- Cheftel, R. I., Munier, R. & Macheboeuf, M. (1952). *Bull. Soc. Chim. biol.* **34**, 380.
- Chefurka, W. (1958). *Proc. 4th int. Congr. Biochem., Vienna*, **12**, 115.
- Consden, R., Gordon, A. H. & Martin, A. J. P. (1944). *Biochem. J.* **38**, 224.
- Frantz, I. D., Zamecnik, P. C., Reese, J. W. & Stephenson, M. L. (1948). *J. biol. Chem.* **174**, 773.
- Gilmour, D. (1941). *J. cell. comp. Physiol.* **18**, 93.
- Halvorson, H. O. (1960). *Advanc. Enzymol.* **22**, 99.
- Heslop, J. P., Price, G. M. & Ray, J. W. (1962). *Biochem. J.* **82**, 24r.
- Hoagland, M. B. (1958). *Proc. 4th int. Congr. Biochem., Vienna*, **8**, 199.
- Humphrey, G. F. (1949). *J. cell. comp. Physiol.* **34**, 323.
- Katz, J. & Chaikoff, I. L. (1955). *Biochim. biophys. Acta*, **18**, 87.
- Phillips, D. M. P. (1949). *Biochim. biophys. Acta*, **3**, 341.
- Price, G. M. (1961a). *Biochem. J.* **80**, 420.
- Price, G. M. (1961b). *Biochem. J.* **81**, 15r.
- Roland, J. F. & Gross, A. M. (1954). *Analyt. chem.* **26**, 502.
- Tauber, H. (1955). *Analyt. chem.* **27**, 287.
- Troll, W. & Lindsley, J. (1955). *J. biol. Chem.* **215**, 655.
- Winteringham, F. P. W. (1958). *Proc. int. Symp. Microchem., Birmingham*, p. 305.
- Winteringham, F. P. W. (1960). *Biochem. J.* **75**, 38.

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Detection and Resolution of Gangliosides in Lipid Extracts by Thin-Layer Chromatography

By J. R. WHERRETT* AND J. N. CUMINGS

Department of Chemical Pathology, Institute of Neurology, The National Hospital, Queen Square, London, W.C. 1

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Ganglioside was the name given by Klenk (1942) to a lipid substance located predominantly in the grey matter of the brain which, on hydrolysis, released cerebroside and other carbohydrate compounds. Among these were identified hexose and neuraminic acid (Klenk, 1942) as well as hexosamine (Blix, Svennerholm & Werner, 1950, 1952; Klenk, 1951). Similar lipid-soluble, neuraminic acid-containing substances extracted and purified by a variety of methods have been described (Folch, Arsove & Meath, 1951a; Svennerholm, 1956a; Bogoch, 1958; Rosenberg & Chargaff, 1958; Folch & Lees, 1959; van Heyningen & Miller, 1961; Payne & Platt, 1961; Svennerholm & Raal, 1961; Trams & Lauter, 1962), which differ with respect to percentage composition and presence of non-glycolipid components, but the relationship between these various preparations is not entirely clear. Evidence that these preparations may contain different combinations of aminoglycolipids (both neuraminic acid and non-neuraminic acid-containing), glycosaminoglycans and glycopeptides has been presented by Svennerholm (1956b) and Bogoch, Belval & Winer (1961). It is now usual, however, to reserve the term ganglioside for glycolipids that contain neuraminic acid, irrespective of the tissue of origin. Ganglioside preparations are extracted from tissues only with lipid solvents

of a mild polar nature and are usually water-soluble, exhibiting in aqueous solution the physical properties of a single large molecule (Folch *et al.* 1951a; Chatagnon & Chatagnon, 1953; Bogoch, 1958; Rosenberg & Chargaff, 1958). Sedimentation behaviour in dimethylformamide as opposed to water (Klenk & Gielen, 1960) and titration data (Klenk, 1942) are consistent with a much smaller molecular weight and the heterogeneity of ganglioside revealed by countercurrent distribution (Meltzer, 1958) and chromatography (Svennerholm, 1956a; Kuhn, 1958, 1959; Kuhn *et al.* 1960; Kuhn, Weigandt & Egge, 1961; Weicker, Dain, Schmidt & Thannhauser, 1960; van Heyningen & Miller, 1961; Klenk & Gielen, 1961a, b, c; Payne & Platt, 1961; Dain, Weicker, Schmidt & Thannhauser, 1962a; Dain, Willis, Sweet & Schmidt, 1962b) would suggest that the physical behaviour in water results from the aggregation of smaller molecular species to form micelles.

