‡ Contribution No. 1385, Sterling Chemistry Laboratory, Yale University.

[1] B. R. Hammond and H. Gutfreund, *Biochem. J.*, **61**, 187, 1955.

[2] H. Gutfreund and J. M. Sturtevant, *Biochem. J.*, **63**, 656, 1956.

[3] H. Lineweaver and D. Burk, *J. Am. Chem. Soc.*, **56**, 658, 1934.

[4] G. S. Eadie, *J. Biol. Chem.*, **146**, 85, 1942.

[5] For a general review of data obtained by Niemann and co-workers see R. J. Foster and C. Niemann, *J. Am. Chem. Soc.*, **77**, 1886, 1955.

[6] B. S. Hartley and B. A. Kilby, *Biochem. J.*, **56**, 288, 1954.

[7] A. K. Balls and F. L. Aldrich, these PROCEEDINGS, **41**, 191, 1955; A. K. Balls and H. N. Wood, *J. Biol. Chem.*, **219**, 245, 1956.

[8] H. Gutfreund, *Discussions Faraday Soc.*, **20**, 167, 1955.

[9] H. Gutfreund, unpublished experiments.

[10] D. W. Thomas, R. V. MacAllister, and C. Niemann, *J. Am. Chem. Soc.*, **73**, 1548, 1951.

[11] S. Moore and W. H. Stein, *J. Biol. Chem.*, **176**, 367, 1948; **211**, 907, 1954; E. C. Cocking and E. W. Yemm, *Biochem. J.*, **58**, xii, 1954.

[12] See *Discussions Faraday Soc.*, vol. **20**, 1955.

[13] The interpretation of an experiment of this type given in our previous publication (*op. cit.*) is in error. The results of that experiment are in qualitative agreement with those of similar experiments reported in the present paper.

---

*STUDIES ON SIALIC ACID OF SUBMAXILLARY MUCOID**

BY RALPH HEIMER† AND KARL MEYER

DEPARTMENT OF MEDICINE, COLUMBIA UNIVERSITY COLLEGE OF PHYSICIANS AND SURGEONS, AND EDWARD DANIELS FAULKNER ARTHRITIS CLINIC OF THE PRESBYTERIAN HOSPITAL, NEW YORK

Communicated by Robert F. Loeb, August 2, 1956

In recent years an increasing body of information has accumulated on compounds of great biological importance, named by some "sialic acid," by others "neuraminic acid," "gynaminic acid," "hemataminic acid," "lactaminic acid," or "serolactaminic acid."[1] These substances, believed to be identical and referred to here as "sialic acid," are encountered as an important constituent of many mucoids, of complex lipids, and of certain oligosaccharides. Sialic acid has been reported in serum, urine, saliva, meconium, brain, milk, colostrum, mammary gland, etc. Increases in serum sialic acid have been established in the presence of inflammatory diseases and cancer. The acid plays a key role in influenza virus hemagglutination. Although the structure of sialic acid has been the object of study in many laboratories, twenty years of activity on the problem have not brought a satisfactory answer. Chief obstacles to success were the difficulty in preparing the naturally occurring material in large quantity and the inability to degrade it to identifiable products. The acid can be isolated after weak acid hydrolysis as well as enzymatic hydrolysis of the proper starting materials. Some investigators[2] have obtained the deacetylated methyl glycoside by metanolysis and hydrolysis. However, enzymatic degradation is preferable for obtaining the compound in good yield and as it probably occurs in nature. Work in this laboratory was undertaken following the isolation of an apparently homogeneous mucoid from submaxillary gland[3] and the hydrolysis of it by an extract of pneumococci.[4] The present communication deals with attempts to establish the mecha-

nism of enzymatic cleavage of BSM by RDE. During this study, an enzymatic degradation of sialic acid also was observed, which sheds new light on the structure of sialic acid.

