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IN VITRO STUDIES OF INTESTINAL DRUG ABSORPTION

DETERMINATION OF PARTITION AND DISTRIBUTION COEFFICIENTS WITH BRUSH BORDER MEMBRANE VESICLES

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Abstract—Brush border membrane vesicles (BBMV) were isolated from rat small intestine and characterized in terms of relative enrichment of specific organelle marker enzymes (20-fold enrichment; 20% yield), contamination by other subcellular organelles (<1%) and functional integrity (Na^+-dependent glucose uptake). Using these vesicles, techniques were developed for the determination of partition and distribution coefficients for the model solutes, nitrobenzene, toluene and benzoic acid. No gender, age or regional variation along the small intestine in partition coefficient (log P) values was detected. There was no temperature (10–40°) or pH (4.5–8.0) dependence in partition coefficients of nitrobenzene and toluene. Fair agreement was obtained for log P and log D values for these two solutes determined with BBMV and those reported with octanol and propylene glycol dipelargonate. Selective removal of proteins, both ecto-brush border and micro-villus core proteins, did not alter the partition coefficients of the three model solutes. In contrast, depletion of the BBMV of non-esterified fatty acids significantly decreased the partition coefficients. Liposomes prepared from BBMV lipid extracts were also used for partition coefficient determinations and gave similar values to intact BBMV; addition of increasing amounts of cholesterol to the lipid extract caused small increases in the partition coefficients of the model solutes in the liposomes. It was concluded that the partition coefficients of the BBMV were related to the lipid and not to the protein composition of the vesicles. The method offers a rapid and reliable means of measuring the partition coefficient of non-protein bound drugs and nutrients in isolated intestinal BBMV and should assist in the subsequent modelling and prediction of intestinal absorption *in vivo*.

Assessment of the intestinal absorption of nutrients and pharmaceuticals requires the extensive use of *in vivo* studies in various animal species including man. Prediction in model systems would, therefore, considerably enhance the design of well-absorbed pharmaceutical agents. The use of partition/distribution coefficients determined in such solvents as isobutanol [1], olive oil [2], octanol [3], propylene glycol dipelargonate (PGDP‡) [4] and iso-octanol-cyclohexane [5] provides a useful prediction of intestinal absorption rates [6, 7] but the correlation is not high and there are several drugs with highly anomalous behaviour. Other groups have used model membranes, e.g. phospholipid-cholesterol membranes [8], black lipid membranes [9], liposomes [10], brain phospholipids [11] and erythrocytes [12] to study the partition of a variety of solutes. As far as we are aware, partition studies have not been described in detail using vesicles and liposome preparations from intestinal brush border membranes. This paper describes the development of such methods using model compounds and investigates some of the variables, both physical and biological, in the system.

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‡ Abbreviations: BBMV, brush border membrane vesicles; NEFA, non-esterified fatty acids; PGDP, propylene glycol dipelargonate; BSA, bovine serum albumin.

MATERIALS AND METHODS

Male and female Sprague–Dawley rats (200–450 g body weight) were supplied by the Clinical Research Centre (Harrow, U.K.) or ICI Pharmaceuticals, Central Animal Breeding Facility (Cheshire, U.K.), and had free access to food and water until being killed. Intestinal BBMV were isolated and characterized for integrity and purity, as described previously [13] by a modification of the Mg^{2+} precipitation procedure of Kesseler *et al.* [14].

Purity was assessed by organelle specific marker enzyme analysis [15]. Based on assays of brush border α-glucosidase, yields of 20% with 20-fold enrichments were regularly achieved. BBMV integrity was confirmed by demonstrating Na^+-dependent glucose uptake [16]. Contaminating organelles in the BBMV fraction, assessed by specific marker enzyme assays (mean ± SD) were: lysosomes (*N*-acetyl-β-glucosaminidase, 0.25 ± 0.17%); mitochondria (succinate dehydrogenase, 0.11 ± 0.1%); nuclei (DNA, <0.01%); golgi (galactosyl transferase, 0.27 ± 0.11%); baso-lateral membrane (Na^+-, K^+-activated Mg^{2+}-dependent ATPase, 0.78 ± 0.12%; 5′nucleotidase, 0.63 ± 0.11%). These results are in agreement with, and supplement results, reported previously from this laboratory [13].

Lipid extracts were prepared with chloroform:methanol, as reported previously [17], and multi-lamellar liposomes prepared as described by Bangham [18]. Protein was assayed by the method

of Bradford [19] with bovine serum albumin as standard. Lipid analyses were as described previously [17] using TLC of lipid extracts in conjunction with specific assays for the individual lipid classes. Other methods were as described and referenced in Results.

