J. Pharm. Pharmacol. 1989, 41:459–463
Received November 25, 1988

Involvement of Sialic Acid in High-affinity Binding of Quaternary Ammonium Compounds by Brush Border Membrane from Rat Intestine

HIROSHI SAITOH, MIKIKO EBINA, SEIKO FUKUDA, KEN ISEKI, KATSUMI MIYAZAKI AND TAKAICHI ARITA*

*Department of Pharmacy, Hokkaido University Hospital, School of Medicine, Hokkaido University, Kita-14-jo, Nishi-5-chome, Kita-ku, Sapporo 060, and * Faculty of Pharmaceutical Science, Mukogawa Women's University, 4–16 Edagawa-cho, Nishinomiya, Hyogo 663, Japan*

Abstract—As one approach to clarify the absorption mechanisms of quaternary ammonium compounds (QACs), their binding characteristics have been studied using brush border membrane vesicles isolated from rat small intestine and liposomes composed of phospholipid and GM_3 ganglioside. The binding of propantheline was significantly decreased when the vesicles were pretreated with neuraminidase. Propantheline and methochlorpromazine bound to the liposomes, the binding for the latter drug being significantly greater than that for propantheline. When GM_3, isolated from rat small intestine, was incorporated into the liposomes their binding capacity for both drugs increased significantly. It is suggested that the binding of QACs to the lipid layer and sialic acid play a role in the high binding of drugs to the intestinal brush border membrane. Furthermore, a sensitive and reproducible high-performance liquid chromatographic method for sialic acid has been developed.

It has been shown that quaternary ammonium compounds (QACs) are absorbed rapidly in the initial phase after dosing (Levine et al 1955; Levine 1966; Turnheim & Lauterbach 1980), although they have poor lipid solubility. Therefore, it has been held that various specialized transport mechanisms could contribute to this process (Levine 1966; Irwin et al 1969; Ruifrok & Mol 1983; Tsubaki & Komai 1986; Saitoh et al 1987, 1988a). However, the details of the absorption mechanisms of QACs remain unclear.

In our previous papers dealing with the transport mechanisms of QACs across the rat intestinal brush border membrane, we have shown that there might be at least two processes in the transport of propantheline, an anti-acetylcholine QAC, across the membrane. Initially propantheline binds rapidly to the brush border membrane (Saitoh et al 1987) and then enters into the epithelium stimulated by the negative transmembrane electrical potential difference (Saitoh et al 1988a). Moreover, we have indicated that the binding of propantheline to the brush border membrane was saturable and was inhibited to varying extents by several QACs such as mepenzolate, methylbenactyzine and butylhyoscine (Saitoh et al 1987). These binding properties of QACs, therefore, suggest the presence of a common binding site on the intestinal brush border membrane.

The enterocyte membrane of the small intestine has been shown to be rich in glycoconjugates such as glycoprotein and glycolipid (Forstner et al 1968; Forstner & Wherrett 1973; Egberts et al 1984). The protein or lipid moiety of the glycoconjugates are thought to be inserted in the hydrophobic membrane matrix, two layers of phospholipid molecules, whereas the carbohydrate part protrudes freely into the extracellular space. It is well known that sialic acids, components of sugar chains, are chiefly responsible for the negative charge of the glycoconjugates, because they always occupy a terminal position in the carbohydrate chains; hence the polar group of the glycoconjugate molecules is negatively charged at a physiological pH.

Correspondence to: K. Miyazaki, Dept. of Pharmacy, Hokkaido University Hospital, School of Medicine, Hokkaido University, Kita-14-jo, Nishi-5-chome, Kita-ku, Sapporo 060, Japan.

