REFERENCES

- Barron, E. J. & Hanahan, D. J. (1958). *J. biol. Chem.* **231**, 493.
- Benson, A. A., Daniel, H. & Wiser, R. (1959). *Proc. nat. Acad. Sci., Wash.*, **45**, 1582.
- Benson, A. A. & Maruo, B. (1958). *Biochim. biophys. Acta*, **27**, 189.
- Carter, H. E., McCluer, R. H. & Slifer, E. D. (1956). *J. Amer. chem. Soc.* **78**, 3735.
- Cooke, R. (1958). *Cholesterol*, p. 486. New York: Academic Press Inc.
- Garton, G. A. (1960). *Nature, Lond.*, **187**, 511.
- Gerson, T. (1961). *J. Chromat.* (in the Press).
- Hilditch, T. P. (1956). *The Chemical Constitution of Natural Fats*, p. 575. London: Chapman and Hall.
- Kates, M. (1953). *Nature, Lond.*, **172**, 814.
- Kates, M. (1959). *Biochem. biophys. Res. Commun.* **1**, 238.
- Kennedy, E. P. (1956). *Canad. J. Biochem. Physiol.* **34**, 334.
- Lepage, M., Mumma, R. & Benson, A. A. (1960). *J. Amer. chem. Soc.* **82**, 3713.
- Morgan, D. M. & Kingsbury, K. J. (1959). *Analyst*, **84**, 409.
- Pollard, A. (1936). *Biochem. J.* **30**, 382.
- Pollard, A., Chibnall, A. C. & Piper, S. H. (1931). *Biochem. J.* **25**, 2111.
- Shorland, F. B. (1961). *J. Sci. Fd Agric.* **12**, 39.
- van Beers, G. J., de Iongh, H. E. & Boldingh, J. (1957). *Int. Conf. Biochem. Probl. Lipids*. Oxford, p. 43.
- Waldron, J. D., Gowers, D. S., Chibnall, A. C. & Piper, S. H. (1961). *Biochem. J.* **78**, 435.
- Weenink, R. O. (1961). *J. Sci. Fd Agric.* **12**, 34.
- Wheeldon, L. W. (1960). *J. Lipid Res.* **1**, 439.
- Zill, L. P. & Harmon, E. A. (1959). *Fed. Proc.* **18**, 359.

Biochem. J. (1962) **82**, 527

Quantitative Separation of Human Brain Glycolipids: Isolation of an Aminoglycolipid

BY L. A. FAILLACE AND S. BOGOCH

The Neurochemical Research Laboratory, Massachusetts Mental Health Center (Boston Psychopathic Hospital), and the Department of Psychiatry, Harvard Medical School, Boston, Mass., U.S.A.*

(Received 4 July 1961)

In recent years a number of methods have been used for the qualitative separation and purification of mammalian brain glycolipids. However, methods by which human brain glycolipids could be quantitatively separated and recovered would be useful for comparison of their concentrations in normal as well as in pathological states.

Since silicic acid had been effective as an adsorbent for the fractionation of selected synthetic and native lipid mixtures (Weiss, 1956; Hirsch & Ahrens, 1958), this was tried as a means of separating a complex mixture of human brain lipids. Quantitative recoveries were obtained which permit comparisons of specimens from discrete areas of the same brain and from different brains. Further information was also obtained about the macromolecular aminoglycolipids (Bogoch, Belval & Winer, 1961) structurally related to a preparation of bovine brain ganglioside previously studied (Bogoch, 1958).

METHODS

Dialysis was performed with prewashed cellophan. Phosphorus was determined by the method of Sperry (1942), nitrogen by a modification of the method of Koch &

McMeekin (1924) as described by Bogoch (1958), hexose by a modified orcinol- H_2SO_4 method (Sorensen & Haugaard, 1933) and neuraminic acid by a modification of the Bial's orcinol reaction (Bohm, Dauber & Baumeister, 1954). Hexosamine was determined after hydrolysis in a sealed tube with N-HCl for 16 hr. by a modified Elson & Morgan (1934) reaction (Bogoch, 1958).

