Linderstrøm-Lang, K. & Jacobsen, C. F. (1941). *J. biol. Chem.* **137**, 443.
Lynen, F. & Reichert, E. (1951). *Angew. Chem.* **63**, 47.
Mozingo, R., Spencer, C. & Folkers, K. (1944). *J. Amer. chem. Soc.* **66**, 1859.
Mozingo, R., Wolf, D. E., Harris, S. A. & Folkers, K. (1943). *J. Amer. chem. Soc.* **65**, 1013.
Neuberger, A. (1937). *Proc. Roy. Soc.* A, **158**, 68.
Newton, G. G. F. & Abraham, E. P. (1950). *Biochem. J.* **47**, 257.
Newton, G. G. F. & Abraham, E. P. (1953). *Biochem. J.* **53**, 597.
Porter, R. R. (1948). *Biochim. biophys. Acta*, **2**, 105.
Sanger, F. (1945). *Biochem. J.* **39**, 507.
Sharp, V. E., Arriagada, A., Newton, G. G. F. & Abraham, E. P. (1949). *Brit. J. exp. Path.* **30**, 444.
Woiwod, A. J. (1949). *J. gen. Microbiol.* **3**, 312.
Wright, L. D., Cresson, E. L., Skeggs, H. R., Peck, R. L., Wolf, D. E., Wood, T. R., Valiant, J. & Folkers, K. (1951). *Science*, **114**, 635.

Anaerobic Reactions of Phospholipins in Brain Suspensions

By G. H. SLOANE-STANLEY
Biochemical Laboratories, Institute of Psychiatry (British Postgraduate Medical Federation, University of London), Maudsley Hospital, London, S.E. 5

(*Received* 25 *August* 1952)

The metabolism of the brain phospholipins of adult mammals has been found to be slow but significant, *in vivo* and *in vitro* (e.g. Perlman, Ruben & Chaikoff, 1937; Hevesy & Hahn, 1940; Fishler, Taurog, Perlman & Chaikoff, 1941; Fries, Schachner & Chaikoff, 1942; Sperry, 1947), and the known phospholipin-splitting activities of brain extracts are low (King, 1931; Rossi, 1935; Thannhauser & Reichel, 1936; Goebel & Seckfort, 1948; Sloane-Stanley, 1952). Kephalin is apparently turned over faster *in vivo* than are other phospholipins (Hevesy & Hahn, 1940; Chargaff, Olsen & Partington, 1940), although no very active brain enzyme attacking kephalin has yet been described; brain lecithinase might be expected to attack kephalin slowly, as does the similar but more active enzyme in kidney (King, 1931, 1934).

It is now known that brain kephalin is not a single compound, but a mixture of phosphatidylethanolamine (with the classical structure of kephalin), phosphatidylserine and diphosphoinositide (a compound of 'inositol metadiphosphate' with one equivalent each of glycerol and fatty acid); large amounts of sodium, potassium, magnesium and calcium are extracted from brain by lipid solvents as salts of the latter two acidic lipids (Folch, 1942, 1947, 1948, 1949*a*, *b*), and may therefore be combined with them *in vivo*.

The apparently slow metabolism of the brain phospholipins as a whole might be due to the rapid turnover of one of the individual members of this complex mixture; such a reaction would be of particular interest if it involved one of the acidic kephalins mentioned above, in view of the physiological importance of the cations associated with these substances. Moreover, phosphatidylserine might be decarboxylated to phosphatidylethanolamine, a neutral substance, without any release of lipid phosphorus; such a reaction would not be detected by studies of phosphate turnover in the phospholipins as a whole.

The experiments described below fell, therefore, into two groups. First, the possibility of a rapid decarboxylation of phosphatidylserine by brain preparations *in vitro* was examined; since no such reaction was in fact detected, these experiments will not be described in detail. Secondly, a search was made for enzymes in brain able to catalyse the rapid hydrolysis of phospholipins; a highly active system, apparently splitting diphosphoinositide, was found. While this reaction was being investigated, two publications from Richter's laboratory appeared, describing the fairly rapid turnover of brain lipid phosphorus in adult mice (Dawson & Richter, 1950) and a kephalinase reaction in excised brain (Tyrrell, 1950), either or both of which could involve the hydrolysis of diphosphoinositide.

Part of this work has already been published in a preliminary report (Sloane-Stanley, 1951).

EXPERIMENTAL

All-glass apparatus was used wherever possible. Homogenizers were of the Potter-Elvehjem type (Umbreit, Burris & Stauffer, 1949), but had smooth pestles without beads sealed on to their bottoms. All pH measurements were approximate, being made with 'capillators' (British Drug Houses Ltd.).

Preparation of lipids

Ox-brain kephalin was prepared and fractionated according to Folch (1942), except that fractions I and II were precipitated together as the 'inositol phosphatide' (Folch, 1949*a*); the other lipids used were by-products from

the preparation of brain kephalin. All lipid fractions were stored as solutions, usually in $CHCl_3$ (mixtures of $CHCl_3$ and methanol for the ether-insoluble material, wet $CHCl_3$ for the 'inositol phosphatide'). Undialysed lipids were used for most of the experiments; for the last group only, with dilute brain suspensions, all the samples used had been dialysed to remove water-soluble impurities, and the emulsions freeze-dried, according to Folch (1942). All fractions of kephalin, and any dialysed lipids, were stored over solid CO_2 (i.e. at about $-70°$); other lipids were kept in a refrigerator at about $-20°$.