RESULTS

Development of method for determining partition coefficients with BBMV

BBMV were suspended in 20 mM HEPES–KOH buffer, pH 7.4, containing 1 mM $MgSO_4$ and 100 mM mannitol, and 100 μL aliquots were added to 1 mL of the same buffer solution containing ^3H_2O as aqueous phase marker ([^{14}C]sucrose was used for studies with ^{3}H-labelled compounds) and [^{14}C]-nitrobenzene, [^{3}H]toluene or [^{14}C]benzoic acid (Amersham International, Amersham, U.K.). Reaction mixtures contained approximately 1.0 μC/mL of radioactivity. After incubation at 37°, membrane and aqueous phases were separated in initial studies by filtration. Millipore filters of various composition including HAWP (mesh 0.45 μm) and Acrodisc (polytetrafluoro-ethane, mesh 0.2 μm) were used. Filters were pre-soaked in 0.15 M NaCl containing non-radio-labelled solute (1 mM or saturated solution). Aliquots of the reaction mixture, filtrate and filters themselves were added to 3 mL Fluoran scintillation fluid (Amersham) and counted for radioactivity. However, this method was abandoned as incubation blank levels of radioactivity in the filters, i.e. after filtration of reaction mixtures lacking BBMV, were unacceptably high (5–10%). When large wash volumes (30 mL) were used to reduce the blank values, loss of BBMV occurred through the filter. Therefore, centrifugation was subsequently used to separate the membrane and aqueous phases. Initial studies used an 8 × 10 mL angle-head rotor in an MSE SS65 ultracentrifuge (100,000 g, 30 min) using a variety of centrifuge tubes including polyallomer (Beckman), polycarbonate (MSE), PTFE (Teflon) and Acetal homopolymer (Derilin). However, solute ([^{3}H]toluene, [^{14}C]nitrobenzene) losses were between 20–50% with damage to the centrifuge tubes. In order to overcome these difficulties and to speed up the separation of the phases, a Beckman Airfuge was used and PTFE-capped titanium reaction-centrifuge tubes were made. This approach proved satisfactory with recovered radioactive solutes of 90 ± 5% after centrifugation at 150,000 g for 15 min at 4°. Recoveries of BBMV as reflected by brush border enzyme assays were >95%.

In the definitive method adopted, 50 μL BBMV suspension was added to 100 μL buffered solute and aqueous markers. Partitioning was carried out at room temperature (22–26°), as no temperature dependence of partition was noted (Fig. 1). Partitioning was routinely carried out for 30 min after preliminary studies showed that complete equilibration occurred within 15 min (Fig. 2). Fig. 3 shows pH-partition dependence of nitrobenzene. There was a small, but statistically not significant, decrease in partition coefficient as the pH increased from 4.5 to 8.0. In contrast, the distribution coefficient for benzoic acid showed a marked pH dependence so that above pH 6.5, insufficient radioactive counts were present in the membraneous phase to enable a value to be calculated. Partition distribution coefficients for the radio-labelled compounds are shown in Table 1 and show good agreement with literature values for partition/distribution coefficients for octanol and PGDP.

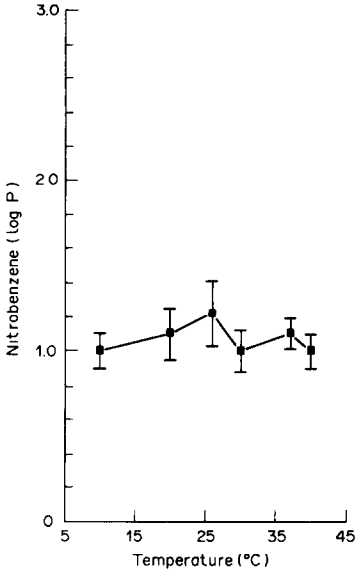

Fig. 1. Temperature dependence of partition coefficient for nitrobenzene in BBMV. Results show means ± SE for 6–15 experiments.

