QUANTITATIVE ESTIMATION OF SIALIC ACIDS

II. A COLORIMETRIC RESORCINOL-HYDROCHLORIC ACID METHOD*, **

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In a previous communication[1] the quantitative estimation of sialic acids with orcinol-hydrochloric acid (Bial's reagent) has been described. With this method the ketohexoses, when tested in dilute solutions, gave a colour that was impossible to distinguish from that of the sialic acids. Thus Bial's reagent cannot be used for the estimation of sialic acids when they occur together with ketohexoses. The amount of ketohexoses is low in animal materials and will not seriously disturb the determination of sialic acids. SPRINGER[2], however, has reported that sialic acids also occur in plant materials, where the content of ketosugars is much greater. Therefore, a systematical investigation was performed to find out whether it would be possible to determine sialic acids when they occur in the same samples as ketohexoses. Reagents used for the estimation of ketohexoses were tested in order to obtain a reagent that gave different colours with ketohexoses and sialic acids. The results of the testing are collected in Table I.

In the resorcinol method of COLE *et al.*[5] sialic acids and fructose gave quite different colours. From the other tests some general trends in the colour formation of sialic acids could be observed. The colour formation was strongly promoted by Cu^{2+} or Fe^{3+}, while alcohols or acetic acid inhibited it to a great extent. On the basis of these general principles a method was worked out. The molar absorbancies of sialic acids and other sugars in the new method were determined and compared to those obtained with Bial's reagent. Mixtures of N-acetylsialic acid and other carbohydrates were analysed by dichromatic readings and the amount of the former was calculated. Some applications of the method on biological materials are mentioned.

EXPERIMENTAL

Materials

Sialic acids. N,O-diacetylsialic acid ($C_{13}H_{21}NO_{10}$,/H_2O/) from bovine submaxillary mucin and N-glycolylsialic acid ($C_{11}H_{19}NO_{10}$) from pig submaxillary mucin were a gift from Professor G. BLIX, Uppsala. N-acetylsialic acid ($C_{11}H_{19}NO_9$) was prepared by the method described by the author[12].

Other carbohydrates. Glucoheptulose was a gift from Professor N. K. RICHTMEYER, Bethesda, and desoxyribose a gift from Professor S. LALAND, Oslo. All the other carbohydrates were commercial preparations. They were all recrystallized before use.

Resorcinol (Merck) was used without further purification.

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** Quantitative Estimation of Sialic Acids I, ref. [1].

TABLE I

THE BEHAVIOUR OF N-ACETYLSIALIC ACID AND FRUCTOSE IN COLORIMETRIC TESTS DEVISED FOR THE ESTIMATION OF KETOSES

The intensity of the colour formed is graduated from + to +++.

Methods	*Colour development*	
	N-Acetylsialic acid 100 μg	*Fructose 50 μg*
ROE (1934)[3] Resorcinol-HCl-ethanol	None	Orange +++
ROE *et al.* (1949)[4] Resorcinol-HCl-ethanol-thiourea	None	Orange-red +++
COLE *et al.* (1954)[5] Resorcinol-HCl-glycerol-Cu^{2+}	Blue-violet +++	Orange +++
KULKA (1956)[6] Resorcinol-HCl-ethanol-Fe^{3+}	Red +	Orange +++
Present method Resorcinol-HCl-Cu^{2+}	Blue-violet +++	Orange +++
POGELL (1954)[7] Skatol-HAc-ethanol	None	Violet +++
PATMALNIEKS AND GARDELL (1956)[8] Thymol-HAc-ethanol	None	Red ++
DISCHE AND BORENFREUND (1951)[9] Cysteine-carbazol-H_2SO_4	None	Violet +++
VAN CREVELD (1927)[10] Diphenylamine-HCl-ethanol	Red-violet +	Blue +++
DISCHE (1930)[11] Diphenylamine-H_2SO_4-HAc	Violet +++	Blue +++

Hydrochloric acid (Merck) AnalaR, density 1.19, at least 36.4 % HCl. Fe^{3+} not more than 0.0001 %.

