the assay mixture. The semicarbazone so formed is then converted into the corresponding 2:4-dinitrophenylhydrazone. The colour of this material in alkaline solution provides a sensitive measure of the enzymic activity.

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Biochem. J. (1961) **78**, 175

The Biochemistry of Aromatic Amines

8. SYNTHESIS AND DETECTION OF DI-(2-AMINO-1-NAPHTHYL) HYDROGEN PHOSPHATE, A METABOLITE OF 2-NAPHTHYLAMINE IN DOGS*

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(*Received* 18 *July* 1960)

Troll, Belman & Nelson (1959*a*) detected a new metabolite of 2-naphthylamine in the urine of dogs. Enzymic studies indicated that this was di-(2-amino-1-naphthyl) hydrogen phosphate. They suggested that this was a potential source of 2-amino-1-naphthol, which is thought to be the active agent in the induction of bladder tumours by 2-naphthylamine. This paper describes the synthesis of the ester and its detection, by paper chromatography, in the urine of dogs dosed with 2-naphthylamine.

EXPERIMENTAL

The dogs used were beagles dosed with 2-naphthylamine five times weekly. They were kept on a diet of canned meat and dog biscuit.

Paper chromatography. Whatman no. 1 paper was employed for descending development with the solvent systems: *a*, butan-1-ol–propan-1-ol–aq. 0·1 N-NH_3 soln. (2:1:1, by vol.); *b*, butan-1-ol–acetic acid–water (2:1:1, by vol.). For the detection of compounds on paper chromatograms the reagents used were: (1) N-HCl (or 50% acetic acid) and $NaNO_2$ (0·5%) followed by hexylresorcinol (0·5% in 2N-NaOH); (2) perchloric acid–molybdate reagent (Hanes & Isherwood, 1949); (3) 0·1 M-$K_2Cr_2O_7$–acetic acid (1:1) followed by $AgNO_3$ (0·1 M) (Knight & Young, 1958); (4) sodium 1:2-naphthaquinone-4-sulphonate (0·02 M); (5) $TiCl_3$ (15%, w/v); (6) *p*-dimethylaminocinnamaldehyde (2 g. in 100 ml. of 6N-HCl and 100 ml. of ethanol) (Harley-Mason & Archer, 1958); (7) Na_2CO_3 (10%) followed by diazotized sulphanilic acid [1·6 ml. of $NaNO_2$ (0·5%) added to 10 ml. sulphanilic acid (0·2% in N-HCl)]. A Chromatolite lamp (Hanovia Ltd.) was used as a source of u.v. light. 2-Amino-1-naphthol was detected in solution by the green derivative which is formed on the addition of conc. aq. NH_3 soln. The derivative can be extracted with benzene, in which solvent it is mauve (Liebermann & Jacobson, 1882). This test is referred to in the text as the ammonia–benzene test.

Materials. 2-Amino-1-naphthyl dihydrogen phosphate was prepared by the oxidation of 2-naphthylamine with permonophosphoric acid (Boyland & Manson, 1957*a*). The ester has R_F values 0·15 in solvent system *a* and 0·7 in solvent system *b*. On paper chromatograms the compound gives a brownish-red colour with hexylresorcinol after diazotization with HCl and $NaNO_2$, but a mauve colour after diazotization with acetic acid and $NaNO_2$.

Di-(2-nitro-1-naphthyl) hydrogen phosphate. The method of Friedmann & Seligman (1950) was used for the synthesis of this compound. Phosphoryl chloride (2·3 ml.) was added to 2-nitro-1-naphthol (10 g.) in sodium-dried benzene (100 ml.), and the mixture was heated under reflux; dry pyridine (4·2 ml.) in dry benzene was added over 20 min. and heating continued for 60 min. The solution was allowed to cool overnight and was then filtered and the filtrate evaporated to dryness *in vacuo*. The residue was dissolved in acetone (100 ml.) and water (50 ml.), heated on a steam bath for 45 min. and allowed to cool overnight, when unchanged 2-nitro-1-naphthol crystallized. This was filtered off and the filtrate evaporated *in vacuo* until a solid began to separate. After cooling, this was collected. By successive evaporation and filtration further crops were collected until they became too oily to filter. At this point the oily material was redissolved by the addition of acetone, and pyridine (5 ml.) was added. The solution was again cooled, when more crystalline material was usually obtained. The combined crops, which consisted of a

