24. Elgee, N. J., Williams, R. H., Lee, N. D., *J. Clin. Invest.*, 1954, v33, 1252.
25. Grodsky, G. M., Forsham, P. H., *ibid.*, 1960, v39, 1070.

Received May 22, 1961. P.S.E.B.M., 1961, v107.

Relation of Sialic Acid to Rh_0 (D) Antigen. (26730)

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Dodd *et al.*(1) reported specific inhibition of Rh_0 (D) antibody by sialic acids. We proposed to examine this inhibition using various neuraminic acid derivatives with Rh_0 (D) saline antibodies and to investigate the effect of neuraminidase, an enzyme which will remove sialic acid from erythrocytes, on agglutination of the cells by specific antiserum.

Materials and methods. N-acetylneuraminic acid (NANA) was prepared from human blood plasma according to Martensson *et al.*(2). Crystallization from glacial acetic acid yielded crystals with a melting point of 184-185°C. No glycolic acid could be detected by the method of Klenk and Uhlenbruck(3). The material moved as a single spot when analyzed by paper chromatography in the following solvents: butanol: acetic acid:water (4:1:5). Rf 0.32; butanol: propanol:0.1 N HCl (1:2:1). Rf 0.45. *Crude bovine sialic acid* (I) was prepared from mucin of bovine submaxillary glands according to Blix *et al.*(4). The dried mucin was suspended in water, sialic acid was liberated by acid hydrolysis and isolated by ion exchange chromatography(5). This material assayed 74% NANA by the resorcinol reaction(6). When examined by paper chromatography, 3 components reacting as sialic acid were observed. A small amount of ninhydrin-positive material was also present. The fastest-moving sialic acid component was identified as N,O-diacetylneuraminic acid by the fact that on further acid treatment it was converted to NANA and also gave a hydroxamic acid test for esters. According to glycolic acid analysis, the material contained 14% N-glycolylneuraminic acid (NGNA). *Crystalline bovine sialic acid* was prepared from crude bovine sialic acid (I) by crystallizing it twice from methanol-ether and once from glacial acetic acid. This material had a melting point of 186-187°C. According to glycolic acid analysis the material contained 16% NGNA. Two distinct spots were observed when the crystals were analyzed by paper chromatography in butanol: propanol:0.1 N HCl. The two spots were identified as NANA, Rf 0.45, and NGNA, Rf 0.34. *Crude bovine sialic acid*(II) was prepared by dissolving bovine submaxillary mucin in water and heating at 100°C for 30 minutes. The liberated sialic acid was isolated by methanol extraction(4). This material contained 61% NANA and 9% NGNA. It showed 5 components reacting as sialic acid when analyzed by paper chromatography. Three components were identified as NANA, NGNA, and N,O-diacetylneuraminic acid. The other 2 components are reported by Blix(7) to be triacetylneuraminic acid and an isomerization product. *Porcine sialic acid* was isolated from porcine submaxillary mucin according to Blix(6). The material contained 63% NANA and 45% NGNA. It showed NANA and NGNA spots by paper chromatography. *Human brain gangliosides* were isolated from acetone powders of fresh human grey matter by extraction with methanol-chloroform (2:1), solvent distribution according to Folch *et al.*(8), dialysis and finally silicic acid chromatography. The final product contained 23% NANA, 31% hexose (as galactose), 5.5% hexosa-

* This investigation was supported by Nat. Inst. Health Grant and by Division of Mental Hygiene and Correction, State of Ohio.

TABLE I. Inhibition of Agglutination of Rh_0 (D) Trypsinized Cells by Complete and Incomplete Antibody.

Inhibitor	Antibody dilution (reciprocal)		Agglutination		
	Incomplete	Complete	Without inhibitor	With inhibitor	Inhibitor conc., μg/0.3 ml
NANA	16		2+	2+	1012
	32		1+	1+	1012
		8	2+	2+	1012
		16	1+	1+	1012
NANA	16		4+	4+	990
	64		2+	2+	990
Crude bovine sialic acid (I)	16		4+	3+	1000
	64		2+	1+	500
Bovine sialic acid (crystalline)		32	3+	3+	830
		64	2+	2+	830
Crude bovine sialic acid (II)	16		4+	4+	996
	64		2+	1+	31
	64		2+	—	62
Porcine sialic acid		32	3+	3+	986
		64	2+	2+	986
Human brain ganglioside		16	4+/S	4+/S	580
		64	2+	1+	580

mine, and 16% sphingosine. *Antisera*—Rh_0 (D) albumin antiserum (incomplete antibody) was obtained from Dade Laboratories, Miami, Fla. Rh_0 (D) saline antiserum (complete antibody) was obtained from Knickerbocker Laboratories, New York. Anti-A serum was obtained from Ortho Pharmaceutical Co., Raritan, N. J. *Pneumococcal neuraminidase,* a highly purified preparation, was kindly supplied by Dr. E. H. Eylar (9). *Inhibition and agglutination tests* were performed as described by Bigley *et al.*(10). The materials to be tested were dissolved in phosphate buffered saline and the pH adjusted to 7.2 $\pm$ 0.2.

Results. As shown in Table I, NANA did not exhibit inhibition in any of the Rh systems tested. Crystalline bovine sialic acid failed to inhibit the agglutination of trypsinized cells with saline antibodies. However, when crystalline bovine sialic acid which had not been neutralized was tested in the same system, lysis occurred in the tube containing the most sialic acid and apparent complete inhibition was observed at a concentration of 500 μg/0.3 ml. Porcine sialic acid also failed to inhibit agglutination.

