THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 234, No. 8, August 1959
Printed in U.S.A.

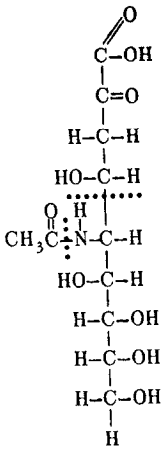
The Thiobarbituric Acid Assay of Sialic Acids*

LEONARD WARREN

From the National Institute of Arthritis and Metabolic Diseases, National Institutes of Health, United States Public Health Service, Bethesda 14, Maryland

(Received for publication, April 6, 1959)

Members of the sialic acid family of compounds occur in a variety of tissues and body fluids (1a, 2) and in bacteria (3), incorporated in conjugated protein. The naturally occurring sialic acids are substituted neuraminic acid derivatives (*N*-acetyl, *N*-glycolyl, *N*,*O*-diacetylneuraminic acid). These are collectively termed "sialic acids." The unsubstituted 9 carbon chain is called neuraminic acid (4) (Scheme 1).



N-Acetyl-neuraminic acid

SCHEME 1

Sialic acids have been detected and measured by several methods such as the orcinol (5–7), resorcinol (8), diphenylamine (9–12), direct Ehrlich (6, 12), tryptophan-perchloric acid (13, 6), hydrochloric acid (14), and sulfuric-acetic acid (15) procedures. Most of these are modifications of assays which had been previously used for the measurement of other carbohydrates. These assays are not only relatively insensitive, but in general have a low specificity and cannot be applied to the direct measurement of sialic acids in tissues and in other unpurified biological materials. Svennerholm (1a) has succeeded in eliminating interfering materials by passing tissue hydrolysates through Dowex 2-acetate columns and thereby partially purifying the sialic acids before measurement. This method, although reliable, is cumbersome and time-consuming.

In 1957, Waravdekar and Saslaw (16) reported a method for the measurement of 2-deoxyribose, in which the periodate oxidation product, malonaldehyde, was coupled with 2-thiobarbituric acid. Since then, Weissbach and Hurwitz (17) have reported that 2-keto-3-deoxy sugar acids can also be assayed by the same method with some modifications. Here the periodate oxidation product was β-formylpyruvic acid.

Although sialic acids are 2-keto-3-deoxy sugar acids, the amino group is always substituted and should not be reactive in the thiobarbituric acid assay. The Waravdekar and Saslaw, and Weissbach and Hurwitz assays on sialic acids do, in fact, lead only to a small amount of color formation.

We have been able to increase color formation from sialic acids considerably by carrying out the periodate oxidation in strong acid solution and by extracting the final colored solution into cyclohexanone. The molecular extinction coefficient thus obtained is 12 times higher than that of the resorcinol method, the most sensitive method used at the present time. The thiobarbituric acid is specific enough to permit accurate direct measurement of the sialic acid content of tissues.

EXPERIMENTAL AND RESULTS

Materials and Methods—*N*-Acetylneuraminic acid was synthesized by the method of Cornforth *et al.* (18) and purified on a Dowex 1-formate column. A sample of *N*-acetylneuraminic acid from blood proteins was kindly supplied by Dr. L. Svennerholm. Methoxyneuraminic acid was generously supplied by Dr. E. Klenk. *N*-Glycolylneuraminic acid and *N*,*O*-diacetylneuraminic acid were isolated from the mucins of pig and beef submaxillary glands, respectively, by the method of Zilliken *et al.* (19). 2-Keto-3-deoxygluconic acid was a gift of Dr. G. Ashwell, and 2-deoxyribose and L-fucose were kindly supplied by Dr. H. G. Fletcher, Jr. Sodium periodate, sodium arsenite, and cyclohexanone were the products of the Fisher Scientific Company. 2-Thiobarbituric acid was obtained from the Eastman Kodak Company and was recrystallized from hot water (25 gm. in 600 ml.).