* Part 7: Boyland & Williams (1960).

mixture of the nitronaphthol and the pyridine salt of the ester, were then extracted several times with hot ether to remove nitronaphthol, the pyridine salt remaining undissolved. After collection the compounds was recrystallized from aqueous acetone to yield (about 2·0 g.) the *pyridine salt* of *di-(2-nitro-1-naphthyl) hydrogen phosphate* as prisms, m.p. 192–195° (decomp.) (Found: C, 57·3; H, 4·0; N, 8·1; P, 6·25. $C_{20}H_{13}O_8N_2P,C_5H_5N$ requires C, 57·8; H, 3·5; N, 8·1; P, 6·0%).

On one occasion after the collection of the mixture of pyridine salt and 2-nitro-1-naphthol, further concentration of the filtrate gave the free acid. Recrystallization from aqueous acetone gave di-(2-nitro-1-naphthyl) hydrogen phosphate as needles, m.p. 187–189° (decomp.) (Found: C, 54·25; H, 3·0; N, 6·5; P, 7·2. $C_{20}H_{13}O_8N_2P$ requires C, 54·55; H, 3·0; N, 6·4; P, 7·05%). The acid was more soluble in acetone than was the pyridine salt. It was readily converted into the pyridine salt. The compound (either as free acid or pyridine salt) had an R_F value of 0·7 in solvent system *a* and gave a pale-yellow spot which darkened slightly in daylight. When sprayed with titanous chloride solution the spot gave a red colour with *p*-dimethylaminocinnamaldehyde. When heated for 7 min. with the perchloric acid–molybdate reagent at 85° and exposed to H_2S for a few minutes, the spot gave a rather feeble blue colour, masked by the yellow colour of the compound. When the compound was heated for 1 hr. at 100° with N-NaOH and the solution was then cooled, red crystals of sodium 2-nitro-1-naphthoxide separated, but paper chromatography showed that hydrolysis was not complete.

Di-(2-amino-1-naphthyl) hydrogen phosphate. Before it was realized that 2-amino-1-naphthol was always formed on the reduction of the nitroester and that the amino ester was stable in alkali, it was thought that the presence of pyridine might be deleterious. Hence the pyridine salt was converted into the sodium salt before reduction. The finely powdered di-(2-nitro-1-naphthyl) hydrogen phosphate (0·67 g.) was triturated with N-NaOH (1·3 ml.) and water (5 ml.). Pyridine and water were removed in a desiccator over H_2SO_4 and the residue was dissolved in ethanol (25%, 25 ml.). The solution was shaken with Adams catalyst (100 mg.) and H_2 at room temperature until reduction was complete (about 2 hr.). The solution was filtered, when it darkened considerably, and 2N-NaOH was added until the pH was about 9·0. The solution was evaporated almost to dryness *in vacuo* at 40–50°. The sodium salt of the amino compound separated out when almost all the water was removed. Ethanol (3 ml.) was added and the product filtered off and washed with a little cold ethanol. Recrystallization from ethanol gave *di-(2-amino-1-naphthyl) sodium phosphate trihydrate* as prisms (90 mg.), m.p. 182–184° (decomp.) (Found: C, 53·3; H, 4·9; N, 5·8; P, 6·8; loss at 100° *in vacuo*, 11·5. $C_{20}H_{16}O_4N_2PNa,3H_2O$ requires C, 52·6; H, 4·9; N, 6·1; P, 6·8; H_2O, 11·8%). The compound was soluble in acetone, water and hot ethanol. The sodium salt was precipitated from aqueous solution by the addition of NaOH. Acidification of an aqueous solution with dilute HCl gave needles. The compound was not hydrolysed by heating in N-NaOH solution but was hydrolysed by heating with 2N-HCl, when 2-amino-1-naphthol was detected. At room temperature the compound was not appreciably hydrolysed by 2N-HCl in 16 hr. The compound had an R_F value of 0·6 in solvent system *a*. The spot became pink on exposure to daylight. It gave a red colour on spraying with *p*-dimethylaminocinnamaldehyde and a pink colour with 1:2-naphthaquinone-4-sulphonic acid. It gave a yellow colour against a brown background with the $K_2Cr_2O_7$–$AgNO_3$ reagent (devised originally for divalent sulphur compounds). When sprayed with the perchloric acid–molybdate reagent and heated at 85° it gave within a few seconds a bright-blue colour. Hence it is not possible to say whether it hydrolysed under the conditions of the test to give phosphate ion which gives a blue colour only after exposure to a reducing agent, e.g. H_2S. 2-Amino-1-naphthyl hydrogen sulphate also gives a blue colour with the perchloric acid–molybdate reagent. When sprayed with $NaNO_2$ and HCl followed by hexylresorcinol a feeble mauve colour was produced. When 50% acetic acid was used in place of HCl the mauve colour with hexylresorcinol was more intense. This method of diazotization was used for detection of the metabolite. When the compound was run in solvent system *b* it gave two fluorescent spots, a major one at R_F 0·7 and a smaller one at R_F 0·9 together with a reddish-brown spot on the solvent front. The major spot gave a reddish-brown colour with hexylresorcinol after diazotization with HCl and $NaNO_2$, and a mauve colour after diazotization with $NaNO_2$ and acetic acid. These reactions were those of 2-amino-1-naphthyl dihydrogen phosphate. If, after drying the paper, it is not completely free of acetic acid, the colour after $NaNO_2$–HCl diazotization was sometimes mauve. The smaller spot gave a very feeble reaction for the di-ester. It was not always present and could sometimes be detected only by the fluorescence. The brown spot at the solvent front was due to oxidation products of 2-amino-1-naphthol, which cannot be run on chromatograms except in the presence of a reducing agent (Boyland & Manson, 1958). (If the di-ester is dissolved in solvent system *b* at room temperature and set aside for 60 min. the aminonaphthol can be detected by the ammonia–benzene test.) Phosphate ion was detected at R_F 0·4 with the perchloric acid–molybdate reagent. The decomposition of the di-ester in the butanol–acetic acid–water solvent was also observed by two-dimensional chromatography with solvent system *b* followed by *a*. The major spot then moved to R_F 0·16 in *a*, identical with the R_F of the mono-ester in this solvent system. Hence in our examination of urines and urine extracts for di-(2-amino-1-naphthyl) hydrogen phosphate by two-dimensional chromatography in solvent system *a* followed by *b*, the spot detected was the mono-ester.