Slight inhibition was observed with crude bovine sialic acid (I), and with the human ganglioside preparation as shown in Table I. The inhibition observed with crude bovine sialic acid (II), using a 1:64 antibody dilution, could not be demonstrated in a 4+ system with a concentration of approximately 1000 μg/0.3 ml. Furthermore, the inhibition obtained in the 2+ system was erratic; the tube containing 1000 μg of material showed no inhibition. Similar results were obtained in repeated testing.

A sample of Type A, Rh positive cells was washed with saline and suspended in Tris buffer, pH 5.4, containing the neuraminidase. A control sample of cells was suspended in the buffer without enzyme and treated identically throughout the procedure. The cells were incubated at 37°C and at 30, 60, and 120 minutes, aliquots removed, cells centrifuged and the supernatant analyzed for liberated sialic acid with the thiobarbituric acid assay(11). A maximum amount of sialic acid had been liberated after incubation for 60 minutes. A packed cell volume and cell count made it possible to calculate that approximately 4×10^7 molecules of sialic acid were liberated per cell. The cells were then washed with saline and a 2% suspension in buffered saline was prepared for the agglutination test. The neuraminidase-treated cells were agglutinated more readily than the non-treated cells (Table II). The treated

TABLE II. Effect of Neuraminidase on Agglutination of Rh_0 (D) Cells with Rh_0 (D) Saline Antiserum.

Antiserum dilution (reciprocal)	4	16	32
Treated cells	4+	3+	2+
Nontreated cells	2+	1+	—

and control cells could not be distinguished when tested with anti-A serum. No inhibition of a maximal (4+) or partial (2+) agglutinating dilution with these cells was observed with NANA at a concentration of 1000 μg/0.3 ml.

Discussion. The specific inhibition of an antigen-antibody system by a compound is generally accepted as evidence of the complementary fit of the antibody and the inhibitor. No inhibition was observed with either of the crystalline sialic acid preparations or with the porcine sialic acid in the systems tested. It should be noted that slight inhibition was observed only with the impure preparations. Due to the heterogeneity of these materials, no conclusion can be made concerning the substance responsible for the slight inhibition observed.

If sialic acid constitutes a portion of the immunological determinate group of Rh_0 (D) antigen, the removal of sialic acid from Rh_0 (D) positive cells would be expected to decrease their affinity for Rh_0 (D) antibody. Pneumococcal neuraminidase was shown to liberate a large amount of sialic acid from the cells. The enzyme-treated erythrocytes were agglutinated more readily by Rh_0 (D) saline antibodies than the non-treated control cells. This phenomenon is similar to the effect produced by trypsin treatment. That trypsin and neuraminidase should affect the cells similarly with respect to Rh agglutination is particularly reasonable in view of recent reports(12,13,14) that a sialic acid-containing peptide is liberated from red blood cells by treatment with trypsin.

The behavior of the neuraminidase-treated erythrocytes and the failure of crystalline sialic acid preparations to inhibit anti-Rh_0 (D) agglutination does not support the supposition that sialic acid resembles the immunological determinate group of Rh_0 (D) antigen.

Summary. Several crude preparations of sialic acid containing different derivatives of neuraminic acid, and 2 crystalline preparations, N-acetylneuraminic acid and bovine sialic acid (containing NANA and NGNA) were tested for their ability to inhibit agglutination of Rh_0 (D) red blood cells by Rh_0 (D) antibody. Two of the crude preparations showed slight inhibition, while no effect was observed with the crystalline preparations. Rh_0 (D) positive erythrocytes were treated with neuraminidase, and sialic acid was shown to be liberated. The neuraminidase-treated cells were more readily agglutinated by Rh_0 (D) antibody than non-treated control cells.

We gratefully acknowledge the advice and cooperation of Drs. M. C. Dodd and N. J. Bigley in these studies.

1. Dodd, M. C., Bigley, N. J., Geyer, V. B., *Science,* 1960, v132, 1398.

2. Martensson, E., Raal, A., Svennerholm, L., *Biochim. et Biophys. Acta,* 1958, v30, 124.

3. Klenk, E., Uhlenbruck, G., *Z. physiol. Chem., Hoppe-Seyler's* 1957, v307, 266.

4. Blix, G., Lindberg, E., Odin, L., Werner, I., *Acta Soc. Med., Upsaliensis,* 1956, v61, 1.

5. Svennerholm, L., *ibid.,* 1956, v61, 75.

6. ———, *Biochim. et Biophys. Acta,* 1957, v24, 604.

7. Blix, G., IVth Intern. Congr. Biochem., Vienna, Austria, Symposium I: Preprint No. 4 (1958).

8. Folch, J., Lees, M., Sloane-Stanley, G. H., *J. Biol. Chem.,* 1957, v226, 497.

9. Eylar, E. H., Ph. D. Thesis, *The Purification and Action of Pneumococcal Neuraminidase,* Harvard Univ., 1958.

10. Bigley, N. J., Chandler, R. W., Dodd, M. C., *J. Immunol.,* 1958, v80, 85.

11. Aminoff, D., *Virology,* 1959, v7, 355.

12. Klenk, E., Uhlenbruck, G., *Z. physiol. Chem., Hoppe-Seyler's* 1960, v319, 151.

13. Mäkelä, O., Miettinen, T., Pesola, R., *Vox Sanguinis,* 1960, v5, 492.

14. Cook, G. M. W., Heard, D. H., Seaman, G. V. F., *Nature,* 1960, v188, 1011.

Received April 26, 1961. P.S.E.B.M., 1961, v107.