6. Data on the composition of cardiolipin preparations and on the mode of hydrolysis of cardiolipin in acid and alkaline conditions are recorded.

7. Glycerophosphoinositide equivalent to about 30% of the inositide present was obtained as crystals from inositide-rich fractions. This inositide contained saturated and unsaturated acids in equimolar proportions: 46% of the fatty acids was present as stearic acid and 15% as C_{20-22} highly unsaturated acids.

We wish to acknowledge the co-operation of Dr A. T. James and Dr J. Webb and thank them for the data obtained by gas chromatography of the fatty acid esters. We wish also to thank Dr A. E. Wilkinson of the Venereal Diseases Reference Laboratory for the serological tests on cardiolipin; British Celanese Ltd. for the gift of cellulose acetate; and the Medical Research Council for a grant to M.G.M. for the assistance of G.M.G.

REFERENCES

Appleton, H. D., La Du, B. N., Levy, B. B., Steele, J. M. & Brodie, B. B. (1953). *J. biol. Chem.* **205**, 803.
Ariyama, N. (1928). *J. biol. Chem.* **77**, 359.
Axelrod, J., Reichenthal, J. & Brodie, B. B. (1953). *J. biol. Chem.* **204**, 903.
Blix, G. (1937). *Mikrochem. Acta*, **1**, 75.
Borgström, B. (1952). *Acta physiol. scand.* **25**, 101.
Burmaster, C. F. (1946). *J. biol. Chem.* **164**, 233.
Debuch, H. (1956). *Hoppe-Seyl. Z.* **304**, 109.
Debuch, H. (1957). *Biochem. J.* **67**, 26P.
Faure, M. & Morelec-Coulon, M. J. (1954). *C.R. Acad. Sci., Paris*, **238**, 411.
Faure, M. & Morelec-Coulon, M. J. (1956). *Ann. Inst. Pasteur*, **91**, 537.
Feulgen, R. & Bersin, T. (1939). *Hoppe-Seyl. Z.* **260**, 217.
Folch, J., Ascoli, I., Lees, M., Meath, J. A. & LeBaron, F. N. (1951). *J. biol. Chem.* **191**, 833.
Gray, G. M. (1957*a*). *Biochem. J.* **67**, 25P.
Gray, G. M. (1957*b*). *Chem. & Ind.* p. 18.
Gray, G. M. (1958*a*). *Biochem. J.* **70**, 425.
Gray, G. M. (1958*b*). Ph.D. Thesis: University of London.
Herb, S. F. & Riemenschneider, R. W. (1953). *Analyt. Chem.* **25**, 953.
Isherwood, F. P. (1946). *Biochem. J.* **40**, 688.
Klenk, E. & Debuch, H. (1954). *Hoppe-Seyl. Z.* **296**, 179.
Klenk, E. & Debuch, H. (1955). *Hoppe-Seyl. Z.* **299**, 66.
Klenk, E. & Friedrichs, E. (1952). *Hoppe-Seyl. Z.* **290**, 169.
Klenk, E. & Krickau, G. (1957). *Hoppe-Seyl. Z.* **308**, 98.
Knight, J. A. & Zook, H. D. (1953). *J. Amer. chem. Soc.* **74**, 4560.
Lakshmanan, T. K. & Lieberman, S. (1954). *Arch. Biochem. Biophys.* **53**, 258.
Lea, C. H. & Rhodes, D. N. (1954*a*). *Biochem. J.* **56**, 613.
Lea, C. H. & Rhodes, D. N. (1954*b*). *Biochem. J.* **57**, 23P.
Lea, C. H., Rhodes, D. N. & Stoll, R. D. (1955). *Biochem. J.* **60**, 353.
Leupold, F. & Buttner, H. (1953). *Hoppe-Seyl. Z.* **292**, 13.
Macfarlane, M. G. & Gray, G. M. (1957). *Biochem. J.* **67**, 25P.
Martland, M. & Robison, R. (1926). *Biochem. J.* **20**, 847.
Pangborn, M. C. (1942). *J. biol. Chem.* **143**, 247.
Pangborn, M. C. (1944). *J. biol. Chem.* **153**, 343.
Pangborn, M. C. (1945). *J. biol. Chem.* **161**, 71.
Pangborn, M. C. (1947). *J. biol. Chem.* **168**, 351.
Pangborn, M. C., Almeida, J. O., Maltaner, F., Silverstein, A. M. & Thompson, W. R. (1955). *W.H.O. Monographs, No. 6: Cardiolipin Antigens*, 2nd ed.
Rapport, M. M. & Alonzo, N. (1955*a*). *J. biol. Chem.* **217**, 193.
Rapport, M. M. & Alonzo, N. (1955*b*). *J. biol. Chem.* **217**, 199.
Rapport, M. M., Lerner, B., Alonzo, N. & Franzl, R. E. (1957). *J. biol. Chem.* **225**, 859.
Rose, A. R., Schattner, F. & Exton, W. G. (1941). *Amer. J. clin. Path. tech. Suppl.* **5**, 19.
Schmidt, G., Benotti, J., Hershman, B. & Thannhauser, S. J. (1946). *J. biol. Chem.* **166**, 505.
Schmidt, G., Ottenstein, B. & Bessmann, M. J. (1953). *Fed. Proc.* **12**, 265.
Stephen, H. (1925). *J. chem. Soc.* p. 1874.
Trappe, W. (1938). *Biochem. Z.* **296**, 180.
Weygand, F., Eberhardt, G., Linden, H., Schafer, F. & Eigen, I. (1953). *Angew. Chem.* **65**, 525.

The Structure of the Plasmalogens of Ox Heart

BY G. M. GRAY
Lister Institute of Preventive Medicine, London, S.W. 1

(*Received* 14 *March* 1958)

Although the natural plasmalogens have not yet been obtained as pure substances, the work of Klenk & Debuch (1954, 1955) and Debuch (1956) has shown conclusively that these plasmalogens contain a fatty acid as well as a fatty aldehyde residue. On treatment of fractions containing both the classical ester phosphatide and the plasmalogen with dilute acid to remove the aldehyde, lysolecithin was obtained from the choline-phosphatide fraction of ox-heart muscle (Klenk & Debuch, 1955) and lysokephalin from the fraction of brain (Debuch, 1956). Klenk & Debuch (1954) had earlier shown that after catalytic hydrogenation of the mixed kephalin fraction the aldehydic properties disappeared, and on alkaline or acid hydrolysis 60% of the phosphorus was present as chimyl or batyl alcohol phosphoric ester, i.e. as glyceryl ether derivatives. They suggested (I) or (II) as

probable structures for the plasmalogens in which the aldehyde was arbitrarily assigned to the α-position by analogy with the α-glyceryl ethers of fish oils. The position of the aldehyde was not determined.