2-Amino-1-naphthyl dihydrogen phosphate and 2-amino-1-naphthol (detected respectively by paper chromatography and the ammonia–benzene test) were always formed during the reduction of the nitro ester. Attempts to isolate the di-ester by dilution of the reduction liquors with water followed by acidification with acetic acid or HCl were unsuccessful, as the product was contaminated with the mono-ester and oxidation products of 2-amino-1-naphthol. The nitro ester was reduced at room temperature with $SnCl_2$ and HCl, and on heating with $FeSO_4$ and $BaCO_3$, but neither method proved a successful means of preparation of the amino ester.

Di-(2-acetamido-1-naphthyl) hydrogen phosphate. The pyridine salt (1·5 g.) of the nitro ester was dissolved in acetic acid (20 ml.). Acetic anhydride (3 ml.) was added and the solution shaken with H_2 and Adams catalyst (100 mg.) at room temperature until the reduction was complete. The solution was poured into water (100 ml.)

and acidified with conc. HCl. A gelatinous precipitate formed (this becomes more crystalline if a crystal of the compound is added to it) and was kept overnight. The product was filtered off, washed with water and crystallized from acetone to yield *di-(2-acetamido-1-naphthyl) hydrogen phosphate* (650 mg.) as plates, m.p. 160–163° (Found: C, 61·5; H, 5·2; N, 5·75; P, 6·3. $C_{24}H_{21}O_6N_2P$ requires C, 62·05; H, 4·6; N, 6·0; P, 6·7%). The ester was soluble in ethanol and hot acetone. It had R_F 0·8 in solvent system *a* and showed as a dark absorbent spot under u.v. light. In solvent system *b* it had R_F 0·87 and did not hydrolyse during chromatography as did the amino ester. Like the amino compound it gave a bright-blue colour on heating with the perchloric acid–molybdate reagent even before exposure to H_2S. After being sprayed with N-HCl and heated for 30 min. at 70° the spot reacted with *p*-dimethylaminocinnamaldehyde to give a red colour. After hydrolysis the spot also gave a blue colour with ammonia vapour due to the 2-amino-1-naphthol formed. The hydrolysed spot gave a reddish-brown colour with hexylresorcinol after diazotization with HCl and $NaNO_2$. After the ester had been warmed for a few minutes with 2N-NaOH, paper chromatography in solvent system *a* revealed the presence of 2-acetamido-1-naphthol at R_F 0·95, identified by the red colour given by diazotized sulphanilic acid or by the blue colour given when the spot was treated with HNO_2. Another product, 2-acetamido-1-naphthyl dihydrogen phosphate, was detected at R_F 0·2 as a dark absorbent spot. Hence, unlike the amino ester, acetamido ester was hydrolysed by alkali.

