Determination of Neuraminic Acid in Crude Brain Lipids

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Strandin, a water-soluble lipid, was first isolated from brain by Folch, Arsove & Meath (1951). Recently, Bogoch (1958) has reported that purified ox-brain strandin (ganglioside) has a molecular weight of about 250 000. The molecule contains fatty acid, sphingosine, glucose, galactose, galactosamine and neuraminic acid (Klenk & Lauenstein, 1953; Bogoch, 1958).

In experiments designed to effect the chromatographic separation of cerebroside from crude brain lipids, the presence of strandin in the latter was considered to be a complicating factor, because of its related composition, and it became important to achieve the complete removal of the strandin before applying chromatography. For this purpose it was necessary to have a reliable method for the determination of strandin.

Neuraminic acid is the most characteristic constituent of strandin, for it has not been found in other lipids. Quantitative determination was therefore based on this component. In addition to the humin reaction described by Folch *et al.* (1951), which is not very sensitive, four other methods are available for its determination, namely reaction with (1) Bial's orcinol reagent, (2) Ehrlich's reagent, (3) diphenylamine and (4) tryptophan and perchloric acid (Werner & Odin, 1952). The sensitivites of reactions (1)–(4) are in the approximate ratio 5:1:2:1 respectively. Since the quantity of neuraminic acid in the lipid fraction extractable from brain amounts only to about 1·5–2·0 μmoles/g. of moist tissue, the very sensitive orcinol reaction has been used in the experiments to be described.

A preliminary report on this work has already been published (Long & Staples, 1959).

EXPERIMENTAL

Materials

Gifts of the following materials were used: synthetic *N*-acetylneuraminic acid (Cornforth, Firth & Gottschalk, 1958) from Miss Patricia Carroll; partially purified ox-brain strandin (Folch *et al.* 1951) from Dr G. H. Sloane-Stanley; ox-brain cerebroside, prepared by Dr R. Rodnight, from Dr G. H. Sloane-Stanley.

Methanol was purified by distillation from solid KOH. $CHCl_3$ was washed with water, dried over $CaCl_2$ and distilled; it was stabilized by the addition of 2% (v/v) of methanol. All other reagents were the best available commercial preparations.

Methods

Brain-lipid extracts. Ox brain, freshly obtained from the Metropolitan Cattle Market, was homogenized with $CHCl_3$–methanol (2:1, v/v) in the proportions of 1 g.: 19 ml. The homogenate was centrifuged and the supernatant filtered. Assuming the brain to contain 80% of water, the final solvent composition would be: $CHCl_3$–methanol–water (approx. 64:32:4, by vol.). Extracts of rat tissues were prepared similarly.

Solvent-partition of lipids. This was done essentially as described by Folch, Lees & Sloane-Stanley (1957). To the lipid extract (150 ml.), in a 250 ml. centrifuge bottle fitted with a ground-glass stopper, 30 ml. of 0·1 M-KCl was added. The mixture was shaken and centrifuged. The upper aqueous methanolic phase (about 80 ml.) was transferred to a 250 ml. volumetric flask. The lower $CHCl_3$-rich phase was then extracted twice by shaking each time with 80 ml. of $CHCl_3$–methanol–0·1 M-KCl (3:48:47, by vol.), followed by centrifuging. The upper phases were combined and diluted to 250 ml. with methanol. The lower $CHCl_3$-rich phase was diluted to 200 ml. with methanol.

Orcinol reaction. This was carried out essentially according to Böhm, Dauber & Baumeister (1954). A measured volume of the test sample was evaporated to dryness in a 10 ml. centrifuge tube, and 1 ml. of water was added, followed by 1 ml. of Bial's orcinol reagent (0·02% of $FeCl_3$ + 0·2% of orcinol in 8 N-HCl). The mixture was heated on a boiling-water bath for 15 min., cooled and extracted with 4 ml. of amyl alcohol. After centrifuging, the upper violet layer was removed and a 3·5 ml. sample was transferred to a 1 cm. glass cell. Ethanol (0·05 ml.) was also added to the cell and the contents were mixed. The presence of the ethanol prevents the solution from becoming turbid owing to the absorption of moisture. Extinctions were measured at wavelengths between 400 and 700 mμ in a Unicam spectrophotometer, SP. 500. Standard *N*-acetylneuraminic acid and a reagent blank were simultaneously carried through the same procedure.

