Evidence for sialidase hydrolyzing gangliosides GM_2 and GM_1 in rat liver plasma membrane

Taeko Miyagi and Shigeru Tsuiki*

Biochemistry Laboratory, Research Institute for Tuberculosis and Cancer, Tohoku University, 4-1 Seiryomachi, Sendai 980, Japan

Received 8 July 1986

Rat liver plasma membrane removed sialic acid from mixed bovine brain gangliosides more efficiently than from sialyllactose and orosomucoid with an optimal pH of 4.5. When individual gangliosides, each labeled with [^{14}C]sialic acid or [^{3}H]sphingosine, were tested, not only GD_{1a} and GM_3 but also GM_2 and GM_1, both of which had been considered to resist mammalian sialidases, were desialylated. The products of GM_2 and GM_1 hydrolysis were identified as asialo-GM_2 and asialo-GM_1, respectively, by thin-layer chromatography.

Sialidase *Ganglioside* *(Rat liver)* *Plasma membrane*

1. INTRODUCTION

We previously demonstrated that in rat liver cytosolic sialidase differs from lysosomal (intralysosomal) sialidase in optimal pH and substrate specificity: the former hydrolyzes oligosaccharides, glycopeptides, glycoproteins and gangliosides at near neutral pH [1] while the latter hydrolyzes only oligosaccharides and glycopeptides at acidic pH [2]. According to [3], however, still another sialidase may be present in rat liver plasma membrane and desialylate gangliosides rather efficiently, although no study has since been made to identify the enzyme. The purpose of the present paper is to show that rat liver plasma membrane in fact possesses sialidase attacking not only GM_3 but also GM_2 and GM_1, which have been shown to resist numbers of mammalian sialidases [4–6] including the one from rat liver cytosol [1].

* To whom correspondence should be addressed

Abbreviations: NeuAc, *N*-acetylneuraminic acid; UDP-Gal, UDP-galactose; UDP-GalNAc, UDP-N-acetylgalactosamine

2. MATERIALS AND METHODS

2.1. *Materials*

Plasma membrane was isolated from the liver of male Wistar rats (200–250 g) according to [7]. Golgi membrane was isolated from rat liver and transplantable rat hepatoma (AH-109A, solid form) [8] as described in [9]. Standard gangliosides GM_1 (porcine brain), GM_2 (Tay-Sachs brain) and GD_{1a} (bovine brain) were kindly provided by Dr A. Makita, Hokkaido University, Sapporo, Japan. GM_1 and GD_{1a} were isolated from bovine brain mixed gangliosides (Sigma, St. Louis, MO) by chromatography on Iatrobeads (Iatron, Tokyo) [10]. GM_3 (human liver) and GM_2 (Tay-Sachs brain) were generous gifts from Drs Y. Suzuki and M. Matsumoto, Shizuoka College of Pharmacy, Shizuoka, and from Dr S. Handa, Tokyo Medical and Dental University, Tokyo, respectively. Lactosylceramide was from Calbiochem (La Jolla, CA) and β-*N*-acetylhexosaminidase (jackbean) from Seikagaku Kogyo (Tokyo). Asialo-GM_1 and asialo-GM_2 were prepared by hydrolyzing GM_1 and GM_2, respectively, with *Arthrobacter ureafaciens* sialidase (Nakarai, Kyoto) in the presence of bile salts [11]. Silica gel thin-layer plates (Kieselgur

Published by Elsevier Science Publishers B.V. (Biomedical Division)
00145793/86/$3.50

60) were purchased from Merck (Darmstadt). CMP-[^{14}C]NeuAc was obtained from New England Nuclear (Boston, MA) and CMP-NeuAc prepared as described in [12]. UDP-Gal and UDP-GalNAc were the products of Sigma. For the sources of other materials, see [1].