0.1 M copper sulphate. 24.97 g of $CuSO_4$, 5 H_2O was dissolved in distilled water to 1 l.

Amyl alcohol. 1 l of *iso*amyl alcohol was mixed with 200 ml of concentrated hydrochloric acid and left for a week. The alcohol was washed with 200 ml of water about ten times and dried with anhydrous potassium carbonate and distilled. The fraction with a b.p. of 130–133° C was used.

Resorcinol reagent. 2 g of resorcinol was dissolved in 100 ml of distilled water (stable for months in a refrigerator). 10 ml of this solution was added to 80 ml of concentrated hydrochloric acid containing 0.25 ml of 0.1 *M* copper sulphate. The volume of the reagent was made up to 100 ml with distilled water. The reagent was prepared at least 4 hours before use. It was stable for a week when stored in the refrigerator.

Blank reagent. It had the same composition as the resorcinol reagent but contained no resorcinol.

Procedure

2 ml of samples containing 10–30 μg of N-acetylsialic acid or a corresponding amount of other sialic acids, were pipetted into three tubes. 2 ml of resorcinol reagent were added to two of the tubes (*test samples*) and 2 ml of blank reagent to the third tube (*sample blank*). In the same manner standard solutions of 0, 15 and 30 μg N-acetylsialic acid were prepared. If the unknown samples also contained other carbohydrates, a mixture of these carbohydrates were run as standards at two different concentration levels. The tubes were heated for 15 minutes at 110° C in an oil bath or in

a boiling water bath. After heating, the tubes were cooled in running water, 5 ml of amyl alcohol were added, the tubes were shaken vigorously and then placed in ice water for 15 minutes. The contents were transferred to centrifuge tubes and spun at 500–1000 r.p.m. for one minute. The tubes were placed in ice water again and left there until the photometrical reading was taken. The amyl alcohol phase was transferred to 50 mm microcells with ballooned pipettes and read at 450 and 580 mμ in a Beckman Spectrophotometer model B against pure amyl alcohol. The readings were completed within one hour after heating.

The absorbancy of the *blank sample* was subtracted from the mean of the *test samples* and the amount of N-acetylsialic acid calculated by means of the equations derived from the standard solutions of N-acetylsialic acid and the carbohydrate mixture.

RESULTS

Optimal conditions for colour production

Composition of the reagent. In the orcinol-hydrochloric acid reaction[1] a final concentration of about 5 *N* acid was found optimal. As it was permissible to mix aliquots of sample and reagent, 10 *N* hydrochloric acid was used in the reagent. The amount of resorcinol used (0.2 g/100 ml reagent) was also the same as the amount of orcinol used. The optimal concentration of Cu^{2+} was determined by analysing the absorbancy of 10 and 40 μg N-acetylsialic acid. Maximum absorbancy was achieved with 0.20–0.25 ml of Cu^{2+}-solution/100 ml reagent. When no oxidants were added to the reagent the absorption curves of N-acetylsialic acid and fructose were much lower and the curve shape of N-acetylsialic acid quite unspecific (Fig. 1).

As the method was intended for the quantitative estimation of sialic acid in biological materials, including lipid extracts, it was necessary to eliminate the influence of fatty materials. Lipids cause an opalescence of the samples, which can be removed by the addition of organic solvents. Addition of miscible alcohols before heating strongly diminished the colour development; the depressing effect of monoalcohols was the largest, of dialcohols less, and of glycerol least. The colouring matter was therefore taken up in amyl alcohol after heating. The colour then turned more blue and the absorption maximum shifted from 570 mμ to 580 mμ. The same phenomenon was also seen when the colouring matter in the orcinol-hydrochloric acid reaction was extracted with the alcohol.

