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DISTRIBUTION IN SPLEEN SUBCELLULAR ORGANELLES OF SIALIDASE ACTIVE TOWARDS NATURAL SIALOGLYCOLIPID AND SIALOGLYCOPROTEIN SUBSTRATES

CARA-LYNNE SCHENGRUND, MARY ANN REPMAN and JOSEPH T. NELSON

Department of Biological Chemistry, The Milton S. Hershey Medical Center, The Pennsylvania State University, Hershey, PA 17033 (U.S.A.)

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Summary

A procedure was devised for the preparation of enriched populations of subcellular organelles from homogenized bovine spleen. The fractions obtained were characterized for arylsulfatase, succinate dehydrogenase, UDPgalactosyltransferase and 5'-nucleotidase activities. The distribution of sialidase (acylneuraminyl hydrolase, EC 3.2.1.18) activity directed towards either endogenous substrate or exogenous ganglioside substrate suggests that it is enriched in the plasma membrane/microsomal fractions. Sialidase activity towards exogenous sialoglycoproteins, isolated from erythrocyte membranes, was enriched in the least dense of the plasma membrane/microsomal-containing fractions. The endogenous sialidase substrates were primarily the sialoglycolipids, hematoside and disialogangliosides. At the pH optimum, 3.8, and 37°C, release of endogenous sialic acid was linear with time for 3 h. At the end of this time, 85% or more of the available endogenous substrate was hydrolyzed.

Introduction

The removal of sialic acid from the cell surface or from circulating sialyl compounds by the action of sialidase (acylneuraminyl hydrolase, EC 3.2.1.18) has been found to alter their properties, e.g. rendering cells more susceptible to phagocytosis [1]; enhancing lymphocyte reactivity [2] and resulting in rapid clearance of desialylated compounds from the plasma [3]. Sialidase activity has been demonstrated in several mammalian organs [4,5] and in various subcellular fractions [6—8]. Several workers have reported the presence of sialidase activity in the plasma membrane of cells [9—11]. The plasma membrane

sialidase appears to act preferentially on available endogenous substrate in the membrane. Exogenous substrate is only hydrolyzed after partial depletion of endogenous substrate [7]. The physiological role of plasma membrane sialidase is not known. It may function in controlling the number of sialyl residues present on the cell surface or on extracellular sialyl compounds which impinge upon it, thereby influencing any of these phenomena.

The presence of sialic acid may affect the splenic functions of (1) clearance of damaged blood cells and foreign substances, (2) reaction with blood-borne antigen to produce antibody, (3) production of lymphocytes, and (4) in some pathological states, the production of erythrocytes and granulocytes. Little has been reported about sialidase activity in the spleen. Carubelli et al. [12] found very low levels of sialidase, active towards sialyllactose, in the 105 000 $\times g$ supernatant obtained from homogenized rat spleen. The present study was undertaken (1) to ascertain whether the spleen has sialidase activity associated specifically with certain membrane fractions, and (2) to determine sialidase activity towards the ganglioside and sialoglycoprotein substrates with which it might interact.