2.2. *Preparation of radioactive gangliosides*

[^{14}C]NeuAc-labeled gangliosides, then abbreviated [^{14}C]gangliosides, were prepared according to [13] but with modifications. Golgi membranes were used as the sources of glycosyltransferases [14]. To obtain [^{14}C]GM_3, the incubation mixture (10 ml) contained CMP-[^{14}C]NeuAc (40 μCi, 200 mCi/mmol), lactosylceramide (8 μmol), Triton X-100 (20 mg), sodium cacodylate (1 mmol, pH 6.2) and AH-109A Golgi membrane (28 mg). When GD_{1a} labeled in the terminal sialic acid residue was being prepared, the reaction mixture (2.5 ml) contained CMP-[^{14}C]NeuAc (10 μCi, 10 mCi/mmol), GM_1 (3 μmol), Tween 80-Triton CF-54 (15 mg, 1/2 by wt), sodium cacodylate (250 μmol, pH 6.2) and liver Golgi (10 mg). After 20 h at 37°C, the mixture was lyophilized, added with 3 ml chloroform-methanol (2:1, v/v) and centrifuged. The extract was then evaporated under N_2 and eluted from a 2.0 × 3.0 cm Sephadex G-25 column with chloroform-methanol-water (60:30:4.5). The radioactive iodine-positive fractions were pooled, evaporated, applied to a 0.25 mm thick silica gel 60 plate and developed with chloroform-methanol-water (60:35:8). The radioactive spot corresponding to GM_3 or GD_{1a} was scraped off and extracted with chloroform-methanol-water (10:10:1). The yields of [^{14}C]GM_3 and [^{14}C]GD_{1a} were 2.0 × 10^6 and 2.2 × 10^5 cpm, respectively. Half the amount of [^{14}C]GM_3 formed was incubated with UDP-Gal (8 μmol), UDP-GalNAc (8 μmol), Triton X-100 (6 mg), Tween 80-Triton CF-54 (12 mg, 1/2 by wt), $MnCl_2$ (50 μmol), sodium cacodylate (300 mol, pH 7.3) and rat liver Golgi (10 mg) in 2.0 ml to obtain [^{14}C]GM_1. After 20 h at 37°C, the [^{14}C]GM_1 formed was isolated as was [^{14}C]GM_3. Two-thirds of the radioactivity of [^{14}C]GM_3 used was recovered as [^{14}C]GM_1. [^{14}C]GM_2 was prepared by incubating half the amount of [^{14}C]GM_1 formed with rat liver β-galactosidase (0.2 U) and sodium acetate (10 μmol, pH 5.0) in 0.1 ml for 36 h at 37°C. The β-galactosidase used was solubilized from the lysosomal fraction [2] and eluted from a Con A-Sepharose column (equilibrated with 10 mM potassium phosphate, pH 6.8, containing 0.5 M NaCl) with 0.5 M methyl-α-mannoside. All these radioactive gangliosides were pure as far as the form of ganglioside was concerned and were diluted to 500 cpm/nmol (GM_3 and GD_{1a}) and 1000 cpm/nmol (GM_1 and GM_2) with the nonradioactive gangliosides before use. [^{3}H]Sphingosine-labeled GM_1, GM_2 and GM_3 were obtained according to [15]. Each of the gangliosides was diluted to 500–3000 cpm/nmol with the corresponding ganglioside.

2.3. *Assay of sialidase activity*

When [^{14}C]gangliosides were used as substrate, the standard assay mixture contained 5–20 nmol substrate, 0.1 mg sodium deoxycholate, 5 μmol sodium acetate, pH 4.5, and 50–300 μg plasma membrane in 0.1 ml. The mixture was incubated at 37°C for 30–120 min, and the sialic acid released was determined according to [16]. For the assay with [^{3}H]gangliosides as substrate, [^{3}H]ganglioside (500 cpm/nmol) replaced ^{14}C-substrate and the asialo ^{3}H-product was quantitated. After incubation, the reaction mixture was partitioned by adding 0.3 ml water and 2 ml chloroform-methanol (2:1). The lower phase was washed with two-thirds of its volume of chloroform-methanol-water (3:48:47), evaporated and chromatographed on a silica gel plate using chloroform-methanol-water (65:25:4) as developer. The area corresponding to asialo ^{3}H-product was scraped off and counted in a liquid scintillation spectrometer. Hydrolysis of nonradioactive substrates was measured as described in [1]. In all these assays, deletion of endogenous substrates by preincubation was not conducted since it resulted in a considerable loss of enzyme activity.