Heating conditions. The testing with the orcinol-hydrochloric acid reagent revealed that the reproducibility was better when lipid-bound sialic acid was estimated at a somewhat higher temperature than at 100° C. The cause of this is still unknown but a better mixing of the rather inhomogeneous samples occurs at the higher temperature. The heating of the samples has therefore been performed at two temperatures, at 110° C in an oil bath, and in a strongly boiling water bath. In the former case closed tubes (Thunberg tubes) were used, in the latter, however, centrifuge tubes. At the higher temperature the maximum absorbancy of N-acetylsialic acid was achieved by heating for 15 minutes. When the heating was performed for the same length of time in a strongly boiling water bath the absorbancy was about 10% lower. The absorbancy, however, increased with a prolonged heating time at least up to 30 minutes, but the absorbancy of hexose increased still more (Table II). To eliminate the influence of hexoses a short heating time would have been desirable, but in that event quantitative values were not obtained, since the colour development was slower for bound than for free sialic acid. Heating for 15 minutes in a boiling water bath is the minimum time recommended for the investigation of protein-bound sialic acid. For lipid extracts and more exact determinations heating at 110° is preferable.

TABLE II

THE RELATIONSHIP BETWEEN ABSORBANCY AND HEATING TIME

The heating was performed in a boiling water bath. The absorbancies after heating at 110° for 15 minutes are also given.

Materials	*Heating at 100° C*				*Heating at 110° C*
	15 min	*20 min*	*25 min*	*30 min*	*15 min*
N-Acetylsialic acid (34.64 μg)	0.899	1.045	1.137	1.200	1.045
Galactose (500 μg)	0.199	0.318	0.383	0.436	0.325
Ribose (50 μg)	0.332	0.477	0.469	0.475	0.475

Stability of the colour formed. When the samples were stored at room temperature the absorbancy diminished by about 5% during the first hour. After this initial decrease, the absorbancy diminished much less rapidly (1%/hour) during the following three hours. The decrease in absorbancy was, however, negligible if the tubes were stored in ice water until the reading was taken.

Sample blank. In order to compensate for non-specific colour in biological materials a sample was run with blank reagent (without resorcinol). The sample blank was subtracted from the test sample before calculation of the sialic acid amount.

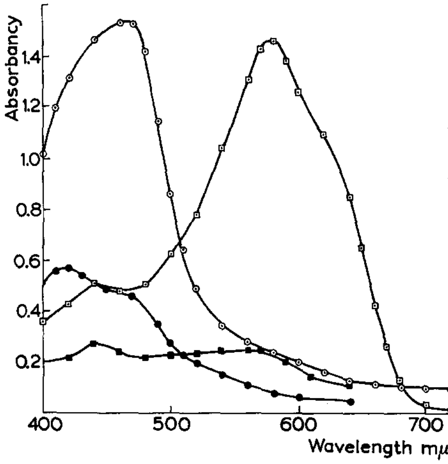

Fig. 1. The absorption spectra of N-acetylsialic acid and fructose with and without Cu^{2+} in the reagent. Without Cu^{2+}: ⊙—⊙ 25 μg, fructose; ⊡—⊡ 50 μg, N-acetylsialic acid. With Cu^{2+}. ●—● 25 μg, fructose; ■—■ 50 μg, N-acetylsialic acid.

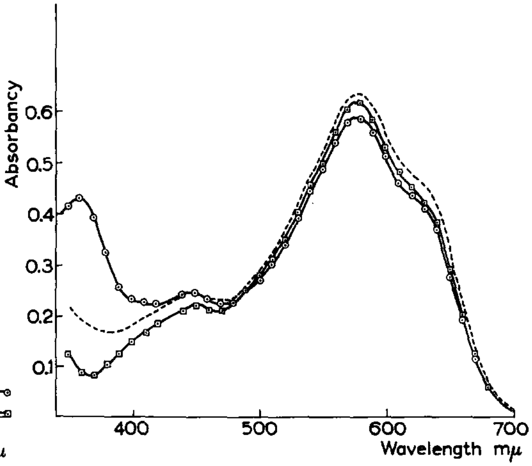

Fig. 2. The absorption spectra of ⊡—⊡ 20.48 μg N-acetylsialic acid; ⊙—⊙ 23.00 μg N,O-diacetylsialic acid; ------ 19.68 μg N-glycolylsialic acid.

