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Structure Studies on the Myxovirus Hemagglutination Inhibitor of Human Erythrocytes*

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We have previously (1–3) described the isolation from erythrocyte stroma of a potent inhibitor of hemagglutination by influenza viruses. The inhibitor has been shown to be a glycoprotein, homogeneous by the criteria of paper and moving boundary electrophoresis, ultracentrifugal analysis, column chromatography on various grades of Sephadex, and end group analysis. It is a glycoprotein of approximately 31,000 molecular weight and contains about 50% carbohydrate. This substance is believed to be identical with the myxovirus receptor site of erythrocytes, since exhaustive pretreatment of the red cells with the viral enzyme, followed by our isolation procedure, results in a material essentially identical in composition with the inhibitor, except for a decreased sialic acid content and a concomitant loss of activity. Recovery studies indicate that this substance is the chief sialic acid-containing component of red cells. By-products of the isolation procedure have little or no inhibitory activity. That this material is probably identical with the M or N blood group, or with both, will be shown in a following publication. It is the purpose of the present work to elucidate further the properties and structure of this inhibitor.

EXPERIMENTAL PROCEDURE

Analytical Procedures—Sialic acid was determined by the thiobarbituric acid method of Warren (4) or by the direct Ehrlich reaction as given by Werner and Odin (5).

Hexosamine was determined by the method of Elson and Morgan (6) as described by Winzler (7).

Hexoses were determined by the orcinol method described by Winzler (8) as modified to eliminate protein precipitation steps.

Acetyl determinations were made according to the method of Ludowieg and Dorfman (9).

Protein determinations were made by the method of Lowry *et al.* (10) by employing the Folin phenol reagent and using bovine or human serum albumin as a standard.

Quantitation of amino acids was accomplished by hydrolysis of 5 to 10 mg of protein with from 2 to 5 ml of glass-distilled 5.7 N hydrochloric acid for 20 hours at 105–108°. The hydrolysate was freed of acid by lyophilization. It was then reconstituted in a suitable buffer and analyzed for amino acids with an ion exchange column as described by Spackman, Stein, and Moore (11) in a Beckman/Spinco amino acid analyzer.

Qualitative determination of the NH_2-terminal amino acid was accomplished by the procedure described by Fraenkel-Conrat, Harris, and Levy (12). The dinitrophenyl protein was hydrolyzed with the 5.7 N hydrochloric acid for 16 hours. The hydrolysate was successively extracted with diethyl ether, and these extracts were pooled and evaporated to dryness. The residue was dissolved in a small volume of distilled water and chromatographed. Attempts at quantitation of dinitrophenylation were made by reconstitution of the ether extracts in a measured volume of slightly acidic distilled water (to render the dinitrophenol colorless), and the optical density was measured at 340 mμ. DNP[1]-aspartic and DNP-leucine were used as standards. Measurements of the extent of dinitrophenylation were also accomplished by dissolving approximately 5 mg of the protein in 0.05 M KCl and adjusting the pH to 8. One hundred microliters of 5% dinitrofluorobenzene were added, and the pH was maintained at 8 by the addition of 0.1 N NaOH. Since each dinitrophenyl group coupled to an amino group results in the liberation of 1 equivalent of hydrogen ion, the process can be quantitated by titration of the hydrogen ions released to maintain a constant pH value.

Borate ions were determined qualitatively by a modification of the method described by Feigl (13) with use of the turmeric reagent. One drop of the reagent (20 g of turmeric boiled with 50 ml of 95% ethanol, filtered, and diluted with 50 ml of distilled water) was added to 1 drop of the test solution and acidified with 1 drop of 1 N hydrochloric acid. A red-brown color that turned blue upon the addition of alkali was indicative of the borate ion or boric acid.

Paper chromatography of amino acids, hexoses, hexosamines, and sialic acid was accomplished by a modification of the solvent system of Jermyn and Isherwood (14): ethyl acetate-pyridine-water in the volume ratios of 10:4:3. Högström's system (15) of methyl ethyl ketone, acetone, water, and formic acid was used for confirmation. Descending chromatography for 20 hours on Whatman 3MM paper was followed by staining of the dried papers with the Moore and Stein (16) ninhydrin reagent for amino acids; with the aniline oxalate reagent of Partridge (17), with ammoniacal silver nitrate (18), or with the periodate-permanganate stain of Lemieux (19) for hexoses and hexosamines; and with a modified Ehrlich reagent (3) or with Warren's (20) thiobarbituric acid reagent for sialic acid.