Although studies of the involvement of sialic acid in uptake or binding of positively charged compounds, such as dopamine (Zaleska & Erecinska 1987), and inorganic particles (Gallagher et al 1987), by biological membranes have been made, there are few reports about the interaction between QACs and sialic acid biological membranes. Therefore, to clarify the binding mechanisms of QACs, we have examined the effect of removal of the surface sialic acid in propantheline binding to rat intestinal brush border membrane vesicles. We have also studied binding of QACs (propantheline and methochlorpromazine) to the lipid membrane and sialic acid of GM_3 ganglioside (*N*-glycolyl), a major glycolipid of rat small intestinal brush border membrane (Forstner & Wherrett 1973), by using the lipid model membrane containing GM_3 or asialo GM_3. Moreover, we have developed a sensitive and reproducible high performance liquid chromatographic method for the determination of low concentrations of sialic acid.

Materials and Methods

Materials

Propantheline bromide, L-α-phosphatidylcholine (from egg yolk), and L-α-phosphatidic acid were purchased from Sigma Chemical Co. (St. Louis, USA). [^{14}C] Methochlorpromazine iodide (sp act 56 mCi mmol^{-1}) was purchased from New England Nuclear Co. (Boston, USA). Neuraminidase (from *Arthrobacter ureafaciens*, E.C.3.2.1.18) was obtained from Nakarai Chemicals Ltd. (Kyoto, Japan). TSK-Gel, Silica Gel 60 TLC plate and Unisil were obtained from Toyo Sodo MFG. Co. (Tokyo, Japan), Analtech Inc. (Newark, USA) and Clarkson Chemical Co. (Williamsport, USA), respectively. 2,4-Dinitrophenylhydrazine, *N*,*N*′-dicyclohexylcarbodiimide and methanolic HCl, reagents for the determination

of GM_3, were purchased from Sigma Chemical Co., Aldrich Chemical Co., (Milwaukee, USA) and Supelco (Bellefonte, USA), respectively. Dialysis tubing (Spectrapor 2, MWCO 12000–14000) was purchased from Spectrum Medical Industries Inc. (Los Angeles, USA). All other reagents were of the highest grade available commercially and used without further purification.

Preparation of brush border membrane vesicles

Brush border membrane vesicles were isolated from the entire small intestine of male Wistar rats (250–300 g) according to the calcium chloride precipitation technique of Kessler et al (1978). The membrane vesicles were suspended with 100 mM D-mannitol, 20mM *N'*-2-hydroxyethylpiperazine-*N'*-2-ethanesulphonic acid (Hepes)/Tris, pH 7·5 (buffer A) or 100 mM D-mannitol, 20 mM 2-(*N'*-morpholino)ethanesulphonic acid (Mes)/Tris, pH 5·5 (buffer B). The purity of the membrane was routinely evaluated by the enrichment of alkaline phosphatase (E.C.3.1.3.1), an enzyme specific to the intestinal brush border membrane. The specific activity of this enzyme increased 12-fold in the final membrane suspension compared with concentrations found in the homogenate of intestinal scrapings. The membrane vesicles were equilibrated for 1 h at 0°C before use.

Neuraminidase treatment

A membrane vesicle suspension in buffer B (0·3–0·4 mg protein mL^{-1}) was incubated at 37°C in a shaking bath with or without neuraminidase (0·05 unit mg^{-1} of membrane protein). At a stated time aliquots of the suspension were cooled at 0°C and centrifuged at 27 000 *g* for 10 min, and supernatants were used for measurements of released sialic acid.

Binding to brush border membrane

A vesicle suspension was treated with neuraminidase for 60 min as described above, centrifuged, and both resulting pellet and control pellet were resuspended in buffer A at 3–4 mg protein mL^{-1}. Aliquots of both suspensions were used for uptake of propantheline (at 25°C) and for measurements of total and protein-bound sialic acid.

The uptake of propantheline into the membrane vesicles was measured by a rapid filtration technique as described previously (Saitoh et al 1987).

Isolation of GM_3 from rat brush border membrane

Pooled brush border membrane vesicles isolated as described above were centrifuged at 27 000 *g* for 20 min. The pellet was homogenized in 20 vol of chloroform–methanol (1:1, v/v) for 30 min and filtered. The filtrate was evaporated to dryness in a rotary evaporator. The residue was taken up in a small volume of methanol and fractionated by ion-exchange chromatography on a column of TSK-Gel DEAE-650S. The conditions were essentially similar to the method described by Ledeen & Yu (1978). GM_3 ganglioside containing *N*-glycolylneuraminic acid, a major ganglioside of rat small intestinal brush border membrane, was finally purified by preparative TLC.