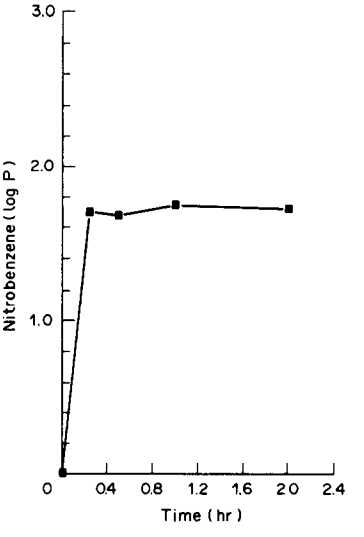

Fig. 2. Time dependence of partition for nitrobenzene in BBMV at room temperature. Results show means of triplicate values for a combined experiment using a single batch of BBMV.

Age, gender and regional variation in partition coefficient

Table 2 shows the results for partition coefficient

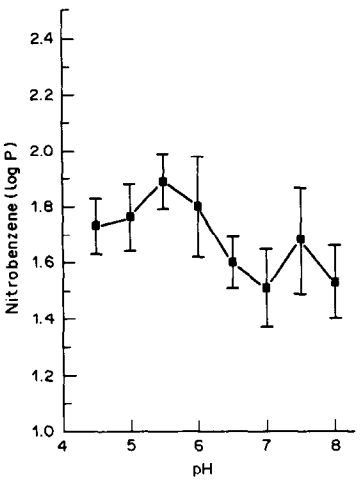

Fig. 3. pH-Dependence of partition coefficient for nitrobenzene. At pH 4.5–6.0, 20 mM sodium citrate buffers and at pH 6.5–8.0, HEPES–KOH buffer were used. Data show means ± SE for 5–15 experiments.

Effect of modifications to BBMV on partition coefficients

BBMV were isolated as described above and pre-incubated with papain to remove some of the ecto-brush border proteins [20], or with lithium diiodosalicylate to extract villus core proteins [21]; partition coefficients were determined for nitrobenzene and toluene. Partial cleavage of the ecto-glycoproteins (16% decrease in protein content) or selective extraction of the brush border protein (60% decrease in protein) did not affect the partition coefficients (Table 3).

The importance of the BBMV lipid components on partition coefficient was investigated by selective depletion of the vesicles of NEFA. The lipid analysis (668 ± 67 μg total lipid/mg protein, mean ± SD for 4–6 analyses) of control BBMV showed that approximately one third of the lipid consisted of NEFA (230 ± 134 μg/mg protein); phospholipid (308 ± 89 μg/mg protein) and cholesterol (126 ± 12 μg/mg protein) accounted for the remainder of the lipid (molar ratio 2:1) [22] with trace amounts of triglyceride (4 ± 3 μg/mg protein).

Selective depletion of NEFA, which are probably formed largely by esterase activity during BBMV isolation or are from dietary sources, can be reduced markedly by overnight fasting and addition of diethyl nitrophenyl phosphate, an esterase inhibitor, to the mucosal homogenization medium, or most potently by pre-incubating the BBMV with fatty acid-free bovine serum albumin, prior to the partition experiments [23, 24]. These manoeuvres reduce the NEFA content of the BBMV by 10, 10, 40 and 90%, respectively, from control values. Table 4 shows the partition coefficients of the two radio-labelled compounds in the control and NEFA-depleted BBMV. Although 24-hr fasting reduces the NEFA content by approximately 40%, the log P values do not show a statistically significant decrease. In contrast, pretreatment of BBMV with BSA reduced the NEFA content by 90% and this was associated with a 40% decrease in log P for both solutes. Further evidence that the lipid composition of the BBMV is a major determinant of drug permeability was obtained by determining log P values using liposomes prepared from brush border membrane lipids. The partition coefficient (log P) for nitrobenzene in BBMV was 1.71 ± 0.16 with a value of 1.45 ± 0.11 in liposomes, similar values to those seen with intact BBMV. Figure 4 shows the effect of increasing the cholesterol content of the liposomes.

Table 1. Partition coefficients for radio-labelled nitrobenzene, toluene and benzoic acid

Solute	pH	Partition coefficient (log P)		
		BBMV	Octanol	PDPG
Nitrobenzene	7.4	1.68 ± 0.28 (17)	1.85	2.11
Toluene	7.4	2.71 ± 0.25 (6)	2.69	2.80
Benzoic acid	5.0	0.53 ± 0.08 (3)	—	—

Log P values are means ± SE for (N) determinations in BBMV, as described in text. Literature values reported for octanol and PDPG [3, 4].