2.4. *Assays of other enzymes*

5′-Nucleotidase [17], Mg^{2+}-ATPase [17], alkaline phosphatase [7], cytochrome oxidase [18], glucose 6-phosphatase [18], catalase [18], acid phosphatase [18], β-*N*-acetylglucosaminidase [18] and sialyltransferase [12] were assayed as described. When β-galactosidase and β-*N*-acetylgalactosaminidase were assayed, 200 nmol of respective 4-MU-glycoside, 10 μmol sodium

acetate, pH 4.5, and plasma membrane sialidase fraction in 0.2 ml were incubated at 37°C. After 30 min, the 4-MU released was measured fluorometrically as described in [1].

2.5. *Identification of sialidase products*

[^{3}H]GM_1 or [^{3}H]GM_2 (3000 cpm/nmol, 15 nmol) was incubated with plasma membrane (0.5 mg protein) in 0.2 ml in the presence of 0.05% deoxycholate at pH 4.5. After 20 h at 37°C, the reaction mixture was subjected to thin-layer chromatography as described for the assay method. Radioactive asialo products were detected by fluorography [19] and standards were visualized with anthrone reagent. In a 10-fold large-scale experiment with cold GM_1 as substrate, the product recovered from the plate was incubated with rat liver β-galactosidase (0.2 U), deoxycholate (0.05 mg) and sodium acetate (10 μmol, pH 5.0) in 0.1 ml for 36 h at 37°C. After partitioning, the evaporated digest was incubated with jackbean β-*N*-acetylhexosaminidase (0.2 U) and sodium citrate (5 μmol, pH 5.0) for 36 h at 37°C. After each step, the digest was applied to a silica gel plate.

3. RESULTS AND DISCUSSION

The plasma membrane was isolated from rat liver homogenate according to [7] and identified by enrichment of plasma membrane markers (5′-nucleotidase, ATPase and alkaline phosphatase) and elimination of markers for other membrane components (cytochrome oxidase for mitochondria, glucose 6-phosphatase for microsomes, catalase for peroxisomes, acid phosphatase and β-*N*-acetylglucosaminidase for lysosomes and sialyltransferase for Golgi) (table 1). When this preparation was assayed for sialidase activity at pH 4.5, mixed gangliosides (bovine brain),

Table 1

Ganglioside-sialidase and several marker enzyme activities in rat liver plasma membrane

Enzymes	Specific activity	Relative specific activity	Recovery
5′-Nucleotidase	36.8 ± 3.5	27.5 ± 4.3	14.7 ± 1.4
Mg^{2+}-ATPase	45.8 ± 1.9	8.5 ± 0.9	6.0 ± 0.2
Alkaline phosphatase	3.4 ± 0.5	17.8 ± 1.9	11.1 ± 0.8
Cytochrome oxidase		0.61 ± 0.21	0.68 ± 0.15
Acid phosphatase		1.22 ± 0.12	0.67 ± 0.17
N-Acetylglucosaminidase		0.60 ± 0.21	0.35 ± 0.13
Catalase		0.81 ± 0.13	0.39 ± 0.07
Glucose-6-phosphatase		0.98 ± 0.02	0.59 ± 0.03
Sialyltransferase		0.87 ± 0.08	0.44 ± 0.05
GM_1-sialidase	0.66 ± 0.17		
GM_2-sialidase	2.58 ± 0.22	15.2 ± 2.1	5.5 ± 0.4
GM_3-sialidase	12.7 ± 1.6	16.1 ± 1.1	9.4 ± 1.1
GD_{1a}-sialidase	12.9 ± 2.0	14.7 ± 0.5	9.2 ± 0.4