Franzl & Rapport (1955) and Rapport & Franzl (1957) found that snake-venom phospholipase *A* removed fatty acid from a plasmalogen-rich lecithin fraction of heart as rapidly as from a pure lecithin prepared from egg yolk. They concluded that in choline plasmalogen the fatty acid must be in the α-position (III), as Hanahan (1954*a*) had shown that phospholipase *A* splits from lecithin only the α-acid. The action of phospholipase *A* on choline plasmalogen has been confirmed in this Laboratory, but although this hydrolysis is strong presumptive evidence that the aldehyde is on the β-carbon atom of the glycerol it is not a strict proof, for it has not been shown that the enzyme would not attack a β-acyl linkage if the α-atom were linked with an aldehyde.

The chemical proof of the β-aldehydo structure given in the present paper (Fig. 1) is based on the fact that choline plasmalogen treated with 90% acetic acid decomposes into free aldehyde and a lysolecithin which must be the β-acyl isomer (IV) if the aldehyde is originally linked on the α-carbon atom or the α-acyl isomer (VII) if the aldehyde is

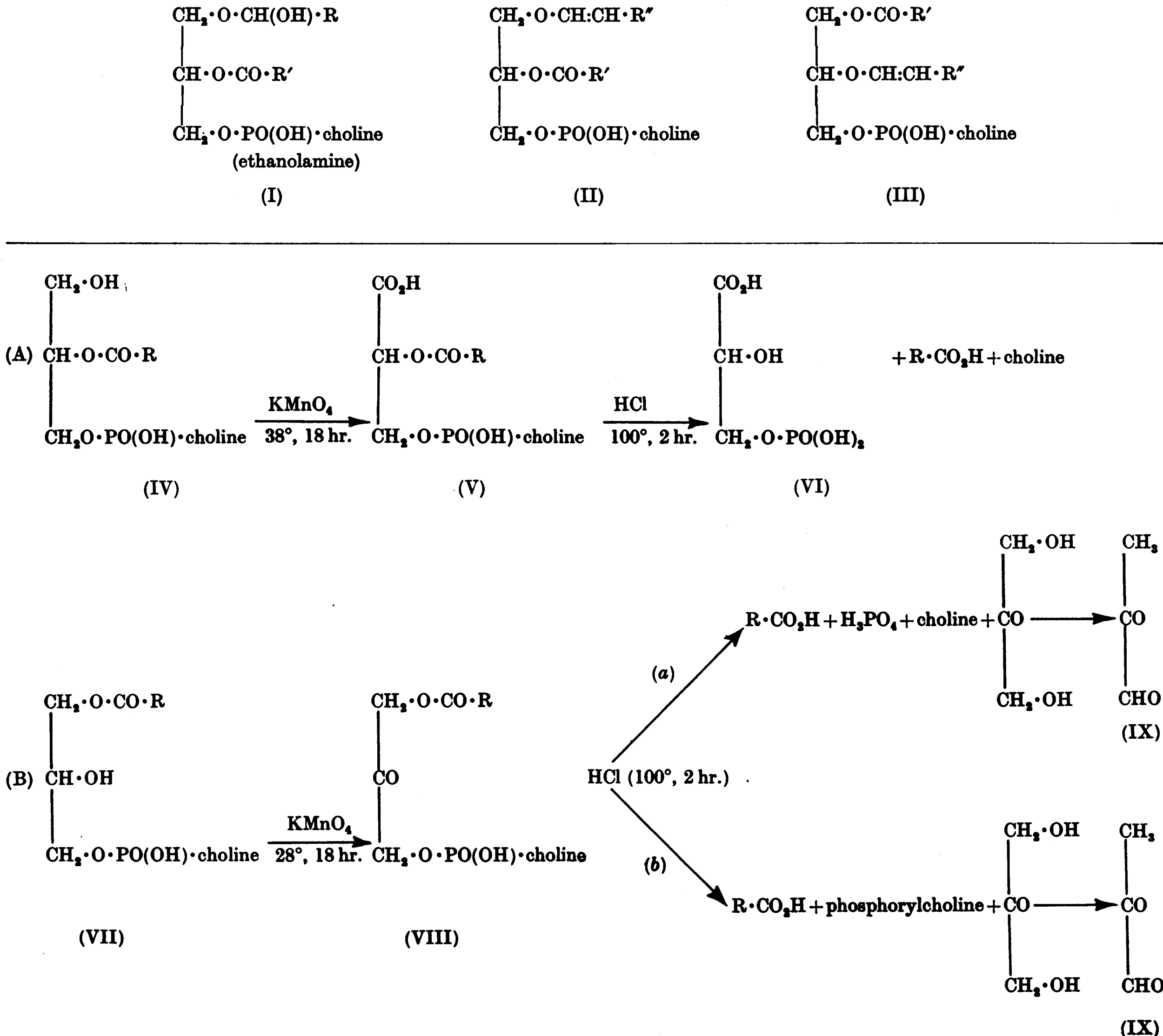

Fig. 1. Oxidation of α- and β-acyl-lysolecithins.

on the β-carbon atom. The β-acyl isomer, which is a known compound, usually obtained by the action of snake venom on lecithin, is oxidized by potassium permanganate to lysolecithinic acid (V), which on acid hydrolysis gives fatty acids, phosphoglyceric acid (VI) and choline (Hanahan, 1954*a*). Oxidation of the α-acyl-lysolecithin (VII), however, should produce a β-oxo compound (VIII), which on acid hydrolysis would give fatty acids and either (*a*) free choline and dihydroxyacetone phosphate, the latter being converted in acid into methylglyoxal (IX) and inorganic phosphate, or (*b*) phosphorylcholine and dihydroxyacetone, the latter giving methylglyoxal (IX). The hydrolysis products of (VII) are in either case different from those of (IV). The plan was to prepare the lysolecithin 'X' from plasmalogen and identify it as the α- or β-acyl isomer, by direct comparison of its properties with the known β-acyl isomer and by identification of the end products of permanganate oxidation and acid hydrolysis.