Binding to lipid membrane by equilibrium dialysis

Control liposomes (3·4 μmol of phosphatidylcholine) and GM_3-liposomes (93 mol % phosphatidylcholine and 7 mol % rat brush border membrane GM_3) were prepared under nitrogen by sonication in 0·01 M phosphate buffer (Na_2HPO_4-KH_2PO_4, pH 7·4) for 30 min at 0°C according to the method of Utsumi et al (1987).

The binding studies were performed at 25°C in cells each of which consisted of two half cells (volume 2·08 mL; membrane surface area 4·15 cm^2) separated by a membrane (Spectrapor 2, MWCO 12000–14000). A mixed solution (1 mL) of an equal volume of substrate (1 nmol mL^{-1}) in the same buffer and each liposome suspension (1·1 μmol lipid mL^{-1}) were added into one side cell (sample side) and 1 mL of a buffer alone was added to the other. In the experiments to assess the mutual inhibition of binding between propantheline and methochlorpromazine, a 25 × concentration of propantheline was added to the sample side cell.

In preliminary experiments, the times required for propantheline and methochlorpromazine dialysed against the buffer to reach equilibrium were determined, and in both cases equilibrium was reached in 3 h. At the end of equilibrium dialysis experiments (3 h), samples were taken from sample side cells, and used for the determination of the concentration of drugs.

Partition coefficients of substances

Propantheline and methochlorpromazine, were dissolved in 10 mL of modified Ringer solution, pH 7·4 (Schulz et al 1966) to give 100 μM solutions. Chloroform (10 mL, saturated with modified Ringer solution) was added. After vigorously shaking for 10 min the phases were separated by centrifugation for 10 min. Initial and final concentrations (after shaking) of drugs in the aqueous phase were determined. The partition coefficient (P) was calculated according to the formula: $P = (Ci - Cf)/Cf$, where Ci = initial concentration of drugs and Cf = final concentration of drugs.

Analytical method

Propantheline was analysed by high-performance liquid chromatography as described previously (Saitoh et al 1987). [^{14}C]Methochlorpromazine was analysed by liquid scintillation counting.

Sialic acid was measured by the thiobarbituric acid method (Warren 1959). But, when the sialic acid concentration was very low, as in its determination in the pellet or supernatant after neuraminidase treatment, the final reacted compound obtained by the thiobarbituric acid method was analysed by HPLC after extraction of reacted compound with acetonitrile. The conditions for HPLC were as follows: a liquid chromatograph (Hitachi 638) equipped with a variable wavelength absorption monitor (875 UV, Japan Spectroscopic Co.); column, 25 cm × 4 mm i.d. stainless steel, packed with Nucleosil 5 NH_2 (Macherey, Nagel & Co. Duren, G.F.R.) and warmed to 50°C; mobile phase 0·13 M KH_2PO_4 solution; flow rate 0·3 mL min^{-1}. The peak was detected by measuring the absorbance at 550 nm and integrated with a Hitachi 833 Data Processor.

For the determination of total sialic acid, the precipitate was resuspended in 1·5 mL of 0·05 M H_2SO_4 homogenized and then hydrolysed for 1·5 h at 80°C. The mixture was neutralized with 0·2 M NaOH and centrifuged at 8 000 *g* for

10 min. The supernatant (1 mL) was used for the determination of sialic acid.

The protein-bound sialic acid was determined after the extraction of aliquots with chloroform–methanol mixtures according to Suzuki (1964). The defatted pellet was subjected to acid hydrolysis, neutralization and centrifugation as described above. The resulting supernatants were analysed for sialic acid.

Sialic acid released during incubations was determined as follows: the supernatant of the incubation samples was applied to a Bond Elute SAX column (0·8 × 1·7 cm, Analytichem International, Habor City, USA) to eliminate interference by D-mannitol. Free sialic acid was then eluted with 2 mL of 1 M sodium acetate and assayed by the thiobarbituric acid method and HPLC as described above.