The plasma membrane fraction as well as the homogenate was assayed for the enzymes indicated. For sialidase assay, [^{14}C]gangliosides served as substrate. Values are means ± SE of 3–7 experiments. Specific activities for the plasma membrane are expressed in μmol (5′-nucleotidase, ATPase and alkaline phosphatase) or nmol (sialidase)/h per mg. Relative specific activity is the specific activity for the plasma membrane fraction/specific activity for the whole homogenate. Recovery: percent recovery of the homogenate activity in the plasma membrane fraction

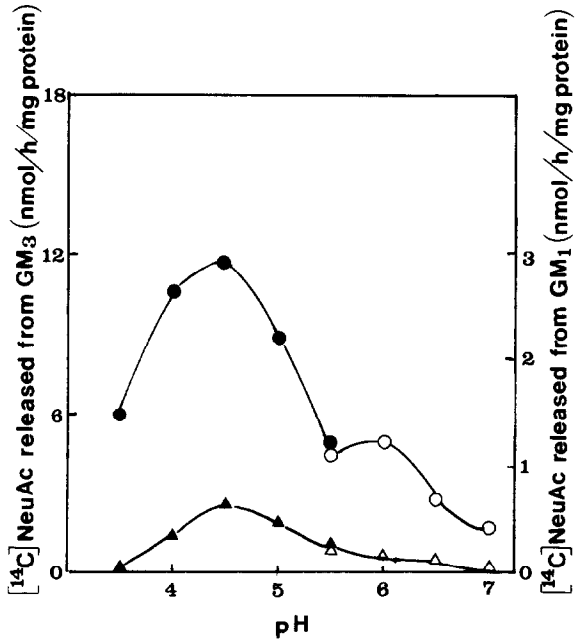

Fig.1. Effect of pH on plasma membrane sialidase. The enzyme was assayed with [^{14}C]GM_3 (●,○) or [^{14}C]GM_1 (▲, △) as substrate at pH values obtained with 50 mM sodium acetate (●, ▲) or sodium cacodylate (○, △).

(2→3)sialyllactose and orosomucoid were hydrolyzed at relative rates of 100, 60 and 3, respectively. This preference for gangliosides distinguishes the membrane sialidase from other sialidases, since the best substrate for intralysosomal and cytosolic sialidase is sialyllactose [1,2]. The activities of the plasma membrane preparation towards individual gangliosides were determined by using [^{14}C]NeuAc- and [^{3}H]sphingosine-labeled substrates. The results with ^{14}C-substrate reported in table 1 indicate that the plasma membrane sialidase is capable of removing sialic acid from GD_{1a}, GM_3 and GM_2. Almost the same results were obtained using ^{3}H-substrate (not shown). Comparison of these activities with those for various marker enzymes in relative specific activity