The fractionation of ganglioside by chromatography on thin layers of silica gel appears to be highly effective. The high resolving power of the method makes it possible to separate the components of a lipid class such as the gangliosides even in the presence of other tissue lipids (Jatzkewitz, 1961). A particular advantage of thin-layer over paper chromatography is that detection methods of high specificity and sensitivity for neuraminic acid may readily be used. In this work the technique

* Present address: Department of Medicine, Toronto General Hospital, 100 College Street, Toronto 2, Canada.

has been developed to give good resolution of neuraminic acid-containing components in crude lipid extracts. Evidence is presented that tentatively identifies most of the components as ganglioside and the patterns given by normal tissues are described.

METHODS

Preparation of tissue extracts. Fresh animal tissues obtained immediately after death were dissected at 4° into portions (1 g.) and these were stored until further use at -18°. The samples were extracted by the procedure of Folch, Ascoli, Lees, Meath & LeBaron (1951*b*), and without washing stage, immediate and complete dispersion being obtained with a power-driven Potter-Elvehjem homogenizer, and were filtered through sintered glass (Wherrett & Mellwain, 1962). Extracts were evaporated to dryness at 40-50° with an air-leak system and redissolved in chloroform-methanol (2:1, v/v) to give a final concentration of 250 mg. of original tissue/ml.

Chromatography. Thin-layer chromatography was carried out with Stahl's (1956, 1958*a*, *b*) apparatus [Camlab (Glass) Ltd.]. Plates (20 cm. by 20 cm.) were spread with a layer (0.25-0.50 mm. thick) of Silica Gel G (Merck) and dried at 110° for 1-6 hr. immediately before use; the best separations were obtained after drying times between 3 and 4 hr. Samples were spotted on lines 1.25 cm. long with Lang-Levy-type constriction pipettes. Plates were run at room temperature in paper-lined tanks, the solvent front being permitted to reach within 4 cm. of the top of the plates.

Solvent system. The following systems have been used: butan-1-ol-pyridine-water (3:2:1, by vol.; Klenk & Gielen, 1961*a*); propan-1-ol-water (7:3, v/v; Kuhn *et al.* 1961); propan-1-ol-aq. $N-NH_3$ soln.-water (6:2:1, by vol.; Weicker *et al.* 1960); chloroform-methanol-water (14:6:1, by vol.), followed by propan-1-ol-aq. 12.5% NH_3 soln. (4:1, v/v; Jatzkewitz, 1961) and chloroform-methanol-water (60:35:8, by vol.; Wagner, Hörhammer & Wolfe, 1961). This last solvent system proved to be the most suitable but

separation of the ganglioside components was enhanced by the substitution of aq. 2.5*N-NH*₃ soln. for water in that system.

Detection. The plates were dried in air, sprayed lightly with resorcinol-HCl reagent (Svennerholm, 1957*a*), covered with a clean second plate and heated at 150° for 8-12 min. Neuraminic acid-containing substances appeared as purple spots against a white background. Heavy spraying and prolonged heating resulted in a browning of the resorcinol-positive spots as well as charring of other lipid compounds present. Glycerides, cholesterol, free fatty acid, polyglycerol phosphatide, cerebroside, phosphatidylethanolamine, sulphatide, phosphatidylcholine, phosphatidylserine, sphingomyelin and monophosphoinositide all migrate in advance of the neuraminic acid-containing compounds. Diphosphoinositide has not been identified.

Reagents and marker substances. Resorcinol (Analar) was recrystallized from benzene-diethyl ether. Reagent-grade chloroform and A.R. methanol were dried with sodium sulphate and potassium carbonate respectively and were then redistilled. All other reagents were of A.R. grade.

