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Quantitative Estimation of Sialic Acids

III. An Anion Exchange Resin Method

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The increasing interest in the distribution of sialic acids in biological materials made it desirable to have a simple method for their analysis. As the colour reactions of the sialic acids are unspecific and influenced by a lot of naturally occurring substances, mainly carbohydrates, a direct analysis of sialic acid is inconvenient for many materials.

In the procedure elaborated the sialic acids are liberated with weak sulphuric acid and retained by a column with Dowex-2 in acetate form. Most of the contaminants are eluted with distilled water and then the sialic acids are displaced with an acetate buffer of pH 4.6. The concentration of sialic acid in the eluates is analysed with the resorcinol method. The optimum conditions for the liberation of the sialic acids are studied in particular.

The method has been satisfactorily applied to the analysis of sialic acids in body fluids and tissues (brain, kidney, liver, pancreas and submaxillary gland).

The concentrations of sialic acids have until now only been determined in biological fluids or isolated watersoluble compounds [1]. For these purposes some colour reactions have been applied to the unhydrolysed material and the colour developed has been determined by photometrical reading. The reactions are not specific but to a greater or lesser extent other biological compounds, mainly carbohydrates, give colours when using the reagents [2–5]. As sialic acids exist together with other carbohydrate components in biological materials, the quantitative figures for sialic acids obtained by direct colorimetry become too high. The positive error given by the other sugars has been corrected by dichromatic readings [3,4] but if the ratio total carbohydrates to sialic acids is great, it is only possible to make an approximate correction. It is also necessary to know the nature of the other sugars and their relative amount which restricts the general application of the procedure of dichromatic readings. There is also an uncertainty as to which degree the colour development of the sialic acids is influenced by substances other than carbohydrates in biological materials. Another disadvantage of direct colorimetric

analyses is that the colour development of bound sialic acids [3] is slower than that of free sialic acids. A prolonged reaction time is, however, unsuitable as the interference from the other carbohydrates then increases considerably [3,4]. It has also become an increasing need for a simple procedure at the analysis of sialic acids in tissues. In the latter case it is in general necessary to split off the sialic acids before they can be assayed with the colour reactions.

For these reasons a quantitative method was elaborated in which the sialic acids were liberated by acid hydrolysis and most of interfering substances removed on columns with an anion exchange resin.

METHODS

Preparation of the ion exchange columns

Dowex 2-X 8, 200—400 mesh, wetscreened through 150 mesh, was heated on the steam bath with three times the volume of 2 N hydrochloric acid. After the resin had settled for 30 min, the supernatant was decanted to remove the fines. After undergoing this treatment four times the resin was stirred up in water and poured into a large tube fitted with a glass filter. The resin was converted to acetate form by passing 2 N sodium acetate through the column until the effluent gave a negative test for chloride. The resin was then slurred up in 1 N acetic acid, transferred to a Büchner funnel and excessive moisture was removed by suction.

For the preparation of the columns about 3 g of the moist resin was suspended in about two volumes of distilled water and poured into a chromatographic tube (inner dimensions 0.7 × 20.0 cm), fitted with a glass filter plate (G 2) at the bottom and widened at the top (2 × 3 cm). The resin was allowed to settle to a height of 6.0 cm and excess resin then poured off. A small filter paper was placed over the resin and above it sand to a height of 0.5 cm. After washing the resin with 10 ml of 0.1 N acetic acid the column was ready for use.

Dowex 50-X 8, 100—200 mesh, was washed slowly several times on a Büchner funnel with 2 N sodium hydroxide, 2 N hydrochloric acid and distilled water in that order. A suspension of the resin in water was poured into a tube of the same dimensions as used for Dowex 2. The height of the column used was about 5 cm.

Regeneration of the columns. Having completed elution the columns were regenerated in the following order. 10 ml of each solution was used.

Dowex 2: 2 N sodium hydroxide, distilled water, 2 N sodium acetate and 0.1 N acetic acid.

Dowex 50: 2 N sodium hydroxide, distilled water, 2 N hydrochloric acid and distilled water.