The Ehrlich and orcinol assays for sialic acids were those of Werner and Odin (6) except for a 10-fold reduction in scale. The resorcinol procedure used was that of Svennerholm (8) and the diphenylamine assay was that of Ayala *et al.* (11). Where indicated, the column method of Svennerholm (1a) for the assay of sialic acids was used with some minor modifications (20).

Thiobarbituric Acid Assay—The procedure in its final form used the following solutions: (*a*) sodium periodate (meta) 0.2 M, in 9 M phosphoric acid; (*b*) sodium arsenite, 10 per cent, in a solution of 0.5 M sodium sulfate-0.1 N H_2SO_4; and (*c*) thiobarbituric acid, 0.6 per cent, in 0.5 M sodium sulfate. All of these aqueous solutions were prepared with warming. Solutions were stored at room temperature and were stable for more than a month.

* A preliminary report of this work has been published (1).

Standard Procedure—To a sample containing up to 0.05 μmole of *N*-acetylneuraminic acid in a volume of 0.2 ml., is added 0.1 ml. of the periodate solution. The tubes are shaken and allowed to stand at room temperature for 20 minutes. Arsenite solution, 1 ml., is added and the tubes are shaken until a yellow-brown color disappears. Thiobarbituric acid solution, 3 ml., is added, the tubes shaken, capped with a glass bead, and then heated in a vigorously boiling water bath for 15 minutes. The tubes are then removed and placed in cold water for 5 minutes. During cooling the red color fades and the solution often becomes cloudy. This does not affect the final reading. Of this solution, 1 ml. is transferred to another tube which contains 1 ml. of cyclohexanone. If desired, the entire 4.3 ml. of aqueous solution can be extracted with 4.3 ml. of cyclohexanone. The tube is shaken twice and then centrifuged for 3 minutes in a clinical centrifuge. The clear upper cyclohexanone phase is red and the color is more intense than it is when in water. Optical densities of the organic phase are determined at 549 mμ in a Beckman model DU spectrophotometer equipped with a micro attachment. Cuvettes with a capacity of 0.6 ml. and a 1-cm. light path are used. If the entire 4.3 ml. of aqueous solution is extracted with 4.3 ml. of cyclohexanone, readings are made in 3-ml. cuvettes (1 cm. light path) in the Beckman spectrophotometer. The procedure is also carried out on 0.2 ml. of water for the blank vessel, and readings are made against this solution.

Color production varies linearly with concentration of *N*-acetylneuraminic acid over the range usually used, 0.01 to 0.06 μmole. The molecular extinction coefficient is 57,000. The amount of *N*-acetylneuraminic acid present in a given sample can be determined from Equation 1:

μMoles *N*-acetylneuraminic acid =

$$\frac{V \times \text{O.D.}_{549}}{57} = \frac{4.3 \times \text{O.D.}_{549}}{57} = 0.075 \times \text{O.D.}_{549} \quad (1)$$

where V is the final volume of the test solution.

The standard deviation of optical density for 0.04 μmole of *N*-acetylneuraminic acid in 14 assays at three different times was 0.9 per cent.

Interfering Substances—When tissues are subjected to the thiobarbituric acid assay for sialic acids an absorption maximum is found at 549 mμ, due to sialic acids. However, there may also be a second absorption maximum at 532 mμ due to 2-deoxyribose for which a correction must be made since the light absorption of this interfering material at 549 mμ is considerable. Other substances such as unsaturated lipides (21) may also yield malonaldehyde upon periodate oxidation and contribute to the optical density at 532 and 549 mμ, but the source of the malonaldehyde does not affect the calculations for correction. For the assay of sialic acids in tissues, readings were made routinely at 532 and 549 mμ and the optical density values were inserted into Equation 2.[1]

μMoles *N*-acetylneuraminic acid

$$= 0.090 \times \text{O.D.}_{549} - 0.033 \times \text{O.D.}_{532} \quad (2)$$

Equation 2 corrects for the optical density at 549 mμ which does not derive from *N*-acetylneuraminic acid. The correction is based upon the observed molecular extinction values of *N*-acetylneuraminic acid and 2-deoxyribose at 532 and 549 mμ. Table I shows the uncorrected and corrected values obtained when four solutions, containing a constant amount of *N*-acetylneuraminic acid in the presence of varying amounts of 2-deoxyribose, were assayed.