Table 2. Age, gender and segmental variation in partition coefficient for nitrobenzene and toluene

	Partition coefficient (log P)	
	Nitrobenzene	Toluene
Females (6 weeks)	1.68 ± 0.28 (17)	ND
Males (6 weeks)	1.85 ± 0.05 (5)	ND
Males (5 months)	1.92 ± 0.07 (6)	ND
Small intestine		
Proximal third	1.70 ± 0.07 (4)	2.76 ± 0.08 (4)
Middle third	1.84 ± 0.06 (4)	2.62 ± 0.09 (4)
Distal third	1.83 ± 0.17 (4)	2.77 ± 0.13 (4)

Mean ± SE values for (N) determinations. Data for male and female rats used entire small intestine as source of BBMV. Data for segments of small intestine used male rats. ND, not determined.

assays using BBMV from male and female rats, both young and mature animals, and from different segments of the small intestine. There were no significant differences for either solute in the various experimental groups. In subsequent experiments, whole small intestine was used as a source of BBMV.

Table 3. Effect of removal of ecto and core proteins on partition coefficients

BBMV	Partition coefficient (log P)	
	Nitrobenzene	Toluene
Control	1.71 ± 0.16 (28)	2.71 ± 0.25 (15)
Papain-treated	1.75 ± 0.22 (11)	2.48 ± 0.20 (11)
L.I.S.-treated	1.66 ± 0.11 (11)	2.37 ± 0.18 (8)

Mean ± SE partition coefficients for (N) preparations. L.I.S., lithium diiodosalicylate.

Table 4. Effect of NEFA-depletion on partition coefficients into BBMV

Treatment	NEFA (% total lipid)	Partition coefficient (log P)	
		Nitrobenzene	Toluene
Control	34	1.71 ± 0.16 (28)	2.71 ± 0.25 (6)
Three days semi-starvation	30	1.75 ± 0.09 (4)	2.52 ± 0.13 (4)
One day fasting	30	1.65 ± 0.05 (4)	2.75 ± 0.13 (4)
DENPP treatment	20	1.61 ± 0.10 (5)	2.39 ± 0.19 (5)
BSA treatment	4	0.91 ± 0.08 (3)*	1.68 ± 0.12 (3)*

Mean ± SE partition coefficients for (N) preparations. Statistical analysis: experimental compared to control values, * $P < 0.05$. BSA was added to wash buffer to deplete vesicles of NEFA; DENPP, diethyl nitrophenyl phosphate added to homogenization and wash buffers to inhibit enzymic hydrolysis of fatty acid esters.

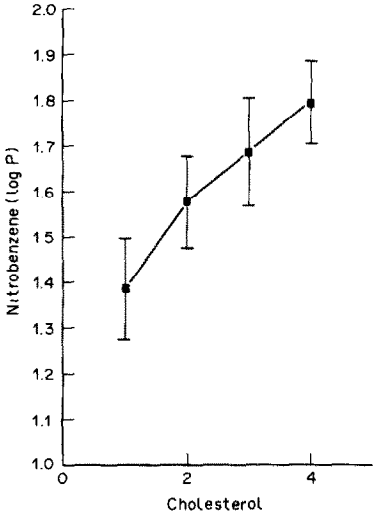

Fig. 4. Effect of increasing cholesterol content of liposomes on partition coefficient of nitrobenzene. Data show mean ± SE for 4–6 determinations. Cholesterol content was determined in the initial BBMV preparation and 1–4 molar excess cholesterol added to the lipid extract prior to preparation of liposomes.

This was accompanied by a progressive increase in log P for nitrobenzene.

DISCUSSION

The technique described in this paper reports the isolation and characterization of BBMV from rat small intestine and the findings are in agreement with previous reports [13, 14]. Characterization of possible contaminating organelles confirms the purity of the vesicle preparation. In the present study, lack of significant contamination by golgi and baso-lateral membranes is reported together with confirmatory data for nuclear, mitochondrial and lysosomal markers in conjunction with peroxisomal and endoplasmic reticulum data reported previously from this laboratory [13].

A technique was developed for the determination of partition and distribution coefficients for model xenobiotics into the vesicles. BBMV from both small intestine and renal tubules have been used extensively to investigate active transport mechanisms and diffusive, passive, and mediated uptake for a variety of physiological substrates and nutrients (see Ref. 25) but this is the first report of the use of intestinal vesicles for studying partition coefficients.

The procedure was very satisfactory and is suitable for use with a variety of drugs and nutrients which do not bind to proteins. Coefficient of variation in the values obtained was 5–10% and at least one complete experiment, including isolation of vesicles, partitioning, centrifugation and solute assays could be undertaken in a normal working day.