RESULTS

Direct orcinol reaction

Fig. 1 shows the absorption curve in amyl alcohol of the reaction product formed on heating 50 μg. (0·16 μmole) of synthetic *N*-acetylneuraminic acid with Bial's orcinol reagent. The two characteristic features of this curve are the absorption maximum at 570 mμ (ϵ, $6{\cdot}0 \times 10^3$) and the flat portion between

440 and 480 mμ (ε, $3{\cdot}8\times10^3$). The latter probably represents the summation of two curves, namely the absorption due to the orcinol-reaction product and that due to a part of the neuraminic acid which undergoes decomposition on heating with HCl alone (Folch *et al.* 1951) to give a brown pigment with absorption maximum at about 420 mμ. The partially purified specimen of ox-brain strandin (165 μg.) gave a curve almost identical in shape with that of *N*-acetylneuraminic acid (Fig. 1).

When the orcinol reaction was applied to the total lipids extractable from 75 mg. of ox brain, the absorption curve (Fig. 1) showed two peaks at 425 and 560 mμ and a pronounced shoulder at about 650 mμ. From this it is clear that the total lipids contain substances other than neuraminic acid which react with the orcinol reagent and that no quantitative conclusions can be drawn about the neuraminic acid content.

Folch *et al.* (1957) reported that when a solution of total brain lipids in $CHCl_3$–methanol–water (64:32:4, by vol.) was shaken with water or a dilute aqueous salt solution (0·2 vol.), the resulting upper aqueous methanolic phase contained strandin. The ox-brain lipid solution was therefore treated with 0·1 M-KCl and partitioned into upper (aqueous methanolic, *A*) and lower ($CHCl_3$-rich, *B*) phases, as described in the Methods section. When amounts of each phase, derived from 75 mg. of ox brain, were separately subjected to the orcinol reaction, the results shown in Fig. 2 were obtained. The curve for the upper-phase material (*A*) somewhat resembled those obtained with synthetic *N*-acetylneuraminic acid and ox-brain strandin (Fig. 1), but the existence of the minor peak at 440–450 mμ, instead of the flat portion in this region, and the inflexion at 650 mμ indicated the presence of small amounts of orcinol-reactive material other than neuraminic acid. From this it can be seen that the determination of the neuraminic acid content of the upper phase (*A*) from the extinction at 570 mμ would probably lead to an erroneously high value.

The lower-phase material (*B*) was also orcinol-reactive (Fig. 2), showing a maximum at 415 mμ, but with some absorption in the region of 570 mμ. It seemed probable that this behaviour was largely due to the presence, in the lower phase, of cerebroside, which on analysis amounted to 2·30 μmoles, since similar types of curves (Fig. 2) were obtained with 1·5 μmoles of cerebroside or galactose.

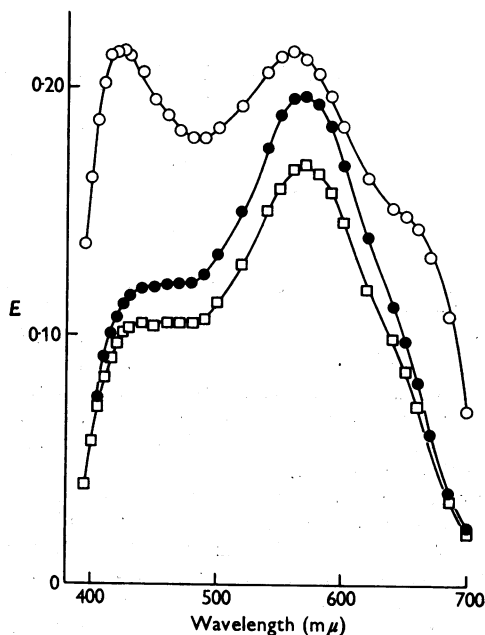

Fig. 1. Absorption spectra of orcinol-reaction products in amyl alcohol. ●, 0·16 μmole of synthetic *N*-acetylneuraminic acid; □, 165 μg. of partially purified ox-brain strandin; ○, mixed ox-brain lipids, derived from 75 mg. of moist tissue. Total vol., 4·8 ml.; 1 cm. light path.