A second method of correction, especially useful in the presence of large amounts of 2-deoxyribose, is to determine the optical densities of the colored solution at 562 mμ as well as at 532 mμ. These values are inserted into Equation 3, which corrects for the absorbance not due to sialic acids.[2]

μMoles *N*-acetylneuraminic acid

$$= 0.138 \text{ O.D.}_{562} - 0.009 \text{ O.D.}_{532} \quad (3)$$

Other methods for the elimination of color due to 2-deoxyribose derivatives can be developed for use in special circumstances. It has been found that when the aqueous solution of chromophores is extracted with two volumes of isoamyl alcohol, 85 per cent of the material with a 532-mμ peak is removed from the aqueous phase whereas only 10 to 15 per cent of the sialic material with the 549-mμ peak is extracted into the organic phase. Subsequent extraction of the aqueous phase with cyclohexanone affords a partially purified sialic acid chromophore. This procedure may be of value when very large amounts of 2-deoxyribose derivatives are present.

Color due to sialic acid may be destroyed by the addition of strong base while the 2-deoxyribose chromophore is stable. This phenomenon could be used to determine the contribution of deoxy sugars to the optical density at 549 mμ.[3]

One μmole of the substances listed below were tested alone and in the presence of 0.04 μmole of *N*-acetylneuraminic acid to see whether they produced color or interfered with color production by *N*-acetylneuraminic acid; glucosamine, *N*-acetylglucosamine, mannosamine, *N*-acetylmannosamine, galactosamine, *N*-acetylgalactosamine, glucose, fructose, galactose, L-fucose, D-mannose, D-ribose, galacturonic acid, diphosphopyridine nucleotide, adeno-

TABLE I

Correction for 2-deoxyribose in the thiobarbituric acid assay

Vessel	Mixtures assayed		Observed amount of *N*-acetylneuraminic acid	
	N-acetylneuraminic acid	2-Deoxyribose	Uncorrected	Corrected
	μmole	*μmole*	*μmole*	*μmole*
1	0.030	0.005	0.035	0.030
2	0.030	0.010	0.038	0.030
3	0.030	0.020	0.047	0.032
4	0.030	0.030	0.056	0.030

[1] The constants of Equation 2 are calculated as follows:

μMoles *N*-acetylneuraminic acid

$$= \left[\frac{\epsilon_3}{\epsilon_2\epsilon_3 - \epsilon_1\epsilon_4} \text{O.D.}_{549} - \frac{\epsilon_4}{\epsilon_2\epsilon_3 - \epsilon_1\epsilon_4} \text{O.D.}_{532}\right] \times 4.3$$

where ϵ_1, and ϵ_2 are the molecular extinction coefficients $\times 10^{-3}$ of *N*-acetylneuraminic acid at 532 and 549 mμ, respectively, and ϵ_3 and ϵ_4 are the molecular extinction coefficients $\times 10^{-3}$ of deoxyribose at 532 and 549 mμ, respectively. $\epsilon_1 = 26$, $\epsilon_2 = 57$, $\epsilon_3 = 133$, $\epsilon_4 = 48$. The final volume of the test solution = 4.3 ml.

[2] Equation 3 is calculated in the same manner as Equation 2.[1] ϵ_1 and ϵ_2 are the molecular extinction coefficients $\times 10^{-3}$ of *N*-acetylneuraminic acid at 532 and 562 mμ, respectively, and ϵ_3 and ϵ_4 are the molecular extinction coefficients of 2-deoxyribose at 532 and 562 mμ, respectively. $\epsilon_1 = 26$, $\epsilon_2 = 32.6$, $\epsilon_3 = 133$, $\epsilon_4 = 8$.