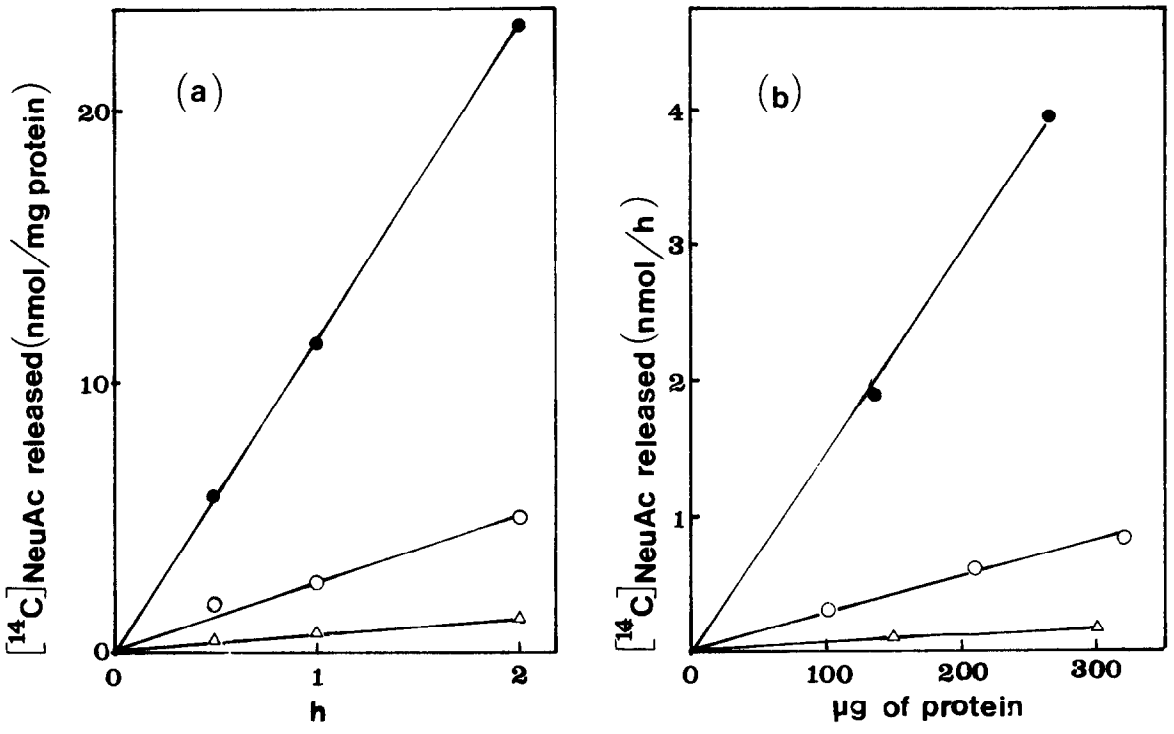

Fig.2. Effect of incubation time (a) and enzyme concentration (b) on plasma membrane sialidase reaction. [^{14}C]GM_3 (●), [^{14}C]GM_2 (○) or [^{14}C]GM_1 (△) was used as substrate.

and percentage yield further suggests that in rat liver, GD_{1a}, GM_3 and GM_2 are hydrolyzed mainly if not entirely in the plasma membrane. We were unable to compute the percentage yield of the GM_1-hydrolyzing activity since in the homogenate, GM_1 was converted to GM_2. This conversion was not detectable in the plasma membrane preparation as demonstrated below, but GM_1 was hydrolyzed at a significant rate (table 1). As shown in fig.1, both the GM_3- and GM_1-hydrolyzing activities were maximum at pH 4.5 though the curve for GM_3 had a shoulder at pH 6.0. Bile salts and Triton X-100 were not essential to these activities, but they stimulated the reactions to variable extents.

Although Brady and his co-workers once reported GM_2-hydrolyzing activity in the intestine [16] and heart [20] of the rat, accumulated evidence suggests that mammalian sialidases lack the activities towards GM_1 and GM_2 [1,4–6]. Additional experiments were therefore carried out to confirm that the plasma membrane sialidase actually attacks GM_1 and GM_2. Fig.2 shows that the hydrolyses of GM_1, GM_2 and GM_3 are all linear against incubation time and enzyme concentration even though the reactions with GM_1 and GM_2 were very slow. In the experiment shown in fig.3a, [^{3}H]GM_1 and [^{3}H]GM_2 were incubated with the plasma membrane preparation and the ^{3}H-products formed were analyzed by thin-layer chromatography and fluorography. When [^{3}H]GM_1 was the substrate, a major spot corresponding to authentic asialo-GM_1 (lane 1) was obtained (lane 6). Likewise, we were able to identify asialo-GM_2 as the desialylation product of GM_2 (cf. lanes 8,2). These asialo spots were not