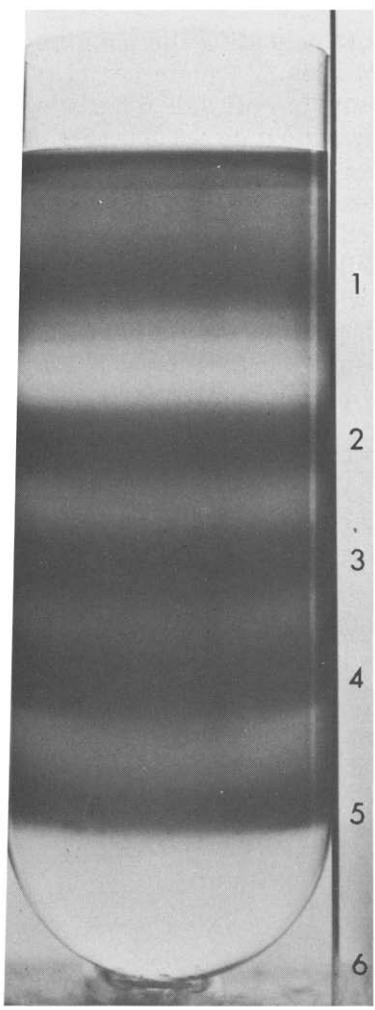
Materials and Methods

Ganglioside standards were isolated from gross gray matter of bovine brain by the partition dialysis method [13]. Individual ganglioside constituents were isolated from the aqueous phase by passage through a silica gel H column and elution with a step gradient of $CHCl_3/CH_3OH/H_2O$ of increasing polarity [14]. Hematoside was isolated in a similar manner from bovine spleen, except that the $CHCl_3$ phase was passed through the silica gel H column. AMP, oxidized horse heart cytochrome *c* (type III), bovine submaxillary mucin (type I), fetuin, and *p*-nitrocatechol sulfate were purchased from Sigma Chemical Company. ^{14}C-labelled UDPgalactose was obtained from New England Nuclear. Human chorionic gonadotropin (HCG) was obtained from Calbiochem, and erythropoietin was initially obtained as a generous gift from Dr. George A. Hayden and subsequently purchased from Connaught Laboratories (Ontario, Canada). Erythrocyte sialoglycoprotein was isolated as described by Marchesi [15]. The material isolated gave rise to four protein bands on SDS-polyacrylamide gel electrophoresis [16]. Two prominent bands were observed of molecular weight approximately 79 000 and 48 000 and two very faint bands corresponding to molecular weights of 30 000 and 21 000. Thin-layer chromatography (TLC) plates, K5 silica gel coated (0.25 mm thick) were obtained from Whatman (Clifton, NJ). Chemicals and solvents were of reagent grade quality and were used without further purification.

Source of tissue and preparation of subcellular fractions. Bovine spleens obtained within 1 h of death were placed on ice and processed within a further hour. The capsule of white connective tissue was opened and the red pulp scraped away from the large vessels and connective tissue and homogenized in 3 vols. of 0.25 M sucrose in 5 mM Tris-HCl buffer (pH 7.8) [17] with two down-and-up strokes in a Potter-Elvehjem homogenizer at 1850 rev./min. The temperature was kept at 4°C during the fractionation procedure. Pellets were prepared by centrifuging at 1000 $\times g$ for 10 min, 17 500 $\times g$ for 55 min, and

100 000 × *g* for 1 h. They were labeled P_1, P_2, and P_3, respectively. Aliquots of the P_2 homogenate were layered onto the top of a discontinuous sucrose gradient and centrifuged in a Beckman SW 25.2 rotor at 52 000 × *g* for 3 h. The gradient consisted of 10 ml 55%, 10 ml 40%, 9 ml 35%. 9 ml 30%, and 9 ml 20% sucrose in 5 mM Tris-HCl buffer (pH 7.8). A typical gradient is shown in Fig. 1.

Characterization of the subcellular fractions. In order to partially remove the sucrose from the membrane fractions, they were diluted 1 : 1 with water, and centrifuged at 100 000 × *g* for 1 h. The pellets were washed once with H_2O,



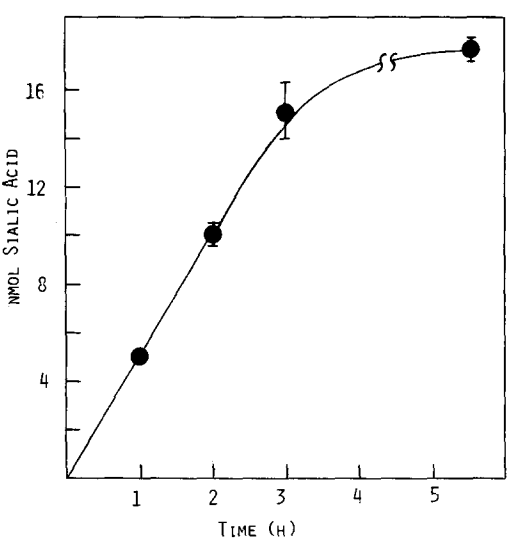


Fig. 1. A typical separation obtained upon centrifugation of fraction P2 on a discontinuous sucrose gradient. The numbers refer to the P2 subfractions recovered from the gradient. Fraction 1 banded at the 0.25 M—20% sucrose interface; 2, at the 20—30% interface; 3, at the 30—35% interface; 4, at the 35—40% interface; 5, at the 40—55% interface; and 6 was recovered as a pellet.