[3] Suggested by Dr. A. Weissbach.

sine triphosphate, aspartic acid, histidine, glutamic acid, folic acid and uridine 5′-monophosphate. None of these produced any color alone or interfered with color formation from *N*-acetylneuraminic acid, except for L-fucose which caused a 35 per cent decrease in optical density. This inhibition is probably due to acetaldehyde, one of the products of periodate oxidation of fucose. Acetaldehyde, 1 μmole, inhibited color formation to about the same extent as 1 μmole of L-fucose; 50 μmoles of sodium formate or sodium acetate did not inhibit color formation from 0.04 μmole of *N*-acetylneuraminic acid.

Factors Which Affect Assay

Periodate Oxidation—As seen in Fig. 1, a strongly acidic environment is required to obtain a maximal molecular extinction coefficient. Strong acid is probably required to remove the acetyl or glycolyl from the amino group before oxidation. We have shown that the substituting group is removed during the reaction, since when *N*-acetylneuraminic acid labeled with C^{14} in the acetyl group is subjected to the thiobarbituric acid assay, the chromophore produced is not radioactive. The mechanism of the reaction is being investigated at the present time.

Color formation rises sharply with increasing amounts of periodate, and a maximal amount of color is produced with 20 to 40 μmoles of sodium periodate per vessel. Considerably less color formation results when the periodate oxidation takes place at 0 or 37° rather than at room temperature (22°). Oxidation in the dark has no effect on the final extinction coefficient. Somewhat lower molecular extinction coefficients result when the sample to be assayed is in a volume greater than 0.2 ml.

Sodium Arsenite Step—An excess of sodium arsenite is used. It has been found that as little as 0.5 ml. of arsenite solution may be used to destroy the periodate present. Vessels to which arsenite has been added have been stored for 4 days at 4° before the thiobarbituric acid heat step, with no decrease in the final optical density reading.

Thiobarbituric Acid Step—A slightly higher optical density can be obtained by decreasing the amount of thiobarbituric acid reagent used in the assay to 2.5 ml. This is the minimal amount which will give a molecular extinction coefficient of 57,000.

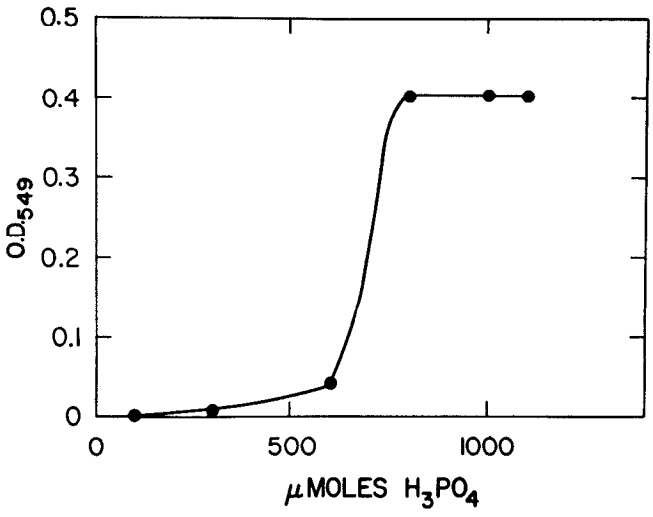


FIG. 1. The influence of acidity on the thiobarbituric acid assay. *N*-Acetylneuraminic acid, 0.03 μmole, was assayed by the method described in the text, except for variation of the phosphoric acid concentration.

