuclei (see Table 3 and Plate 2) does not exclude their occurrence in the nuclear membrane, since the fate of this membrane during fractionation was not traced. The observation on the formation of small vesicles from the intact brush border by treatment in a Waring Blendor is at variance with the results of Miller & Crane (1961), who prepared brush-border fractions by using a Waring Blendor for homogenization, but is in agreement with the results of Carnie & Porteous (1962*b*). These authors reported that, depending on the type of homogenizer

employed, invertase could be recovered in the low-speed sediment or the microsomal fraction, indicating that the brush border may break up into smaller vesicles.

In the past, several authors have reported on the intracellular localization of some of the above-mentioned enzymes in the mucosa of the small intestine. In some instances, conflicting evidence was presented. The evaluation of previous results and comparison with the present data is difficult for two reasons. First, the characterization of the subcellular fractions was seldom sufficient to allow unambiguous conclusions to be drawn. Secondly, different species were investigated and it is likely that there may be significant differences in enzymic composition of the epithelial lining of the small intestine of carnivores, herbivores and omnivores. To demonstrate this point the reports on invertase, alkaline phosphatase and 'leucine aminopeptidase' may be discussed.

Miller & Crane (1961) and Holt & Miller (1962) were the first to report that, in the epithelial layer of the small-intestinal mucosa of the hamster, invertase is a brush-border enzyme. A similar intracellular distribution of invertase was reported for the small-intestinal mucosa of the rabbit (Carnie & Porteous, 1962*b*; Gitzelmann, Davidson & Osinchak, 1964). The results obtained in the present investigation with preparations from the intestinal mucosa of the guinea pig are in agreement with those quoted above. A microsomal localization of this enzyme was reported for the intestinal mucosa of the hog (Borgström & Dahlqvist, 1958). In fact, most of the invertase activity was recovered in the first low-speed sediment, an observation that Borgström & Dahlqvist (1958) ascribed to unbroken cells rather than to the sedimented brush border. Similarly, of the subcellular fractions from rat small-intestinal mucosa, the mitochondrial and microsomal fractions were considered to contain most of the invertase activity (Dahlqvist, 1962), but the first low-speed sediment was not included in the survey of the intracellular distribution.

Histochemical studies had indicated that invertase activity was localized in small granules throughout the cytoplasm of the epithelial cell of rat small-intestinal mucosa, the concentration of these granules being somewhat higher in the perinuclear and apical regions (Dahlqvist & Brun, 1962). In these studies, the tissue slices were incubated for at least 3 hr., during which time there was a considerable degree of autolysis, particularly of the brush border. Nitro-BT, the dye used by Dahlqvist & Brun (1962), was recently shown to be of very limited value in histochemical investigations, especially when autolysis occurred (Meier-Ruge, 1965). Thus, when considering all the observations on

the intracellular distribution of invertase, there is more convincing evidence for a predominant localization of invertase in the brush border than for its occurrence in other subcellular structures.

The reports on the intracellular distribution of alkaline phosphatase are somewhat more contradictory. A predominantly microsomal localization of this enzyme was reported for the intestinal mucosa of the rat (Allard, de Lamirande & Cantero, 1957; Triantaphyllopoulos & Tuba, 1959; Robinson, 1963; Ailhaud *et al.* 1963), the calf (Morton, 1954) and the rabbit and guinea pig (Hers, Berthet, Berthet & de Duve, 1951). In contrast, Holt & Miller (1962) had described alkaline phosphatase as a typical brush-border enzyme in the intestinal mucosa of the hamster. Clark & Porteous (1963) had come to the same conclusion in their investigation of rabbit small-intestinal mucosa. With the exception of the work of Morton (1954), who discarded the first low-speed sediment in his fractionations, no explanation can be given that might reconcile these opposing views.

The intracellular distribution of alkaline phosphatase observed in the present investigation of guinea-pig small-intestinal mucosa could be best interpreted by assuming that, although most of the enzyme was in the brush border, an occurrence in other subcellular structures seemed likely. The view of a localization of alkaline phosphatase in more than one subcellular structure is supported by the results of histochemical studies. Ever since Gomori (1941) described a phosphatase activity in the surface of human small intestine, many workers have either confirmed this observation or extended it to other tissues including the epithelial layer of the intestinal mucosa of the mouse (Deane & Dempsey, 1945; Brandes, Zetterqvist & Sheldon, 1956), the guinea pig (Bourne, 1943; Jervis, 1963), the rat (Emmel, 1945; Johnson & Kugler, 1953; Jervis, 1963), the rabbit (Jervis, 1963) and the cat and dog (Johnson & Kugler, 1953). In most of these investigations it was pointed out that alkaline-phosphatase activity was seen in bilaminar layers of the brush border as well as in the apical region of the cytoplasm, which in some instances was referred to as the Golgi zone. Thus, though there is reasonable agreement between histochemical observations and our results, more work is required to investigate the apparent discrepancies in the intracellular distribution of alkaline phosphatase in the intestinal mucosa of the various species mentioned above.