Extraction of complex lipid mixture. Human cerebral hemispheres, 6–12 hr. *post mortem*, were freed of investing meninges and blood vessels, separated into grey and white matter and extracted with hot methanol (Bogoch, 1958). The filtered hot methanol extract of cerebral material was kept at 4° for 48 hr. The precipitate formed was filtered in the cold, taken up at room temperature with chloroform-methanol (2:1, v/v), approx. 0.5 ml./g. wet wt. of cerebral tissue, and partitioned with 12.2% of water. The upper and lower phases were removed and the lower phase was partitioned with 5.6% of water. The combined upper phase contained the bulk of the brain ganglioside and other hexosamine-rich aminoglycolipids (Bogoch *et al.* 1961) and the resultant lower phase contained other glycolipids and phospholipids. The lower-phase material was dried by vacuum distillation at 60° and taken up with minimal amounts of chloroform, and kept at 4° until chromatographed. The total yield of lower-phase material obtained by this method is $23.1 (\pm 0.13)$ (range: 21.0–24.7) mg./g. wet wt.

Preparation and charging of column. The size of the column was dependent upon the amount of material to be chromatographed. A ratio of 8 mg. of material for every gram of silicic acid was the maximum amount that could

* Now Foundation for Research on the Nervous System, 36 The Fenway, Boston, Mass., U.S.A.

be charged on the columns without impairing their resolving power. The columns were prepared as described by Weiss (1956). For a 2 g. sample, 250 g. of silicic acid, Baker's Reagent grade, which had been dried overnight at 105°, was thoroughly mixed with 500 ml. of chloroform-methanol (2:1, v/v). This suspension was slowly added to columns (40 mm. × 60 cm.) with a coarse-porosity sintered disk. Additional chloroform-methanol (2:1, v/v) was added until a total of 1250 ml. was used. When the level of the solvent had reached the adsorbent surface, chloroform totalling 1000 ml. was drained through the column. If this did not result in a column which was translucent and free of opacities, the material was discarded.

The human cerebral lipids dissolved in 4–10 ml. of chloroform were added to the column and washed in with 10 ml. of chloroform.

Elution. A stepwise elution procedure was adopted, solvents of increasing polarity (Hanahan, Dittmer & Warashina, 1957) being used. Chloroform, mixtures of chloroform with increasing amounts of methanol and finally pure methanol, were the eluents used (Table 1). Elution was continued with each eluent until quantitative exhaustion of all the solids elutable with each solvent mixture had been achieved. The next solvent mixture was added just before the surface of the silicic acid was exposed. Since variation in temperature will adversely affect the adsorption of lipids on silicic acid (Hirsch & Ahrens, 1958), all chromatography was carried out in a constant-temperature room (25 ± 2°). The complete fractionation of a 2 g. sample of cerebral-lipid mixture on a 250 g. silicic acid column required approx. 500 hr. of continuous solvent flow. That a procedure of this duration might result in some alteration of the lipid material on the column must be considered. Attempts to increase the rate of flow by using constant dry nitrogen pressure resulted in a marked decrease in the resolving power of the column and overlapping of fractions. The eluates were dried *in vacuo* at 60°, taken up with minimal amounts of chloroform-methanol (2:1, v/v) and centrifuged for 2 min. This procedure was necessary in order to separate any silicic acid which may have contaminated the effluent, as occurred when methanol comprised 15–25% of the eluent. After centrifuging the solution was decanted and stored at 4°.

Table 1. *Stepwise elution scheme for lipids of human cerebral grey matter*

Solvents A–K represent chloroform-methanol mixtures and the proportions (v/v) of these are shown in parentheses.

Fraction no.	Solvent	Total vol. eluted (ml.)
I	Chloroform	960
II	A (16:1)	665
III	B (14:1)	910
IV	C (12:1)	475
V	D (10:1)	425
VI	E (7:1)	444
VII	F (6:1)	1312
VIII	G (3:1)	445
IX	H (2:1)	958
X	J (1:2)	1103
XI	K (1:4)	526
XII	Methanol	335

RESULTS

Many solvent mixtures of chloroform and methanol were tried in order to find the optimum ratios which might remove selectively individual lipids from the silicic acid column. In some fractionations up to 14 fractions were obtained. However, owing to the overall duration of the fractionation, some of the eluents which yielded fractions containing only small amounts of material were eliminated in subsequent experiments.