The experimental work entailed the preparation of the known β-acyl-lysolecithin as a control, and was extended to an analogous proof of the structure of ethanolamine plasmalogen. It also provided material for comparison of the constituent fatty acids of the plasmalogens and ester phosphatides of ox heart. A preliminary report of part of this work has been made (Gray, 1957).

EXPERIMENTAL

Methods. The analytical methods and general chromatographic procedures used and the preparation of the phospholipid fractions were described in the preceding paper (Gray & Macfarlane, 1958).

Position of the aldehyde group in choline plasmalogen

Preparation of lecithin and lysolecithin X. A typical choline-phosphatide fraction, a mixture of lecithin and choline plasmalogen, was obtained from ox-heart phospholipid by chromatography on silicic acid as described by Gray & Macfarlane (1958). The material (400 mg. of P; molar ratio P:choline, 1:1; plasmalogen value, 47%) was dissolved in 20 ml. of 90% (v/v) acetic acid and incubated at 38° for 18 hr. All the plasmalogen was split into free aldehyde and lysolecithin, but the breakdown of the lecithin, judged from the amount of water-soluble P formed, was negligible. The solution was diluted with 200 ml. of ether and shaken with sufficient NaOH solution to neutralize most of the excess of acid. The ether layer containing the lecithin, lysolecithin and aldehyde was shaken with several changes of water to remove the remaining acid; the combined aqueous layers contained 9·3 mg. P. Troublesome emulsions which formed sometimes during the washing were most readily broken by addition of chloroform and centrifuging. The solution of phospholipids and aldehydes was dried with Na_2SO_4 and the solvent was removed by vacuum distillation. The residue was dissolved in 95 ml. of methanol–chloroform (1:1, v/v); total P, 382 mg. The solution was chromatographed on a column containing 300 g. of silicic acid in methanol–chloroform (1:1, v/v) and this solvent was used for elution. Fractions of 25 ml. were collected automatically. The initial band quickly separated into three bands. The first (yellow) moved with the solvent front and contained all the free aldehyde and only 3 mg. of P. The two bands left were just visible by their slightly coloured fronts. As the fractions were collected, every second one was analysed for P and every fourth one for fatty acid ester groups. A typical separation curve is shown in Fig. 2. The sharpness of the separation, denoted by the decrease in the ratio of fatty acid ester to P from 2:1 at the peak of the second band (lecithin) down to 1:1 in the third band (lysolecithin), can be clearly seen. The fractions (total P, 363 mg.; recovery 94%) were pooled into two main fractions containing the lecithin and the lysolecithin X respectively and the intermediate fractions containing 20 mg. were discarded. The analyses of the main fractions are given in Table 1. The lecithin fraction was tested with the Feulgen reagent after hydrolysis in the usual test and gave no plasmal reaction. The recovery of the lysolecithin (P 163 mg.) was in good agreement with the amount expected from the original plasmalogen value.

Preparation of β-acyl-lysolecithin. The plasmal-free lecithin prepared above (1 g.; 40 mg. of P) was dissolved in 50 ml. of ether, and 2·2 mg. of mocassin venom (phospholipase *A*) in 0·5 ml. of 5 mM-$CaCl_2$ was added. A white precipitate of the β-acyl-lysolecithin formed as the reaction proceeded. After 4 hr. at room temp. the mixture was centrifuged, the supernatant removed and the precipitate washed several times with 10 ml. of anhydrous ether, dried under vacuum and dissolved in chloroform. The solution contained 39·2 mg. of P; the molar ratio P: fatty acid ester

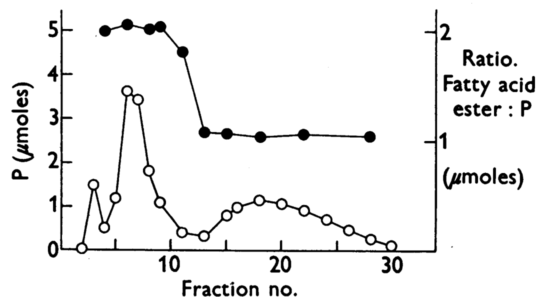

Fig. 2. Chromatographic separation of lecithin and α-acyl-lysolecithin. ○, Total phosphorus; ●, ratio of fatty acid ester groups to P. Adsorbent, silicic acid. Solvent, chloroform–methanol (1:1, v/v).

Table 1. *Analysis of lecithin and lysolecithin X separated on a silicic acid column*

	Lecithin	Lysolecithin X
Total P (mg.)	178	163
P (% on dry wt.)	4·15	5·9
N (% on dry wt.)	1·82	2·76
Molar ratio P:N	1:0·97	1:1·03
Molar ratio P: fatty acid ester	1:2·02	1:1·02
Double bond/mol. fatty acid ester	1·04	2·22

was 1:1·15, showing that the lysolecithin was contaminated with 15% of unchanged lecithin. This was removed by fractionating on silicic acid with methanol–chloroform 1:1 (v/v) as solvent; 30 mg. of P as pure β-acyl-lysolecithin was obtained.

Hydrogenation of lysolecithins. The lysolecithin X derived from plasmalogen and the β-acyl-lysolecithin were hydrogenated before oxidation. A sample of approximately 200 mg. (12·5 mg. of P) was dissolved in 10 ml. of dry chloroform–ethanol (1:4, v/v) and 15 mg. of platinum oxide (Adams catalyst) was added. The solution was shaken at room temp. for 2 hr. in hydrogen (1 atm.). On completion of the hydrogenation, which was tested by estimation of the iodine values, the catalyst was filtered off and the solutions were evaporated to dryness. The hydrogenated lysolecithins were less soluble in ethanol than the original lysolecithins and as solids were more powdery and less waxy.

Oxidation of hydrogenated β-acyl-lysolecithin to lysolecithinic acid. The solution of the hydrogenated β-acyl-lysolecithin was evaporated and the residue dissolved, readily, in 20 ml. of 0·1 M-sodium acetate buffer, pH 5·8 (Davidson, Long & Penny, 1956). Potassium permanganate (180 mg.) dissolved in 10 ml. of the buffer was added and after 18 hr. at 38° the mixture, which contained a large precipitate of MnO_2, was centrifuged. The magenta-coloured supernatant was removed and the precipitate washed three times with 15 ml. of hot water. The washings were added to the first supernatant and the excess of permanganate was destroyed with the minimum of sodium metabisulphite.