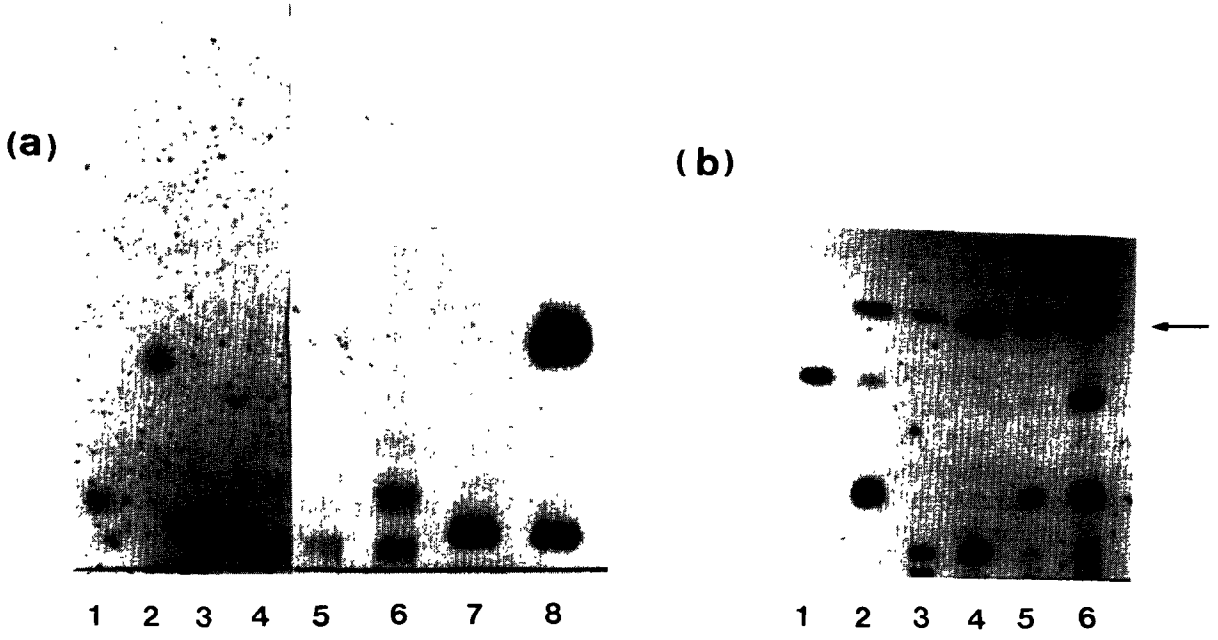

Fig.3. Identification of the products in plasma membrane sialidase reactions. (a) Thin-layer chromatography of the reaction product with [^{3}H]sphingosine-labeled GM_1 or GM_2 as substrate. The plate was developed with chloroform-methanol-water (65:25:4) as described in the text. ^{3}H-labeled compounds were detected by fluorography [19] and standards were visualized with anthrone reagent. Lanes: 1, asialo-GM_1; 2, asialo-GM_2; 3, GM_2; 4, GM_1; 5, GM_1 + boiled plasma membrane; 6, GM_1 + plasma membrane; 7, GM_2 + boiled plasma membrane; 8, GM_2 + plasma membrane. (b) The reaction product with GM_1 as substrate was sequentially digested with β-galactosidase and β-*N*-acetylhexosaminidase as described in the text. The plate was developed with chloroform-methanol-water (65:25:4) and then stained with anthrone. Lanes: 1, lactosyl ceramide; 2, asialo-GM_2 + lactosylceramide; 3, GM_1 + asialo-GM_1; 4, original product (from GM_1); 5, after digestion with β-galactosidase; 6, after digestion with β-*N*-acetylhexosaminidase. The arrow indicates deoxycholate included in the reaction mixture.