Relatively few investigations have been made on the intracellular distribution of acid phosphatase. Gitzelmann *et al.* (1964) recovered 34.9% of the acid phosphatase of homogenates of rabbit intestinal mucosa in the nuclear fraction and the corresponding value quoted by Holt & Miller (1962) for prepara-

tions from hamster intestinal mucosa was 17.6%. We recovered 54.9% of the original activity in the low-speed sediment from homogenates of guinea-pig intestinal mucosa. If expressed as percentage of the total phosphatase activities recovered, this value would become 19.1%. Though it is not questioned that acid phosphatase is, to a large extent, localized in the cytoplasm, it would seem essential to establish whether the enzyme found in the low-speed sediment represents a true component of the brush border. The results describing the enzymic composition of the purified brush-border fraction (see Table 3) seem to indicate that acid phosphatase is a true component of the microvilli. However, Gitzelmann *et al.* (1964) observed that, in tissue slices, the microvilli region did not stain in the histochemical test for acid phosphatase but that a staining was obtained with isolated microvilli, and they concluded that with the isolated microvilli an adsorption of acid phosphatase had taken place.

The first reports on the occurrence of leucine aminopeptidase appeared only recently. Holt & Miller (1962) regarded it as a brush-border enzyme, whereas Robinson (1963) recovered most of the aminopeptidase in the cell sap. In the intestinal mucosa of the guinea pig we found an intracellular distribution similar to that of alkaline phosphatase, i.e. though mainly in the brush-border fraction its occurrence in other subcellular structures was very likely. These results are not necessarily contradictory since Robinson (1963) used L-leucylglycine as substrate whereas in the other studies L-leucyl- β -naphthylamide was employed. There is good evidence that in a number of tissues two aminopeptidases occur that differ from each other with respect to their reaction rates with L-leucylglycine or L-leucyl- β -naphthylamide as substrate (Patterson, Hsiao & Keppel, 1963; Fleischer *et al.* 1964; Sylvén & Snellman, 1964). Histochemical evidence suggests that the enzyme hydrolysing the naphthylamide derivative is located in the brush-border region of rat intestinal mucosa (Nachlas, Morris, Rosenblatt & Seligman, 1960).

No conclusive evidence could be obtained for the occurrence of lysosomes in the intestinal mucosa of the guinea pig, though the distribution of a number of acid hydrolases between the mitochondrial and microsomal fractions seems to suggest rather than exclude their occurrence (see Table 6). The work of Doell & Kretschmer (1962) had indicated that there are two β -galactosidases in rabbit intestinal mucosa that differed from each other with respect to pH optimum and substrate specificity. Hsu & Tappel (1964) compared the intracellular distribution of a number of acid hydrolases in subcellular fractions prepared from rat liver and rat small-intestinal mucosa respec-

tively. Unfortunately, a Waring Blender was used in the initial homogenization of the intestinal preparations and distilled water in the later stages.

The results obtained on the subfractionation of the microsomal membranes are similar to those reported by Dallner (1963) and Dallner *et al.* (1963) for preparations from rat liver. The separations of the microsomal membranes reported in the present paper should be regarded as a first step only towards a fuller understanding of the biochemical function of these membranes. Initially, the distribution of other enzymes within the submicrosomal fractions should be studied to obtain marker enzymes that may be characteristic for each type of membrane and thus be of help in further fractionation studies. Aliesterase (Underhay, Holt & de Duve, 1956), cystine reductase (Myers & Worthen, 1961), α -hydroxy acid decarboxylase (Levis & Mead, 1964) and the Na⁺-activated adenosine triphosphatase may prove to be useful marker enzymes. Nucleoside diphosphatase, cytochrome *b₅*, the carbon monoxide-binding pigment and DT diaphorase have been studied by Dallner (1963). Of the above-mentioned enzymes, cystine reductase and DT diaphorase were assayed in the present work, but cystine reductase was found to be inactive under the conditions used, and the inhibition of DT diaphorase by dicoumarol was unreliable.

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