After removal of the MnO_2 by centrifuging, the colourless solution had a faint silky sheen and contained 11·7 mg. total P (94% recovery); orthophosphate, 2·3% of recovered P; molar ratio P to fatty acid ester 1:1. The solution was acidified to pH 1–2 with HCl and the precipitate was collected by centrifuging; a little more precipitate was obtained by adding an equal volume of acetone to the supernatant. The precipitates were combined, washed quickly with a little cold water, then with acetone and dried *in vacuo* to a white powder. This was dissolved in chloroform and analysed. A sample was also evaporated to dryness, dissolved in benzene–methanol (19:1, v/v) and titrated with 0·02N-sodium ethoxide. The molar ratio P : fatty acid ester : carboxylic acid found was 1:1·02:1·06, consistent with the structure of lysolecithinic acid (V).

Oxidation of hydrogenated lysolecithin X derived from plasmalogen. The hydrogenated material (11·5 mg. of P) was dissolved in 20 ml. of 0·1 M-acetate buffer, pH 5·8, giving a cloudy solution which cleared only on warming (cf. β-acyl-lysolecithin). Potassium permanganate (180 mg.) in 10 ml. of buffer was added and the mixture kept at 38° for 16 hr. It was then centrifuged and the precipitated MnO_2 washed with four 15 ml. portions of hot water. It was necessary to use hot water (above 60°) as the oxidized material was fairly insoluble in cold water and seemed to be held in some way by the MnO_2. The washings were combined with the original supernatant solution and the excess of permanganate destroyed by addition of the minimum amount of sodium metabisulphite. A further small precipiate of MnO_2 was removed by centrifuging. As the warm clear solution cooled to room temp. a considerable amount of flocculent white precipitate formed, which redissolved readily on warming. The solution contained 10·9 mg. of total P (95% recovery); inorganic P, 3% of recovered P; molar ratio P to fatty acid ester 1:1.

The solution was cooled to 0° and the precipitate which formed was removed by centrifuging. The supernatant solution was concentrated in a vacuum desiccator to half its volume and cooled in ice–water; an equal volume of ice-cold acetone was added to it and the precipitate which formed was added to the first one. This material, containing 9·1 mg. of P (79% recovery; a little more precipitate could be obtained by further concentration and extraction of the mother liquor), was dried under vacuum to a fine white powder, insoluble in ether, *cyclo*hexane, acetone and methanol, and fairly soluble in chloroform at room temperature but tending to come out of solution at 0°. P, 5·6% dry wt.; molar ratio P to fatty acid ester 1:1; no carboxylic acid group was present.

Acid hydrolysis of the oxidation products. Samples (about 1·0 mg. of P) of the lysolecithinic acid and the oxidation product X_0 of lysolecithin X were hydrolysed for 2 hr. at 100° in 2N-HCl. After cooling in ice–water the solidified fatty acids were removed either by filtration or extraction with ether. A small portion of each of the aqueous solutions was diluted with an equal volume of water and set aside for estimation of methylgloxal. The larger portions were neutralized with NaOH solution and samples were analysed for total and inorganic P and free choline. The results in Table 2 show that only very small amounts of free choline and orthophosphate were produced from X_0, while lysolecithinic acid gave as expected the calculated amount of choline as free choline. As the oxidation products were different, the lysolecithin X must have been the β-acyl isomer VII.

Identification of P-containing products of hydrolysis. Samples of the neutralized hydrolysis products, together with blanks, were incubated with bone phosphatase free from diesterase, in bicarbonate buffer as veronal interferes with the choline determination. After 1 hr. at 38°, pH 8·6, 2·0 ml. of 10% trichloroacetic acid was added and the acid filtrate analysed for orthophosphate and free choline. Table 3 shows that the P-containing product of acid hydro-

Table 2. *Products of acid hydrolysis of lysolecithinic acid and* X_0

All results are given in μmoles.

	Lysolecithinic acid	X_0
Total P	2·16	2·98
Total choline*	2·16	2·98
Inorganic P	0·162	0·164
Free choline	2·14	0·208†

* Calc. from total P.

† Corrected from observed value for presence of excess of phosphorylcholine (Webster, 1956).

Table 3. *Hydrolysis with phosphatase of the acid-hydrolysis products of lysolecithinic acid and* X_0

Samples (0·5 ml.) + 1·0 ml. of $NaHCO_3$–Na_2CO_3 buffer, pH 8·6 + 0·2 ml. of phosphatase + 0·3 ml. of water; 40 min. at 38°. All results are given in μmoles.

	Lysolecithinic acid	X_0
Total P	0·855	1·32
Total choline (calc.)	0·855	1·32
Inorganic P	0·85	1·35
Free choline	0·84*	1·36

* Choline already freed by previous acid hydrolysis.

lysis of X_0 was phosphorylcholine, as the total P present and the equivalent amount of choline were found as orthophosphate and free choline after the action of the phosphomonoesterase. The phosphoglyceric acid in the hydrolysed solution of lysolecithinic acid (which already contained free choline) was hydrolysed by the enzyme, as expected. The small amounts of inorganic P (6–8%) found in the acid hydrolysis products (Table 2) were consistent with amounts expected from the hydrolysis of phosphoglyceric acid or phosphorylcholine in the conditions (2 hr. in 2N-HCl at 100°).

Another sample of lysolecithinic acid (P 6 mg.) was hydrolysed with acid as above and the phosphoglyceric acid present in the hydrolysed solution was isolated by concentrating 5 ml. to 2 ml., acidifying with 0·5 ml. of 5N-HCl and adding excess of $BaCl_2$ followed by 1 vol. of ethanol. The acid Ba salt was purified twice by dissolving in 0·5 ml. of N-HCl and reprecipitating with 1 vol. of ethanol; the final precipitate was washed with ethanol and dried over P_2O_5. The yield was about 60% (50 mg.) of the theoretical. This phosphoglyceric acid was optically inactive in presence of 8% ammonium molybdate solution ($[\alpha]_D$ found for a sample of 3-D-phosphoglyceric acid in molybdate was −360°).