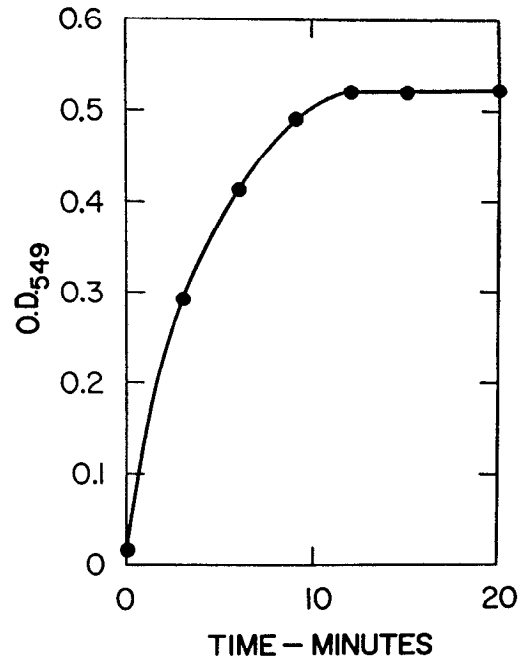


FIG. 2. Development of color with time of heating in thiobarbituric acid assay. *N*-Acetylneuraminic acid, 0.04 μmole, was used in the assay.

Fig. 2 demonstrates increasing color formation with time in the heating bath.

Unsuccessful attempts have been made to use solutions of thiobarbituric acid in organic solvents. The greater solubility of thiobarbituric acid in certain organic solvents would allow the assay to be carried out in a smaller final volume and would intensify color without further additions (see below).

If barbituric acid is substituted for 2-thiobarbituric acid in the assay a pink color is obtained. The absorption peaks of *N*-acetylneuraminic acid and 2-deoxyribose chromophores are at 506 and 485 mμ, respectively. The molecular extinction coefficients are considerably lower than when 2-thiobarbituric acid is used. No color was produced when 5-nitrobarbituric acid or 5,5′-diethylbarbituric acid replaced 2-thiobarbituric acid.

Organic Solvent Extraction and Use of Sodium Sulfate—It has been found that when the chromophore is extracted into cyclohexanone there is a marked increase in the intensity of the color. Color-forming resonance of the chromophore is apparently less in polar solvents than it is in relatively nonpolar organic solvents. The addition of many water-soluble organic solvents to the aqueous solution such as ethanol also intensify color.

Although the salt depresses the color intensity in aqueous solution, it facilitates the transfer of chromophore into the organic phase. The presence of sodium sulfate in the sodium arsenite and thiobarbituric acid solutions leads to a 28 per cent increase in the observed molar extinction coefficient by making the extraction of chromophore by cyclohexanone more complete.

Characteristics of Chromophore—Fig. 3 illustrates the absorption characteristics of the *N*-acetylneuraminic acid and 2-deoxyribose chromophores. The absorption maxima are at 549 and 532 mμ, respectively. The color formed with 2-keto-3-deoxygluconic acid has a spectrum that is identical with that of *N*-acetylneuraminic acid. The colors are very stable in acidic aqueous solution and in cyclohexanone. Optical densities remain constant for several hours.

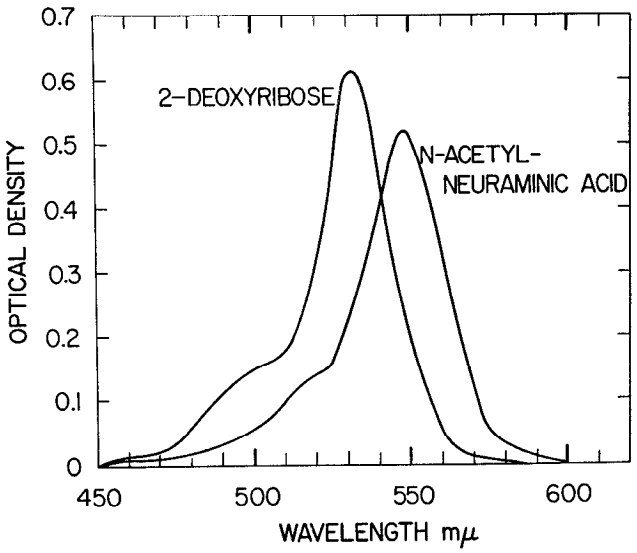


FIG. 3. The absorption spectra of *N*-acetylneuraminic acid (0.04 μmole) and 2-deoxyribose (0.02 μmole) in the thiobarbituric acid assay.