Determination of hemagglutination and hemagglutination inhibition titers was accomplished as described in an earlier publication (3).

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[1] The abbreviations used are: DNP, 2,4-dinitrophenyl; DFB, 2,4-dinitrofluorobenzene.

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RESULTS

Examination of the chemical analysis (Table I) of the inhibitor reveals that there is essentially a 1:1:1 correspondence of sialic acid to hexosamine to hexose on a molar basis. We have shown previously that the sialic acid is essential for biological activity and that at least part of the sialic acid is in a terminal position. Demonstration that *all* of the sialic acid is in a terminal position was accomplished through the following experiments. A sample of the glycoprotein weighing 4.66 mg was dissolved in 3 ml of phosphate-buffered NaCl (0.1 M phosphate in 0.9% NaCl) at pH 7.4, and 0.4 ml of the solution was removed for initial determination of free and total sialic acid. Three milliliters of undiluted influenza virus vaccine, PR-8 strain, containing 0.75 unit of neuraminidase activity per ml, were added to the substrate solution. One unit of neuraminidase activity is defined as the amount of enzyme that will hydrolyze 1 μmole of *N*-acetylneuraminic acid from an excess of neuraminlactose in 1 minute at 37° at pH 7.0. Aliquots of 0.2 ml, in duplicate, were removed and tested for free sialic acid at time intervals of 2.5, 5, 10, 15, 30, 45, 60, 120, 180, 240, and 1020 minutes. The identical experiment was performed with Asian influenza vaccine having the same enzymatic activity as measured against neuraminlactose under the conditions defined above. The results are shown in Fig. 1. It has been established reasonably well that the viral enzyme acts upon ketosidically linked sialic acids (21–24). In the present work, incubation mixtures of the vaccine and protein were chromatographed on paper; only a single substance, identifiable with Ehrlich's reagent and the thiobarbituric acid reagent, and moving with the R_F of authentic *N*-acetylneuraminic acid, was found. No other substances identifiable with aniline oxalate, ninhydrin, periodate-permanganate, or ammoniacal silver nitrate were seen. From the results with Asian influenza vaccine, it must be assumed that all of the sialic acid in the protein is in a terminal position. The same conclusion was reached in experiments in which it could be shown by acid hydrolysis with 0.1 N sulfuric acid at 75–78° for 45 minutes that all of the sialic acid could be freed without concomitant liberation of any other substituent.

TABLE I

Composition of inhibitor

The hexosamines, galactosamine and glucosamine, appear in the ratio of 3–4 to 1, respectively, based upon the relative amounts found in amino acid hydrolysates and upon the intensity of spots on paper chromatography, after hydrolysis for quantitative total hexosamine determination. The hexose is galactose.

Constituent	Per cent	Moles per 31,000 g of protein
Sialic acid	23.0	23
Hexosamines	13.0	22
Hexose	13.1	22
Fucose	1.1	2
Nitrogen	10.1	

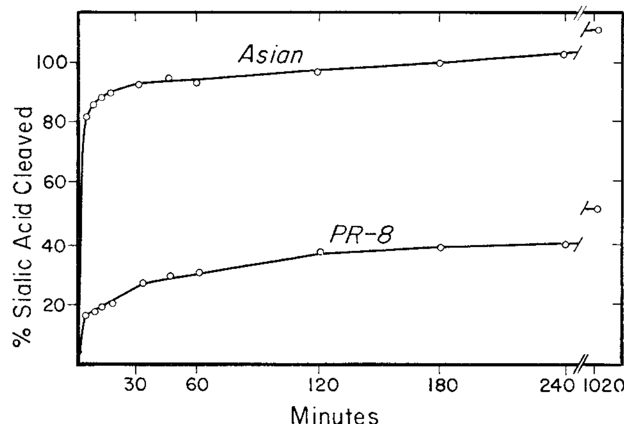

FIG. 1. The incubation mixture contained 4.66 mg of the inhibitor in 3 ml of buffer at pH 7.4 with 3 ml of Asian or PR-8 strain of influenza vaccine. Samples were removed at 0, 2.5, 5, 10, 15, 30, 45, 60, 120, 180, 240, and 1020 minutes.