Fig. 2. Rate of sialic acid release by spleen plasma membrane/microsomal sialidase from endogenous substrate. The bars represent the standard deviation for duplicate determinations.

except when arylsulfatase activity was being measured (in this case, the wash was omitted). The pellets were then homogenized in H_2O and aliquots used for subsequent assays. The plasma membrane-associated enzyme, 5'-nucleotidase (EC 3.1.3.5) was assayed according to the method of Touster et al. [17], and released phosphate by the method of Fiske and SubbaRow [18]. Succinate dehydrogenase (EC 1.3.99.1), a mitochondrial marker, was determined as previously described [7]. Arylsulfatase (EC 3.1.6.1) activity, used as a measure of lysosomal content was assayed according to the procedure described by Horvat and Touser [19] using 2-hydroxy-5-nitrophenylsulfate as the substrate. Golgi enrichment was monitored by determining UDPgalactosyltransferase (EC 2.4.1.22) activity using the method described by Fleischer [20]. Appropriate controls were run for each assay.

Endogenous sialidase activity was determined by incubating the membrane preparations (up to 3 mg protein) in 1 ml 0.02 M sodium acetate buffer (pH 3.8) at 37° C for 50 min. Sialidase activity was measured at pH 3.6—5.6 and was optimum at pH 3.8. Endogenous release was linear with time for 3 h (Fig. 2). To determine exogenous sialidase activity, samples were incubated under the same conditions for at least 3 h to allow for release of 85% or more of the endogenous substrate. The sample was then centrifuged at 4° C at 12 000 $\times g$ for 20 min, and the supernatant discarded. The pellet was homogenized in the same buffer and incubated for 90 min at 37° C with 0.4 mg of disialo- and trisialogangliosides (in a molar ratio of 85 : 15), or with the desired sialoglycoprotein in 1 ml total volume. The sialoglycoprotein concentration was determined by taking an amount containing sialic acid residues equivalent to those in the ganglioside sample. To determine the amount of sialic acid present in the sialoglycoprotein samples, they were incubated in 1 ml 0.1 N H_2SO_4 for 1 h at 80° C to release bound sialic acid, which was measured by the thiobarbituric acid assay [21]. Appropriate enzyme and substrate controls were run. At the end of the reaction times (endogenous and exogenous) samples to be assayed for released sialic acid were adjusted to pH 7, passed through Dowex 1-X10 columns [22] and free sialic acid determined using the thiobarbituric acid assay [21].

Protein concentration was determined by the method of Lowry et al. [23] using bovine serum albumin as the standard.

Gangliosides were extracted from the aqueous fractions using $CHCl_3/CH_3OH$ (2 : 1, v/v) and identified qualitatively by TLC, developed in $CHCl_3/CH_3OH$/ 2.5% aq. NH_3 (65 : 35 : 8, v/v/v) for 3 h [24] and spots visualized with resorcinol spray [25]. Quantitative determination of gangliosides was by the method of Suzuki [26].

Results

The subcellular distribution of the arylsulfatase, succinate dehydrogenase, UDPgalactosyltransferase and 5'-nucleotidase activities is shown in Table I. Numerous membrane preparations were made and assayed. Each gave similar enzyme distribution patterns. The arylsulfatase activity recovered in the P2 gradient fractions was concentrated in the fraction recovered in the 55% sucrose (P2-6) and the succinate dehydrogenase in the fraction recovered in the

TABLE I

DISTRIBUTION OF ARYLSULFATASE, SUCCINATE DEHYDROGENASE, 5'-NUCLEOTIDASE, AND UDPGALACTOSYLTRANSFERASE ACTIVITIES IN BOVINE SPLEEN SUBCELLULAR FRACTIONS

The fractions P_1, P_2 and P_3 were obtained upon differential centrifugation of the homogenate. The fractions designated P2-1 through P2-6 refer to the fractions obtained upon centrifugation of fraction P2 on the discontinuous sucrose gradient (Fig. 1). Specific activities for each of the enzymes assayed are defined as follows: arylsulfatase, μg nitrocatechol liberated/h per mg protein; succinate dehydrogenase, ΔA_{550nm} (due to reduction of cytochrome *c*)/min per mg protein; 5'-nucleotidase, μmol phosphate released/20 min per mg protein; and UDPgalactosyltransferase, nmol transferred galactose/h per mg protein. Relative specific activity is defined as percent total activity/percent total protein in a set of fractions. P_1, P_2 and P_3 comprise one set of fractions and P2-1 through P2-6 the second set of fractions. Duplicate assays were made on each fraction assayed from a preparation. n.m., not measureable (activity was too low for accurate measurement).