Formation of methylglyoxal by acid hydrolysis of X_0. The small portions of the acid-hydrolysed solutions of lysolecithinic acid and X_0 set aside previously were tested for methylglyoxal by the method of Ariyama (1928). The solutions were neutralized with NaOH just before testing. Samples of 0·5 ml. together with a blank containing water and a standard containing 0·25 μmole of methylglyoxal were placed in tubes and to each tube 0·2 ml. of Benedict's uric acid reagent (phosphoarsenotungstate), 0·1 ml. of M-KCN and 0·3 ml. of M-Na_2CO_3 were added in that order. The hydrolysed X_0 sample gave a deep-blue colour; the amount was about 45% of that expected on the P content. However, if methylglyoxal is heated in HCl with traces of manganese (which were still present in X_0) the colour yield is decreased. The blanks and the hydrolysis products from the lysolecithinic acid gave no colour with the reagent.

Conclusion. The oxidation products of the lysolecithin derived from choline plasmalogen by removal of the aldehyde chain is the β-oxo compound (VIII), differing from the oxidation product (lysolecithinic acid, V) of β-acyllysolecithin (IV) in that on acid hydrolysis it gives phosphorylcholine and methylglyoxal, instead of phosphoglyceric acid and free choline. The original plasmalogen had therefore the α-acyl-β-aldehydo structure (III).

Position of the aldehyde group in ethanolamine plasmalogen

The method used to prove the position of the aldehyde group in ethanolamine plasmalogen was the same as that used for choline plasmalogen, i.e. by identification of the lysokephalin formed by the action of weak acid on the plasmalogen as the α-acyl isomer. This was done by oxidation with permanganate and identification of the hydrolysis products. The procedure was complicated by the necessity of protecting the amino group by acetylation before oxidation.

Preparation of phosphatidylethanolamine and lysokephalin X. A typical kephalin fraction from ox heart (349 mg. of P), containing 33% of the P as ethanolamine plasmalogen, was dissolved in 12 ml. of 90% (v/v) acetic acid and kept at 38° overnight. The phospholipids and aldehyde were extracted into ether and the ethereal solution was well washed with water as before, dried and evaporated. The residue was taken up in methanol–chloroform (1:49, v/v) and the solution was chromatographed on 300 g. of silicic acid in the same solvent, which was used for elution. The free aldehyde passed rapidly through the column and when the eluate gave no reaction for aldehyde (by spot test with $HgCl_2$ and the Feulgen reagent) the solvent was changed to methanol–chloroform (1:4, v/v). The brown band of phospholipid at the top of the column then separated into a fast-moving brown band and a very slow-moving yellow band. After elution of the brown band the solvent was changed to methanol–chloroform (2:3, v/v) and the yellow fraction was eluted. The analyses (Table 4) showed that the brown and yellow fractions contained the kephalin and the lysokephalin X respectively.

The lysokephalin fraction was evaporated to dryness, dissolved in a little chloroform and cooled in ice, and ice-cold ether was added. The white precipitate was separated and washed with ether by centrifuging, dried under vacuum and dissolved in chloroform to give a very pale yellow solution, total P 97 mg. The recovery of purified lysokephalin, based on the plasmalogen value of the original mixture, was 84%, and of the phosphatidylethanolamine 92%.

Acetylation of the amino group of the lysokephalin. As the hydroxyl group on the glycerol moiety had to be left free, the selective method of White (1940) for *N*-acetylation was used. Approximately 200 mg. (0·44 m-mole) of lysokephalin X (12 mg. of P) was dissolved in 2·0 ml. of dry methanol; 70 mg. (0·42 m-mole) of silver acetate and 57 mg. (0·52 m-mole) of acetic anhydride were added and the flask was shaken at room temp. for 3·5 hr. The solution was refluxed for 5 min. and filtered hot. The residue was washed with a little methanol and the washings added to the main solution. Analysis for P and amino N showed that acetylation was 85% complete. The solution was taken to dryness and the residue dissolved in 2·0 ml. of methanol–chloroform (1:9, v/v), and chromatographed on 4·5 g. of silicic acid in this solvent. The *N*-acetylated lysokephalin was quickly eluted in the first few fractions, followed by the lysokephalin. The solvent was evaporated from the *N*-acetylated material, leaving a straw-coloured syrup which was dissolved in 4·0 ml. of chloroform (total P, 10·0 mg.; amino N, less than 2% of total).

Hydrogenation of N-*acetyl-lysokephalin X.* The material (9 mg. of P) was dissolved in 15 ml. of chloroform–ethanol (1:2, v/v) and shaken with 8 mg. of platinum oxide (Adams catalyst) for 2 hr. in hydrogen at 1 atmosphere and room temp. After filtration the solution contained 9 mg. of

Table 4. *Analysis of kephalin and lysokephalin X separated on a silicic acid column*

	Kephalin	Lysokephalin X
Total P (mg.)	216	97
P (% on dry wt.)	4·1	6·4
N (% on dry wt.)	1·88	2·96
Amino N/total N (%)	106	102
Molar ratio P:N	1:1·01	1:1·02
Molar ratio P:fatty acid ester	1:2·01	1:1·0
I_2 value (double bonds/mol. fatty acid)	1·79	3·2

P; molar ratio P to fatty acid ester, 1:1; iodine value, nil; amino N/total N, less than 2%.

Oxidation of hydrogenated material. The material was evaporated to dryness (8·8 mg. of P) and 15 ml. of 0·1M-acetate buffer (pH 5·6) was added, giving a white emulsion. Potassium permanganate (150 mg.) was added and the mixture was kept at 38° for 18 hr. It was then centrifuged and the supernatant solution (7·5 mg. of P) removed. The precipitate was washed twice with hot water, which recovered 1·3 mg. more of P. The first supernatant was cooled in ice and an equal volume of ice-cold acetone was added; the flocculent precipitate was recovered by centrifuging and dried under vacuum, to a fine white non-sticky powder. The acetone supernatant was concentrated and a further amount of precipitate recovered on addition of acetone. The combined precipitates were dissolved in chloroform and a small amount of inorganic salt removed by centrifuging; the solution of the oxidation products contained 6·8 mg. of P (recovery 91%).