EXPERIMENTAL

Isolation of BSM.—BSM was prepared by extraction at 0° of acetone powders of bovine submaxillary gland with aqeuous 6 *N* urea, adjusted to pH 9. The clear extracts obtained after centrifugation were acidified to pH 3.5, at which point a mucin clot appeared. The latter was purified by dissolving in equal amounts of *N* calcium acetate and 0.5 *N* acetic acid, and stirring with a mixture of chloroform and amyl alcohol (3:1, v:v) until fully deproteinized. The aqueous phase was fractionated by addition of ethanol. Mucoids precipitated between 46 and 78 per cent ethanol contained the same proportion of sialic acid. The homogeneous mucoid precipitated at 54 per cent ethanol was eventually selected for further study, as it accounted for 60 per cent of the total yield (9 gm. of mucoid from 100 gm. of acetone powder).

Enzymes.—RDE was prepared from filtrates of *Vibrio cholerae* in the laboratories of Sharp and Dohme, Philadelphia, Pennsylvania, and from autolysates of pneumococcus, type II, according to methods described elsewhere.[5]

Enzymatic Hydrolysis of BSM.—An aqueous solution of 600 mg. of BSM was incubated with 30 mg. of enzyme (either source), kept 16–48 hours under toluene at 37° without addition of buffer solution. The digest was dialyzed against frequently changed distilled water. The dialyzable and nondialyzable portions were isolated and analyzed after concentration and lyophilization.

Isolation of Sialic Acid.—The dialysate was shaken with Dowex 50 (H+) and chromatographed on Dowex 1–10 (formate). Sialic acid was eluted with 0.07 *N* formic acid in a single peak. After evaporation under reduced pressure, followed by lyophilization, the eluate was dissolved in absolute methanol and slowly brought to turbidity by addition of ether and petroleum ether. Crystals appeared on standing near 0° after a few days. In other experiments, slow evaporation of the solvent in a desiccator at room temperature resulted in the appearance of crystalline deposits on the walls of the vessel, which were then harvested and repeatedly recrystallized.

Analytical Methods.—Sialic acid was evaluated by Bial's orcinol, Ehrlich's dimethylaminobenzaldehyde, and Tracy's manometric technique.[6] Hexosamine was determined by the methods of Elson and Morgan,[7] as well as of Gardell;[8] reducing values by hypoiodite[9] and ferricyanide[10]; and nitrogen by the Markham modification of the micro-Kjeldahl procedure. Elementary analyses, acetyl determinations (*p*-toluenesulfonic acid), and methoxyl analyses were carried out by Schwarzkopf Microanalytical Laboratories, Woodside, New York.

RESULTS AND DISCUSSION

Inhibition of Influenza Virus by BSM.[11]—One to two micrograms of BSM inhibited 5 hemagglutinating units of indicator virus (PR8, heated 56° for 30 minutes); 2–5 μg. of BSM inhibited 5 hemagglutinating units of indicator virus LEE and active NWS virus. The inhibitory capacity of BSM was abolished by prior treatment with active influenza virus, by RDE, and by crystallized trypsin (Tryp-

tar, Armour) but was unaffected by culture filtrates of *Clostridium tertium*. Urinary mucoprotein (Tamm and Horsfall) was used as a standard of comparison in assays of BSM, 1–2 μg. inhibiting 5 hemagglutinating units of the same indicator virus.

Characteristics of BSM.—Table 1, Column I, lists chemical analyses. BSM was completely water-soluble. A 0.35 per cent solution had a relative viscosity of 3.65 at 37°. Its specific rotation was −4° at 27°. The ultraviolet spectrum showed no maxima. BSM was homogeneous and monodisperse as tested by electrophoresis (pH 4.5 and 8.6) and by ultracentrifugation[12] (pH 7). Tests for sulfate and phosphate were negative. Maximum reducing values after acid hydrolysis (*N* HCl, 100°, 4 hours) were equivalent to 16 per cent glucose. Hexosamines were identified as galactosamine and glucosamine (ratio 6:1, 15.4 per cent by the method of Gardell). Dische's carbazole test for uronic acid was negative. Tests for hexose (anthrone) and fucose gave insignificant values (1.3 and 0.5 per cent,