Fraction	Enzyme activity							
	Arylsulfatase		Succinate dehydrogenase		5'-Nucleotidase		UDPgalactosyl-transferase	
	Spec. act.	Relative spec. act.	Spec. act.	Relative spec. act.	Spec. act.	Relative spec. act.	Spec. act.	Relative spec. act.
Total homogenate	66		0.026		0.24		133	
P_1	104	2.3	0.016	0.63	0.13	0.60	79	0.79
P_2	55	1.2	0.065	2.5	0.35	1.64	217	2.16
P_3	18	0.40	0.005	0.19	0.40	1.88	145	1.45
P2-1	24	0.43	n.m.	n.m.	0.73	2.32	71	0.58
P2-2	19	0.36	n.m.	n.m.	0.74	2.32	267	2.20
P2-3	30	0.54	0.024	0.47	0.38	1.18	302	2.49
P2-4	50	0.91	0.056	1.1	0.26	0.83	139	1.14
P2-5	69	1.3	0.085	1.65	0.20	0.62	20	0.16
P2-6	125	2.3	n.m.	n.m.	0.34	1.07	22	0.14

40—55% sucrose interface (P2-5). In contrast, the 5'-nucleotidase activity was enriched in the fractions banding at the 0.25 M—20% (P2-1) and 20—30% (P2-2) sucrose interfaces, and the UDPgalactosyltransferase activity in the fractions banding at the 20—30% (P2-2) and 30—35% (P2-3) sucrose interfaces.

Sialidase activity towards endogenous substrate was determined as a function of time and of the amount of protein taken. Sialic acid release was essentially linear for the first 3 h, at which point about 85% of the available substrate had been released (Fig. 2). The rate of release decreased sharply after that time. Increasing the amount of protein to 3.5 mg/assay had no effect on the rate of endogenous release (Fig. 3). The preferred endogenous substrate for the membrane-bound sialidase were hematoside and the disialogangliosides. Comparison of lipid- ($CHCl_3/CH_3OH$ extractable) and protein-bound sialyl residues before and after incubation suggested that less than 10% of the total sialic acid released from endogenous substrate was from sialoglycoproteins. Changes in the concentration of specific sialoglycolipids during incubation were measured quantitatively [26] and the results are shown in Table II. The subcellular distribution of endogenously directed sialidase activity is shown in Table III.

Exogenously directed sialidase activity was directly proportional to the amount of protein in the sample assayed (Fig. 3). With 0.4 mg added ganglioside substrate sialic acid release was linear for more than 90 min and