In addition to the good qualitative separation obtained, consistent with that previously observed with more limited mixtures of lipids (Weiss, 1956; Hanahan *et al.*, 1957; Hirsch & Ahrens, 1958; Long & Staples, 1961), excellent quantitative recoveries were achieved in terms of dry weight, phosphorus, hexose and nitrogen. The mean percentage recoveries were: 104.4 ± 3.99 (range: 99.3–105.4) for dry wt.; 104.1 ± 5.69 (range: 98.1–113.1) for nitrogen; 100.1 ± 1.58 (range: 98.4–102.9) for hexose; 96.7 ± 0.91 (range: 95.1–97.9) for phosphorus. The chromatographic technique as here used therefore provides the possibility of comparing quantitatively the absolute amounts of these separate fractions in individual human brain specimens.

The analytical results (Table 2) show the reproducibility of the method. Duplicate specimens of cerebral grey matter from the same human brain were fractionated on identical silicic acid columns, similar conditions of temperature and amount of solvent flow being maintained. The results also illustrate the similarities as well as the variations that have been noted between individual human brains.

In a typical chromatographic separation of human cerebral grey material (Table 2), 26.5% of the total sample was not adsorbed on the silicic acid column, being eluted with the first washing of chloroform. This fraction contained only trace amounts of phosphorus (0.002%), hexose (0.03%) and nitrogen (0.04%).

Hexose (Table 2) is present in trace amounts in all fractions. However, four fractions, III, VII, IX and X, contain 92.8% of the total hexose. Fraction III, eluted with chloroform-methanol (14:1, v/v), is rich in hexose, containing 46.2% of the total hexose. This fraction, which is 7.1% of the total weight, contains hexose 24%, nitrogen 1.33% and is free of phosphorus. The percentage composition of these constituents suggests that the bulk of this fraction is a glycosphingolipid.

This method also provides a means of partially separating phospholipids from non-phospholipids (Table 2). Fractions I–VI contain only a trace of phosphorus, whereas 92.5% of the total phosphorus is found in fractions VII, IX and X.

Table 2. *Quantitative recovery of dry weight, nitrogen, hexose and phosphorus in duplicate specimens of cerebral grey matter from each of two human brains*

Eluting solvents A-K represent chloroform-methanol mixtures and the proportions (v/v) of these are shown in parentheses.

Fraction no.	...	I	II	III	IV	V	VI	VII	VIII	IX	X	XI	XII
Eluting solvent	Chloroform	A (16:1)	B (14:1)	C (12:1)	D (10:1)	E (7:1)	F (6:1)	G (3:1)	H (2:1)	J (1:2)	K (1:4)	Methanol	
Dry wt. (mg.)	530.0	54.2	155.0	17.5	15.0	20.0	437.5	18.8	272.5	552.5	18.0	10.0	
	492.5	55	142.0	23.8	12.5	11.7	402.5	36.3	265.0	600	9.4	8.1	
	515.0	34.5	155.0	14.2	8.2	—	265.0	72.8	270.0	625	—	26.3	
	545.0	42.5	155.0	10.8	8.8	—	285.0	73.3	260.0	625	—	26.3	
Nitrogen (mg.)	0.44	0.42	2.25	0.29	0.35	0.91	6.88	0.64	3.88	10.00	0.22	0.08	
	0.20	0.40	1.90	0.28	0.20	0.96	6.57	0.61	3.63	10.75	0.19	0.06	
	0.24	0.61	2.25	0.31	0.40	—	4.70	1.50	4.38	12.13	—	0.52	
	0.39	0.61	2.15	0.29	0.35	—	4.75	1.20	4.31	12.00	—	0.52	
Hexose (mg.)	0.53	0.73	34.60	1.35	0.56	1.40	16.25	0.52	7.00	12.38	0.44	0.40	
	0.16	0.50	34.20	1.37	0.62	0.81	14.25	0.61	7.00	13.25	0.48	0.40	
	0.19	0.38	28.25	0.75	0.54	—	9.70	1.98	4.50	6.63	—	0.39	
	0.14	0.46	28.25	0.89	0.50	—	10.75	2.00	4.25	6.88	—	0.41	
Phosphorus (mg.)	0.43	0.03	0.03	0.06	0.08	0.13	14.88	0.59	10.88	23.00	0.37	0.05	
	0.02	0.07	0.00	0.07	0.05	0.11	14.63	0.73	10.13	23.00	0.25	0.00	
	0.00	0.14	0.32	0.18	0.13	—	8.40	1.95	12.00	24.00	—	0.97	
	0.00	0.10	0.30	0.20	0.14	—	8.40	2.30	11.88	24.00	—	0.98	

Fraction X, eluted with chloroform-methanol (1:2, v/v) and the largest fraction in terms of dry weight (30 % of the total), contains 44.6 % of the total phosphorus as well as 17.9 % of the total hexose.