TABLE 1

ANALYSES OF BSM AND OF RDE-TREATED BSM FRACTIONS*

	BSM (I) (Mg.)	Nondialysable (II) (Mg.)	Dialysable (III) (Mg.)	Recovery (II) + (III) (Per Cent)
Weight	595.5	486.7	114.1	101%
Sialic acid:				
Ehrlich	148 (24.8%)	73.5 (15%)	36.2 (31.7%)	74%
Bial	160 (27.8%)	77.3 (15.9%)	36.3 (31.8%)	71%
Tracy	188 (31.6%)	92.5 (19%)	69.7 (61.2%)	86%
Total hexosamine (Elson-Morgan)	109.8 (18.6%)	94.4 (19.4%)	9.1 (8%)	95%
Hypoiodite (glucose) without hydrolysis)	23.8 (4%)	13.6 (2.8%)	34.3 (30%)	201%
$Fe(CN)_6$ (glucose) (without hydrolysis)	23.8 (4%)	43.6 (8.9%)	5.9 (5.2%)	206%
Nitrogen	54.2 (9.1%)	44.6 (9.2%)	3.7 (3.2%)	90%
Inorganic (Ca^{++})	....	....	9.5 (8.3%)	..

* 595.5 mg. BSM incubated with 30 mg. RDE, 48 hours, 37° C. (Fraction II corrected for contribution of 30 mg. RDE).

respectively). The acetyl content of BSM was 9.80 per cent. (*Theory:* 6.87 per cent if sialic acid is monoacetylated, 9.66 per cent if sialic acid contains an additional *O*-acetyl group. Theoretical values were calculated on the basis of 17.0 per cent hexosamine [av.] and 28.1 per cent sialic acid [av.].) This result indicates that BSM contains diacetylated (bovine) sialic acid.[13] When BSM was left standing at room temperature for 1 hour in the presence of 0.02 *N* Na(OH), the alkali-labile *O*-acetyl group appeared to have been hydrolyzed, as evidenced by an acetyl value of 7.53 per cent of reisolated BSM with unchanged sialic acid content (27 per cent). The amino acids after hydrolysis with 6 *N* HCl for 16 hours and determination by two-dimensional paper chromatography were valine, proline, alanine, threonine, serine, glycine, glutamic acid, aspartic acid, and lysine.

Action of RDE on BSM.—Digestion of BSM with RDE was followed by increases in hypoiodite consumption. The pH optimum of the reaction was between pH 4.5 and 6.0. No hydrolysis occurred below pH 4.0. In 3 hours, 53 per cent of BSM sialic acid was hydrolyzed in 0.003 *M* phosphate buffer, pH 6, 37° C. Digestion was accompanied by a sharp decrease in relative viscosity of BSM from 3.65 to 2.30 in 10 minutes, sloping off to 1.94 in 120 minutes. The high viscosity retained indicates that even after loss of approximately 50 per cent sialic acid, the

molecule retained considerable asymmetry. No significant changes in pH or increase in free amino or free hexosaminidic end groups[14] occurred.

Analysis of the nondialyzable mucoid core (see Table 1) indicated that this portion was in the main responsible for the increased ferricyanide reducing values observed during incubation. On the other hand, the core showed no comparable increase in hypoiodite consumption. Reduction of the core by sodium borohydride, however, caused no appreciable change in either reducing value. Ferricyanide reducing values varied with the amount of sialic acid released, being nearly 18 per cent (as glucose) when all the sialic acid had been removed from BSM by acid hydrolysis (0.5 N H_2SO_4, 2 hours, 100°). The hypoiodite reducing value of BSM freed of sialic acid was not greater than of unaltered BSM. Sodium borohydride still was ineffective in causing a change in the reducing values. These data indicate the failure of either enzyme or acid to lay open reducing end groups within the mucoid core concomitant with the hydrolysis of sialic acid. The nature of the group responsible for the ferricyanide reduction is not known.