Some characteristics of the partitioning process were determined. Surprisingly, no significant temperature dependence of log P was found suggesting that membrane fluidity and order were not major determinants, at least for the compounds studied. This was useful as it indicated that strict temperature control was unnecessary during the partitioning and

separation steps. Uptake and equilibration of the solutes was relatively rapid being complete within 15 min. In addition, for the neutral solutes, there was no significant pH dependency. This suggested an association of the solutes with uncharged elements in the BBMV. This observation is important as it suggests that partitioning of the ionized solutes and weak acids and bases can be determined without any pH-dependent BBMV alterations. pH-Dependent uptake of weak acids and bases will, therefore, largely reflect their pK_a in bulk solution, and this was confirmed by the studies with benzoic acid (pK_a 4.2) where only at pH 5.0 could an accurate log D be determined. At neutral pH, no significant association with the BBMV could be demonstrated. However, correcting for the pH dependence of log D, a calculated value of −1.9 was obtained for benzoic acid at pH 7.4.

The present studies indicate clearly that the partitioning is largely confined to the lipid phase of the membrane, although BBMV are composed of approximately equal amounts of protein and lipid. Removal of the proteinaceous elements of the BBMV had no effect on the log P, even for the relatively polar nitrobenzene. This result indicates that the unstirred aqueous layer in the glycoproteins surrounding the BBMV [26] did not significantly interfere with the partitioning.

The partition coefficients are dependent on the uptake of significant amounts of solute by the BBMV and, therefore, hydrophilic compounds with negative partition coefficients can not be studied. In practice, compounds with partition coefficients less than +0.3 cannot be reliably studied as the amount of BBMV and radio-labelled solute required would be prohibitive.

The values obtained for log P and log D with BBMV agree reasonably well with values reported for the model amphipathic solvents, octanol and PGDP [4], but more extensive studies with compounds of widely differing partition coefficients are needed. Preliminary studies from our laboratory [27] indicate a reasonably close correlation between partition coefficients in BBMV and in these two solvents for values up to log P = 3; for compounds with higher values, significant discrepancies were observed with the BBMV giving consistently smaller values.

Studies on variation of partition coefficient of the model solutes along the small intestine reveal little regional variation and do not indicate lower values for ileum compared with jejunum. Thus, the greater absorptive capacity of the jejunum compared to the ileum is unlikely to reflect this initial uptake step of the absorptive process [28, 29] and other differences including cell number [30], absorptive surface area, permeability [31] and blood flow [32] are likely to be more relevant as well as, of course, intralumenal factors.

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REFERENCES

1. Collander R, The distribution of organic compounds between isobutanol and water. *Acta Chem Scand* **4**: 1085–1098, 1950.
2. Collander R, The partition of organic compounds between higher alcohols and water. *Acta Chem Scand* **5**: 774–786, 1951.
3. Leahy DE, Intrinsic molecular volume as a measure of the cavity term in linear solution energy relationships: octanol–water partition coefficients and aqueous solubilities. *J Pharm Sci* **75**: 629–636, 1986.
4. Leahy DE, Taylor P and Wait AR, Model solvent systems for QSAR. Part I. Propylene glycol dipelargonate (PGDP). A new standard solvent. *Quant Struct–Act Relat* **8**: 17–31, 1989.
5. Young RC, Mitchell RC, Brown TH, Ganellin CR, Griffiths R, Jones M, Rana KK, Saunders D, Smith IR, Sone NE and Wilks TJ, Development of a new physicochemical model for brain penetration and its application to the design of centrally-acting H_2 receptor histamine antagonists. *J Med Chem* **31**: 656–671, 1988.
6. Nook T, Doelker E and Bori I, Role of bile and bile salts in drug absorption. *Int J Pharm* **43**: 119–129, 1988.
7. Collado EF, Fabra-Campass S, Peris-Ribera JG, Casabo VG, Martin Villoclre A and Pia-Delfina JM, Absorption–partition relationships for a true homologus series of xenobiotics as a possible approach to study mechanisms of surfactants in absorption II. Aromatic amines in rat small intestine. *Int J Pharm* **44**: 187–196, 1988.
8. Tobias JM, Agin DP and Pavlowski R, Phospholipid–cholesterol membrane model. Control of resistance by ions or current flow. *J Gen Physiol* **45**: 989–1001, 1962.
9. Tien HT and Howard RE, Biomolecular lipid membranes. *Tech Surf Colloid Chem Phys* **1**: 109–211, 1972.
10. Cohen BE and Bangham AD, Diffusion of small non-electrolytes across liposomal membranes. *Nature* **236**: 173–174, 1972.
11. Mueller P, Rudin DO, Tien HT and Wescott WC, Formation and properties of biomolecular lipid membranes. *Prog Surf Sci* **1**: 379, 1964.
12. Klocke RA, Andersson KK, Rotman HH and Forster JJ, Permeability of human erythrocytes to ammonia and weak acids. *Am J Physiol* **222**: 1004–1014, 1972.
13. Wilkes JM, Garner A and Peters TJ, Studies on the localisation and properties of rat duodenal HCO_3-ATPase with special reference to alkaline phosphatase. *Biochim Biophys Acta* **924**: 159–166, 1987.
14. Kesseler M, Acuto O, Storelli C and Murer H, A modified procedure for the rapid preparation of efficiently transporting vesicles from small intestinal brush border membranes. *Biochim Biophys Acta* **506**: 136, 1978.
15. Peters TJ, Analytical subcellular fractionation of jejunal biopsy specimens: methodology and characterisation of the organelles in normal tissue. *Clin Sci* **51**: 557–574, 1976.
16. Hopfer U, Nelson K, Perroto J and Isselbacher KJ, Glucose transport in isolated brush border membrane from rat small intestine. *J Biol Chem* **248**: 25–32, 1973.
17. Cairns SR and Peters TJ, Micro methods for quantitative lipid analysis of human liver needle biopsy specimens. *Clin Chim Acta* **127**: 373–382, 1983.
18. Bangham AD, DeGier J and Greville GE, Single bilayer vesicles prepared without sonication: physicochemical properties. *Chem Phys Lipids* **1**: 225–232, 1967.
19. Bradford MM, A rapid and sensitive method for the quantitation of microgram quantities of protein utilising the principle of protein-dye binding. *Anal Biochem* **72**: 248–254, 1976.
20. Colbeau A and Maroux S, Integration of alkaline phosphatase in the intestinal brush border membrane. *Biochim Biophys Acta* **511**: 39–51, 1978.
21. Riendeau D, Lemaine J, Maestracci D and Lessard L,