A gift of *N*-acetylneuraminic acid was obtained from Dr L. Svennerholm and samples of purified ganglioside that had been prepared by method 11 of Booth (1962) were received from Mr D. Booth and from Dr D. B. Gammack.

RESULTS

Fig. 1*A* shows the pattern of resorcinol-reacting bands in an extract of ox cerebral cortex. After the extract is partitioned by the procedure of Folch, Lees & Sloane-Stanley (1957) there is an absence of bands when the 'lower phase' is used (Fig. 1*B*), but they are present in the 'upper-phase' extract (Fig. 1*C*). The pattern present resembles that given by the purified ganglioside markers, two of which are shown in Fig. 1 (*D* and *E*). Both in fresh extracts and in purified ganglioside preparations from ox brain a constant pattern has been observed.

Fig. 1. Resorcinol-reacting components of ox grey matter compared with those of purified ganglioside: *A*, of the total crude lipid extract; *B*, of the 'lower phase' (Folch *et al.* 1957); *C*, of the dialysed 'upper phase'; *D*, 100 μ g. of a purified preparation of ganglioside containing 0.1% of P and 28.2% of *N*-acetylneuraminic acid; *E*, 100 μ g. of a purified preparation of ganglioside containing 0.1% of P and 28.2% of *N*-acetylneuraminic acid. Both ganglioside preparations were ninhydrin-negative. Components of the patterns are indicated but in *C*, *D* and *E* the 'slow' band is missing, as is indicated in the text.

This pattern consists of five major bands, designated G Ia, G Ib, G Ic, G II and G III, together with four minor bands here called 'slow', 'intermediate 1', 'intermediate 2' and 'fast', as is seen in Fig. 1E. The 'slow' band was seen in most extracts of fresh tissue, but it was not present in freshly prepared ganglioside in chloroform-methanol, although it appeared in such solution upon standing at room temperature for several weeks. This band also appeared after treatment of the purified ganglioside markers with 0.05N-acid at 80° and its position on the chromatogram corresponded to that of the free neuraminic acid marker.

Possible interfering contaminants were also chromatographed. Glucose, galactose, fructose, sucrose, glucosamine and galactosamine all gave with the detecting reagent a golden-brown colour, which could not be confused with the purple colour given by neuraminic acid. Ribose and deoxyribose gave blue colours; R_F of ribose was of the same order as G III, whereas that of deoxyribose was of the same order as the 'fast' component.

When individual components of the purified ganglioside, detected on the plate by bromothymol blue (Jatzkewitz, 1961), were extracted from the gel, they maintained their identity on subsequent rechromatography (Fig. 2) with no apparent inter-conversion of these components.

The neuraminic acid-reacting components from extracts of various normal nervous tissues were

examined. Ten different anatomical regions of ox brain (frontal cortex, parietal cortex, caudate nucleus, hippocampal uncus, superior colliculus, thalamus, globus pallidus, subcortical white matter, tegmentum of the midbrain and the retina) all showed a complete pattern and, except for the retina, variations in the relative intensities of the bands one to another were not seen. In the retina the G III band was considerably diminished and the 'intermediate 2' band greatly increased in intensity when compared with the other regions of the brain. A similar pattern was also observed in the retina of the dog. The intensity in staining of the whole pattern varied from one area to another in that the grey areas showed much more intensity than did the white matter. Extracts of the cerebral cortex of seven mammals (rat, coypu, guinea pig, rabbit, sheep, monkey and man) and the whole brain of the mouse were also examined and compared. The neuraminic acid patterns were remarkably similar except that the 'intermediate 1' band was clearly seen only in the sheep, monkey and man, and the intensity of the 'fast' component, which was not detected in the mouse, rat or coypu, paralleled brain size in the remainder of the mammals.

Similarly, no major alterations in the patterns from the human cortical grey matter at different ages (0, 2, 8 and 12 months, 6 and 14 years) were shown, with the exception that G Ib appeared to increase in amount with age. G Ic was present at all ages. The main components of normal brain-ganglioside pattern (G Ia, b, c, G II, G III and 'fast') were also detected in the spleen and kidney of the guinea pig. Both of these tissues showed additional resorcinol-reacting 'fast' components (one in the spleen, two in the kidney), whereas in liver and muscle a single fast band only was detected with a positive-resorcinol reaction.