The dialyzable portion, on the other hand, was responsible for the increase in hypoiodite consumption recorded during enzymatic incubation. The liberation of sialic acid was responsible for hypoiodite reduction. The fact that color reactions used for assay of sialic acid in the dialysate gave low recoveries, whereas Tracy's method did not, eventually led to the detection of another enzyme contained in RDE, which is responsible for a conversion of sialic acid to N-acetylglucosamine and another yet unknown compound. This will be discussed later. The ratio of sialic acid (Tracy) to hypoiodite consumption was nearly unity. Therefore, the major event during enzymatic hydrolysis of BSM attendant to the cleavage of sialic acid is the liberation of a glycosidic bond. Further evidence for this came from experiments with sodium borohydride. The latter caused no change in colorimetrically measured sialic acid in either BSM or the mucoid core, whereas the dialysate treated similarly lost all its Bial's and Ehrlich's color. This indicates that the reducing end of sialic acid must be linked to the mucoid. Furthermore, since there was no change in the free amino group content of BSM following enzymatic hydrolysis, the bond split must be O-glycosidic rather than N-glycosidic, as suggested by Gottschalk.[15] The name "sialidase" is proposed for the enzyme responsible for this action. On the other hand, since the destruction of receptor sites on mucoids capable of inhibiting influenza virus hemagglutination can be mediated by enzymes other than sialidase, it is proposed that the general term "RDE" be retained to describe those cases where destruction of biological activity alone is determined.

Characterization of Isolated Sialic Acid.—Sialic acid isolated as the methyl ester from the dialysate gave the following analysis:

	C	H	N	OCH_3	COCH
Calculated for $C_{13}H_{23}NO_{10}$	44.14	6.56	3.96	8.78	12.49
Found	44.94	6.66	4.08	8.41	12.20

Melting point: 169°–171° uncorr. $[\alpha]_D^{24} = -32°$ (water)
Yield: 40 per cent of colorimetrically estimated sialic acid, or a total of 10 per cent of BSM

The rotation as well as the infrared spectrum of this compound were nearly identical with the ones reported for sialic acid free of methoxyl groups.[16] The crystalline

material is believed to be a methyl ester. The ester gave a strong ferric hydroxamate test, in contrast to sialic acid. On paper chromatography in butanol, acetic acid, and water (50:12:25), the ester exhibited two spots, R_f 0.48 and 0.58 (sialic acid, R_f 0.24). Esterification occurred in crystallization, as the spot of R_f 0.24 was the only one found in paper chromatograms of the dialysate. The ester gave a brown spot with the benzidine trichloroacetic acid spray reagent,[17] whereas the color of the acid spot was blue. Hypoiodite titration equivalent to 1 mole of aldehyde, as well as the failure of the compound to exhibit an ultraviolet spectrum, exclude the proposed 2 keto-, 3 deoxy-, 5 deoxy-, 5 amino-, 5-acetaminononononic acid[15] as a possible structure of sialic acid.

Enzymatic Degradation of Sialic Acid.—Table 2 enumerates the changes occurring upon incubation of sialic acid with RDE. The loss of Bial's orcinol as well as the increase in hexosamine were found to be a function of time and enzyme concentration. The stoichiometric relationship between the loss of sialic acid (0.084 μM) and the appearance of hexosamine (0.089 μM), accompanied by an increase in reducing value (0.152 μM), is interpreted as an indication of internal glycosidic splitting. One product of the cleavage was N-acetylglucosamine, while the other has yet to be identified. Evidence for the former was obtained by paper chromatography as well as by absorption-spectrum analysis. Using Partridge's hexos-

TABLE 2

NET CHANGES IN ANALYSIS OF SIALIC ACID FOLLOWING INCUBATION WITH RDE* (μM)

	Initial	48 Hours	Change
Bial's orcinol (sialic acid)	0.260	0.176	−0.084
Hexosamine	.013	.102	+0.089
Hypoiodite	0.244	0.396	+0.152

* 1 mg. of methyl sialate incubated with 5 mg. of RDE (*Vibrio cholerae*), 48 hours, 37° C., under toluene, 0.003 *M* phosphate buffer, pH 5. Values expressed in micromoles after subtraction of enzyme blanks.