When all of the fractions obtained were examined for neuraminic acid, this substance was found in small amounts (0.75 %) only in fraction X. Previous work in this Laboratory on bovine brain (Bogoch & Faillace, 1961) has suggested that this fraction might contain aminoglycolipids which had not passed quantitatively into the upper phase during preliminary partition. The separation of this material from the rest of the lipid material of fraction X was accomplished by rechromatographing on silicic acid a large sample of fraction X and eluting with the following mixtures of chloroform-methanol: 2:1, 1:1, 1:1.5 and 1:2 v/v. All the neuraminic acid was eluted with chloroform-methanol (1:1, v/v). This fraction was dried *in vacuo* at 60°, emulsified with water and exhaustively dialysed. The non-dialysable material, containing all of the neuraminic acid, was freeze-dried and then extracted with diethyl ether. The diethyl ether-insoluble fraction was dried, taken up with chloroform-methanol (2:1, v/v) and partitioned twice with water as before, it now passing quantitatively to the upper phase. This upper-phase material (I) was also obtained from unfractionated lower-phase material by silicic acid chromatography on elution only with chloroform, chloroform-methanol (2:1, v/v), chloroform-methanol (1:1, v/v) and methanol. All of the neuraminic acid was

eluted again with chloroform-methanol (1:1, v/v) and (I) purified as above by dialysis, diethyl ether extraction etc. This greatly facilitated the separation of the desired material and reduced the length of time of solvent flow, thus decreasing the possibility of degradation on the column. By means of this simplified procedure, from the 2 g. of original lower-phase human cerebral-grey-matter material, 20 mg. of material (I) was obtained. Analysis of (I) showed that it contained neuraminic acid 18–20 %, hexose 23.8 %, hexosamine 10.8 %, phosphorus 0.3 % and nitrogen 2.4 %.

DISCUSSION

After the hot methanol extraction of human cerebral-grey-matter lipids and preliminary partition between chloroform-methanol and water, the utilization of column chromatography with silicic acid as an adsorbent for lower-phase lipids has resulted in the quantitative and reproducible separation of several lipid fractions. This affords the opportunity of comparing quantitatively the concentrations of several glycolipid fractions between individual human brains in normal as well as in pathological states.

The aminoglycolipid substance (I) isolated from human grey matter in small quantities contains the amounts of neuraminic acid, hexosamine and hexose consistent with one unit of the double-unit structure of a preparation of bovine brain ganglioside (Bogoch, 1958). The preparation of bovine

brain ganglioside whose structure was consistent with two single linear units each consisting of (neuraminic acid-galactosamine-galactose-glucose-sphingosine-stearic acid) and joined by a bridge consisting of (neuraminic acid-galactose) was obtained from the upper phase after multiple partitions in which the chloroform lower-phase material was constantly discarded (Bogoch, 1958). The single unit above contains 1 molecule each of neuraminic acid and hexosamine, 2 molecules of hexose and 3 molecules of nitrogen. The molar ratios found for the human substance (I) for neuraminic acid, hexosamine, hexose and nitrogen are 1.0:1.0:2.18:2.7. The slightly low nitrogen value may be due to the error of the method or to the presence of a persistent trace of phosphorus. Substance (I) remains in the lower phase and associated with non-hexose-containing lipids during many varied separation procedures, whereas the double-unit substance passes to the upper phase.

Klenk & Gielen (1960), using a different isolation procedure, presented evidence for one substance composed of a single linear unit in agreement with that in our 'double unit' above. Other related aminoglycolipids have been described (Svennerholm, 1957; Rosenberg & Chargaff, 1958; Kuhn, 1959; Weicker, Dain, Schmidt & Thannhauser, 1960; Bogoch *et al.* 1961). The demonstration in the present paper of a substance (I) in human brain whose analysis is consistent with that of a single unit of our preparation of bovine brain ganglioside, isolated from the lower-phase material previously discarded in the purification of the 'double-unit' substance, is therefore of interest.