Acid hydrolysis of the oxidation product of N-*acetyl-lysokephalin.* Two samples of the oxidized material (1·0 mg. of P) were hydrolysed, one in 2N- and one in 5N-HCl for 2 hr. at 100°. The solutions were cooled in ice–water, filtered and neutralized. After 2 hr. in 2N-HCl, 85% of the amino group was deacetylated and 21% of the P was orthophosphate. (By contrast, when a sample of the lysokephalin was oxidized without acetylation, and then hydrolysed 2 hr. in 2N-HCl, only 9% of the P was converted into orthophosphate, but some breakdown of the amino group occurred.) After 2 hr. in 5N-HCl deacetylation was complete (molar ratio P:amino N, 1:1·05).

The neutralized hydrolysis product was tested for methylglyoxal, with positive results; the amount was only about 50% of that expected on the P content. All the P was present as a phosphomonoester, estimated enzymically. The remaining solution was treated with $BaCl_2$; the precipitate contained only inorganic phosphate. The filtered solution was then treated with an equal volume of ethanol, but no precipitate formed. It was concluded from the absence of any insoluble barium salt that no phosphoglyceric acid was present, and from the presence of a very soluble phosphomonoester and methylglyoxal that the hydrolysis products were phosphorylethanolamine and methylglyoxal, together with fatty acids and acetic acid. The oxidation product was therefore the *N*-acetyl-β-oxo-lysokephalin, analogous to (VIII). The original plasmalogen therefore had the α-acyl-β-aldehydo structure, giving the α-acyl-lysokephalin on removal of the aldehyde.

Nature of the aldehydes in the choline plasmalogen of heart muscle

The aldehydes were isolated quantitatively from lecithin–choline plasmalogen and kephalin–ethanolamine plasmalogen fractions as their dimethylacetal derivatives and tested for unsaturation. The iodine values (equivalent to 0·12 and 0·17 double bond/aldehyde for choline plasmalogen and ethanolamine plasmalogen respectively) showed that most of the aldehydes were saturated.

Isolation as the 2:4-*dinitrophenylhydrazone derivatives.* A typical lecithin–choline plasmalogen mixture was treated with 90% acetic acid at 38° for 18 hr., and the free aldehydes were separated as described above (p. 427) from the residual phospholipids on a silicic acid column. The chloroform solution of the aldehydes was evaporated to dryness and the residue dissolved in dry ethanol. The 2:4-dinitrophenylhydrazones were prepared (Allen, 1930) and recrystallized from hot ethanol.

Fractionation of the dinitrophenylhydrazone. The method of Kramer & Van Duin (1954) for chromatographic fractionation was tested with an artificial mixture of palmitaldehyde and stearaldehyde 2:4-dinitrophenylhydrazones, which were well separated. A sample of the plasmalogen derivatives (0·5 mg.) was put on a silicic acid column [15 g., prepared by Isherwood's (1946) method] mixed with 16 ml. of nitromethane; height 29 cm., diameter 1·1 cm. The column was developed with light petroleum (b.p. 40–60°) equilibrated with nitromethane. The band separated into two bands corresponding in position to those of the stearal and palmital derivatives, the rear band (palmital) being obviously larger. The bands were collected separately and the material dissolved in equal volumes of chloroform. By comparison of the colour value in the EEL colorimeter, filter 621, the second band was approximately four times as concentrated as the first. The results indicated that the aldehydes of choline plasmalogen consisted of about 80% of palmitaldehyde and 20% of stearaldehyde, but the method did not exclude the presence of traces of unsaturated aldehydes or of aldehydes of odd-numbered chain length.

Gas-phase chromatography. The mixed aldehydes were examined as their dimethylacetal derivatives and synthetic palmitaldehyde dimethylacetal was used as a reference compound. This 'pure' standard was found to be only 77·5% of palmitaldehyde with three closely associated aldehydes (4·4, 11·25 and 4·35%) as the main impurities. These impurities, however, would make little if any detectable difference in the Feulgen colour reaction of this standard from the colour given by 100% palmitaldehyde.

The mixture of the natural aldehydes was shown to contain 41% of palmitaldehyde (the major component) with two lower aldehydes (16 and 9·5%, identical with two of the 'impurities' in the palmitaldehyde standard) making up 66·5% of the total. The remaining 33·5% was distributed between 15 aldehydes; seven of these, one of which was stearaldehyde, were of higher molecular weight than palmitaldehyde, and accounted for 19% of the total aldehydes. The amount of stearaldehyde present appeared to be less than 5%.

Fatty acids of the plasmalogens

During the fractionation of lecithin–choline plasmalogen mixtures by partition chromatography (Gray & Macfarlane, 1958) it was observed that the iodine value of the front fractions rich in choline plasmalogen was greater than that of the back fractions, indicating that the fatty acids in the

Table 5. *Unsaturation of fatty acids in phospholipids of heart muscle*

Phospholipids	Double bonds/ fatty acid*
Lecithin	1·04
β-Acyl-lysolecithin	0·34
α-Acyl-lysolecithin†	2·20
Kephalin	1·79
α-Acyl-lysokephalin‡	3·20

* μmoles of iodine taken up per μmole of fatty acid ester.
† From choline plasmalogen.
‡ From ethanolamine plasmalogen.

plasmalogens were more unsaturated. This fact was confirmed by estimation of the iodine values of the pure lecithin and kephalin, the pure α- and β-acyl-lysolecithins and the α-acyl-lysokephalin prepared during the present work. The results in Table 5 show that the average number of double bonds/mol. of fatty acid is higher in the plasmalogen derivatives (one fatty acid) than in the ester phosphatides, but the interpretation is not clear unless the proportion of saturated and unsaturated acids is known. The compounds were therefore saponified and the fatty acids fractionated quantitatively by lead salts, and reanalysed.

Samples of the phospholipids (100–200 mg.) were saponified in 6·0 ml. of aqueous N-NaOH in a stoppered tube filled with N_2, at room temp. overnight. The mixtures were warmed at 45° for 15 min. to liquefy them and acidified with 4·0 ml. of 5N-HCl. The fatty acids were extracted with ether, and the ethereal solution washed with water and evaporated. The acids were dissolved in 1·5 ml. of 95% ethanol, and 5·0 ml. of 95% ethanol containing 100 mg. of lead acetate was added at boiling point. The mixture was allowed to cool slowly overnight, and the insoluble lead salts were collected by centrifuging and washed three times with 2·0 ml. of 95% ethanol. The washings were added to the soluble fraction, which was evaporated. The fatty acids were recovered by decomposing the insoluble and soluble lead salts with 2N-HNO_3 and 2N-HCl respectively, ethanol solution being washed with water and dried by repeated evaporation with benzene. After weighing, the acids were dissolved in methanol, and samples taken for iodine values (Trappe, 1938) and alkali isomerization (Herb & Riemenschneider, 1953).