GM_3 was analysed by HPLC as described previously (Miyazaki et al 1986). Protein was determined by the method of Lowry et al (1951).

Results

Determination of sialic acid by HPLC

The final reacted compound obtained by the thiobarbituric acid method was eluted at 14 min. The calibration curve of peak area of standard sialic acid (0·5, 1·0, 2·0, 3·0, 5·0 μg mL^{-1}) was linear with a correlation coefficient of 0·9998. The coefficient of variation at 1·0 μg mL^{-1} was 4·8% (n = 5) and the limit of sensitivity for quantitation was 0·5 μg mL^{-1}.

Release of sialic acid

Fig. 1 shows the time course of release of sialic acid from the brush border membrane vesicles treated with or without neuraminidase (0·05 unit (mg protein)$^{-1}$). The effect of the enzyme reached a plateau at 120 min, and the amount of released sialic acid at 120 min, the difference between the amount of sialic acid in the pellets treated with and without (initial) neuraminidase, was about 78%.

To provide an insight into the possible site of action of neuraminidase on the brush border membrane preparation, the content of released protein-bound and lipid-bound sialic acid, which is a subtraction of total and protein-bound sialic acid, was determined. The content of protein-bound sialic acid was 35·6 nmol (mg protein)$^{-1}$ while the lipid-bound pool was 13·8 nmol (mg protein)$^{-1}$.

Binding to the brush border membrane

To examine the involvement of membrane sialic acid in the binding of propantheline by the brush border membrane vesicles, the time course of binding of propantheline was studied using control and neuraminidase-treated vesicles. As shown in Fig. 2, propantheline bound rapidly to the control vesicles, but was significantly less bound when the vesicles were pretreated with neuraminidase for 60 min (0·05 unit (mg protein)$^{-1}$).

In a previous study (Saitoh et al 1987), we have confirmed that most of the uptake of propantheline by the brush border membrane vesicles was based on binding to the membrane and that the effect of accumulation within the intravesicular space was negligible.

Binding to liposome

The equilibrium binding of propantheline and methochlorpromazine to liposomes, and the effect of GM_3, are shown in Fig. 3. Both drugs bound to the liposomes, the binding of methochlorpromazine being significantly greater compared with that of propantheline. It was also found that GM_3 significantly increased the binding capacity of the liposomes for both drugs (Fig. 3). Moreover, for methochlorpromazine there was a significant change in binding capacity between the liposomes containing GM_3 and asialo GM_3.

To examine the mutual inhibition between propantheline and methochlorpromazine, the effect of propantheline on the binding of methochlorpromazine to the liposomes containing GM_3 was studied. There were no significant changes in the binding capacity between liposomes containing GM_3 and control liposomes or those containing asialo GM_3.

In this experiment, we determined GM_3 content within

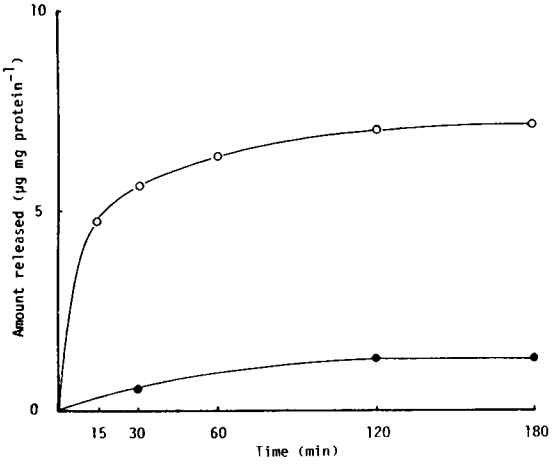

FIG. 1. Time course of release of sialic acid from the brush border membrane vesicles treated (○) and untreated (●) with neuraminidase (0·05 unit (mg protein)$^{-1}$) at 37°C. Incubation medium was 20 mM Mes/Tris buffer solution containing 100 mM D-mannitol (pH 5·5). Each point represents the mean of two experiments.