TABLE II

Distribution of acetyl groups in inhibitor

Sixteen milligrams of the inhibitor were incubated with 1 ml of Asian influenza vaccine, and the mixture was dialyzed. The dialyzable material was lyophilized, desiccated, and analyzed.

	Micromoles per 16 mg. of protein
Dialyzable:*	
Acetyl	4.2
Sialic acid	4.6
Total:	
Acetyl	13.8
Hexosamine	11.6
Sialic acid	11.0

* The ratio of acetyl to sialic acid in the dialyzable portion is 4.2/4.6 = 0.92.

The acetyl groups were determined to verify the nature of the sialic acid contained in the protein. Sixteen milligrams of the inhibitor were incubated for 4 hours with 1 ml of Asian influenza vaccine (0.75 unit) and 1 ml of phosphate-buffered NaCl adjusted to pH 6.5 at 37°. The incubation mixture was then dialyzed in the cold against 100 ml of distilled water for 2 days with agitation. The dialyzable material was collected and lyophilized. Because of the high salt concentration, quantitative recovery of the lyophilized material was difficult. The reconstituted residue was analyzed for sialic acid, and aliquots were transferred to vials, dried by vacuum desiccation, and allowed to react with methanolic hydrochloric acid according to the procedure of Ludowieg and Dorfman (9) for acetyl determination. Untreated protein was also used for determination of total acetyl groups as well as for the determination of sialic acid and hexosamine. *N*-Acetylglucosamine samples were used to verify the method. In addition, several samples of orosomucoid were run for comparison, and a value of 0.86 μmole of acetyl per mg of protein was obtained. The results are summarized in Table II. Essentially a 1:1 molar ratio exists between the dialyzable sialic acid and acetyl groups. Of significance is the fact that the total protein did not contain enough acetyl groups to permit the acetylation of both the hexosamine and the neuraminic acid. Since the neuraminic acid appears to be present as the acetyl derivative, the hexosamine must be largely nonacetylated.

Borohydride Reduction Studies—As one way of determining whether there were any free reducing groups and to gain some insight into the linkages of the various carbohydrates, a series of studies was carried out in which aqueous sodium borohydride

was used as a reducing agent. With few exceptions, this reagent will specifically reduce carbonyl functions. Samples of the inhibitor weighing 4 to 5 mg were dissolved in 0.25 ml of phosphate-buffered NaCl, pH 7.1, and 0.25 ml (1 unit per ml) of polyvalent A and B influenza vaccine or an additional 0.25 ml of phosphate-buffered NaCl was added. For more complete cleavage of sialic acid, the same weight of samples was dissolved directly into 0.5 ml of a partially purified extract of the receptor-destroying enzyme from *Vibrio cholerae* (0.0032 unit). These were incubated at 37° for 2 hours (virus) or 16 hours (receptor-destroying enzyme). Thirty milligrams of sodium borohydride dissolved in 0.5 ml of distilled water were added to each incubation mixture, and these were permitted to stand for 20 hours at room temperature with occasional shaking. This quantity of sodium borohydride represents an 80-fold excess, on a molar basis, if all of the reducing groups of all of the carbohydrate components are free. The excess sodium borohydride was destroyed by the addition of glacial acetic acid in amounts adequate to lower the pH to 5 with the complete cessation of hydrogen evolution. Because it was found that the borate product formed interfered with the determination of hexosamine, the reduced solutions were passed through a G-25 Sephadex column, 1 × 10 cm. Fractions, 1 ml each, were collected; these were tested for protein by Lowry's method, and for borate by using the turmeric reagent. In every instance, the protein was free from borate compounds. Analyses were made of sialic acid, hexosamine, and hexose in each tube containing protein. From these determinations was calculated the total recovery of unreduced constituent. The results are shown in Table III.