Hydrolysis of the samples

Samples, containing 25—200 μg of sialic acid, were hydrolysed in small tubes having glass stoppers in 5 ml of 0.1 N sulphuric acid at 80°C and in 5 ml of 0.05 N sulphuric acid at 90°C for one hour. (When lipid extracts were investigated the lipids were dissolved in 0.5—1.0 ml of methanol by heating and the sulphuric acid was added initially in drops to give a fine emulsion.) Having been heated, the tubes were cooled to room temperature and the hydrolysates filtered into the column with Dowex 2. If the sample contained more than 0.1 mequiv. of inorganic cations, the hydrolysate was filtered into the tube with Dowex 50 which was placed immediately above the column with Dowex 2, so that the effluent from the first tube dropped into the Dowex 2 column. After filtration the hydrolysis tubes and the columns were washed twice with 5 ml of water.

Standards of 0, 100 and 200 μg N-acetylsialic acid were treated under the same conditions as the samples in each experiment.

Elution of sialic acids

The sialic acids were eluted from the Dowex 2 columns with 8 ml of 1 M acetic acid-sodium acetate buffer (pH 4.6). The effluent was collected in a 10 ml cylinder. Distilled water was added to the 10 ml mark.

Analysis of sialic acids

Resorcinol method. — Reagent. 0.2 g of resorcinol A.R. was dissolved in 10 ml distilled water. 80 ml conc. hydrochloric acid and 0.25 ml 0.1 M copper sulphate were added, and the volume was made up to 100 ml with distilled water.

Procedure: 2 ml of effluent in duplicates was pipetted into a centrifuge tube (16 × 120 mm) and 2 ml of resorcinol reagent added. The tubes were heated for 15 min in a bath of boiling water. After cooling in tap water, 5 ml of *iso*amyl alcohol was added and the tubes were vigorously shaken and chilled for 10 min in a bath of ice water. After centrifuging the amyl alcohol phase was transferred to 50 mm microcells and read in a Beckman Spectrophotometer model B at 450 and 580 mμ. In the eluates of hydrolysed tissues also ribose esters occurred. Their influence on the absorbancy values at 580 mμ was calculated from the reading at 450 mμ.

RESULTS

Stability of sialic acids in acid milieu. For the quantitative estimation of sialic acids only acid hydrolysis is applicable, as alkaline hydrolysis transforms part of the sialic acids to 2-carboxypyrrole [6,7]. The sialic acids are easily split off in acid milieu but the problem is to achieve such mild conditions that the destruction of sialic acids is negligible. The stability of the sialic acids was tested by heating N-acetylsialic acid under varying conditions and afterwards analysing the hydrolysates with the resorcinol method:

After heating N-acetylsialic acid, dissolved in distilled water, for one hour in a bath of boiling water, the absorbancy diminished by 1—2 %. When the substance was dissolved in 0.05 N and 0.1 N sulphuric acid the absorbancy diminished by 3 and 5 %, respectively. In these tests the concentration of N-acetylsialic acid was 20—40 μg/ml; if the concentration was 10-fold the destruction increased to about twice this value. Therefore, in all the following tests the low concentration of sialic acid was used. When the heating was performed at a lower temperature, 80—90°C, the destruction of N-acetylsialic acid was less. After heating for one hour with distilled water the absorbancy

Table 1. The recovery of N-acetylsialic acid (Ac. sial.) and N-glycolylsialic acid (Glyc. sial.) from Dowex 2 columns after heating with sulphuric acid under varying conditions.