Paper chromatography of dog urines

The urinary bladder of a dog dosed with 2-naphthylamine was emptied by catheterization. The dog was then given 2-naphthylamine (200 mg.) by mouth, followed by water (100 ml.). After 45 min., the bladder was emptied and thereafter at 30 min. intervals until 6½ hr. after the time of dosing. The samples were kept at 0° until examined by paper chromatography the same day with solvent system *a*; 0·025 ml. of urine was used. 2-Amino-1-naphthyl hydrogen sulphate was the only detectable metabolite in the first two specimens of urine (8 and 20 ml. respectively). Succeeding specimens (9, 5, 3, 5, 6 and 2 ml.) contained also 2-naphthylamine, 2-amino-1-naphthyl glucosiduronic acid, 2-amino-1-naphthyl hydrogen sulphate and 2-amino-6-naphthyl glucosiduronic acid. These metabolites were identified by their R_F values and colour reactions (Booth, Boyland & Manson, 1955; Boyland & Manson, 1957*b*; Boyland & Manson, 1958). Troll, Belman, Nelson, Levitz & Twombley (1959*b*) have shown that 2-amino-1-naphthyl glucosiduronic acid is a metabolite of 2-naphthylamine in dogs.

Five other metabolites were present which gave mauve colours with hexylresorcinol after diazotization with $NaNO_2$ and HCl or acetic acid. On a chromatogram in which the R_F of 2-amino-1-naphthyl hydrogen sulphate was R_F 0·5, these metabolites had R_F values of 0·42, 0·34, 0·2, 0·13 and 0·07 in solvent system *a*. Only the last one was detectable when a sample of urine which had been acidified with HCl was kept for 1 hr. at room temperature and then run in solvent system *a*. When a sample of urine was made alkaline with aq. NH_3 soln. (sp.gr. 0·88), kept for 1 hr. and re-examined, these metabolites were still present. On two-dimensional chromatograms developed with solvent system *a* and then *b* all the spots except the one with R_F 0·07 in solvent *a* lay in line with 2-amino-1-naphthyl hydrogen sulphate at R_F 0·7 after the second development. The very slow-running spot had an R_F value of 0·42 in solvent system *b*. It is probable that the compounds which can no longer be detected after acidification of the urine and which appear to yield 2-amino-1-naphthyl hydrogen sulphate in solvent system *b* are labile conjugates of the amino group of this ester (cf. Boyland, Manson & Orr, 1957).

In the fifth (2¼ hr.) and subsequent specimen of the urine a fluorescent compound was present with an R_F value of 0·6 which gave an immediate colour with *p*-dimethylaminocinnamaldehyde and a mauve colour with hexylresorcinol after diazotization with $NaNO_2$ and acetic acid. After diazotization with $NaNO_2$ and HCl only a feeble colour was given. The urines containing the metabolite were combined, adjusted to pH 4·0 and continuously extracted with ether for 3 hr. After removal of the ether the residue was examined by two-dimensional chromatography with solvent system *a* followed by *b*. The spots were treated with NH_3 vapour before running. A fluorescent compound was located which was identical in colour reaction with 2-amino-1-naphthyl dihydrogen phosphate. It had the same R_F values as the di-ester in solvent system *a* and appeared to be hydrolysed in the solvent system *b*. A mixture of the synthetic di-ester and the ether residue gave a single spot on paper chromatograms. 2-Naphthylamine, a trace of 2-amino-1-naphthyl hydrogen sulphate and 2-amino-1-naphthyl glucosiduronic acid were also present on chromatograms of the urine extract, together with another metabolite. This had the same R_F value as the sulphuric ester in the first solvent system but separated from it in solvent *b* with R_F 0·93. This gave a yellow colour with hexylresorcinol after diazotization and a positive reaction to the $K_2Cr_2O_7$–$AgNO_3$ reagent. This is believed to be an aminonaphthyl mercapturic acid (unpublished work).