Destruction of the sugars or change in ratios of the sialic acid to hexose or of hexosamine to hexose was insignificant after treatment of the native inhibitor with sodium borohydride, which indicates that reducible groups were absent and also that no degradation occurred during the alkaline conditions of the reduction. However, when sialic acid was removed and the protein then was treated with sodium borohydride, a striking reduction of the hexosamine took place. The *extent* of the reduction was identical with the molar equivalents of sialic acid

TABLE III

Sodium borohydride treatment of native and desialysed inhibitor

Approximately 5 mg of native or enzymatically desialyzed inhibitor were treated with 30 mg of $NaBH_4$ for 20 hours at room temperature and then passed through a G-25 Sephadex column, 1 × 10 cm. Fractions 1 ml each, were collected, and recoveries were calculated from total constituent found. Figures were adjusted to exactly 5 mg of sample weight.

Treatment	Micromoles recovered per 5 mg			Sialic acid remaining	Ratio:	
	Sialic acid	Hexosamine	Hexose		Sialic acid to hexose	Hexosamine to hexose
				%		
None	3.56	3.33	3.45	100	1.03	0.97
$NaBH_4$	3.12	3.79	3.20	100	0.98	1.18
Polyvalent A + B vaccine, $NaBH_4$	1.80	1.72	3.70	50	0.49	0.47
Receptor-destroying enzyme, $NaBH_4$	0.80	0.61	3.48	22	0.23	0.19

TABLE IV

Amino acid composition of inhibitor

Amino acid	Micromoles per mg of protein	Moles per 31,000 g of protein	No. of residues per mole of protein (nearest integer)
Lysine	0.099	3.0	3
Histidine	0.106	3.3	3
Arginine	0.112	3.5	3
Aspartic acid	0.164	5.1	5
Threonine	0.383	11.8	12
Serine	0.377	11.6	12
Glutamic acid	0.278	8.6	9
Proline	0.183	5.7	6
Glycine	0.187	5.8	6
Alanine	0.187	5.8	6
Valine	0.213	6.6	7
Isoleucine	0.122	3.8	4
Leucine	0.126	3.9	4
Tyrosine	0.100	3.1	3
Phenylalanine	0.098	3.0	3
Methionine	0.040	1.2	1

removed. Finally, it should be noted that the protein-bound hexose was almost quantitatively recovered.

Some preliminary experiments on the chromatography of the hydrolytic products of desialyzed, sodium borohydride-treated inhibitor were carried out. One milligram of authentic galactosamine hydrochloride, reduced with 2 mg of sodium borohydride for 16 hours, was acidified to destroy the remaining sodium borohydride and passed through a Dowex 1 column in the formate form to remove borate ions. The galactosaminitol thus obtained served as a standard for comparison. Upon chromatography of reduced, hydrolyzed protein, a new spot, identifiable by the periodate-permanganate reagent and with a relative mobility of 0.62 compared to galactosamine, was found. The reduced galactosamine had a mobility relative to that of galactosamine of 0.55. The new substance and the galactosaminitol reacted slightly with ninhydrin but not with aniline oxalate. A very faint spot, not yet identified, and moving slightly slower than galactose was also observed with the treated hydrolyzed protein.

Amino Acid Analysis of Inhibitor—Amino acid analyses were carried out on several samples of the receptor substance. The results are shown in Table IV.

Determination of NH_2-terminal Amino Acid—A sample of the inhibitor weighing 8.5 mg was allowed to react with dinitrofluorobenzene according to the Fraenkel-Conrat method for a period of 2 hours. An aliquot was subjected to electrophoresis on paper at pH 8.6, and a single band was observed to migrate. No other ninhydrin-staining substance could be detected. Another aliquot was used to see whether dinitrophenylation affected the inhibitory titer. There was no change in hemagglutination inhibitory activity. The remainder of the DNP-protein was extracted, hydrolyzed, and re-extracted with ether. Upon both chromatography and electrophoresis, two spots were observed. One spot became colorless when acidified and had the mobility of dinitrophenol; the other spot had the mobility of DNP-aspartic acid. In the pyridine-ethyl acetate-water chromatographic system, the ratio of mobility of both authentic DNP-aspartic acid and the unknown spot to that of dinitrophenol was 0.52.