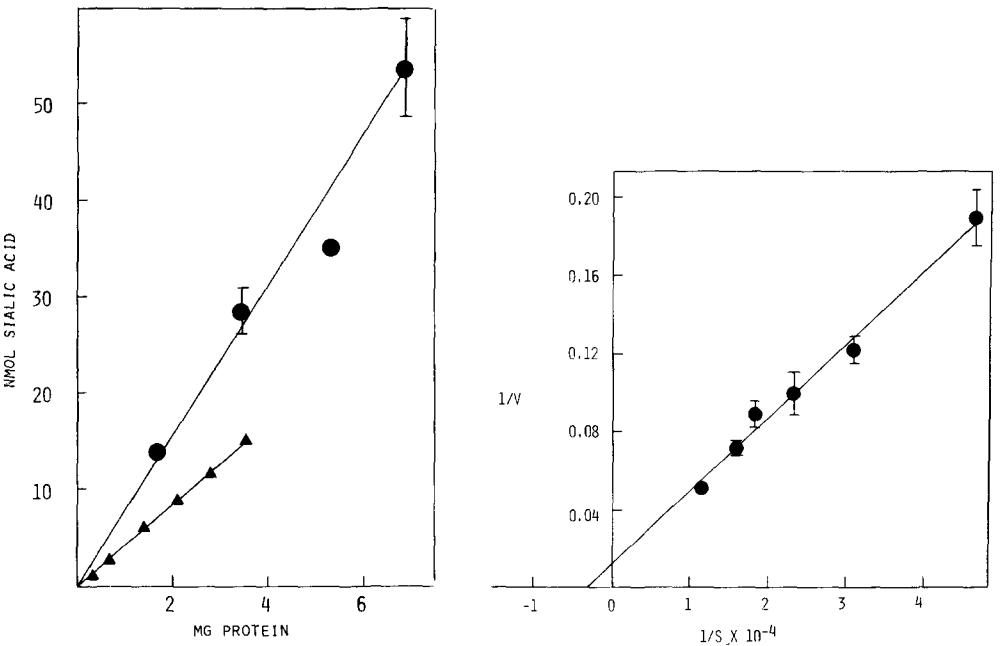


Fig. 3. Relationship between quantity of membrane protein used and sialic acid released by spleen plasma membrane/microsomal sialidase from endogenous (△———△) and exogenous (added ganglioside) (○———○) substrates. Endogenous release was measured after a 3 h reaction time and exogenous release after a 90 min reaction time. The standard deviation for the duplicate assays is indicated by bars when it was greater than the area covered by the symbol used.

Fig. 4. Lineweaver-Burk plot of preincubated spleen plasma membrane/microsomal sialidase acting upon exogenous ganglioside substrate, consisting of 95 mol% di- and 5 mol% monosialoganglioside. Samples were incubated at 37°C for 30 min. The value obtained for the K_m is $3 \cdot 10^{-4}$ M.

with 0.1 mg added ganglioside substrate release was linear for at least 40 min. The rate of sialic acid release did not increase when the ganglioside substrate concentration was increased above 0.2 mg/ml reaction mixture. The K_m was

TABLE II

THE EFFECT OF ENDOGENOUSLY DIRECTED SPLEEN PLASMA MEMBRANE/MICROSOMAL SIALIDASE ACTIVITY ON THE INDIVIDUAL GANGLIOSIDE COMPONENTS

The abbreviations used for the gangliosides are those proposed by Svennerholm [33]. Data are expressed as mol%. The relative mol% of the ganglioside constituents were determined for lipid samples extracted from equivalent amounts of protein by the method of Suzuki [26]. —, absence of measurable quantities of that ganglioside component.

Ganglioside	Time (h)				
	0	1	2	3	4
G_{M3}	65	65	59	58	51
G_{M2}	6.8	5.6	5.9	7.1	8.8
G_{M1}	7.5	11	20	35	40
G_{D1a} and G_{D2}	17	18	15	—	—
G_{D1b}	3.6	—	—	—	—

TABLE III

DISTRIBUTION OF SIALIDASE ACTIVITY IN SUBCELLULAR FRACTIONS OBTAINED FROM BOVINE SPLEEN

The fractions designated P2-1 through P2-6 refer to the fractions obtained upon centrifugation of fraction P2 on the discontinuous sucrose gradient (Fig. 1). Duplicate assays were made on each fraction from each preparation. Specific activity for sialidase directed toward the indicated substrates is defined as follows: endogenous, nmol sialic acid released/50 min per mg protein; gangliosides and erythrocyte sialoglycoprotein, nmol sialic acid released/ 90 min per mg protein. Numerous preparations were assayed and similar enzyme distribution patterns were obtained. n.m., not measurable (activity too low for accurate determination).

Fraction	Sialidase activity							
	Endogenous substrate		Exogenous substrate					
			Gangliosides		Erythrocyte membrane sialoglycoproteins			
					Bovine		Human	
	Spec. act.	Relative spec. act.	Spec. act.	Relative spec. act.	Spec. act.	Relative spec. act.	Spec. act.	Relative spec. act.
Total homogenate	2.1		0.66		0.58		0.25	
P_1	n.m.	—	0.26	0.25	0.32	0.51	0.08	0.22
P_2	4.0	1.9	3.2	3.0	1.2	1.9	0.96	2.8
P_3	4.6	2.2	0.56	0.53	0.95	1.5	0.41	1.2
P2-1	5.7	1.1	4.6	1.5	2.6	2.8	3.2	4.9
P2-2	10	2.1	5.1	1.6	0.86	0.93	0.64	1.0
P2-3	8.9	1.8	4.6	1.5	0.91	0.98	0.28	0.43
P2-4	7.4	1.5	3.0	0.96	0.79	0.85	0.35	0.52
P2-5	3.0	0.62	0.68	0.22	0.59	0.64	0.32	0.47
P2-6	1.1	0.22	n.m.	—	0.05	0.05	0.10	0.14