detectable when the previously boiled plasma membrane preparation was employed (lanes 5,7). Under these conditions, formation of GM_2 and GM_3 from GM_1 and of GM_3 from GM_2 was negligible (see fig.3a). The results obtained were similar when chloroform-methanol-water (60:35:8) was used as the solvent to achieve better resolution of gangliosides (not shown). It should be noted that the plasma membrane fraction exhibited low β-galactosidase and β-*N*-acetylgalactosaminidase activities with the respective 4-MU-glycosides as substrate. These indicate that the plasma membrane fraction possesses sialidase which removes sialic acid from GM_1 and/or GM_2 directly, and not with the mediation of β-galactosidase and β-*N*-acetylgalactosaminidase. To confirm further the identity of the desialylation product of GM_1, it was scraped off the plate and subjected to stepwise digestion with exogenous β-galactosidase followed by β-*N*-acetylhexosaminidase. As demonstrated in fig.3b, the products of the first and second digestions coincided with asialo-GM_2 and lactosylceramide, respectively. From the observations shown in fig.3a,b, it is evident that the major desialylation product formed from GM_1 by the plasma membrane sialidase was asialo-GM_1.

The present results clearly show that rat liver plasma membrane removes sialic acid from not only GD_{1a} and GM_3 but also GM_2 and GM_1, which have been considered to resist hydrolysis by mammalian sialidase [1,4–6]. Although it is likely that a single sialidase desialylates all of these gangliosides, the data for the pH effect and subcellular distribution may suggest that more than a single sialidase is involved in attacking gangliosides in rat liver plasma membrane. The solubilization and purification of these activities are now in progress.

ACKNOWLEDGEMENTS

We wish to thank Drs Akira Makita and Shinsei Gasa, Hokkaido University, School of Medicine, for helpful advice and discussion. We also wish to acknowledge the excellent technical assistance of Mr Kimio Konno and Mrs Chikako Ito.

REFERENCES

[1] Miyagi, T. and Tsuiki, S. (1984) Eur. J. Biochem. 141, 75–81.
[2] Miyagi, T. and Tsuiki, S. (1985) J. Biol. Chem. 260, 6710–6716.
[3] Schengrund, C.-L., Jensen, D.S. and Rosenberg, A. (1972) J. Biol. Chem. 247, 2742–2746.
[4] Tettamanti, G. and Zambotti, V. (1968) Enzymologia 35, 61–74.
[5] Leibovitz, Z. and Gatt, S. (1968) Biochim. Biophys. Acta 152, 136–143.
[6] Ohman, R., Rosenberg, A. and Svennerholm, L. (1970) Biochemistry 9, 3774–3782.
[7] Ray, T.K. (1970) Biochim. Biophys. Acta 196, 1–9.
[8] Miyagi, T., Goto, T. and Tsuiki, S. (1984) Gann 75, 1076–1082.
[9] Dewald, B. and Touster, O. (1973) J. Biol. Chem. 248, 7223–7233.
[10] Momoi, T., Ando, S. and Nagai, Y. (1976) Biochim. Biophys. Acta 441, 488–497.
[11] Sugano, K., Saito, M. and Nagai, Y. (1978) FEBS Lett. 89, 321–325.
[12] Miyagi, T. and Tsuiki, S. (1982) Eur. J. Biochem. 126, 253–261.
[13] Tallman, J.F., Fishman, P.H. and Henneberry, R.C. (1977) Arch. Biochem. Biophys. 182, 556–562.
[14] Keenan, T.W., Morre, D.J. and Basu, S. (1974) J. Biol. Chem. 249, 310–315.
[15] Schwarzmann, G. (1978) Biochim. Biophys. Acta 529, 106–114.
[16] Kolodny, E.H., Kanfer, J., Quirk, J.M. and Brady, R.O. (1971) J. Biol. Chem. 246, 1426–1433.
[17] Emmelot, P., Bos, C.J., Benedetti, E.L. and Rumke, Ph. (1964) Biochim. Biophys. Acta 90, 126–145.
[18] Yamamoto, K., Ikehara, Y., Kawamoto, S. and Kato, K. (1980) J. Biochem. (Tokyo) 87, 237–248.
[19] Randerath, K. (1970) Anal. Biochem. 34, 188–205.
[20] Tallman, J.F. and Brady, R.O. (1973) Biochim. Biophys. Acta 293, 434–443.