References p. 611.

Application of the method to sialic acids and other carbohydrates

Sialic acids. Three naturally-occurring sialic acids are known, all with the same basic structure[13]. They have tentatively been named N-acetylsialic acid ($C_{11}H_{19}NO_9$), N,O-diacetylsialic acid ($C_{13}H_{21}NO_{10}, H_2O$) and N-glycolylsialic acid ($C_{11}H_{19}NO_{10}$)[12]. Several other names for the same substances have been used by other researchers; this has been discussed in a recent paper[1].

The absorbancy indices of the three different acids were determined. As can be seen in Table III the molar absorbancy index (A_M) of N-glycolylsialic acid was 20% higher than the indices of the other two. The same difference was also seen in Bial's reaction. In comparison with the indices found with the latter method, the absorbancy indices with the resorcinol reagent were about 50% higher. Beer's law was valid up to an absorbancy of 1.5 when 50 mm microcells were used. The same absorbancy coefficients were found if the readings were performed in 10 mm cells. The method was also tested with greater concentrations of sialic acids in the samples. When 10 mm cells were used, there was a departure from the linearity for absorbancies greater than 0.5, amounting to 7.5% at an absorbancy of 0.9.

TABLE III

THE MOLAR ABSORBANCY INDICES (A_M) OF SIALIC ACIDS AND OTHER CARBOHYDRATES AT 580 mμ IN THE RESORCINOL REACTION

For comparison, the molar absorbancy indices of the other compounds are also given in percentage of the index of N-acetylsialic acid ($C_{11}H_{19}NO_9$). Further, the same data for the orcinol-hydrochloric acid reaction are tabulated.

Materials	*Colour*	*Resorcinol reaction*				*Orcinol reaction*	
		Heating at 100° C		*Heating at 110° C*		*Heating at 106° C*	
		A_M	*Percentage of* $C_{11}H_{19}NO_9$	A_M	*Percentage of* $C_{11}H_{19}NO_9$	A_M	*Percentage of* $C_{11}H_{19}NO_9$
N-Acetylsialic acid	Blue	8020	100	9520	100	5895	100
N,O-Diacetylsialic acid	Blue	7980	99.5	9498	99.8	5798	98.4
N-Glycolylsialic acid	Blue	9545	119.0	11280	118.5	7060	119.7
Galactose	Yellow	48	0.6	74	0.8	85	1.4
Glucose	Yellow	59	0.7	86	0.9	97	1.6
Mannose	Yellow	67	0.8	115	1.2	131	2.2
Arabinose	Red-violet	207	2.6	567	6	600	10
Ribose	Red-violet	996	12.4	1395	14.7	1554	26.4
Glucuronolactone	Red-violet	278	3.5	530	5.6	585	9.9
Desoxyribose	Olive green	953	11.9	938	9.9	442	7.5
Fructose	Yellow	1544	19.2	1660	17.4	1602	27.2
Glucoheptulose	Blue-green	2950	36.8	3465	36.0	7050	120
L-Fucose	Red-violet	28	0.35	38	0.4	53	0.9
2-Deoxyglucose	Yellow-red	1417	17.7	1634	17.2	—	—
Digitoxose	Red	2920	36.4	3330	34.5	4440	75

The absorption spectra of the acids were also estimated and showed a good agreement between 500–700 mμ (Fig. 2). The differences in the curve-shapes seen at shorter wavelengths may depend on an admixture of small amounts of other carbohydrates, since sugars forming furfural and furfurol analogues have an optimum around 450 mμ. The method is thus also a sensitive indicator of small amounts of contaminating sugars in a sialic acid preparation.