The di-ester was also detected in the urine of two dogs after subcutaneous injection of 2-naphthylamine (180 mg. in 5 ml. of arachis oil), being present in the catheter specimen from 3 to 6 hr. after administration. After the injection of 2-naphthylamine into two dogs which had not previously received the amine, the di-ester was not

found in the urine. This is probably of no significance as the compound could not always be detected in the urine of continuously dosed dogs even when the dose was increased to 400 mg. Di-(2-amino-1-naphthyl) hydrogen phosphate could not be detected in 24 hr. specimens of urine or ether extracts of these. These urines, however, were not collected under sterile conditions and probably had some faecal contamination.

Troll *et al.* (1959*a*) infer that the metabolite is alkali-labile because of the appearance of free 2-amino-1-naphthol in the urine after it was made or became alkaline. Treatment of the urine samples and urine extracts which contained di-(2-amino-1-naphthyl) hydrogen phosphate with alkali did not cause any noticeable loss of the metabolite when chromatograms before and after treatment were compared. The synthetic ester is also stable to alkali. In addition, with the ether extract residue, no hydrolysis to 2-amino-1-naphthol was detected. Nevertheless, when some of the urine samples (2 ml.) obtained by catheter were made alkaline with ammonia (0·1 ml., sp.gr. 0·88), and shaken with benzene (0·3 ml.), the benzene became mauve after about 3 hr. at room temperature. In an experiment in which eight dogs were used and the urine was collected by catheter 3 hr. after dosing, no di-ester was detected in any of the samples or in ether extracts of these, but three of the urines gave positive ammonia–benzene tests.

Incubation of di-(2-amino-1-naphthyl) hydrogen phosphate with dog urine. Three samples of combined 24 hr. specimens from four dogs that had been dosed with 2-naphthylamine were adjusted to pH 5·0, 7·0 and 8·5 respectively. Di-(2-amino-1-naphthyl) hydrogen phosphate could not be detected in the samples. The di-ester (3 mg.) was added to 1 ml. samples of the urine at each pH and incubated for 16 hr. at 37°. 2-Amino-1-naphthol was not detected after 4 hr. or at the end of the incubation and chromatograms of the urine did not indicate any loss of the ester.

DISCUSSION

Examination by paper chromatography of the urine of dogs dosed with 2-naphthylamine has shown a metabolite with the properties of di-(2-amino-1-naphthyl) hydrogen phosphate to be present. This confirms the findings of Troll *et al.* (1959*a*). However, neither the metabolite nor the synthetic compound appears to be alkali-labile. Our results seem to indicate that the appearance of 2-amino-1-naphthol in alkaline dog urines may be due to the hydrolysis of another metabolite.

This new metabolite is remarkable in that the phenol is conjugated as a phosphoric ester and in that two phenolic residues are joined in the compound. Although the mammalian body contains much more phosphate than sulphate, the latter is more commonly used for conjugation and excretion of foreign phenols. The present authors had examined urine of animals treated with 2-naphthylamine specifically for 2-amino-1-naphthyl phosphate some years ago and failed to detect it.

Troll *et al.* (1959*a*) suggested that di-(2-amino-1-naphthyl) hydrogen phosphate is a probable cause of bladder cancer, because it is excreted by dogs which develop bladder cancer, and not by rabbits, in which species 2-naphthylamine does not induce bladder cancer. The compound is unusual in being soluble in ether and presumably in the cell lipids so that it might penetrate the bladder membrane and be hydrolysed to the immediate carcinogen in the bladder mucosa. This hypothesis needs further investigation but it appears to provide a possible explanation of the mechanism of the carcinogenic action of 2-naphthylamine.

SUMMARY

1. Di(2-amino-1-naphthyl) hydrogen phosphate has been synthesized by catalytic reduction of di(2-nitro-1-naphthyl) hydrogen phosphate.

2. The compound has been detected in the urine of dogs dosed with 2-naphthylamine.

3. The dog urine also contains 2-amino-1-naphthyl hydrogen sulphate, 2-naphthylamine, 2-amino-1-naphthyl glucosiduronic acid, 2-amino-6-naphthyl hydrogen sulphate and 2-amino-6-naphthyl glucosiduronic acid.