Material	Sulphuric acid N	Temp. C°	Time of heating, h	No. of expts	Recovery, % M. ± S.E.M.
Ac.sial.	0.05	90	1	10	94.5 ± 0.6
Glyc.sial.	0.05	90	1	10	94.2 ± 0.6
Ac.sial.	0.05	90	2	4	89
Ac.sial.	0.10	80	1	10	95.0 ± 0.5
Glyc.sial.	0.10	80	1	10	94.3 ± 0.7
Ac.sial.	0.10	80	2	4	90
Ac.sial.	0.20	80	1	4	93

was unchanged. When heated for one hour with sulphuric acid no measurable decrease in absorbancy was found for a concentration of sulphuric acid $\leq$ 0.05 N at 90°C, $\leq$ 0.2 N at 80°C and $\leq$ 0.4 N at 70°C. The stability of N-acetylsialic acid was somewhat lower in hydrochloric acid than in sulphuric acid of the same strength. Trichloroacetic acid (10 %) and formic acid (1 N) did not destroy the sialic acid at 80 or 90°C, but the concentrations necessary for the quantitative release of bound sialic acid from, *e. g.*, serum proteins were too high for the capacity of the ion exchange resin. It was thus found that sulphuric acid was the most convenient acid for the hydrolysis. Addition of sodium chloride in a concentration of up to 10 mequiv/l did not give any measurable increased destruction of N-acetylsialic acid.

Recovery of sialic acids after the resin procedure. N-acetylsialic acid and N-glycolylsialic acid were hydrolysed with 0.05 N sulphuric acid at 90°C and with 0.1 N and 0.2 N sulphuric acid at 80°C for 1 and 2 h. The hydrolysates were then poured on the anion exchange resin columns, and after washing with water the sialic acids were eluted with 8 ml of acetate buffer as described. The recovery of the sialic acids is shown in Table 1. There was thus a difference in the recoveries of sialic acids between direct colorimetry and colorimetry after previous resin purification. Analysis of sialic acids in the effluents of the hydrolysate and the wash water were negative. Nor were any sialic acids eluted after a further addition of 10 ml of acetate buffer. This may be explained by a partial destruction of the sialic acids during the heating resulting in products still reacting with the colour reagents. However, they may be so strongly adsorbed to the resin that it is impossible to eluate them with the buffer used. At the isolation of N-acetylsialic acid with the resin technique [8], small fractions of Bial-positive substances were eluted after the main fraction. In paper partition chromatography the R_F-values for all these sialic acids were the same as those of N-acetylsialic acid. Odin and Berggård [9] found, however, when samples of sialic acids were heated with acids, that Bial-positive substances were formed which run faster than N-acetylsialic acid. Therefore it seems likely that the latter compounds will be attached so firmly to the anion resin, that they are not eluted.

It should also be pointed out that special precautions must be taken when isolating the sialic acids, which should be used as standard substances. When the sialic acids were prepared with the anion exchange method [8] and the eluates concentrated to a small volume on the water bath before lyophilization, 2–5 % of the sialic acids were changed and could no longer be attached to the resin. By eluting the sialic acid with acetate buffer, removing the sodium ions with a cation exchange resin in hydrogen form, extracting the acetic acid with diethyl ether, and lyophilizing more dilute solutions, the change of the sialic acids was negligible. For routine work it is however more simple to calculate the concentration of authentic sialic acid in a standard substance by estimating its amount. Unhydrolysed samples of the standard substances were analysed with the resin method and the yield of sialic acid determined. As the recovery of the sialic acids obtained with the described isolation procedure was 100 % after resin treatment the difference in absorbancy for the common standard substances with direct colorimetry and after resin treatment may be attributed to the change of the sialic acids during the isolation.

The destruction of the sialic acids during the liberation were also evaluated by heating biological samples for varying times. With serum and brain tissue

Table 2. Liberation of sialic acids from serum with sulphuric acid. 0.2 ml of serum was hydrolysed with 5 ml of sulphuric acid under varying conditions. The hydrolysates were treated according to the standard procedure.

Temp.	Sulphuric acid	Absorbancy			
		Heating time			
	N	1/2 h	1 h	2 h	3 h
80°C	0.05	0.906	0.904	0.894	0.875
	0.10	0.907	*0.924*	0.882	0.835
	0.20	0.888	0.890	0.850	—
90°C	0.05	0.922	*0.935*	0.881	0.817
	0.10	0.886	0.899	0.819	—

the yield of sialic acid decreased with about 5 % per hour when heated under standard conditions.

