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Sialic Acid of Human Blood Platelets *

MORTON A. MADOFF,† SHIRLEY EBBE,‡ AND MARIO BALDINI

(From the Infectious Disease Service and the Blood Research Laboratory, Pratt Clinic–New England Center Hospital, and the Department of Medicine, Tufts University School of Medicine, Boston, Mass.)

Sialic acid has been demonstrated in the blood platelets of man and other species (1, 2), but its function in these elements is unknown. Jerushalmy, Kohn, and de Vries (3) and others (4, 5) have shown that Myxoviruses are adsorbed to and eluted from blood platelets. Bacterial neuraminidase, which liberates free sialic acid by cleavage of the α-ketosidic linkage between terminal sialic acid and galactose or galactosamine (6), interferes with these platelet adsorption-elution phenomena (3) in a manner qualitatively similar to its effect on erythrocytes (7) and leukocytes (8). The electrophoretic mobility of platelets is reduced after neuraminidase treatment (3), again mimicking the behavior of the other blood elements (9, 10).

Our investigations have yielded previously unknown data concerning the type and distribution of sialic acid in the platelet and its availability to viral and bacterial neuraminidases.

Materials and Methods

Human group O, Rh-positive blood was collected by antecubital puncture into plastic bags containing EDTA anticoagulant [1] (1.5% disodium ethylenediaminetetraacetate in 0.7% NaCl). The plasma was separated by centrifugation at 290 × *g* at 4° C for 25 minutes in an International PR-2 refrigerated centrifuge. This platelet-rich plasma was again centrifuged in the same manner to further reduce the number of erythrocytes and leukocytes; the platelet button was obtained by centrifugation at 1,350 × *g* at 4° C for 30 minutes. The platelets were washed 3 times by resuspension in approximately 50 vol of cold Tris saline buffer (0.135 M NaCl, 0.02 M Tris, pH 7.4) with 0.02% Triton [2] and centrifugation at 1,350 × *g* at 4° C for 30 minutes. They were finally resuspended for study in Tris saline buffer containing 0.002 M $CaCl_2$, final concentration. All glassware was siliconized.

Platelet counts were done by the direct method using phase contrast microscopy (11). The platelet suspensions were diluted quantitatively to give concentrations suitable for counting. Contamination with erythrocytes and leukocytes was measured directly on the undiluted final platelet suspension. This did not exceed 200 leukocytes per mm^3 or 1,500 erythrocytes per mm^3, and the contribution of their sialic acid to the quantitation of platelet sialic acid was not considered further.

Platelet protein was measured in dilutions of fractions of the final suspensions by the Folin-phenol method of Lowry, Rosebrough, Farr, and Randall (12) with bovine serum albumin [3] as standard.

Neuraminidase of *Vibrio cholerae,* purified by the method of Mohr and Schramm (13),[4] was concentrated by pressure ultrafiltration (14) to contain 600 U of enzyme activity per ml, 1 U being equal to the amount of enzyme necessary to liberate 1 μg of sialic acid from acid alpha-1 glycoprotein [5] in 15 minutes at 37° C.

Free sialic acid was measured by the thiobarbituric acid method of Warren (15). Crystalline *N*-acetylneuraminic acid (NANA)[6] was used as a reference standard with each assay. Optical density readings in a Beckman DU spectrophotometer at 532 mμ were subtracted from the readings at 549 mμ, as suggested by Warren (15), to calculate free sialic acid. No interfering substances (16, 17) were encountered.

Total sialic acid values were obtained by hydrolysis of

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† Research career development awardee, National Institute of Allergy and Infectious Diseases, U. S. Public Health Service.

‡ Research fellow, The Medical Foundation, Inc. Present address: St. Elizabeth's Hospital, Brighton, Mass.

[1] Platelet Pack JP-1, Fenwal Laboratories, Inc., Morton Grove, Ill.

[2] WR-1339, Winthrop Laboratories, New York, N. Y.

[3] Cohn Fraction V, California Biochemical Corp., Los Angeles, Calif.