Other carbohydrates. Several other carbohydrates were also tested and their molar absorbancy indices calculated and compared to that of N-acetylsialic acid. The determinations were performed after heating at both 100° and 110° C for 15 minutes. Ketosugars and 2-deoxy sugars had somewhat greater percentage absorbancy when heated at the lower temperature, while the aldosugars had considerably greater percentage absorbancy at the higher temperature. The aldohexoses and 6-deoxyhexoses had very low absorbancy indices and in rather great amounts only caused a minor error. The absorbancies of the ketohexoses were still high but their influence was eliminated by reading the samples at a second wavelength. Pentoses, glucuronic acid, 2-deoxyglucose and digitoxose had in addition to the maximum absorbancy around 450 mμ, a second maximum at 580 mμ. This is obvious from Fig. 3 in which the absorption spectra of N-acetylsialic acid and some other carbohydrates are shown. The method is thus unsuitable for samples containing pentoses, glucuronic acid and 2-deoxyhexoses; in those cases the diphenylamine method of DISCHE[11] is to be preferred.

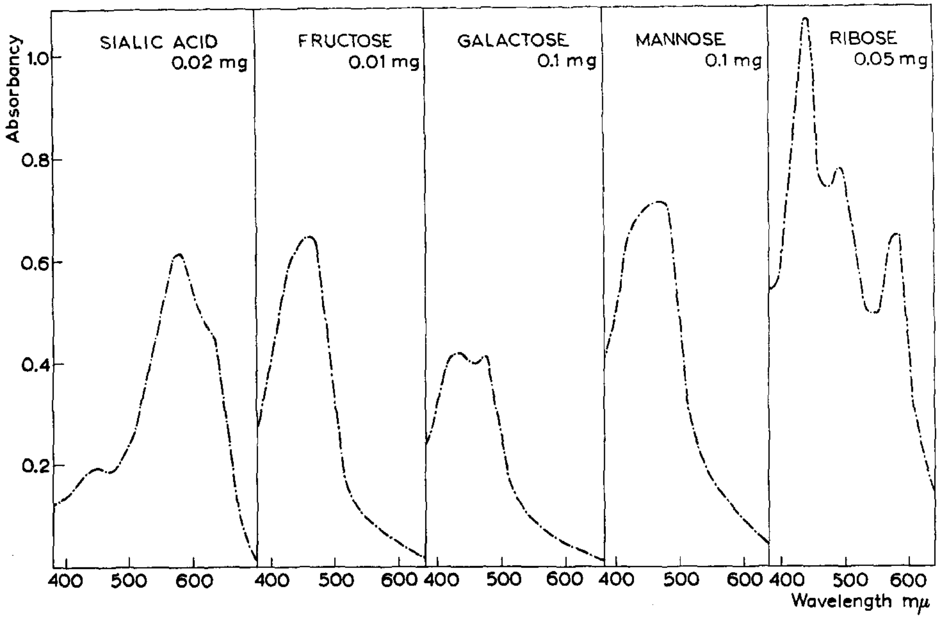

Fig. 3. The absorption spectra of N-acetylsialic acid and other carbohydrates.

Mixtures of N-acetylsialic acid and other carbohydrates

Since biological materials besides sialic acids contain other carbohydrates, mixtures of N-acetylsialic acid and galactose, and N-acetylsialic acid and fructose were analysed with the resorcinol reagent. The samples were read at 450 and 580 mμ and the concentration of N-acetylsialic acid calculated from simultaneous equations. The recovery of N-acetylsialic acid from known mixtures is shown in Tables IV and V. If mixtures of other carbohydrates interfere with the determinations a correction can be made, provided that the approximate composition of this mixture is known[14].