Liberation of sialic acids. Serum and brain tissue were hydrolysed with varying concentrations of sulphuric acid at 70°C, 80°C and 90°C for 1—3 h. The optimum conditions were tested by analysing the sialic acid content in the eluates from the anion exchange resin columns. The largest concentration of sialic acid was found in the materials by heating with 0.1 N sulphuric acid at 80°C for one hour or with 0.05 N acid at 90°C for one hour. In Table 2 the figures found for serum are given. At 70°C only 80—90 % of the optimum concentrations of sialic acid were achieved.

In the experiment listed the largest absorbancy was found after heating at 90°C but in most experiments maximum absorbancy was achieved after heating at 80°C. When lipid extract was hydrolysed the higher temperature often gave the largest value, while the lower temperature was optimal for tissues with a low concentration of sialic acids. It is therefore preferable to hydrolyse the samples both with 0.1 N sulphuric acid at 80°C and with 0.05 N acid at 90°C.

In recovery experiments pure N-acetylsialic acid was added to the samples before hydrolysis. The recovery of N-acetylsialic acid added to serum was 95.5 % $\pm$ 0.7. The recovery of N-acetylsialic acid added to different tissues was from 90 to 98 %. The lower the concentration of sialic acid, the lower was the recovery. It is therefore preferable to add the standard of sialic acid to each new material which is investigated if exact figures are wanted.

Anion exchange resin column. Dowex 2 X-8, 200—400 mesh, was used throughout. The resin was in the preliminary experiments used both in formate and acetate form. The capacity of the resin in retaining sialic acid was somewhat larger when it was in acetate form. Up to 5 ml of 0.4 N sulphuric acid could be added to the column without any sialic acid occurring in the effluent. If the material, however, contained more than 0.2 mequiv of inorganic cations the capacity of the resin to retain sialic acids was measurably lower. Therefore, the hydrolysed sample was first run through a Dowex-50 resin (H^+-form) when the concentration of inorganic cations was more than 0.1 mequiv.

Table 3. Elution of N-acetylsialic acid from Dowex 2 columns. 225 μg of N-acetylsialic acid was hydrolysed with 5 ml of 0.1 N sulphuric acid at 80°C for 1 h and quantitatively transferred to the resin column. Elution was performed under varying conditions. The effluent was analysed with the resorcinol method, and the total absorbancy determined.

	Acetate buffer pH 4.6			
Volume	Molarity			
(ml)	0.2	0.5	0.8	1.0
	Absorbancy			
4	—	1.25	4.60	5.45
6	—	5.50	5.85	6.10
8	0.600	6.00	6.20	6.25
10	—	—	6.20	6.15
20	—	—	6.15	6.10

Elution of sialic acid. An acetate buffer of pH 4.6 was used for the quantitative elution of sialic acid. At pH 4.6 the stability of the sialic acids is optimal, as judged from the reading of the samples in ultraviolet light [9]. The sialic acids were quantitatively eluted with 8 ml of 1 M acetate buffer (Table 3). The yield of sialic acid was neither enlarged by increasing the volume and the strength of acetate nor by changing the pH of the buffer. With a lower concentration of the acetate buffer or with a smaller volume the elution was not quite quantitative.

Sialic acid determination. Of the colorimetric methods applied for the determination of sialic acids the resorcinol method is the most sensitive one and give the lowest standard error. Therefore, it was used as the standard method. The colour development with the reagent was not influenced by an acetate concentration of up to 2 M in the samples. At the determination of sialic acid in body fluids as serum, lymph, milk, synovial fluids and transudates no other carbohydrate was eluted together with the sialic acids. However, when tissues were hydrolysed also nucleic acids were released and the phosphate esters of ribose were retained by the columns and eluted together with the sialic acids. The ribose esters were displaced somewhat slower than the sialic acids but it was impossible to eluate sialic acid quantitatively without a large part of the ribose also being eluted. A rather accurate calculation of the interference from ribose could be made by dichromatic readings, but it would be preferable to analyse the samples with another method, too. Several methods have been applied but none has been quite satisfactory. With the diphenylamine reagents [11,12] ribose had a low absorbancy, but some unknown substances in the tissues gave a false absorbancy of sialic acid. The direct Ehrlich reaction [2] was rather specific but it was insensitive with a very large standard error. A good reproducibility was also obtained with the orcinol method [3] but the absorbancy of ribose was high. The reactions mentioned will be discussed further in a forthcoming paper on the determination of sialic acid in nervous tissue [10].