The chemistry of these substances is of increasing interest, since bovine brain ganglioside has viral receptor properties (Bogoch, 1957*a*; Bogoch, Lynch & Levine, 1959) and it stimulates the contraction of *Venus mercenaria* heart (Bogoch & Bogoch, 1959). These experimental findings supported the hypothesis that brain ganglioside is involved in membrane receptor and transmission functions in the nervous system (Bogoch, 1957*b*, 1958, 1960; Bogoch *et al.* 1959; Bogoch *et al.* 1961), an hypothesis for which the work with brain-ganglioside preparations (i) of van Heyningen (1959) on tetanus toxin, (ii) of G. Trams & H. Spyropoulos (1960, personal communication) on peripheral nerve and (iii) of McIlwain (1960, 1961) on stimulation of brain slices by electrical pulses have added further evidence.

SUMMARY

1. A chromatographic method with silicic acid and a stepwise elution scheme for the separation of human cerebral-grey-matter glycolipid and phospholipid fractions is described.

2. Quantitative recoveries for 12 distinct fractions obtained from human cerebral material is reported.

3. The reproducibility of this method is demonstrated and the similarities and variations between individual human brains are noted.

4. An aminoglycolipid has been isolated from grey matter of human brain containing neuraminic acid, hexosamine and hexose in the approximate molar ratios 1:1:2, conforming to a single unit of the 'double-unit' structure for one preparation of bovine brain ganglioside previously described (Bogoch, 1958).

The assistance of Mr Peter C. Belval is gratefully acknowledged. Dr D. H. Flashman provided the brain specimens. These studies were done with the support of a grant (M-2095) from the U.S. Public Health Service, and during the tenure of a postdoctoral research fellowship to one of us (L.A.F.) from the National Institute of Mental Health, U.S. Public Health Service.

REFERENCES

- Bogoch, S. (1957*a*). *Virology*, **4**, 458.
 Bogoch, S. (1957*b*). *Nature, Lond.*, **180**, 197.
 Bogoch, S. (1958). *Biochem. J.* **68**, 319.
 Bogoch, S. (1960). *Neurology*, **10**, 439.
 Bogoch, S. & Bogoch, E. S. (1959). *Nature, Lond.*, **183**, 53.
 Bogoch, S., Belval, P. & Winer, D. (1961). *Nature, Lond.*, **190**, 152.
 Bogoch, S. & Faillace, L. A. (1961). *Nature, Lond.*, **190**, 150.
 Bogoch, S., Lynch, P. & Levine, A. S. (1959). *Virology*, **7**, 161.
 Bohm, P., Dauber, St. & Baumeister, L. (1954). *Klin.-ther. Wschr.* **32**, 289.
 Elson, L. A. & Morgan, W. T. J. (1934). *Biochem. J.* **28**, 988.
 Hanahan, D. J., Dittmer, J. C. & Warashina, E. (1957). *J. biol. Chem.* **228**, 685.
 Hirsch, J. & Ahrens, E. (1958). *J. biol. Chem.* **233**, 311.
 Klenk, E. & Gielen, W. (1960). *Hoppe-Seyl. Z.* **319**, 283.
 Koch, F. C. & McMeekin, T. L. (1924). *J. Amer. chem. Soc.* **46**, 2066.
 Kuhn, R. (1959). *Naturwissenschaften*, **46**, 43.
 Long, C. & Staples, D. A. (1961). *Biochem. J.* **78**, 179.
 McIlwain, H. (1960). *Biochem. J.* **76**, 16*p*.
 McIlwain, H. (1961). *Biochem. J.* **78**, 24.
 Rosenberg, A. & Chargaff, E. (1958). *J. biol. Chem.* **232**, 1031.
 Sorensen, M. & Haugaard, G. (1933). *Biochem. Z.* **260**, 247.
 Sperry, W. M. (1942). *Industr. Engng Chem. (Anal.)*, **14**, 88.
 Svennerholm, L. (1957). Symp. on *Cerebral Lipidoses*, pp. 139, 145. Ed. by van Bogaert, L., Cumings, J. N. & Lowenthal, A. Oxford: Blackwell Scientific Publications.
 van Heyningen, W. E. (1959). *J. gen. Microbiol.* **20**, 291, 301, 310.
 Weicker, H., Dain, J. A., Schmidt, G. & Thannhauser, S. J. (1960). *Fed. Proc.* **19**, 219.
 Weiss, B. (1956). *J. biol. Chem.* **223**, 523.