[4] Courtesy of Dr. G. Ruhenstroth-Bauer, Max. Planch. Institut für Biochemie, München, Germany.

[5] Courtesy of Dr. E. H. Eylar, University of Southern California School of Medicine.

[6] Purified from Cohn fraction IV-4. Courtesy of Dr. E. H. Eylar.

platelets in 0.1 N H_2SO_4 at 80 to 82° C for 1 hour (mild acid hydrolysis) before measuring by the Warren method. Since further hydrolyses were found, in preliminary studies, to result in an additional yield of sialic acid of less than 5%, they were not used.

Influenza PR8 virus grown in embryonated eggs was partially purified by a single cycle of adsorption-elution with human group O, Rh-positive red cells, spun at 40,000 × *g* at 4° C for 90 minutes in a Spinco Model L ultracentrifuge, and resuspended in cold Tris saline buffer immediately before use.

Sialic acid for chromatography was prepared by mild acid hydrolysis of platelets, as described. The hydrolyzed platelet remnants were spun down (21,000 × *g* for 2 hours), the supernatant fluid neutralized with 0.1 M $Ba(OH)_2$, and the precipitate removed. This supernatant liquid was passed through columns of Dowex-1-formate (1 × 10 cm), which were then washed with water and 0.05 M formic acid. Sialic acid was eluted with 0.6 M formic acid, lyophilized, and redissolved in water. Ascending paper chromatography (on Whatman 1 filter paper) was performed in two solvent systems: *n*-butanol : *n*-propanol : 0.1 N HCl (1 : 2 : 1) and ethanol : water : concentrated ammonia (80 : 20 : 1). The sialic acids were spotted in 35 to 50 μl containing 50 to 200 μg of these substances.

Crystalline *N*-acetylneuraminic acid and *N*-glycolylneuraminic acid (NGNA) from porcine submaxillary mucin [7] (80% pure) were applied to each paper as standards. The sialic acids were localized with thiobarbituric acid spray reagent (18). Preliminary studies showed that as little as 3 μg of sialic acid could be detected by these methods.

Spectral curves of the cyclohexanone extracts from the Warren assay (15) were obtained with a Cary model 15 recording spectrophotometer [8] over the wave length region from 350 to 650 mμ against a cyclohexanone "blank."

Platelet electrophoretic mobility was measured by direct microscopic observation in a microelectrophoresis apparatus with a rectangular cell oriented in the "lateral" position. The microscope was fitted with a 22 × KsCel Leitz objective, working distance 1.6 mm, and a 10 × Spencer eyepiece provided with a reticule. On the platinum electrodes a two-way switch permitted reversal of current. The entire apparatus was thermostated to ± 0.4° C. The electrophoresis buffer contained 0.201 mole of sucrose, 0.0059 mole of Na_2HPO_4, 0.0108 mole of NaH_2PO_4, and 0.0441 mole of NaCl per L, ionic strength 0.072, pH 6.4. For isoelectric point determinations, the pH was adjusted with H_3PO_4, NaH_2PO_4, Na_2HPO_4, and NaOH, and the ionic strength was kept near 0.07. Mobility measurements were made at 25° C and corrected to water at this temperature by multiplying by 1.28 the relative viscosity of the phosphate-sodium chloride-sucrose buffer.

[7] Sigma Chemical Co., St. Louis, Mo.

[8] Courtesy of Dr. Edwin Astwood, New England Center Hospital, Boston, Mass.

Preliminary measurements showed that the distribution of mobilities with depth gave the expected symmetrical parabola where the depth is the distance along the optical axis which is intersected by the two faces of the cell. Platelet mobilities were measured at the first stationary level only, 0.211 × depth. In each sample, mobilities of 10 to 15 platelets were timed in each direction. Mobility was computed from this equation:

$$\text{microns/second/volt/centimeter} = Vobs\ Ks\ q/I,$$

where *Vobs* is the observed velocity in microns per second, *Ks* is the specific conductance, *q* is the cross-sectional area of the cell, and *I* is the current.