TABLE II

Summary of molar extinction coefficients

Compound	Ehrlich	Diphenylamine	Orcinol	Resorcinol	Thiobarbituric acid
N-Acetylneuraminic acid...	2030	4040	4340	4700	57,000
N-Glycolylneuraminic acid.	2060	3340	5060	5450	46,000
Methoxyneuraminic acid...	2180	2270	5700	5600	0
2-Keto-3-deoxygluconic acid					79,000
2-Deoxyribose.....					133,000

Color due to *N*-acetylneuraminic acid is destroyed in basic solution whereas the absorption maximum of 2-deoxyribose color moves to a higher wave length. The chromophores of both *N*-acetylneuraminic acid and 2-deoxyribose in cyclohexanone are destroyed in a few minutes at 100°. The *N*-acetylneuraminic acid chromophore does not fluoresce in aqueous or organic solvents but does have an orange fluorescence when dry on paper.

Molecular Extinction Coefficients—Table II is a summary of molecular extinction coefficients of sialic acids in five assays. *N*-Acetylneuraminic acid in the thiobarbituric acid method has a molecular extinction of 57,000, 12 times that in the resorcinol method and 28 times that in the direct Ehrlich assay. The ε value of *N*-glycolylneuraminic acid in the thiobarbituric acid assay is 19 per cent lower than that of *N*-acetylneuraminic acid. This may reflect a difference in the ease with which the glycolyl and acetyl groups are removed from the amino of neuraminic acid. Methoxyneuraminic acid gives no color in the thiobarbituric acid assay and all attempts to remove the 2-substituent, so that a positive test can be obtained, have been unsuccessful. The oxygen atom on the second carbon of neuraminic acid must be free for a positive test. Sialic acids bound to carbohydrates in tissues, presumably at the 2-position do not react in the thiobarbituric acid assay without prior hydrolysis. Mild hydrolysis is required to free sialic acids and make them reactive in the thiobarbituric acid assay.

Our preparations of *N*,*O*-diacetylneuraminic acid were impure and did not yield reliable extinction values. However, it is likely that the ε of *N*,*O*-diacetylneuraminic acid is the same as that of *N*-acetylneuraminic acid because mild acid treatment, which is known to convert diacetyl to *N*-acetylneuraminic acid (22), did not alter the molar absorption coefficient of our diacetylneuraminic acid preparations. Svennerholm has shown that the ε values of *N*-acetylneuraminic acid and *N*,*O*-diacetylneuraminic acid are approximately the same in the orcinol (7) and resorcinol assays (8). Colominic acid, a polymer which is made largely of *N*-acetylneuraminic acid residues (3), gave only a faintly positive reaction in the assay. The small amount of color produced may be due to contaminating free *N*-acetylneuraminic acid, or possibly end groups may be reactive in the thiobarbituric acid test.

Assay of Sialic Acids in Tissue Hydrolysates

Since the thiobarbituric acid assay measures only free sialic acids, tissue homogenates were heated at 80° for 1 hour in 0.1 N H_2SO_4. This procedure is known to release bound sialic acids without degradation (1a, 20). In this study we compared the thiobarbituric acid method with the column method of Svennerholm, the most reliable method of measuring sialic acid in biological materials previously used. In general the procedure followed was to hydrolyze a fluid or tissue in a volume of 4 ml. with a final concentration of H_2SO_4 of 0.1 N. After hydrolysis 3.7 ml. were placed on a Dowex 1-acetate column whereas 0.3 ml. was used directly in the thiobarbituric acid assay divided among three vessels with differing amounts of hydrolysate in each vessel. The eluates from the Dowex 1-acetate columns were measured by the resorcinol assay according to the method of Svennerholm (1a, 8).