Table 4. The concentration of sialic acid in different biological materials. All values are expressed as N-acetylsialic acid ($C_{11}H_{19}NO_9$).

Samples	Number of analyses	N-acetylsialic acid mg per 100 g wet weight	dry weight
Serum, human	25	63.0	
» rabbit	2	64.1	
» sheep (pooled)	2	63.6	
Joint fluid, human	2	80.8	
Milk, human	20	118.8	
» cow (pooled)	2	12.2	
» cow's colostrum	2	180.7	
Semen, human	4	105.5	
Brain, calf (total)	3	82.0	465
» pig (total)	3	84.9	446
» » (grey matter)	3	95.8	630
» sheep (total)	3	87.1	417
Kidney, human	4	35.5	291
» bovine	2	29.9	254
» calf	2	48.7	243
» horse	2	24.9	128
» pig	2	33.5	234
Liver, human	2	16.0	114
Pancreas, sheep	3	41.5	159
Parotis gland, horse	2	29.2	104
Submaxillary gland, elk	2	138.5	591
» » horse	3	111.5	482
» » pig	3	659	2 732

Accuracy. The standard error of the whole procedure was calculated from duplicate determinations of 20 sera. 0.2 ml of serum was hydrolysed with 5 ml of 0.1 N sulphuric acid at 80°C for one hour. The standard deviation was $\pm$ 1.4 %. In parallel determinations the sialic acid concentration was also determined by a direct analysis of alcohol-precipitated sera and the absorbancy of the hexoses was corrected by dichromatic readings. The standard deviation with the latter procedure was considerably larger, $\pm$ 3.6 %. Normal as well as pathological sera were analysed. The agreement between the two procedures was good. The standard error was also calculated on the analyses of sialic acid in fresh brain tissue of human fetuses. The standard deviation was there $\pm$ 1.6 %.

APPLICATION OF THE METHOD

The resin separation method has been applied to hydrolysates of several biological materials. The hydrolysis was performed with 5 ml of 0.1 N sulphuric acid at 80°C and 5 ml of 0.05 N sulphuric acid at 90°C for one hour. In

general there was a good agreement between the two procedures but for some tissues with a rather low concentration of sialic acid (e.g. kidney and liver) the value found with 0.05 N sulphuric acid was 5—10 % lower. In these cases only the figures found with the stronger sulphuric acid were used for the calculations of the sialic acid content.

All the figures are calculated as N-acetylsialic acid. In the physiological state the sialic acids often contain at least one O-acetyl groups as well. Besides, instead of the N-acetyl group the sialic acids may partly be substituted by an N-glycolyl group. In fact only in human sources the N-glycolylsialic occurs in negligible amounts. In all the other species investigated (beef, horse, pig and sheep) the N-glycolylsialic acid constitutes from 10—90 % of the total amount of sialic acids [10]. The molar absorbancy index of N-glycolylsialic acid is 15—20 % greater * than that of N-acetylsialic or N,O-diacetylsialic acid in the common colorimetric methods for sialic acids [3-5]. Therefore the figures for sialic acid in many cases are too high especially in beef tissues and pig submaxillary gland where N-glycolylsialic acid constitutes at least 50 %.

All the figures are calculated from the values of the hydrolysed and resin-treated standard samples of N-acetylsialic acid. In this procedure about 5 % of the standard substance was destroyed but in some tissues (liver and kidney) the destruction of sialic acid was probably larger. However, no correction was made for this excess destruction.

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* *Added in proof:* With a recently prepared sample of N-glycolylsialic acid the molar absorbancy index was 30 % greater than that of N-acetylsialic acid.