The release of sialic acid from platelets by influenza virus, neuraminidase, and mild acid hydrolysis and the effects of platelet disruption and dextran were studied as follows. Final platelet suspensions, prepared as described previously and containing approximately 10^7 platelets per mm^3, were mixed thoroughly and divided into several samples. A measured sample was removed from each for protein determination.

a) One sample was subjected to mild acid hydrolysis.

b) Two specimens were prewarmed to 37° C in flasks in a reciprocal shaker bath. After removal of a zero-time sample, 300 U of neuraminidase was added to one sample and 10,000 hemagglutinating doses of influenza virus to the other. At suitable times, samples were removed from each flask, plunged into similar amounts of ice-cold Tris saline buffer, and spun immediately at high speed at 4° C. The supernatant liquids were frozen in dry ice-alcohol and stored at −20° C until sialic acid was measured.

c) Virus, prechilled to 4° C, was added to a platelet sample in an ice bath for 1 hour to permit virus adsorption. A sample was removed and the specimen transferred to a 37° C shaker bath and handled in the same way as the virus-treated platelets in *b*).

d) A platelet suspension was diluted with an equal amount of 6% dextran,[9] incubated at 37° C for 30 minutes, centrifuged at high speed for 30 minutes, then washed twice and resuspended in Tris saline buffer with calcium. The suspension was divided into 3 aliquots from which samples for protein determination were taken; these aliquots were subjected to acid hydrolysis or neuraminidase or virus treatment for 2 hours. They were then centrifuged and the supernatant fluids stored for sialic acid assay.

e) One sample was, six times successively, frozen in dry ice-alcohol and thawed in a 37° C bath. The frozen-thawed suspension was divided into several portions from which samples for protein were removed and treated by acid hydrolysis for 1 hour or virus or neuraminidase for 2 hours. One sample was centrifuged (21,000 × *g* for 2 hours) and the supernatant liquid assayed for sialic acid, directly and after acid hydrolysis.

Platelet samples not subjected to any treatment and appropriately diluted neuraminidase and virus were in-

[9] Dextran, 6% wt/vol in saline, Abbott Laboratories, Chicago, Ill.

cluded in each study. These were incubated identically to the test specimens, and samples were removed at the same times for sialic acid measurement.

Human group O, Rh-positive erythrocytes, and stroma prepared quantitatively by the method of Tishkoff, Robscheit-Robbins, and Whipple (19) were included as further controls and subjected to the same general procedures as the platelets. The sialic acid of erythrocytes has been studied extensively (9, 20, 21), and the amount of this substance that should be released under given conditions can be predicted with reasonable confidence. In every study, the amount of sialic acid released after neuraminidase treatment of intact erythrocytes for 2 hours or acid hydrolysis of equivalent amounts of