Table 6 shows that slightly more than half of the fatty acids in the ester phosphatides and nearly all the acids in the α-acyl lyso compounds derived from plasmalogen were unsaturated. The degree of unsaturation was very much higher in the ethanolamine derivatives than in the choline derivatives. Table 7 shows the percentage composition of the unsaturated fatty acids based on the absorption at 233, 268, 315 and 346 mμ after alkali isomerization, from which values ascribed to linoleic acid, linolenic acid, arachidonic acid and C_{20-22} polyenoic acids were calculated, the remainder calculated by difference being ascribed to oleic acid. The unsaturated acids in the lecithin and kephalin were slightly more than 50% of the total fatty acids. It is usually considered that the unsaturated acids occur on the α-position. The β-acyl-lysolecithin had in fact a very low iodine value (Table 5). The unsaturated acids shown in Table 7 therefore probably substantially make up the α-acyl component of the lecithin, choline plasmalogen, kephalin and ethanolamine plasmalogen of ox heart respectively.

DISCUSSION

The plasmalogens and the ester phosphatides used in this study were representative of the complete range present in ox-heart phosphatides, because the quantitative extraction and fractionation of the phosphatides was followed by hydrolysis of the whole lecithin and kephalin fractions obtained. The results show that the aldehyde residue is apparently exclusively linked to the β-carbon atom of the glycerol in the plasmalogen molecule. This confirms

Table 6. *Percentage and iodine number of saturated and unsaturated acids of phospholipids*

Fatty acids were separated as the insoluble lead salts.

	Source of fatty acids			
	Lecithin	α-Acyl-lysolecithin*	Kephalin	α-Acyl-lysokephalin†
	Saturated fatty acids			
Wt. (mg.)	26·7	3·6	43·7	7·7
% of total	43·0	3·5	46·0	7·0
Iodine no.	8	89	11	55
	Unsaturated fatty acids			
Wt. (mg.)	35·0	97·7	51·5	102·2
% of total	57·0	96·5	54·0	93·0
Iodine no.	153	187	269	260

* From choline plasmalogen. † From ethanolamine plasmalogen.

Table 7. *Composition of unsaturated fatty acids of phospholipids by alkali isomerization*

	Percentage of total unsaturated acids			
Acid	Lecithin	α-Acyl-lysolecithin*	Kephalin	α-Acyl-lysokephalin†
Oleic‡	55·2	40·0	33·0	18·6
Linoleic	32·0	39·0	13·6	35·0
Linolenic	7·3	8·0	1·5	11·0
Arachidonic	5·2	10·0	41·5	23·0
C_{20} polyene	0·3	3·0	10·4	12·4

* From choline plasmologen. † From ethanolamine plasmalogen. ‡ By difference.

the conclusions reached by Rapport & Franzl (1957) from the action of phospholipase *A* on choline plasmalogen of ox heart and shows that the ethanolamine plasmalogen has a similar structure.

In ox heart almost all the aldehydes isolated from the plasmalogens were saturated compounds. Gas-phase chromatography confirmed that palmitaldehyde was the major component of the aldehydes isolated from choline plasmalogen, and showed the presence of seventeen other components, fifteen of them occurring in amounts of only 1–5 %. This sensitive analytical procedure illustrates very clearly the complexity of the natural plasmalogens.

The fatty acids in the plasmalogens were found to be mainly (95 %) unsaturated acids. As a result of the work of Hanahan (1954*b*, *c*) and Rhodes & Lea (1956) with phospholipase *A* it was shown that in lecithin and kephalin the α-position was mainly occupied by unsaturated fatty acids and the β-position mainly by saturated fatty acids. This general structural arrangement was found in phosphatides from various sources (liver, brain, egg yolk). The positions of the saturated and unsaturated aliphatic chains in the plasmalogens of ox-heart are the same as those in the ester phosphatides. There is therefore a very close similarity of structure between these two classes of compounds (cf. also Long & Penny, 1957).

Marinetti & Erbland (1957) reported that the plasmalogens isolated from pig heart had the aldehyde residue linked exclusively in the α-position. Their method of proof was different from that described in this paper and involved a periodate-oxidation procedure and infrared measurement. Their results imply that the plasmalogens from different species of animals may have different structures. If there is as close a relationship between the plasmalogens and ester phosphatides of pig heart as there is in ox heart one would expect a corresponding difference in the positions of the saturated and unsaturated fatty acids in the ester phosphatides. A systematic investigation of various tissues of different species of animal is needed to obtain a clearer picture of plasmalogen structure and composition.

Kephalin and ethanolamine plasmalogen contain a higher proportion of the highly unsaturated acids, arachidonic acid and C_{20} polyethenoic acids than do choline plasmalogen or lecithin. Phosphatidylethanolamine from egg is also almost twice as unsaturated as phosphatidylcholine from the same source (Rhodes, 1957), and a similar difference appears in the kephalin and lecithin fractions from human brain (Klenk, Debuch & Daun, 1953; Debuch, 1956).

The compositions of the unsaturated fatty acids from choline plasmalogen determined by alkali isomerization (Table 7) are in substantial agreement with the results of Klenk & Krickau (1957). A comparison of the unsaturated acids from lecithin and choline plasmalogen (Table 7) shows a higher degree of unsaturation in the plasmalogen arising from the greater amount of linoleic, arachidonic and C_{20} polyethenoic acids. The ethanolamine plasmalogen, however, does not show a general increase in unsaturation over kephalin, because the larger amounts of linoleic acid and linolenic acid in the plasmalogens are offset by the very large amount of arachidonic acid in the kephalin.

SUMMARY

1. The lysolecithin derived from the choline plasmalogen of ox heart by removal of the aldehyde residue was identified as the α-acyl isomer by oxidation with permanganate and identification of the acid hydrolysis products.

2. The lysokephalin similarly derived from kephalin plasmalogen was identified as α-acyl-lysokephalin.

3. The aldehyde residue in the plasmalogens of ox heart is therefore linked to the β-carbon atom of the glycerol moiety.

4. The aldehydes isolated from the choline plasmalogen were mainly saturated, the major component being palmitaldehyde. The fatty acids present in choline and ethanolamine plasmalogen were mainly (95 %) unsaturated acids.