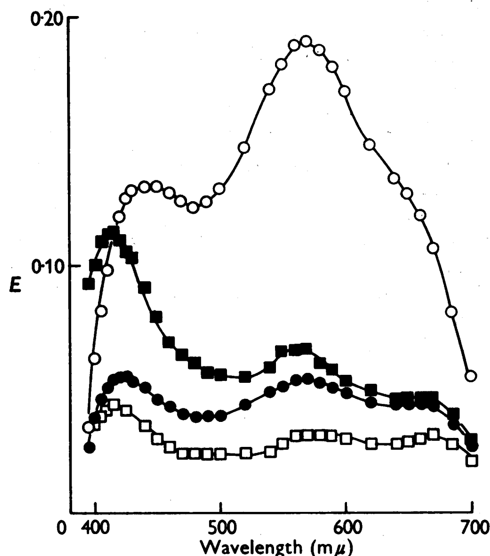

Fig. 2. Absorption curves of orcinol-reaction products in amyl alcohol. ○, Upper aqueous methanolic phase derived from 75 mg. of moist ox brain; ■, lower $CHCl_3$-rich phase derived from 75 mg. of moist ox brain; ●, cerebroside (1·5 μmoles); □, galactose (1·5 μmoles).

Effect of acid treatment before applying the orcinol reaction

As previously mentioned, Folch *et al.* (1951) developed a method for the estimation of strandin whereby the lipid sample was heated with conc. HCl and the resulting brown decomposition product

was determined spectrophotometrically after dilution with acetone. When synthetic *N*-acetylneuraminic acid was first heated with either acid or alkali and the orcinol reaction subsequently applied, extensive decomposition was found to have taken place (Fig. 3). The sample of ox-brain strandin behaved similarly under acidic conditions, but was completely stable to treatment with hot alkali. These findings suggest that only free neuraminic acid is susceptible to alkaline decomposition, and that the stability of strandin is due to the alkali-resistance of the glycosidic bond which links neuraminic acid to the rest of the molecule. It will also be seen from Fig. 3 that the characteristic change, produced by prior treatment with HCl on the orcinol reaction of neuraminic acid, is the fall in extinction at 570 mμ.

In order to determine whether the lower-phase material (*B*) from the partition experiment (Folch *et al.* 1957) contained any neuraminic acid, use was made of this acid-lability of neuraminic acid derivatives. A sample of lower-phase material (*B*), corresponding to 75 mg. of moist ox brain, was evaporated to dryness and the lipid residue was heated with 1 ml. of 4N-HCl on a boiling-water bath for 30 min. After cooling, the contents of the tube were extracted with a mixture of methanol (1·6 ml.) and $CHCl_3$ (3·2 ml.). The upper aqueous methanolic phase which separated on standing was discarded. The lower phase was extracted twice with 2 ml. portions of $CHCl_3$–methanol–water (3:48:47, by vol.) and the upper phases were again discarded. The lower $CHCl_3$-rich phase (*C*) was then evaporated to dryness and treated with the orcinol reagent in the usual way. An equivalent sample of the original lower phase (*B*), which had not been heated with acid, was also subjected to the orcinol reaction. The results of this experiment are shown in Fig. 4. Although there are slight differences in the extinctions below 470 mμ and above 590 mμ, the curves are superimposable between these

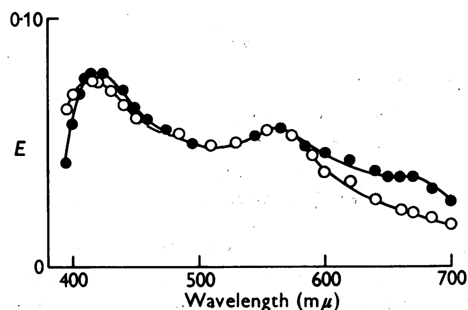

Fig. 4. Absorption spectra of orcinol-reaction products of $CHCl_3$-rich phase derived from 75 mg. of moist ox brain before and after heating with 4N-HCl at 100° for 30 min. ●, Untreated; ○, after hot-acid treatment.

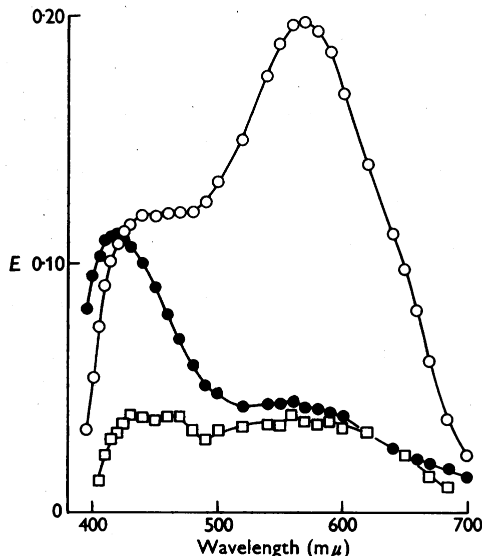

Fig. 3. Effect of heating *N*-acetylneuraminic acid with acid or alkali on the absorption spectra of the orcinol-reaction products. All samples originally contained 0·16 μmole of *N*-acetylneuraminic acid. ○, Untreated; ●, preliminary treatment with 4N-HCl at 100° for 30 min.; □, preliminary treatment with N-NaOH at 100° for 30 min.