Sensitivity and accuracy

The molar absorbancy of N-acetylsialic acid, calculated on the amyl alcohol phase, was 8,000 and 9,500 for 15 minutes heating in a boiling water bath and oil bath of 110° C, respectively. This means that the absorbancy of 1 μg of N-acetylsialic acid is about 0.030. The sensitivity of the new method is 50% greater than that of the orcinol method.

The standard deviation calculated from 20 samples of N-acetylsialic acid, each containing 34.64 μg of the acid, was ± 0.8% when heated at 110° C and ± 1.0% when heated at 100° C. The standard deviation was in general twice as great in biological materials.

The absorbancies of the standards have been very constant during the year that the method has been employed, so that standards are not necessary in each run if the conditions applied are strictly followed.

TABLE IV

DETERMINATION OF SIALIC ACID IN MIXTURES WITH GALACTOSE BY DICHROMATIC READINGS

The solutions were heated at 100° C for 15 minutes in open tubes. The absorbancies were determined in a Beckman Spectrophotometer model B with a slit-width of 0.3–0.4 mm, 50 mm cells. The readings were done against a reagent blank.

N-Acetylsialic acid μg	*Galactose* μg	*Absorbancy* 450 mμ	580 mμ	*N-Acetylsialic acid* *found** μg	*recovered* (%)
34.64	0	0.295	0.875	34.64	—
25.98	100	0.398	0.668	25.67	99
17.32	200	0.340	0.490	17.62	102
8.66	300	0.320	0.275	8.60	100
0	400	0.355	0.083	—	—

* The calculations were done from equations derived from the first and last samples.

TABLE V

DETERMINATION OF SIALIC ACID IN MIXTURES WITH FRUCTOSE BY DICHROMATIC READINGS

The solutions were heated at 100° C for 15 minutes in a boiling water bath. The absorbancies were determined in a Beckman Spectrophotometer model B with a slit-width of 0.3–0.4 mm The readings were done against amyl alcohol.

N-Acetyl sialic acid μg	*Fructose* μg	*Absorbancy* 450 mμ	580 mμ	*N-Acetyl sialic acid* *found** μg	*recovered* (%)
0	0	0.072	0.014	—	—
21.44	0	0.258	0.558	21.44	—
16.08	5.00	0.539	0.485	16.39	102
10.72	10.00	0.774	0.390	10.74	100
5.36	15.00	1.010	0.305	5.51	103
0	20.00	1.255	0.215	—	—

* The calculations were done from equations derived from the first and last samples.

Application of the method for biological samples

The method has been employed in a number of analytical problems:

(a) Quantitative estimation of N-acetylsialic acid in serum, milk and cerebro spinal fluid and in methanol-chloroform extracts of nervous tissue[14]. (b) Study of the release of N-acetylsialic acid from mucoproteins and gangliosides[14]. (c) Analysis of the effluent from anion-exchange columns in the isolation of N-acetylsialic acid from different sources[12].

For the assay of protein-bound N-acetylsialic acid in body fluids, besides sialic acid standards, equimolar solutions of mannose-galactose were used as standards. It was found that 92–98% of the absorbancy at 580 mμ was due to N-acetylsialic acid, if the heating conditions were 100° C for 15 minutes. No significant difference was seen between samples from normal and pathological cases. A rather accurate and rapid determination of sialic acid in, for example, serum proteins can thus be done only by reading the samples at 580 mμ and afterwards correcting the values by multiplying by 0.95.

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SUMMARY

A new method for the quantitative determination of sialic acids is described. Optimal conditions for the colour formation were investigated. The molar absorbancy indices of N-acetylsialic acid, N-glycolylsialic and N,O-diacetylsialic acid and several naturally-occurring carbohydrates were determined. The method is about 50 % more sensitive than the orcinol-hydrochloric acid method generally used. The influence of other carbohydrates is also considerably lower with the resorcin reagent. The recovery of N-acetylsialic acid from known mixtures with galactose and fructose was determined. The determination of sialic acid in body fluid proteins is briefly discussed.

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