A comparison was made of the sialic acid concentrations of various tissues and fluids determined by several other methods (Table III). For the calculations it is assumed in all cases that

TABLE III

Sialic acid content of biological materials by different assays

Tissue	No. of samples	Thiobarbituric acid method	Column method	Direct Ehrlich	Resorcinol	Orcinol	Diphenylamine
		μmoles/100 gm.					
Grey matter, beef brain.....	1	392	398	1447	710	747	770
White matter, beef brain.....	1	125	132	2230	349	424	1290
Bull semen.....	2	600	620	1382	1305	913	2316
Human semen....	8	268	272	650			
Beef liver.....	1	149	189	855	623	495	919
Mouse liver.....	1	97	89				
Mouse adrenal...	1	128	230				
Mouse spleen....	1	164	179				
Rat submaxillary gland.....	1	193	202				
Guinea pig thyroid.....	1	646	603				
Egg yolk (hen)...	1	136	132	1160	735	633	810
Egg white (hen)..	1	98	81	333	136	95	210
Human cerebrospinal fluid*....	2	4.2	4.8				

* These specimens had been frozen and stored for several months before assay.

the form of sialic acid present is *N*-acetylneuraminic acid (mol. wt. = 309). In most cases excellent agreement between the column and thiobarbituric acid methods was obtained whereas values obtained by other methods were high. The mouse adrenal specimen was so small that determinations were made at the limit of resolution of the column and thiobarbituric acid methods. This may account for the discrepancy between the results of the two methods. The concentration of sialic acid in cerebrospinal fluid reported in the literature is far higher than ours; this may be due to the relatively nonspecific assay used in the previous study (23). The levels of sialic acids in specimens of normal and pathological cerebrospinal fluid are now being determined in collaboration with Dr. R. K. Jakoby.

A difference of results between the column and thiobarbituric acid assays might also arise because all calculations are based upon the molar extinction coefficients of *N*-acetylneuraminic acid. As seen in Table II the molecular extinction coefficient of *N*-acetylneuraminic acid is 16 per cent lower than that of *N*-glycolylneuraminic acid in the resorcinol assay (column method) whereas it is greater in the thiobarbituric acid assay. Thus, differences between the values obtained by the resorcinol and thiobarbituric acid assays might arise, since the proportions of the *N*-glycolyl and *N*-acetyl derivatives in these tissues is not known. However, the close agreement between the methods indicates that *N*-acetylneuraminic is probably the predominant form of sialic acid present. This is known to be so in human semen (20) where the results of the two methods agree within 1.5 per cent.

DISCUSSION

It is presumed that sialic acids form β-formylpyruvic acid upon periodate oxidation, since the thiobarbituric acid chromophores of sialic acids and 2-keto-3-deoxygluconic acid behave similarly. Their absorption spectra are identical (λ_{max} = 549 mμ). Both are soluble in cyclohexanone and only slightly soluble in isoamyl alcohol and both are unstable in basic solution in contrast to the malonaldehyde chromophore. The chromophores of both have the same R_F in two different solvent systems.[4]

The effect of the pyranose ring of *N*-acetylneuraminic acid in the thiobarbituric acid assay is unknown but it is doubted that it has a significant influence on the reaction. The data presented here are consistent with the structures of the sialic acids proposed by Gottschalk (24).

The assay described differs from the others in that the sialic acids must be free. By carrying out the assay on hydrolyzed and unhydrolyzed samples, the levels of free, bound, and total sialic acids in biological materials can be determined.

Recently, after this manuscript had been prepared a letter was published which described an assay for sialic acid that used thiobarbituric acid (25). No definite extinction values were given but from the data it appears that the method is no more sensitive for sialic acid than the 2-deoxyribose assay of Waravdekar and Saslaw (16) or the 2-keto-3-deoxy sugar acid assay of Weissbach and Hurwitz (17). Both of these are 12 times less sensitive than the procedure described in this paper.