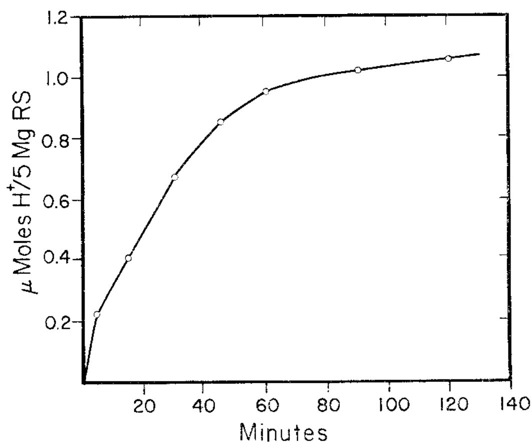

FIG. 2. Titration of hydrogen ions released during dinitrophenylation of 5 mg of the inhibitor. The pH was maintained at 8. *RS* indicates an inhibitor preparation.

When the dinitrophenylation of 5 mg of inhibitor was followed quantitatively by titration of hydrogen ions released, the curve shown in Fig. 2 was obtained. A value of 0.16 μmole of H^+ released during the most rapid phase could be obtained by extrapolation to zero time.

DISCUSSION

Because a molar ratio of unity exists between the various carbohydrate components, it is tempting to speculate that these might be sequentially linked. Since all of the sialic acid can be removed either by graded acid hydrolysis or by enzymes, the sialic acid must occupy a terminal position. We have no explanation for the observation shown in Fig. 1 that the PR-8 strain of influenza vaccine did not remove all of the sialic acid under experimental conditions in which removal by Asian virus vaccine was complete. However, it may be noted that the PR-8 strain of virus occupies a much less active position in the receptor gradient table as determined by us[2] with a method similar to that originally employed by Burnet, McCrea, and Stone (25).

At present, there are at least three naturally occurring sialic acids reported. These are the *O,N*-diacetyl, *N*-glycolyl, and *N*-acetyl derivatives of neuraminic acid. From the chromatographic and acetyl determination data presented, it would appear that the inhibitor contains only *N*-acetylneuraminic acid. This is in accord with the conclusions of Yamakawa (26), who found only *N*-acetylneuraminic acid in human erythrocytes.

Acetyl determinations of the intact inhibitor have been made many times with a good degree of reproducibility, and we believe the acetyl value to be correct. Moreover, it should be noted that the acetyl value obtained from orosomucoid, the substance used for comparison, of 0.86 μmole per mg of protein corresponds to 37 μmoles of acetyl groups per mole of protein on the assumption of a molecular weight of 41,000 for orosomucoid (27). According to the structure proposed by Winzler and Inouye (28), all of the neuraminic acid (18 moles per mole of orosomucoid) and two-thirds of glucosamine ($\frac{2}{3} \times 27$ moles per mole of orosomucoid) are believed to be acetylated, or 36 moles of acetyl groups per mole of orosomucoid. Our values for acetyl determinations, then, are in excellent agreement with the previous findings (28) for orosomucoid, and we have every reason to be confident that the values obtained with the inhibitor are also valid. If all of the sialic acid in the inhibitor is the monoacetyl derivative of neuraminic acid, as the data suggest, there are not enough acetyl groups to account for the complete acetylation of the hexosamine. Further investigation of this point is presently under way.

The native protein shows no loss of any carbohydrate component upon attempted reduction with sodium borohydride. Therefore, the carbonyl functions of sialic acid, hexosamine, and hexose must be linked in the intact protein. When the sialic acid is enzymatically removed and the altered protein is similarly treated with sodium borohydride, a reduction in hexosamine occurs that corresponds to the amount of sialic acid removed. We interpret this as an indication that the sialic acid is linked through its anomeric carbon (C-2) to the anomeric carbon (C-1) of the hexosamine.

If the chains exist as discrete disaccharide units (or trisaccharides with galactose), there would be 22 such units in the glycoprotein. No one amino acid is present in molar quantities equivalent to the number of such units. It is interesting to note, however, that the most abundant amino acids, serine and threonine, together correspond to 23 moles per mole of the glycoprotein. These hydroxyamino acids, along with aspartic and glutamic acids, are the most likely sites of linkage of the peptide chain to the carbohydrates.

SUMMARY

Certain aspects of the structure of an inhibitor of hemagglutination have been investigated. Sialic acid has been shown to occupy a terminal position, since all of it can be liberated enzymatically without the concomitant release of any other substances identifiable by aniline oxalate, ammoniacal silver nitrate, ninhydrin, or Ehrlich's reagent. The sialic acid is probably the *N*-acetyl derivative of neuraminic acid because its chromatographic movement is identical with that of authentic *N*-acetylneuraminic acid and because of a 1:1 correspondence of acetyl groups to sialic acid. By reduction with sodium borohydride subsequent to the enzymatic removal of sialic acid, it can be shown that there is a decrease in the hexosamine content that corresponds to the lost sialic acid. Similar attempted reduction of the native inhibitor results in no loss of any carbohydrate component.