Selective release of inner core proteins from intestinal microvillus membrane by lithium diiodosalicylate. *Mol Cell Biochem* **71**: 45–62, 1986.
22. Forstner GG, Tanaka K and Isselbacher KJ, Lipid composition of the isolated rat intestinal microvillus membrane. *Biochem J* **109**: 51–59, 1968.
23. Simpson RJ, Venkatesan S and Peters TJ, Brush border membrane non-esterified fatty acids. Physiological levels and significance for mucosal iron uptake in mouse proximal intestine. *Cell Biochem Funct* **7**: 165–171, 1989.
24. Simpson RJ, Moore R and Peters TJ, Significance of non-esterified fatty acids in iron uptake by intestinal brush border membrane vesicles. *Biochim Biophys Acta* **941**: 39–47, 1988.
25. Lentze MJ and Sterchi EE (Eds.), *Mammalian Brush Border Membrane Proteins*, p. 253. Georg Thieme, Stuttgart, 1988.
26. Ito S, The enteric surface coat on cat intestinal microvilli. *J Cell Biol* **27**: 475–491, 1965.
27. Alcorn CJ, Simpson RJ, Leahy DE and Peters TJ, Use of isolated intestinal brush border membrane vesicles as a model for membrane partitioning of drugs. *Clin Sci* **76**: 42, 1989.
28. Brasitus TA, Kwo-Yih Y, Holt PR and Schachter D, Lipid fluidity and composition of intestinal microvillus membranes isolated from rats of different ages. *Biochim Biophys Acta* **778**: 341–348, 1984.
29. Brasitus TA and Schachter D, Lipid composition and fluidity of rat enterocyte basolateral membranes regional differences. *Biochim Biophys Acta* **774**: 138–146, 1984.
30. Batt RM and Peters TJ, Absorption of galactose by the rat small intestine *in vivo*: proximal distal kinetic gradients and a new method to express absorption per enterocyte. *Clin Sci* **50**: 499–509, 1976.
31. Fortran JS, Recter FC, Ewton MF, Souter N and Kinner J, Permeability characteristics of human small intestine. *J Clin Invest* **44**: 1935–1944, 1965.
32. Preedy VR, Venkatesan S, Peters TJ, Nolt DM, Yates J and Jenkins SA, Effect of chronic ethanol ingestion tissue RNA and blood flow in skeletal muscle with comparative reference to bone and tissues of the gastrointestinal tract of the rat. *Clin Sci* **76**: 243–247, 1989.