We wish to thank Mr Watson of the Buckston Browne Farm, Downe, Kent, for his help in maintaining the dogs. We also thank the Microanalytical Laboratory, Imperial College of Science and Technology, and Mr P. Baker, of Wellcome Research Laboratories, for the analyses. This investigation has been supported by grants to the Chester Beatty Research Institute (Institute of Cancer Research: Royal Cancer Hospital) from the Medical Research Council, the British Empire Cancer Campaign, the Jane Coffin Childs Memorial Fund for Medical Research, the Anna Fuller Fund and the National Cancer Institute of the National Institutes of Health, U.S. Public Health Service.

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Chromatographic Separation of Brain Lipids

CEREBROSIDE AND SULPHATIDE

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(*Received* 18 *July* 1960)

In recent years chromatography on silicic acid columns has been widely used for the resolution of the lipid mixtures extractable from living cells [egg yolk (Lea, Rhodes & Stoll, 1955; Rhodes & Lea, 1957); liver (Hanahan, Dittmer & Warashina, 1957); plasma (Garton & Duncan, 1957); spermatozoa (Lovern, Olley, Hartree & Mann, 1957); heart (Gray & Macfarlane, 1958; Gray, 1958)]. Alumina columns have also been employed in some cases [yeast (Hanahan & Jayko, 1952); egg yolk (Rhodes & Lea, 1957)]. The lipids of brain and spinal cord, which contain both cerebroside and sulphatide, have not been investigated by chromatography on alumina, and silicic acid chromatography has been applied only to enriched material, after the removal of the bulk of the other lipids by solvent fractionation (Weiss, 1956; Payne & Platt, 1958).

The purpose of the present work was to examine the chromatographic behaviour of the cerebroside and sulphatide, present in the lipid mixture extractable by chloroform–methanol from rat brain, with both alumina and silicic acid columns. Some of the early findings have already been reported in preliminary form (Long & Staples, 1959*a*).

EXPERIMENTAL

Materials

Lipids. Partially purified samples of ox-brain cerebroside (prepared by Dr R. Rodnight, Institute of Psychiatry, Maudsley Hospital, London) and of ox-brain sulphatide (Lees, Folch, Sloane-Stanley & Carr, 1959) were given by Dr G. H. Sloane-Stanley of this Department. The cerebroside sample was free from sulphatide; the sulphatide specimen had a sulphate : galactose molar ratio of 0·89. Lecithin and phosphatidylethanolamine were obtained from egg yolk by the method of Rhodes & Lea (1957). Lysolecithin was prepared according to Long & Penny (1957). Samples of phosphatidylserine and sphingomyelin were given by Dr J. N. Hawthorne and Dr June Olley respectively. Synthetic dihydrocerebroside (palmitoyl) was a gift from Dr D. Shapiro, Weizmann Institute, Rehovoth, Israel.

Solvents. Methanol was refluxed for 30 min. with NaOH pellets and granulated zinc, and distilled through a fractionating column. Chloroform was shaken five times with water, and the lower phase was distilled through a fractionating column; the water present distilled over first as a $CHCl_3$–water azeotrope and was discarded. The freshly distilled $CHCl_3$ was immediately treated with methanol, to give $CHCl_3$–methanol (98:2, v/v).

Chromatographic materials. Silicic acid (A.R. 100-mesh powder, Mallinckrodt Chemical Works; purchased from Bell and Croyden Ltd., 50 Wigmore Street, London, W. 1) was sieved and the material passing a 240 British Standards sieve was discarded. The silicic acid was then activated by heating at 120° for 24 hr. Alumina (Brockmann, grade II) was obtained from Savory and Moore Ltd., London. Two batches were used, *A* and *B*; these appeared to be similar in chromatographic properties, except that type *B* had a greater adsorptive affinity for sulphatide. The cellulose powder used was Whatman no. 1.

All other chemicals were the best available commercially.

Methods

Extracts of rat-brain lipid. The whole brains of one to four adult Wistar albino rats, of either sex, were homogenized with $CHCl_3$–methanol (2:1, v/v), 19 ml. of solvent/g. of brain being used. The homogenate was filtered through a sintered-glass funnel (porosity 2), and the residue was washed with 5 ml. of solvent/g. of brain. The combined filtrates were freed from water-soluble substances by solvent partition with 0·1 M-KCl, according to the method of Folch, Lees & Sloane-Stanley (1957), as described for ox