Examination of the amino acid analyses indicates that there are 16 amino acids present; serine and threonine are the two most abundant ones, and these two appear in total molar quantity approximately equal to the number of moles of sialic acid, hexosamine, and hexose.

REFERENCES

1. KATHAN, R. H., JOHNSON, C. A., AND WINZLER, R. J., *Acta Soc. Med. Upsaliensis*, **64**, 290 (1959).
2. KATHAN, R. H., WINZLER, R. J., AND JOHNSON, C. A., *Federation Proc.*, **19**, 338 (1960).
3. KATHAN, R. H., WINZLER, R. J., AND JOHNSON, C. A., *J. Exptl. Med.*, **113**, 37 (1961).
4. WARREN, L., *J. Biol. Chem.*, **234**, 1971 (1959).
5. WERNER, I., AND ODIN, L., *Acta Soc. Med. Upsaliensis*, **57**, 230 (1952).
6. ELSON, L. A., AND MORGAN, W. T. J., *Biochem. J.*, **27**, 1824 (1933).
7. WINZLER, R. J., in D. GLICK (Editor), *Methods of biochemical*

[2] Unpublished data.

analysis, Vol. II, Interscience Publishers, Inc., New York, 1955, p. 292.
8. Winzler, R. J., in D. Glick (Editor), *Methods of biochemical analysis, Vol. II*, Interscience Publishers, Inc., New York, 1955, p. 290.
9. Ludowieg, J., and Dorfman, A., *Biochim. et Biophys. Acta*, **38**, 212 (1960).
10. Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randall, R. J., *J. Biol. Chem.*, **193**, 265 (1951).
11. Spackman, D. H., Stein, W. H., and Moore, S., *Anal. Chem.*, **30**, 1190 (1958).
12. Fraenkel-Conrat, H., Harris, J. I., and Levy, A. L., in D. Glick (Editor), *Methods of biochemical analysis, Vol. II*, Interscience Publishers, Inc., New York, 1955, p. 370.
13. Feigl, F., *Spot tests, Vol. 1*, Elsevier Publishing Company, New York, 1954, p. 311.
14. Jermyn, M. A., and Isherwood, F. A., *Biochem. J.*, **55**, 402 (1949).
15. Högström, G., *Acta Chem. Scan.*, **11**, 743 (1957).
16. Moore, S., and Stein, W. H., *J. Biol. Chem.*, **211**, 907 (1954).
17. Partridge, S. M., *Nature (London)*, **158**, 270 (1946).
18. Partridge, S. M., *Biochem. J.*, **42**, 238 (1948).
19. Lemieux, R. V., and Bauer, H. F., *Anal. Chem.*, **26**, 920 (1954).
20. Warren, L., *Nature (London)*, **186**, 237 (1960).
21. Gottschalk, A., in F. F. Nord (Editor), *Advances in enzymology, Vol. 20*, Interscience Publishers, Inc., New York, 1958, p. 135.
22. Gottschalk, A., and Graham, E. R. B., *Biochim. et Biophys. Acta*, **34**, 380 (1959).
23. Mayron, L. W., Robert, B., Winzler, R. J., and Rafelson, M. E., Jr., *Arch. Biochem. Biophys.*, **92**, 475 (1961).
24. Howe, C., Rose, H. M., and Schneider, L., *Proc. Soc. Exptl. Biol. Med.*, **96**, 88 (1957).
25. Burnet, F. M., McCrea, J. F., and Stone, J. D., *Brit. J. Exptl. Pathol.*, **27**, 228 (1946).
26. Yamakawa, T., *J. Biochem. (Tokyo)*, **43**, 867 (1956).
27. Smith, E. L., Brown, D. M., Weimer, H. E., and Winzler, R. J., *J. Biol. Chem.*, **185**, 569 (1950).
28. Winzler, R. J. and Inouye, T., *Abstracts of the 139th national meeting of the American Chemical Society, St. Louis, March 1961*, p. 4C.