determined for sialidase acting on added ganglioside substrate (in a molar ratio of 95% di-, 5% monosialoganglioside) at concentrations below the critical micelle concentration (0.18 mg/ml) and was $3 \cdot 10^{-4}$ M (Fig. 4).

Spleen sialidase exhibited essentially no activity towards human chorionic gonadotropin, bovine submaxillary mucin or fetuin. Erythropoietin was susceptible to the action of the membrane-associated sialidase. Sialoglycoprotein isolated from either bovine or human erythrocyte plasma membranes was also susceptible to the action of the membrane-associated sialidase. The distribution of sialidase active towards erythrocyte sialoglycoprotein and added ganglioside substrate is shown in Table III.

Discussion

The method presented for the subcellular fractionation of bovine spleen cells yielded fractions enriched in plasma and microsomal membranes, relatively free of mitochondrial and lysosomal contaminants. This is demonstrated by the distribution of succinate dehydrogenase, arylsulfatase and 5′-nucleotidase activities. Although UDPgalactosyltransferase activity was maximal in P2-3, there was considerable contamination of the plasma membrane/microsomal fractions with Golgi. The fractionation procedure can be completed in 1 day

and if desired, can be performed on a preparative scale using a zonal rotor. The results obtained were similar when either fresh or frozen spleen was used. The 3 h centrifugation time for the discontinuous gradient was essential in order to obtain a clean separation of the fractions. Shorter times resulted in a smeared enzyme distribution and less distinct bands.

Comparison of the distribution of sialidase activity in the P2 subfractions with that of the marker enzymes (Tables I and III) shows that sialidase activity directed towards endogenous substrate, while present in all fractions, is enriched in those fractions containing plasma and microsomal membranes as well as those containing Golgi. Sialidase activity directed toward added ganglioside substrate showed a similar distribution. Bands P2-1 through P2-3 (Fig. 1) contained 70% of the total recoverable ganglioside sialidase activity. P2-1 and P2-2 contained more than half of this activity or over 40% of the total activity recovered in all of the P2 subfractions. The presence of 40% or more of the sialidase activity in the less dense plasma membrane/microsomal fractions is compatible with our previous finding that approx. 40% of the total sialidase activity of transformed fibroblasts is localized on the outer surface of the plasma membrane [10]. The ganglioside sialidase activity is probably not attributable to Golgi contamination since Kishore et al [8] have reported that in Golgi preparations obtained from rat liver, the sialidase is inactive towards disialogangliosides. Sialidase activity towards added sialoglycoprotein (isolated from either human or bovine erythrocyte membranes) was enriched in the lightest gradient fraction suggesting that this activity is also associated with the plasma membranes and microsomes.

The properties of the spleen plasma membrane/microsomal sialidase are similar to those found for sialidase associated with the plasma membranes of cells from other organs. The sialidase has an acid pH optimum which might function as a control of the enzymatic activity. The K_m of $3 \cdot 10^{-4}$ M obtained using predominantly disialoganglioside substrate (95%) is similar to the value of 10^{-4} M obtained by Tallman and Brady [27] using G_{D1a} and G_{D1b} as substrates for rat heart sialidase. Öhman et al. [28] showed that human brain sialidase acted on gangliosides below the critical micelle concentration. In this study no change in the rate of reaction was observed for the spleen plasma membrane/microsomal sialidase when the substrate concentration was increased above the critical micelle concentration. The similarity of properties obtained for the spleen sialidase to those obtained for synaptic plasma membrane sialidase from bovine brains [7] and plasma membrane sialidase from rat liver [9,29] suggest a possible similarity of function for the plasma membrane/microsomal sialidase.