SUMMARY

A colorimetric assay has been developed for sialic acids in which sialic acids are oxidized with sodium periodate in concentrated phosphoric acid. The periodate oxidation product is coupled with thiobarbituric acid and the resulting chromophore is extracted into cyclohexanone. The thiobarbituric acid assay is reproducible, sensitive (ϵ = 57,000 for *N*-acetylneuraminic acid), and considerably more specific than other methods and permits accurate direct analysis for sialic acids in tissue hydrolysates. The method is unique in that it only measures unbound sialic acid.

Acknowledgment—The author is indebted to Dr. H. Tabor for helpful discussions and advice throughout the course of this work. The able technical assistance of Mrs. Mary L. Smith is also gratefully acknowledged.

REFERENCES

1. Warren, L., *Federation Proc.*, **18**, 347 (1959).
1a. Svennerholm, L., *Acta Chem. Scand.*, **12**, 547 (1958).
2. Chatagnon, L., and Chatagnon, P., *Presse méd.*, **63**, 1194 (1955).
3. Barry, G. T., *J. Exptl. Med.*, **107**, 507 (1958).
4. Blix, F. G., Gottschalk, A., and Klenk, E., *Nature*, **179**, 1088 (1957).
5. Klenk, E., and Langerbeins, H., *Z. physiol. Chem.*, **270**, 185 (1941).
6. Werner, I., and Odin, L., *Acta Soc. Med. Upsaliensis*, **57**, 230 (1952).
7. Svennerholm, L., *Arkiv Kemi*, **10**, 577 (1957).
8. Svennerholm, L., *Biochim. et Biophys. Acta*, **24**, 604 (1957).
9. Dische, Z., *Mikrochemie*, **8**, 4 (1930).
10. Saifer, A., and Gerstenfeld, S., *J. Lab. Clin. Med.*, **50**, 17 (1957).
11. Ayala, W., Moore, L. V., and Hess, E. L., *J. Clin. Invest.*, **30**, 781 (1951).
12. Pigman, P., Hawkins, W. L., Blair, M. G., and Holley, H. L., *Arthritis and Rheumatism*, **1**, 151 (1958).
13. Siebert, F. B., Pfaff, M. L., and Siebert, M. V., *Arch. Biochem. Biophys.*, **18**, 279 (1948).
14. Folch, J., Arsove, S., and Meath, J. A., *J. Biol. Chem.*, **191**, 819 (1951).
15. Hess, E. L., Coburn, A. F., Bates, R. C., and Murphy, P., *J. Clin. Invest.*, **36**, 449 (1957).
16. Waravdekar, V. S., and Saslaw, L. D., *Biochim. et Biophys. Acta*, **24**, 439 (1957).
17. Weissbach, A., and Hurwitz, J., *J. Biol. Chem.*, **234**, 705 (1959).
18. Cornforth, J. W., Firth, M. E., and Gottschalk, A., *Biochem. J.*, **68**, 57 (1958).
19. Zilliken, F., Braun, G. A., and Gyorgy, P., *Arch. Biochem. Biophys.*, **63**, 394 (1956).
20. Warren, L., *J. Clin. Invest.*, **38**, 755 (1959).
21. Bernheim, F., Bernheim, M. L. C., and Wilbur, K. M., *J. Biol. Chem.*, **174**, 257 (1948).
22. Blix, G., Lindberg, E., Odin, L., and Werner, I., *Acta Soc. Med. Upsaliensis*, **61**, 1 (1956).
23. Bogoch, S., *A. M. A. Arch. Neurol. Psychiat.*, **80**, 221 (1958).
24. Gottschalk, A., *Nature*, **176**, 881 (1955).
25. Aminoff, D., *Virology*, **7**, 355 (1959).

[4] Unpublished results.

The Thiobarbituric Acid Assay of Sialic Acids

Leonard Warren

J. Biol. Chem. 1959, 234:1971-1975.