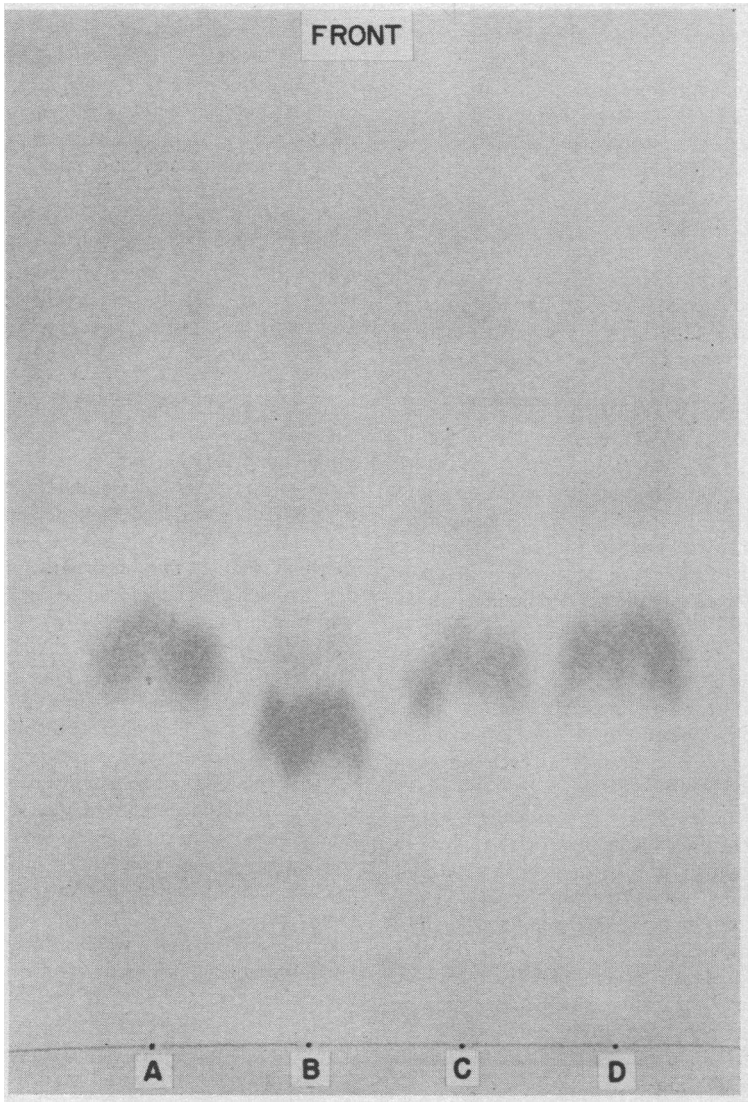

FIG. 1. CHROMATOGRAM OF PLATELET SIALIC ACID. A = Pure *N*-acetylneuraminic acid (NANA); B = *N*-glycolylneuraminic acid (NGNA), 80% pure; C = platelet sialic acid obtained by mild acid hydrolysis and chromatography on Dowex-1-formate columns; and D = mixture of platelet sialic acid and pure NANA. Chromatogram developed in *n*-butanol : *n*-propanol : 0.1 N HCl (1 : 2 : 1). R_f of NANA = 0.27 to 0.39, NGNA = 0.29 to 0.31, platelet sialic acid = 0.36 to 0.38. Spots located with thiobarbituric acid spray reagent (18). Chromatography in ethanol : water : concentrated ammonia (80 : 20 : 1) resulted in R_f of NANA = 0.26, NGNA = 0.18, platelet sialic acid = 0.28.

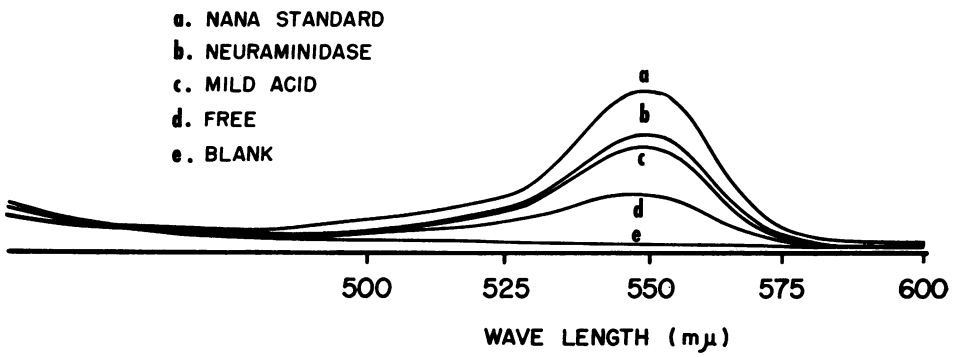

FIG. 2. TRACINGS OF ABSORPTION SPECTRA OF COLORS OBTAINED IN THIOBARBITURIC ACID ASSAY OF PLATELETS. Cyclohexanone blank (e) and pure *N*-acetylneuraminic acid standard (a) compared with platelets hydrolyzed by neuraminidase (b) and mild acid (c) and the unhydrolyzed supernatant fluid (d) of disrupted platelets.

stroma for 1 hour was equivalent to 128 to 141 μg per ml of packed erythrocytes. Virus released 54 to 65% of this amount from intact red blood cells. Since these conform to predicted values, we feel that suitable conditions existed during the platelet procedures.