Fig. 2. Chromatographic behaviour of isolated components of the resorcinol-positive pattern of a ganglioside preparation. Purified ganglioside of ox cerebral cortex (7 mg.) was spotted on a silica-gel plate as a 18 cm. streak and chromatographed. The pattern was detected by spraying with bromothymol blue (Jatzkewitz, 1961) and the gel containing each of the three major components (G I, G II and G III) was recovered separately together with portions of the intervening gel by extraction for 4 hr. in a Soxhlet thimble with chloroform-methanol (1:2, v/v). Portions of each of the recovered fractions and of the original ganglioside were chromatographed. A and E, 100 μ g. of purified ganglioside; B, fraction G I; C, fraction G II; D, fraction G III.

DISCUSSION

In interpreting these chromatograms, one must take into account the uneven solvent front and considerable variation of R_F values which depend, among other factors, on the composition of the mixture undergoing chromatography. However, in addition to specific detection methods, the characteristics of the patterns observed and the strict order of migration of components make useful observations possible.

The preliminary acetone-extraction and subsequent thorough dialysis involved in the preparation of the purified ganglioside preparations used (Booth, 1962) should exclude significant interference from pentoses, so that all the bands observed in purified preparations should contain neuraminic acid. It is possible, however, that even after

extensive purification, non-ganglioside, neuraminic acid-containing substances will be present in ganglioside preparations (Svennerholm, 1956*b*). Therefore resemblance of patterns in crude extracts to the purified ganglioside preparations may not be taken to confirm the identity of the resorcinol-reacting bands; nevertheless, all gangliosides will be visualized.

Although they exhibit marked sensitivity to acid conditions (Folch, Meath & Bogoch, 1956; Bogoch, 1958; Svennerholm & Raal, 1961), gangliosides, as judged by neuraminic acid release, are quite stable under the alkaline conditions prevailing during chromatography (Svennerholm, 1957*b*; Long & Staples, 1959). It seems unlikely that the pattern observed results from degradation of a macromolecular polymer, in that the extraction procedure is mild, minimal further treatment is involved and evidence of breakdown during chromatography as indicated by release of neuraminic acid or interconversion of components was not observed. However, the constancy of the pattern irrespective of cellular origin points to a close combination of each of the native components *in vivo*. Nevertheless, it has been demonstrated both by enzymic and by non-enzymic methods that interconversion of ganglioside fractions is possible (Bernheimer & van Heyningen, 1961; Svennerholm & Raal, 1961; Kuhn *et al.* 1961).

Previous results (Jatzkewitz & Mehl, 1960; Weicker *et al.* 1960; Jatzkewitz, 1961; Klenk & Gielen, 1961*b*; Kuhn *et al.* 1961; Wagner *et al.* 1961; Dain *et al.* 1962*a, b*) cannot readily be compared with those reported here because of the different sources of material and preparative procedures as well as varying chromatographic and detection procedures employed. However, from the reported behaviour of gangliosides on thin-layer chromatography and on silicic acid columns (van Heyningen & Miller, 1961; Svennerholm & Raal, 1961), and with consideration of the behaviour of other polar lipids on silicic acid (Rouser, Bauman, Kritchevsky, Heller & O'Brien, 1961) and on Silica Gel G (Merck) (Mangold, 1961), one may predict that the slower bands observed in our chromatograms contain more neuraminic acid or hexose residue or both per mole than the faster ones. Variations in the degree of saturation and polarity of the fatty acid moiety can also be expected to affect the separation (Mangold, 1961). There is a possibility that some of the bands observed may contain different ionic states of the same ganglioside, but this is not likely because the carboxyl group of neuraminic acid should be highly dissociated in the basic solvent system used.

The retina, which has a highly specialized function, shows a ganglioside pattern specifically different from other brain regions and, further, a more

complex ganglioside pattern is seen with increase in complexity of the mammalian species. Ganglioside in the kidney has not been previously described.