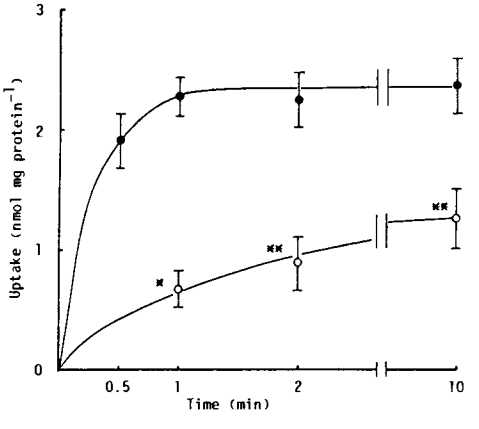

FIG. 2. Time course of propantheline binding to brush border membrane vesicles pretreated (○) and untreated (●) with neuraminidase (0·05 unit (mg protein)$^{-1}$). Incubation medium was 20 mM Hepes/Tris buffer solution containing 100 mM D-mannitol and 2·5 mM propantheline (pH 7·5). Each point represents the means ± s.e.m. of four to five experiments. * $P < 0·01$; ** $P < 0·05$, significantly different from untreated vesicles.

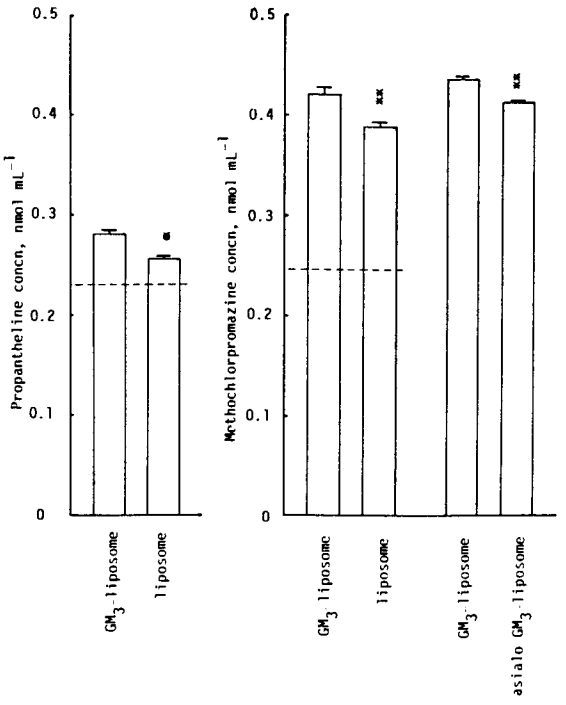

FIG. 3. Binding of propantheline and methochlorpromazine to liposomes after 3 h incubation at 25°C. Columns represent mean values with vertical bars showing s.e.m. and $n \geq 4$ for each group. Dotted line indicate the equilibrium concentration of drugs in the absence of liposome. * $P < 0.01$; ** $P < 0.005$, significantly different from GM_3-liposome.

liposomes after separation of liposomes from free GM_3 using Sephadex G 50 gel filtration. The trap ratio of GM_3 within liposomes was about 96%. Moreover, we confirmed that liposomes and GM_3 did not permeate across the dialysis membrane used in this binding study.

Discussion

Previously (Saitoh et al 1987, 1988a), we have shown that QACs bind rapidly to the brush border membrane of the rat small intestine. We also indicated that the binding of propantheline to the brush border membrane was saturable and inhibited to varying extents by several QACs (Saitoh et al 1987). Moreover, we observed that the Scatchard plot of the methochlorpromazine to the brush border membrane indicated two types of binding sites (Saitoh et al 1988b). These binding properties of QACs, therefore, suggest the presence of a common binding site for such organic cations on the brush border membrane.