Results

Paper chromatography. The free sialic acid liberated from platelets by mild acid hydrolysis moved in both solvent systems with an R_f identical to that of authentic NANA (Figure 1). No spots corresponding to NGNA or unknown substances (17) were seen. Elution of the NANA spot yielded 94 to 97% of the sialic acid applied to the paper. Only minute amounts of other sialic acids would have escaped detection.

Spectral curves. The absorption spectra of the cyclohexanone extracts from the Warren assay of pure *N*-acetylneuraminic acid, the supernatant liquid of unhydrolyzed frozen-thawed platelets, and the products of hydrolysis of frozen-thawed platelets by mild acid and pure neuraminidase all show maxima at 549 mμ (Figure 2). This indicates that the same 2-keto-3-deoxy sugar acid groups contributed the chromogen obtained with thiobarbituric acid. As noted previously, all calculations were corrected for possible interference by substances absorbing at 532 mμ. No other significant peaks were noted. The findings support the validity of the Warren method in these studies in that the values obtained were almost certainly due to sialic acid.

Quantitation of platelet sialic acid. Platelet concentration in each study was determined by protein measurement (11); the sialic acid quantities were related in terms of micrograms of sialic acid per milligram of platelet protein. Although platelet counts were also performed, the protein

TABLE I

Sialic acid in human platelets

Platelet procedure	No. of experiments	μg sialic acid/mg platelet protein			
		Free*	% of total	Total†	% of total
Intact	12	0.1	<1	15.5 ± 1.3	100
Frozen-thawed	4	1.3 ± 2.1	8–14	15.1 ± 1.5	97
Frozen-thawed supernatant liquid	4	1.3 ± 2.2	8–14	4.1	27
Intra-RDE‡ (2 hours)	6	9.5 ± 0.3	61		
Frozen-thawed-RDE‡ (2 hours)	4	11.4 ± 0.5	74		
Intact-virus (2 hours)	6	6.5 ± 0.4	42		
Intact-virus (24 hours)	4	9.9 ± 0.7	64		
Platelet control (2 hours)	6	0.1			
Platelet control (24 hours)	4	1.7			

* Determined by Warren (15) method without prior mild acid hydrolysis.
† Determined by Warren method after mild acid hydrolysis (0.1 N H_2SO_4, 80° C, 1 hour).
‡ RDE (receptor destroying enzyme) = neuraminidase.

determined in individual platelet samples was consistently reproducible, in the range of 15 to 21 mg protein per 10^{10} platelets. These values are on the low side but within the sets of values reported by others (*22, 23*).

Mild acid hydrolysis of 12 platelet samples revealed a mean value for total sialic acid of 15.5 ± 1.3 μg (SD) per mg of platelet protein, with a range of values from 13.5 to 17.6 μg. Freezing-thawing of the samples before hydrolysis did not change these values significantly.

When platelet specimens were frozen-thawed and analyzed directly for free sialic acid, certain features were, however, noted. Whereas intact platelets showed essentially no free sialic acid, frozen-thawed platelets yielded 1.3 to 2.1 μg of free sialic acid, approximately 8 to 14% of the total available (Table I). This free acid was probably inside the platelet. The clear supernatant liquid obtained by centrifugation of the frozen-thawed material contained 4.1 μg of acid-hydrolyzable and, again, 1.3 to 2.2 μg free sialic acid.

Only 61% of the total sialic acid was available for release from intact platelets by neuraminidase, which would suggest that not all of the platelet sialic acid was located on the platelet surface. Neuraminidase could release only 11.4 μg, or 74% (Table I), from platelets disrupted by freezing, however, suggesting that, in addition to location, further factors, such as steric configuration or unusual binding, rendered part of the sialic acid unavailable for enzyme action.

During an identical period of incubation with influenza PR8 virus, only 6.5 μg sialic acid was released by the action of viral enzyme, or approximately two-thirds as much as by bacterial neuraminidase. Although this amount increased to 9.9 μg on prolonged incubation, spontaneous platelet disintegration was occurring and probably contributing both free and total sialic acid to the virus-treated specimens. Confirmation was provided by control platelet specimens, showing the release of 1.7 μg of sialic acid into the suspending medium (Table I).