This simple technique can be used to assist in the localization of the site of the metabolic lesion in diseased tissue (Muldner, Wherrett & Cumings, 1962), where qualitative disturbances of ganglioside may be present. For example, in the infantile form of amaurotic familial idiocy (Tay-Sachs type) accumulation of the minor 'fast' component was accompanied by a decrease of the other components, whereas in the juvenile form of amaurotic familial idiocy (Batten type) a normal pattern was observed.

The technique as applied to neuraminic acid compounds can serve as a useful method for further isolation and characterization of gangliosides and it is believed could prove useful in the study of the metabolic activity of gangliosides.

SUMMARY

1. Crude lipid extracts of brain and other tissues have been chromatographed on thin layers of silica gel.

2. A resorcinol-hydrochloric acid detection spray, highly specific for neuraminic acid, revealed complex patterns in neural tissues, spleen and kidney.

3. Comparison of patterns from tissue extracts with those obtained from purified ox cerebral cortex-ganglioside preparations supported the tentative identification of eight of the bands as ganglioside.

4. All procedures used were of a relatively mild nature and it was therefore likely that each of the bands isolated was a molecular species of ganglioside. The pattern was a highly constant one and it is suggested that all the species exist *in vivo* in close combination. A distinctly anomalous quantitative distribution of pattern components was observed only in retina.

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REFERENCES

- Bernheimer, A. W. & van Heyningen, W. E. (1961). *J. gen. Microbiol.* **24**, 121.
- Blix, G., Svennerholm, L. & Werner, I. (1950). *Acta chem. scand.* **4**, 717.
- Blix, G., Svennerholm, L. & Werner, I. (1952). *Acta chem. scand.* **6**, 358.
- Bogoch, S. (1958). *Biochem. J.* **68**, 319.
- Bogoch, S., Belval, P. C. & Winer, D. (1961). *Nature, Lond.*, **190**, 152.
- Booth, D. A. (1962). *J. Neurochem.* **9**, 265.

- Chatagnon, C. & Chatagnon, P. (1953). *Bull. Soc. Chim. biol., Paris*, **35**, 1319.
- Dain, J. A., Weicker, H., Schmidt, G. & Thannhauser, S. J. (1962a). In *Cerebral Sphingolipidoses*, p. 289. Ed. by Aronson, S. M. & Volk, B. W. New York: Academic Press Inc.
- Dain, J. A., Willis, R., Sweet, E. I. & Schmidt, G. (1962b). *Fed. Proc.* **21**, 282.
- Folch, J., Arsove, S. & Meath, J. A. (1951a). *J. biol. Chem.* **191**, 819.
- Folch, J., Ascoli, I., Lees, M., Meath, J. A. & LeBaron, F. N. (1951b). *J. biol. Chem.* **191**, 833.
- Folch, J. & Lees, M. (1959). *Amer. J. Dis. Child.* **97**, 730.
- Folch, J., Lees, M. & Sloane-Stanley, G. H. (1957). *J. biol. Chem.* **226**, 497.
- Folch, J., Meath, J. A. & Bogoch, S. (1956). *Fed. Proc.* **15**, 254.
- Jatzkewitz, H. (1961). *Hoppe-Seyl. Z.* **326**, 61.
- Jatzkewitz, H. & Mehl, E. (1960). *Hoppe-Seyl. Z.* **320**, 251.
- Klenk, E. (1942). *Hoppe-Seyl. Z.* **273**, 76.
- Klenk, E. (1951). *Hoppe-Seyl. Z.* **288**, 216.
- Klenk, E. & Gielen, W. (1960). *Hoppe-Seyl. Z.* **319**, 283.
- Klenk, E. & Gielen, W. (1961a). *Hoppe-Seyl. Z.* **323**, 126.
- Klenk, E. & Gielen, W. (1961b). *Hoppe-Seyl. Z.* **326**, 144.
- Klenk, E. & Gielen, W. (1961c). *Hoppe-Seyl. Z.* **326**, 161.
- Kuhn, R. (1958). *Proc. 4th int. Congr. Biochem., Vienna*, **1**, 67.
- Kuhn, R. (1959). *Naturwissenschaften*, **46**, 43.
- Kuhn, R., Egge, H., Brossmer, R., Gauhe, A., Klesse, P., Lochinger, W., Röhm, E., Trischman, H. & Tschampel, D. (1960). *Angew. Chem.* **72**, 805.
- Kuhn, R., Weigandt, H. & Egge, H. (1961). *Angew. Chem.* **73**, 580.
- Long, C. & Staples, D. A. (1959). *Biochem. J.* **73**, 385.
- Mangold, H. K. (1961). *J. Amer. Oil Chem. Soc.* **38**, 708.
- Meltzer, H. L. (1958). *J. biol. Chem.* **233**, 327.
- Müldner, H., Wherrett, J. R. & Cumings, J. N. (1962). *J. Neurochem.* **9**, 607.
- Payne, S. N. & Platt, B. S. (1961). *J. Neurochem.* **7**, 5.
- Rosenberg, A. & Chargaff, E. (1958). *J. biol. Chem.* **232**, 1031.
- Rouser, G., Bauman, A. J., Kritchevsky, G., Heller, D. & O'Brien, J. S. (1961). *J. Amer. Oil Chem. Soc.* **38**, 544.
- Stahl, E. (1956). *Pharmazie*, **11**, 633.
- Stahl, E. (1958a). *Chemikerztg.* **82**, 323.
- Stahl, E. (1958b). *Parfüm. u. Kosmet.* **39**, 564.
- Svennerholm, L. (1956a). *Nature, Lond.*, **177**, 524.
- Svennerholm, L. (1956b). *Acta chem. scand.* **10**, 694.
- Svennerholm, L. (1957a). *Biochim. biophys. Acta*, **24**, 604.
- Svennerholm, L. (1957b). *Acta Soc. Med. upsalien.* **62**, 1.
- Svennerholm, L. & Raal, A. (1961). *Biochim. biophys. Acta*, **53**, 422.
- Trams, E. G. & Lauter, C. J. (1962). *Biochim. biophys. Acta*, **60**, 350.
- van Heyningen, W. E. & Miller, P. (1961). *J. gen. Microbiol.* **24**, 107.
- Wagner, H., Hörhammer, L. & Wolfe, P. (1961). *Biochem. Z.* **334**, 175.
- Weicker, H., Dain, J., Schmidt, G. & Thannhauser, S. J. (1960). *Fed. Proc.* **19**, 219.
- Wherrett, J. R. & McIlwain, H. (1962). *Biochem. J.* **84**, 232.