Sialic acids constitute the most common terminal residue of intestinal microvillus membrane glycoproteins and glycolipids (Forstner & Wherrett 1973; Egberts et al 1984). In this study, we have examined the involvement of sialic acid in the (high-affinity) binding of a QAC, a positively charged compound, by rat intestinal microvillus membrane. The binding of propantheline was markedly inhibited by pretreatment of the brush border membrane vesicles with neuraminidase. We also observed that the content of sialic acid released from glycoprotein was higher than that from glycolipid. These results presented herein indicate that the microvillus membrane sialoglycoproteins are more susceptible to hydrolysis with exogenous neuraminidase than sialoglycolipid, and both sialoglycoconjugates, especially glycoprotein, might contribute to the binding of propantheline to the brush border membrane. Perhaps sialic acid might be an indispensable constituent of the binding site for QACs. This binding is in agreement with the observation of Zaleska & Erecinska (1987) that sialoglycoconjugates, especially glycoprotein, may be involved in high affinity uptake of dopamine by the synaptosomal membrane.

Moreover, it was possible to determine low concentrations of sialic acid reproducibly and sensitively by the HPLC method we describe.

To further elucidate the involvement of sialic acid in the binding of QACs, binding experiments using equilibrium dialysis were carried out with liposomes composed of phospholipid and GM_3 isolated from rat intestine. In that experiment we did not use glycoprotein as a model of sialoglycoconjugate because of the difficulties of isolation and incorporation into the lipid layer. Both propantheline and methochlorpromazine bound to the liposomes, and incorporated GM_3 significantly increased the binding capacity of the liposomes. Moreover, the binding activity of GM_3 for methochlorpromazine was inhibited in the presence of propantheline. The involvement of phosphatidylserine, a negatively charged phospholipid, in the binding of gentamicin (Kubo et al 1986) and methochlorpromazine (Elferink 1977), which are positively charged compounds, by liposomes has been shown. These observations and the present results suggest that the negatively charged components of the membrane surface may play a role in the binding of positively charged compounds to the biological membrane.

In another experiment, we observed that the binding of methochlorpromazine to the brush border membrane was significantly higher than that of propantheline (Saitoh et al 1988b). Our present results agree with this observation. Although the reason for the difference observed in the affinity to the liposomes between two drugs should be further examined, it is suggested that the structure of methochlorpromazine and the partition coefficients of the two drugs are associated with their affinity to the lipid layer. Methochlorpromazine has the same tricyclic structure as chlorpromazine and it is well known that the phenothiazines such as chlorpromazine and tricyclic antidepressive substances bind to the lipid layer (Cater et al 1974; Zimmer & Schulze 1981). Moreover, the partition coefficients of methochlorpromazine and propantheline in chlorform–modified Ringer solution (pH 7·4) system were 1·0 and 0·4, respectively.

In conclusion, it is suggested that the binding of QACs to the lipid layer and sialic acid might play a role in the high binding of QACs to the intestinal brush border membrane.

References

Cater, B. R., Chapman, D., Hawes, S. M., Saville, J. (1974) Lipid phase transitions and drug interactions. Biochim. Biophys. Acta 363: 54–69

Egberts, H. J. A., Koninkx, J. F. J. G., van Dijk, J. E., Mouwen, J. M. V. M. (1984) Biological and pathobiological aspects of the glycocalyx of the small intestinal epithelium. A review. The Veterinary Quarterly 6: 186–199

Elferink, J. G. R. (1977) The asymmetric distribution of chlorpro-

mazine and its quaternary analogue over the erythrocyte membrane. Biochem. Pharmacol. 26: 2411–2416

Forstner, G. G., Wherrett, J. R. (1973) Plasma membrane and mucosal glycosphingolipids in the rat intestine. Biochim. Biophys. Acta 306: 446–459

Forstner, G. G., Tanaka, K., Isselbacher, K. J. (1968) Lipid composition of the isolated rat microvillus membrane. Biochem. J. 109: 51–59

Gallagher, J. E., George, G., Brody, A. (1987) Sialic acid mediates the initial binding of positively charged inorganic particles to alveolar macrophage membranes. Am. Rev. Respir. Dis. 135: 1345–1352

Irwin, G. M., Kostenbauder, H. B., Dittert, L. W., Staples, R., Misher, A., Swintosky, J. V. (1969) Enhancement of gastrointestinal absorption of a quaternary ammonium compound by trichloroacetate. J. Pharm. Sci. 58: 313–315