The release with time of free sialic acid by treatment of intact platelets with purified neuraminidase or influenza virus is illustrated in Figure 3. Sialic acid release was essentially linear, except for the 24-hour virus-treated specimen which, as noted, represented at least in part an artifact of platelet disruption. Adsorption of virus to platelets in the cold before incubation at 37° C resulted, as expected, in a slightly accelerated early release of sialic acid. The overall results were unchanged.

Prior incubation of platelets in a 3% final concentration of dextran at 37° C for 30 minutes did not interfere with the total sialic acid released by mild acid hydrolysis (Table II). The activities of the bacterial and viral neuraminidases were both reduced by approximately 20%, from

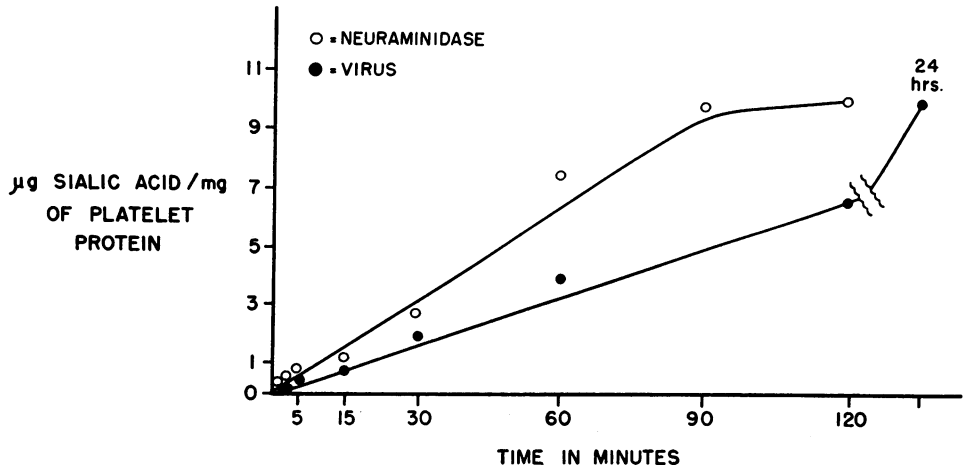

FIG. 3. RELEASE OF SIALIC ACID FROM INTACT HUMAN PLATELETS BY INFLUENZA PR8 VIRUS AND NEURAMINIDASE. Value shown for sialic acid release after 24 hours incubation with virus is not corrected for that released by spontaneous platelet disintegration, which was occurring.

TABLE II

Effect of dextran on release of sialic acid from platelets

Procedure	No. of experiments	μg sialic acid/mg platelet protein	
		Normal	Dextran*
Mild acid hydrolysis	6	16.4	16.6
Neuraminidase (2 hours)	4	10.1	8.3
Influenza PR8 virus (2 hours)	4	6.6	4.8

* Platelets incubated at 37° C for 30 minutes in 3% final concentration of dextran before hydrolysis by acid, neuraminidase, or virus.

10.1 to 8.3 and from 6.6 to 4.8 μg sialic acid per mg platelet protein, respectively.

Electrophoretic mobility. The electrophoretic mobility of human platelets in a lateral cell at pH 6.4 was reduced after neuraminidase treatment from -1.14 to -0.66 μ per second per volt per cm. The isoelectric point of normal platelets under these conditions was found to be 3.88. Experiments to determine the effects of neuraminidase and aldehydes on the isoelectric point of platelets were attempted, but neuraminidase-treated platelets exhibited great instability at markedly acidic or alkaline pH values, precluding any valid interpretation of these mobility data. These studies were therefore abandoned.