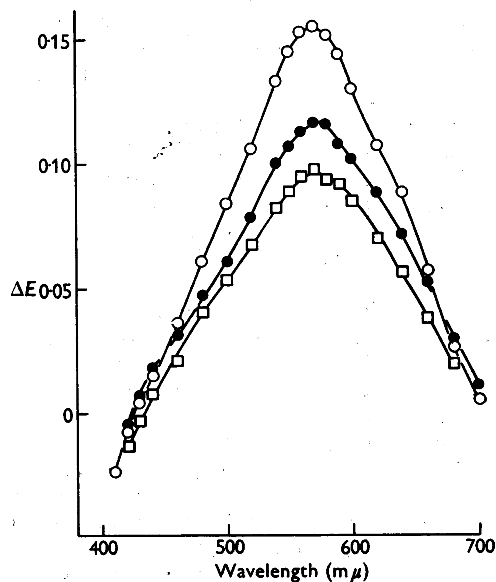

Fig. 5. 'Difference' absorption spectra of orcinol-reaction products (untreated minus acid-treated). ○, *N*-Acetylneuraminic acid (0·16 μmole); □, partially purified strandin (135 μg.); ●, upper aqueous methanolic phase derived from 75 mg. of moist ox brain.

Table 1. *Neuraminic acid content of lipid extracts of rat tissues*

Tissues were homogenized with $CHCl_3$–methanol (2:1, v/v) and determinations made as described in text.

Tissue	Difference extinction (ΔE_{570}/75 mg. of fresh tissue)	*N*-Acetylneuraminic acid (μmoles/g. of fresh tissue)
Brain (grey matter)	0·118, 0·137	1·54, 1·83
Brain (white matter)	0·072, 0·068	0·94, 0·90
Brain (cerebellum)	0·066, 0·078	0·86, 1·04
Lung	0·003	0·04
Skeletal muscle	0·002	0·02
Kidney	0·002	0·02
Uterus	0·002	0·02
Spleen	0·001	0·01
Adipose tissue	0·001	0·01
Heart	0	0
Intestine (small)	0	0
Liver	0	0

wavelengths. The absence of any fall in extinction at 570 mμ, as a result of the hot-acid treatment, has been taken to show conclusively that the lower-phase material (*B*) was free from neuraminic acid.

Since it follows that the whole of the neuraminic acid content of the original lipid extract must be present in the upper phase (*A*), it only remained to develop a suitable method for its determination. This has also been achieved by making use of the selective destruction of neuraminic acid during a preliminary acid hydrolysis. In Fig. 5 are shown the 'difference' curves obtained with synthetic *N*-acetylneuraminic acid, partially purified ox-brain strandin and the upper-phase material (*A*) from 75 mg. of ox brain. The difference extinctions (ΔE) were obtained by deducting the extinction of an acid-treated sample from that of an equal amount of an untreated sample, after both had been subjected separately to the orcinol reaction. It will be seen from Fig. 5 that there are no important differences between the shapes of these three curves, all of which show maxima at 570 mμ. When different concentrations of strandin or *N*-acetylneuraminic acid were used, the ΔE values at 570 mμ were always directly proportional to concentration, at least up to 0·16 μmole of neuraminic acid, which was the highest concentration tested. From these curves, the partially purified ox-brain strandin was calculated to be 68 % pure, assuming that strandin contains 30·9 % of neuraminic acid (Bogoch, 1958). Similarly, ox-brain lipid was calculated to contain 1·64 μmoles of *N*-acetyl-neuraminic acid/g. of moist tissue.

It has been found that neuraminic acid determinations, based on this same principle, can also be made equally well with a simple photoelectric colorimeter of the EEL (Evans Electroselenium Ltd.) type with a no. 626 filter in place of a spectrophotometer.

Neuraminic acid content of lipid extracts of rat tissues

Folch *et al.* (1951) reported that tissues other than brain contained little or no strandin, but the method of determination used by these workers, the humin reaction, was relatively insensitive. With the more sensitive method now available, we have redetermined the neuraminic acid content of lipid extracts from several rat tissues.