Kessler, M., Acuto, O., Storelli, C., Murer, H., Muller, M., Semenza, G. (1978) A modified procedure for the rapid preparation of efficiently transporting vesicles from small intestinal brush border membrane. Their use in investigating some properties of D-glucose and choline transport systems. Biochim. Biophys. Acta 506: 136–154

Kubo, M., Gardner, M. F., Hostetler, K. Y. (1986) Binding of propanolol and gentamicin to small unilamellar phospholipid vesicles. Biochem. Pharmacol. 35: 3761–3765

Ledeen, R. L., Yu, R. K. (1978) in: Marks, N., Rodnight, R. (eds) Research Method in Neurochemistry. Vol. 4, Plenum Publishing Corp., New York, pp 371–410

Levine, R. R. (1966) Mechanisms of intestinal absorption as they relate to quaternary ammonium compounds. Arzneimit-Forsch. 16: 1373–1375

Levine, R. M., Blair, M. R., Clark, B. B. (1955) Factors influencing the intestinal absorption of certain monoquaternary anticholinergic compounds with special reference to benzomethamine [*N*-diethylaminoethyl-*N'*-methyl-benzilamide methobromide (MC-3199)]. J. Pharmacol. Exp. Ther. 114: 78–86

Lowry, O. H., Rosebrough, N. G., Farr, A. L., Randall, R. J. (1951) Protein measurement with the Folin phenol reagent. J. Biol. Chem. 193: 265–275

Miyazaki, K., Okamura, N., Kishimoto, Y., Lee, Y.C. (1986) Determination of gangliosides as 2,4-dinitrophenylhydrazides by high-performance liquid chromatography. Biochem. J. 235: 755–761

Ruifrok, P. G., Mol, W. E. M. (1983) Paracellular transport of inorganic and organic ions across the rat ileum. Biochem. Pharmacol. 32: 637–640

Saitoh, H., Kobayashi, Y., Miyazaki, K., Arita, T. (1987) A highly sensitive HPLC method for the assay of propantheline used to measure its uptake by rat intestinal brush border membrane vesicles. J. Pharm. Pharmacol. 39: 9–12

Saitoh, H., Kawai, S., Miyazaki, K., Arita, T. (1988a) Transport characteristics of propantheline across rat intestinal brush border membrane. Ibid. 40: 176–180

Saitoh, H., Kawai, S., Iseki, K., Miyazaki, K., Arita, T. (1988b) Binding of organic cations to brush border membrane from rat small intestine. Ibid. 40: 776–780.

Schultz, S. G., Fuisz, R. E., Gurran, P. F. (1966) Amino acid and sugar transport in rabbit ileum. J. Gen. Physiol. 49: 849–866

Suzuki, K. (1964) A simple and accurate micromethod for quantitative determination of ganglioside patterns. Life Sci. 3: 1227–1233

Tsubaki, H., Komai, T. (1986) Intestinal absorption of tetramethylammonium and its derivatives in rats. J. Pharmacobiodyn. 9: 747–754

Turnheim, K., Lauterbach, F. (1980) Interaction between intestinal absorption and secretion of monoquaternary ammonium compounds in guinea pigs–a concept for the absorption kinetics of organic cations. J. Pharmacol. Exp. Ther. 212: 418–424

Utsumi, T., Aizono, Y., Funatsu, G. (1987) Receptor-mediated intraction of ricin with lipid bilayer of ganglioside G_{M1}-liposomes. FEBS Lett. 216: 99–103

Warren, L. (1959) The thiobarbituric acid assay of sialic acids. J. Biol. Chem. 234: 1971–1975

Zaleska, M. M., Erecinska, M. (1987) Involvement of sialic acid in high-affinity uptake of dopamine by synaptosomes from rat brain. Neuroscience Letters 82: 107–112

Zimmer, G., Schulze, P. (1981) Membrane action of tricyclic drugs. Arzneimet-Forsch. 31: 1389–1392