Discussion

Under most circumstances, sialic acid occurs in a bound form linked, through intermediary carbohydrates, to protein (6). The presence of free sialic acid has been reported previously, notably in the thyroid gland (24) and the cerebrospinal fluid (25, 26). Saifer and Gerstenfeld (27) have contended, however, that the substances responsible for the chromogen obtained when the Warren reaction is applied to unhydrolyzed spinal fluid are not "free" sialic acid but rather a different, unidentified material with absorption maxima at 442 mμ and 510 mμ. The latter has a sufficiently broad peak to give significant readings at 549 mμ. Materials of this nature were not detected in spectrophotometer recordings of the chromogens derived from unhydrolyzed supernatant fluids of disrupted platelets, indicating the presence of true unbound sialic acid within these blood elements. The source of this free acid is not known. Neuraminidase activity, present in several tissues (28–31), has not been reported in platelets.

N-acetylneuraminic acid is the only type of sialic acid thus far identified in human tissues. That only this variety should be present in human blood platelets is not, therefore, surprising. By comparison with human erythrocytes, the amount of sialic acid in platelets is large. If we assume a platelet surface area of 28.3 μ^2 (3) and a protein concentration of 18 mg per 10^{10} platelets, there are 19.1×10^5 molecules of sialic acid per μ^2 of platelet surface. This is approximately eleven times the concentration in human erythrocytes and is, in fact, greatly in excess of the amount in the red cells of any species studied previously (9). Only 61% of the total sialic acid of intact platelets could be liberated by neuraminidase. A significant proportion may exist in a "ganglioside-linked" form (32) resistant to enzyme attack.

Examination of the effects of dextran on the enzymatic release of sialic acid was suggested by the findings of Ross and Ebert (33) that dextran lowered the isoelectric point of platelets in citrate buffer. Previous experiments (9) have shown that the carboxyl group of sialic acid is primarily responsible for the negative surface charge of erythrocytes. In our study, removal of sialic acid was also associated with a marked decrease in the net negative charge of platelets. Ross and Ebert (33) postulated that dextran might lower platelet mobility by conjugating with cell surface proteins and, by so doing, changing their orientation. Similarly, neuraminidase release of sialic acid from platelets might be reduced by dextran conjugation with protein, producing either steric hindrance of enzymatic sites or reorientation, thus presenting sialic acid groups insusceptible to neuraminidase action.

Sialic acids have been demonstrated in most blood cells (3, 9, 10) and in other body tissues and fluids (34). Their precise function is not known. Removal of sialic acid is associated with changes in the antigenic character and survival of erythrocytes (35, 36), leukocyte metabolism (37), and the adhesiveness of malignant cells to vascular endothelium (38). The occurrence of similar effects in platelets has not been explored. Our study confirms the contribution of sialic acid to the surface charge of the blood elements (3, 9, 10). This is a descriptive phenomenon, however, rather than one of known functional significance. Gottschalk (39) has suggested that certain aspects

of the viscosity of body fluids are dependent on the presence of sialic acid and that the binding of gonadotropic hormones and erythropoietin to effector sites may also be a function of this substance.

Platelets have a marked capacity to adsorb plasma proteins to their surface (40). These proteins may not be removed, even with the extensive washing procedures we employed, and it is entirely conceivable that a significant although relatively constant amount of the sialic acid measured in these studies was contributed by adsorbed substances.

Summary

The sialic acid of platelets from human group O, Rh-positive blood was shown by paper chromatography to consist entirely of *N*-acetylneuraminic acid. Mild acid hydrolysis of platelets revealed 15.5 μg sialic acid per mg of platelet protein. Free sialic acid comprised 8 to 14% of the total in frozen-thawed material. The supernatant fluid of disrupted platelets contained 1.6 μg free and 4.1 μg total sialic acid per mg of protein. Purified neuraminidase of *Vibrio cholerae* released 9.5 and 11.4 μg per mg protein from intact and disrupted platelets, respectively. Release of sialic acid by influenza virus was slower but essentially identical on prolonged incubation. Prior exposure of platelets to dextran decreased by 21% the amount of sialic acid released by virus or bacterial enzyme. The electrophoretic mobility of platelets was reduced from − 1.14 to − 0.66 μ per second per volt per cm by neuraminidase treatment.

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