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The Activation of Sulphate by Extracts of Cornea and Colonic Mucosa from Normal and Vitamin A-Deficient Animals

By C. A. PASTERNAK, SHIRLEY K. HUMPHRIES AND ANTOINETTE PIRIE
Department of Biochemistry and Nuffield Laboratory of Ophthalmology, University of Oxford

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Mammalian cornea can incorporate [³⁵S]sulphate into mucopolysaccharide *in vivo* (Odeblad & Boström, 1952; Dohlman, 1957) and *in vitro* (Aurell, Dohlman & Rodén, 1956; Wortman & Strominger, 1957; Wortman, 1960, 1961), and therefore presumably contains the enzymes adenosine triphosphate-sulphate adenylyltransferase (EC 2.7.7.4) and adenosine triphosphate-adenylylsulphate 3'-phosphotransferase (EC 2.7.1.25), which catalyse the activation of inorganic sulphate by ATP (Robbins & Lipmann, 1956) to form adenosine 3'-phosphate 5'-sulphatophosphate ('active sulphate'). We have now demonstrated the activation of sulphate in extracts of calf, rabbit, guinea-pig and rat corneal epithelium and stroma.

An early sign of vitamin A deficiency is keratinization of the corneal epithelium followed by liquefaction of the corneal stroma; when Varandani, Wolf & Johnson (1960) reported that the activation of sulphate by colonic mucosa was decreased in rats made deficient in vitamin A and that activity could be restored by addition of vitamin A *in vitro*, we examined corneal extracts for a similar effect. We have been unable to detect any significant decrease, as a result of vitamin A-deficiency, in the activation of sulphate by extracts of rat cornea, rat colonic mucosa, rabbit corneal epithelium or rabbit colonic mucosa. The activation of sulphate by rabbit corneal stroma was possibly decreased, but could not be restored by the addition of vitamin A *in vitro*.