A sample (0·2 g.) of fresh tissue was homogenized in a Potter–Elvehjem-type homogenizer with 3·8 ml. of $CHCl_3$–methanol (2:1, v/v) and the suspension was centrifuged. The supernatant was transferred to a 50 ml. distillation flask. The residue was washed twice with 2 ml. of solvent and the washings were combined with the original supernatant. After evaporating the extract to dryness, the lipid residue was dissolved in about 2 ml. of $CHCl_3$–methanol (2:1, v/v). Any insoluble material was removed by filtration, and the filtrate and washings were diluted to 3 ml. This lipid extract was then partitioned by the method of Folch *et al.* (1957), as described in the Methods section. Two samples of upper phase, each corresponding with 75 mg. of moist tissue, were taken for determination of the difference extinction at 570 mμ, after treatment with the orcinol reagent. The results obtained (Table 1) confirm that neuraminic acid is present only in lipid extracts of brain. Grey matter from the cortex contained about twice the amount of *N*-acetylneuraminic acid, on a moist-weight basis, as did white matter or cerebellum.

DISCUSSION

The primary aim of the work described was to effect the complete removal of strandin from crude brain lipids, with a view to carrying out sub-

sequent chromatographic separations of the individual constituents. The partitioning of brain lipids between aqueous methanolic and chloroform-rich phases, according to the method of Folch *et al.* (1957), resulted in the complete transfer of the strandin into the upper phase. In unpublished work, we have shown that negligible amounts of other lipids are lost in this way. However, the water-soluble non-lipid impurities do pass into the upper phase (cf. also Folch *et al.* 1957). The carbohydrate content is about 30% greater than would be expected from the composition of strandin (Bogoch, 1958), and this excess probably represents water-soluble sugars and their derivatives.

The presence of this extra carbohydrate in the upper phase almost certainly accounts for the extra peak at 440 mμ (Fig. 2), after the orcinol reaction. Although small in absolute amount, this carbohydrate would also contribute significantly to the absorption at 570 mμ, so giving an erroneously high value for the neuraminic acid content based on direct readings of E_{570}. The use of 'difference' extinctions, depending on the selective destruction of neuraminic acid by hot-acid treatment, satisfactorily removes this interference.

SUMMARY

1. Synthetic *N*-acetylneuraminic acid and partially purified ox-brain strandin have been subjected to Bial's orcinol reaction and the characteristics of the absorption spectra have been studied.

2. Crude ox-brain lipids under similar conditions showed anomalous spectra, which were largely traced to the presence of cerebroside.

3. Partitioning of ox-brain lipids between aqueous methanolic and chloroform-rich phases caused the strandin to pass completely into the upper aqueous methanolic phase. The neuraminic acid content of this phase was determined by measuring the 'difference' extinction at 570 mμ of the orcinol-reaction products (untreated minus acid-treated).

4. Neuraminic acid has been determined in the lipids of several rat tissues. It was found only in the brain.

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Metabolism of Polycyclic Compounds

14. THE CONVERSION OF NAPHTHALENE INTO COMPOUNDS RELATED TO *TRANS*-1:2-DIHYDRO-1:2-DIHYDROXYNAPHTHALENE BY RABBITS*

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The conversion of aromatic hydrocarbons into dihydrodihydroxy compounds by 'perhydroxylation' was first recognized when Boyland & Levi (1935, 1936) isolated (+)- and (−)-1:2-dihydro-1:2-dihydroxyanthracene, and the corresponding glucosiduronic acids, from the urine of rabbits and rats respectively which had been dosed with anthracene. Boyland & Wolf (1950) showed that phenanthrene is converted by rats into *trans*-9:10-dihydro-9:10-dihydroxyphenanthrene and by rabbits into this diol and into (−)-*trans*-1:2-dihydro-1:2-dihydroxyphenanthrene. Young (1947) found that naphthalene is converted by rats into (−)-*trans*-1:2-dihydro-1:2-dihydroxynaphthalene and Booth & Boyland (1949) obtained both the (±) and the (−)-forms of this diol from rabbits treated with naphthalene. It has been shown that naphthalene is converted into 1:2-dihydro-2-hydroxy-1-naphthylglucosiduronic acid in rabbits (Corner, Billett & Young, 1954) and in rats (Corner & Young, 1954). The product from rabbits yielded (±)-*trans*-1:2-dihydro-1:2-dihydroxynaphthalene on hydrolysis

* Part 13: Booth & Boyland (1958).