The presence of sialidase activity, associated with the plasma membrane/microsomal fractions of spleen cells, directed toward normal cell surface components suggests a possible function for sialidase in the normal utilization of sialylated compounds. Desialylation of plasma sialoglycoproteins has been shown to result in their more rapid clearance by the liver [3]. A protein has been isolated from rat liver which is specific for the binding of desialylated serum glycoproteins [30]. One of the functions of the spleen is the removal of damaged red blood cells from the circulation. Erythrocytes, partially desialylated by sialidase treatment are cleared more rapidly from the circulation and are found in the liver and spleen [31,32]. One possible role for the

splenic sialidase is to remove surface sialyl residues from the circulating red blood cells thereby enhancing their removal by the spleen.

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References

1 Lee, A. (1968) Proc. Soc. Exp. Biol. Med. 128, 891—894
2 Han, T. (1972) Transplantation 14, 514—517
3 Ashwell, G. and Morell, A.G. (1975) Adv. Enzymol. 41, 99—128
4 Morgan, E.H. and Laurell, C.-B. (1963) Nature 197, 921—922
5 Mahadevan, S., Nduaguba, J.C. and Tappel, A.L. (1967) J. Biol. Chem. 242, 4409—4413
6 Tulsiani, D.R.P. and Carubelli, R. (1970) J. Biol. Chem. 245, 1821—1827
7 Schengrund, C.-L. and Rosenberg, A. (1970) J. Biol. Chem. 245, 6196—6200
8 Kishore, G.S., Tulsiani, D.R.P., Bhavanandan, V. P. and Carubelli, R. (1975) J. Biol. Chem. 250, 2655—2659
9 Visser, A. and Emmelot, P. (1973) J. Membrane Biol. 14, 73—84
10 Schengrund, C.-L., Rosenberg, A. and Repman, M.A. (1976) J. Cell. Biol. 70, 555—561
11 Cruz, T.F. and Gurd, J.W. (1978) J. Biol. Chem. 253, 7314—7318
13 Carubelli, R., Trucco, R.E. and Caputto, R. (1962) Biochim. Biophys. Acta 60, 196—197
13 Folch, J., Arsove, S. and Meath, J.A. (1951) J. Biol. Chem. 191, 819—831
14 Schengrund, C.-L., Lausch, R.N. and Rosenberg, A. (1973) J. Biol. Chem. 248, 4424—4428
15 Marchesi, V.T. (1972) Methods Enzymol. 28, 252—254
16 Laemmli, U.K. (1970) Nature 227, 680—685
17 Touster, O., Aronson, N.N., Jr., Dulaney, J.T., and Hendrickson, M. (1970) J. Cell Biol. 47, 604—618
18 Fiske, C.H. and SubbaRow, Y. (1925) J. Biol. Chem. 66, 375—400
19 Horvat, A. and Touster, O. (1967) Biochim. Biophys. Acta 148, 725—740
20 Fleischer, B. (1974) Methods Enzymol. 31, 180—191
21 Warren, L. (1959) J. Biol. Chem. 234, 1971—1975
22 Horvat, A. and Touster, O. (1968) J. Biol. Chem. 243, 4380—4390
23 Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randall, R.J. (1951) J. Biol. Chem. 193, 265—275
24 Wagner, H., Hörhammer, L. and Wolff, P. (1961) Biochem. Z. 334, 175—184
25 Svennerholm, L. (1957) Biochim. Biophys. Acta 24, 604—611
26 Suzuki, K. (1964) Life Sci. 3, 1227—1233
27 Tallman, J.F. and Brady, R.O. (1973) Biochim. Biophys. Acta 293, 434—443
28 Öhman, R., Rosenberg, A. and Svennerholm, L. (1970) Biochemistry 9, 3774—3782
29 Schengrund, C.-L., Jensen, D.S. and Rosenberg, A. (1972) J. Biol. Chem. 247, 2742—2746
30 Pricer, W.E., Jr. and Ashwell, G. (1976) J. Biol. Chem. 251, 7539—7544
31 Gregoriadis, G., Putnam, D., Louis, L. and Neerunjun, D. (1974) Biochem. J. 140, 323—330
32 Durocher, J.R., Payne, R.C. and Conrad, M.E. (1975) Blood 45, 11—20
33 Svennerholm. L. (1963) J. Neurochem. 10, 613—623