amine spray reagent,[18] authentic N-acetylglucosamine as well as the material obtained from the enzymatic degradation had identical R_f values. Acid hydrolysis converted this compound into glucosamine, having an R_f value indistinguishable from authentic D-glucosamine HCl when tested in two solvent systems. After ninhydrin oxidation, both compounds, when tested by the procedure of Stoffyn and Jeanloz,[19] gave arabinose. Sialic acid therefore is believed to contain a glucosamine moiety. Additionally, absorption spectra of the Elson-Morgan hexosamine chromogens were compared. Sialic acid does not show the absorption spectrum of the chromogen of N-acetylglucosamine, and acid-hydrolyzed sialic acid similarly does not resemble glucosamine. Upon incubation with enzyme, however, spectra typical for the chromogen of N-acetylglucosamine and glucosamine (after acid hydrolysis) appeared. Appropriate enzyme blanks did not exhibit the Elson-Morgan chromogen. The spectra were, furthermore, re-examined 48 hours after initial color development, to rule out anomalous hexosamine chromogens obtained by interaction of aldehyde and amine groups other than hexosamine.[20] The increase in reducing value following the degradation of sialic acid is interpreted as due to a cleavage of a glycosidic bond, linking N-acetylglucosamine with a yet to be characterized aldehyde. This aldehyde should contain an α-hydroxycarboxylic acid moiety, accounting for the low pK of sialic acid as well as for the stoichio-

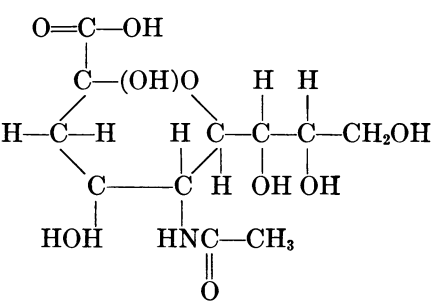
metric liberation of CO_2 with acid hydrolysis. This fragment is $C_4H_6O_4$, if sialic acid is $C_{12}H_{21}NO_{10}$. Only two structures can be written for this, namely, malic acid semialdehyde or α-hydroxy, α-methylmalonic acid semialdehyde. Since sialic acid contains a free hexosaminidic end group,[16] the glycosidic bond could be only on carbons 3–6 of the hexosamine moiety. Any acceptable structural proposal for sialic acid, however, must accommodate the fact that all efforts to obtain hexosamine by acid hydrolysis have remained unsuccessful, apparently because humin formation precedes glycolysis.

SUMMARY

A homogeneous mucoid (BSM) of bovine submaxillary gland has been described. RDE, by a mechanism of O-glycosidic cleavage, splits BSM to yield sialic acid. Sialic acid is a glycoside, yielding, upon incubation with filtrates of *V. cholerae*, N-acetylglucosamine and a yet uncharacterized reducing compound.

Addendum.—After completion of this manuscript, the following additional information has been obtained by us:

Sialic acid is split by extracts of *V. cholerae* to yield equimolar amounts of N-acetylglucosamine and pyruvic acid. The presence of pyruvic acid has been established by following the enzymatic incubation by the 2,4-dinitrophenylhydrazine reaction for pyruvic acid. Sialic acid does not interfere with this determination. The absorption spectra of the 2,4-dinitrophenylhydrazones of pyruvic acid and of the material liberated by the enzyme were identical. Evidence points to the splitting of sialic acid by an aldolase mechanism, indicating that the compound has a structure similar to the one proposed by Gottschalk:[15]



Similarly to α-keto acids, the compound reacts with semicarbazide at room temperature, although the rate of reaction is substantially slower than with other α-keto acids. This indicates a pyranose ring structure, which is also suggested by the result of periodate studies.[16] The hypoiodite reduction of sialic acid in the absence of an aldehyde group is anomalous. Sialic acid is attached glycosidically to the mucoid core, not through its aldehydic reducing end but through its carbonyl group located at C-2.

* This work was supported by a grant from the National Institutes of Health, Bethesda, Maryland.

† Taken in part from a thesis to be submitted by Ralph Heimer in partial fulfillment of the requirements for the Ph.D. degree, Faculty of Pure Science, Columbia University, New York. Abbreviations used: BSM = bovine submaxillary mucoid; RDE = receptor destroying enzyme.