5. The composition of the unsaturated fatty acids of the plasmalogens and of the kephalin and lecithin of ox heart was examined by alkali isomerization. The ethanolamine phosphatides were more highly unsaturated than the choline phosphatides, and contained large amounts of C_{20-22} polyenoic acids.

I wish to thank Dr A. T. James for carrying out the gas-phase chromatographic analysis of the aldehyde dimethylacetals, and the Medical Research Council for a grant.

REFERENCES

Allen, C. F. H. (1930). *J. Amer. chem. Soc.* **52**, 2955.
Ariyama, N. (1928). *J. biol. Chem.* **77**, 359.
Davidson, F. M., Long, C. & Penny, I. F. (1956). *Biochemical Problems of Lipids*, p. 253. Ed. by Popják, G. & Le Breton, E. London: Butterworths Scientific Publications.
Debuch, H. (1956). *Hoppe-Seyl. Z.* **304**, 109.
Franzl, R. E. & Rapport, M. M. (1955). *Fed. Proc.* **14**, 213.
Gray, G. M. (1957). *Biochem. J.* **67**, 25P.
Gray, G. M. & Macfarlane, M. G. (1958). *Biochem. J.* **70**, 409.
Hanahan, D. J. (1954*a*). *J. biol. Chem.* **207**, 879.
Hanahan, D. J. (1954*b*). *J. biol. Chem.* **211**, 313.
Hanahan, D. J. (1954*c*). *J. biol. Chem.* **211**, 321.
Herb, S. F. & Riemenschneider, R. W. (1953). *Analyt. Chem.* **25**, 953.

Isherwood, F. A. (1946). *Biochem. J.* **40**, 688.
Klenk, E. & Debuch, H. (1954). *Hoppe-Seyl. Z.* **296**, 179.
Klenk, E. & Debuch, H. (1955). *Hoppe-Seyl. Z.* **299**, 66.
Klenk, E., Debuch, H. & Daun, L. H. (1953). *Hoppe-Seyl. Z.* **292**, 241.
Klenk, E. & Krickau, G. (1957). *Hoppe-Seyl. Z.* **308**, 98.
Kramer, P. J. G. & Van Duin, H. (1954). *Rec. Trav. chim. Pays-Bas*, **73**, 63.
Long, C. & Penny, I. F. (1957). *Biochem. J.* **65**, 382.
Marinetti, G. V. & Erbland, J. (1957). *Biochim. biophys. Acta*, **26**, 429.
Rapport, M. M. & Franzl, R. E. (1957). *J. biol. Chem.* **225**, 851.
Rhodes, D. N. (1957). *Biochem. J.* **68**, 380.
Rhodes, D. N. & Lea, C. H. (1956). *Nature, Lond.*, **177**, 1129.
Trappe, W. (1938). *Biochem. Z.* **296**, 180.
Webster, G. R. (1956). *Biochim. biophys. Acta*, **20**, 432.
White, T. (1940). *J. chem. Soc.* p. 428.

Electrophoresis in a Density Gradient

BY P. A. CHARLWOOD AND A. H. GORDON
The National Institute for Medical Research, The Ridgeway, Mill Hill, London, N.W. 7

(*Received* 14 *April* 1958)

In the method of zone electrophoresis a means for the stabilization of the medium against convection has to be provided. That a density gradient of sucrose might be suitable for this purpose was suggested by Consden, Gordon & Martin (1946). Since then several attempts, discussed by Svensson, Hagdahl & Lerner (1957), have been made to achieve separations on this principle. In such systems it is comparatively easy to obtain good separations of substances if they are present in such small amounts as not to contribute significantly to the density of the initial solution, or if they are of approximately the same molecular size as the substance used for making the gradient. These conditions can be met quite satisfactorily with dyestuffs in a sucrose gradient, but not so easily with proteins. With large molecules, instability of the initial zone develops rapidly, even though its density has been adjusted so that it is slightly less than that of the solution on which it rests. This happens because the decrease in density due to diffusion of large molecules out of the zone is more than compensated by the more rapid diffusion of sucrose into the zone. As a result, the zone becomes relatively dense and droplets are formed. Fortunately this difficulty has now been overcome by Svensson *et al.* (1957), who have shown that droplet formation in the initial zone does not occur if an appropriate amount of a dense, rapidly diffusing substance such as potassium bromide is present in the protein solution. Most of Svensson's work was carried out either with dyestuffs (Svensson & Valmet, 1955) or with normal or pathological sera (Svensson *et al.* 1957). The aim of the present work has been to examine the reproducibility and completeness of the separation of proteins in a modification of the apparatus used by Svensson *et al.* (1957). For this purpose human albumin and artificial mixtures of human albumin and haemoglobin have been used.

Complete separation of many of the proteins of human and animal sera can, of course, be achieved readily by the method of zone electrophoresis described by Porath (1954). Probably the most serious disadvantage of this method is the adsorption of traces of the proteins on the cellulose matrix of the column. Evidently the sucrose-gradient column avoids all difficulties due to adsorption, except on the glass of the apparatus. Since the possibility of adsorption of virus particles on a solid matrix cannot be ignored, it is interesting to note that Cramer, Lerner & Polson (1957) have recently employed sucrose-gradient electrophoresis for a problem of this kind. We report here an application to the slow-reacting substance A of Brocklehurst (1955, 1956), which possesses the property of stimulating guinea-pig gut.

EXPERIMENTAL

The apparatus (Fig. 1), basically the same as that of Svensson *et al.* (1957), incorporates some novel features. It was constructed with all-glass joints, held together by springs. The joints at the top of the electrode vessels were inverted to eliminate accumulation of large gas bubbles, which otherwise were periodically detached during electrophoresis, producing slight but undesirable movement of the liquid. Filling of the gradient tube was made automatic. The liquid from the mixer was fed through a long capillary tube which was held in contact with the wall of the column about an inch above the meniscus. This capillary was raised intermittently by means of a screwed-rod mechanism actuated by two contacts attached to the capillary tube and touching the liquid surface. Virtually no current passed between the contacts, since the control was exercised through a sensitive electronic relay.

With sucrose columns, sections 2 and 4 (Fig. 1) were first filled with plain buffer. Next the capillary tube 7 was