[1] G. F. Springer, *Klin. Wochschr.*, **33**, 347, 1955.

[2] E. Klenk, *Z. physiol. Chem.*, **268**, 50, 1941.
[3] K. Meyer, *Advances in Protein Chemistry*, **2**, 249, 1945.
[4] K. Meyer, E. Chaffee, G. L. Hobby, and M. H. Dawson, *J. Exptl. Med.*, **73**, 309, 1941.
[5] M. M. Rapport, A. Linker, and K. Meyer, *J. Biol. Chem.*, **192**, 283, 1951.
[6] I. Werner and L. Odin, *Acta Soc. med. Upsaliensis*, **57**, 230, 1952.
[7] L. A. Elson and W. T. J. Morgan, *Biochem. J.*, **27**, 1924, 1933.
[8] S. Gardell, *Acta chem. Scand.*, **7**, 207, 1953.
[9] M. McLeod and R. Robison, *Biochem. J.*, **23**, 517, 1929.
[10] O. Schales and S. S. Schales, *Arch. Biochem.*, **8**, 285, 1948.
[11] We are indebted to Dr. Calderon Howe, of the Department of Microbiology, Columbia University College of Physicians and Surgeons, New York, for these studies.
[12] We wish to thank Dr. Baruch S. Blumberg for performing the ultracentrifugation studies.
[13] G. Blix, E. Lindberg, L. Odin, and I. Werner, *Nature*, **175**, 340, 1955.
[14] W. T. J. Morgan and L. A. Elson, *Biochem. J.*, **28**, 988, 1934.
[15] A. Gottschalk, *Yale J. Biol. and Med.*, **28**, 525, 1956.
[16] E. Klenk, H. Faillard, F. Weygand, and H. H. Schöne, *Z. physiol. Chem.*, **304**, 35, 1956.
[17] J. S. D. Bacon and J. Edelman, *Biochem. J.*, **48**, 114, 1951.
[18] S. M. Partridge, *Biochem. J.*, **42**, 238, 1948.
[19] P. J. Stoffyn and R. W. Jeanloz, *Arch. Biochem.*, **52**, 373, 1954.
[20] J. Immers and E. Vasseur, *Acta chem. Scand.*, **6**, 363, 1952.

---

*AN IDENTICAL DUPLEX STRUCTURE FOR POLYNUCLEOTIDES**

BY JERRY DONOHUE AND GUNTHER S. STENT

DEPARTMENT OF CHEMISTRY, UNIVERSITY OF SOUTHERN CALIFORNIA, LOS ANGELES, AND VIRUS LABORATORY, UNIVERSITY OF CALIFORNIA, BERKELEY

Communicated by W. M. Stanley, August 7, 1956

A structure for the sodium salt of deoxyribonucleic acid (DNA) conceived by Watson and Crick[1] consists of two polynucleotide chains, which are running in opposite directions and are wound about a common helical axis. In order to satisfy the geometrical requirements of this structure, the occurrence of the bases adenine, guanine, thymine, or cytosine on one chain requires the respective occurrence of thymine, cytosine, adenine, or guanine on the other, so that the two chains bear a complementary relationship to each other. The planes of the bases lie perpendicular to the axis of this double helix, 3.4 A apart. This structure is in agreement with the analytical and X-ray data for DNA. It is possible to construct helical two-chain models for polynucleotides[2] other than the above complementary duplex. These models share with the Watson-Crick structure the features of two sugar-phosphate chains winding about a common helical axis and hydrogen-bonded base pairs lying perpendicular to that axis. The models differ from the Watson-Crick formulation in the manner in which the purine and pyrimidine bases of the two opposite chains are hydrogen-bonded to each other.

We wish to describe in this communication a further example of a hypothetical double helix: a duplex built up of two polynucleotide chains of *identical*, rather than complementary, base sequence. Along one chain adenine, guanine, cytosine, and thymine (uracil) may follow one another in any arbitrary order. The base pairing is shown in Figure 1, where it is seen that each base on one chain forms two hydrogen bonds with an identical base on the second chain and that the