Analytical methods

Phosphate. Total phosphorus in a sample was determined, after digestion with 60% (w/v) $HClO_4$, according to Weil-Malherbe & Green (1951), except that a 1:1 (v/v) mixture of *iso*butanol and benzene (Martin & Doty, 1949) was used as solvent. Inorganic phosphate was also preferably determined by the method of Weil-Malherbe and Green (1951), with the slight change mentioned; in the earlier stages of this work, the procedure of Lowry & Lopez (1946) was used.

The samples used for the analysis of lipids were in the form of aqueous emulsions, prepared by suspending the residues from samples of the $CHCl_3$ solutions (evaporated to dryness in homogenizer tubes *in vacuo*) in ice-cold water. For the estimation of inorganic phosphate, the filtrate from such an emulsion, to which an equal vol. of approx. 0·9 N-$HClO_4$ had been added, was treated according to Weil-Malherbe & Green (1951); when the method of Lowry & Lopez (1946) was used for the final determination, the emulsion was first precipitated by the addition of an equal vol. of 10% (w/v) trichloroacetic acid.

Table 1. *Inorganic phosphate in brain lipids*

(Lipids from 584 g. fresh ox brain, separated according to Folch (1942, 1949*a*).)

Lipid fraction	Residue weight (g.)	Inorganic P (%)
Acetone extract	13·9	—
Ethanol extract	13·3	0·0007
Light petroleum extract	14·0	0·55
Ether-insoluble fraction of light petroleum extract	4·3	0·06
'Ethanol-soluble impurity' of light petroleum extract	3·3	0·02
Crude kephalin	4·7	1·45
'Inositol phosphatide' of kephalin	2·8	1·75
Fraction III of kephalin (mainly phosphatidylserine)	0·8	0·02

During the preparation of one batch of kephalin, a search was made for labile phosphate, by heating a lipid sample from each stage of the process for 10 min. in N-HCl at 100°, cooling, filtering and estimating the inorganic phosphate in the filtrate according to Lowry & Lopez (1946). No evidence for the existence of labile phosphate in the lipids was obtained, but the results of the inorganic phosphate estimations were unexpected and are given in Table 1. It will be seen that the inorganic phosphate accompanying the inositol phosphatide (cf. Folch, 1949*a*) could be detected in the preceding cruder mixtures at all stages of the preparation but not in the by-products; in particular, the ethanol extract, made before the brain residue was extracted with light petroleum, contained only a negligible amount of inorganic phosphate.

Total N. Aqueous emulsions of lipids were prepared as for phosphate estimations, and their total N determined according to Hiller, Plazin & Van Slyke (1948), with the apparatus of Markham (1942) for the final distillation of the NH_3. For the determination of N in filtrates from manometric experiments, a method capable of estimating 0·1–2·0 μmoles of N in up to 2 ml. of an aqueous solution was developed, based on that of Shaw & Beadle (1949), using digestion tubes similar to those described by Borsook & Dubnoff (1939), and standard no. 1 size Conway units (Conway, 1947) for the final diffusion and titration of the NH_3. During the concentration and digestion of the samples, for which the volume of digestion mixture (Shaw & Beadle, 1949) was increased to 0·2 ml., the tubes had to be covered with an inverted beaker nearly touching the acid in the bath, to prevent the absorption of NH_3 from the air.

Serine and ethanolamine. A method based on that of Artom (1945) was used but proved to be not wholly satisfactory.

Inositol. (*a*) *Chemical method.* The method was adapted from that of Platt & Glock (1943), depending on the oxidation of inositol by HIO_4, which also gives a measure of the glycerol present. The main alterations to the original procedure were that the scale was reduced about 20-fold, the samples were not treated with yeast to remove fermentable carbohydrate and the ion-exchange resins for the removal of interfering substances were Zeo-Karb 315 and De-Acidite E (Permutit Co. Ltd.); these were ground and sieved to the 60–90 mesh size and used in one-fifth of the amounts suggested by the original authors in tubes of about 5 mm. internal diameter. Lastly, the period of hydrolysis with HCl, used for the estimation of the total inositol in a sample, was increased to at least 16 hr. Recoveries of 1·5 μmoles of inositol in the 'total inositol' procedure averaged 98·8%; correcting for this loss, sodium phytate (Ciba Laboratories Ltd.) gave an average of 99% of the inositol content calculated from the formula $C_6H_6O_{24}P_6Na_{12}.38H_2O$ (Posternak, 1921), which suggested that its hydrolysis was complete. The results obtained with duplicate samples never differed by more than 12·5%, and frequently agreed to within 2%. The estimation of glycerol was less reliable; recoveries averaged 74·8%, and duplicates differed by from 5 to 17%. Glycerophosphate was about 80% hydrolysed under these conditions, as shown by estimation of the inorganic phosphate liberated. The amounts of apparent inositol and glycerol found in extracts from manometer flasks which had contained only bicarbonate-saline were very small.

(*b*) *Biological method.* The second method used for the determination of inositol was, like that described by Woolley (1941*a*), a microbiological assay using a yeast for which inositol is a growth factor. The method (as yet unpublished) was developed by Dr E. R. Dawson of the Distillers Company Ltd., who kindly supplied the details of the procedure, a sample of the strain of yeast required (*Saccharomyces carlsbergensis* 4228) and the following description of the growth medium: 'The medium was essentially that described by Atkin, Schultz, Williams & Frey (1943) for the assay of pyridoxine, except that 2·5 mg./l. of pyridoxine hydrochloride replaced the inositol while nicotinic acid and $(NH_4)_2HPO_4$ were added, as suggested by Hopkins & Pennington (1947).' The method of calculating the results was less rigorous than that recommended by Dr Dawson, since the yeast assay was principally required to

check the specificity of the HIO_4 method, and the amount of each extract available was limited; the inositol concentrations in the 'unknown' solutions were read off from a standard curve drawn from the results obtained with known concentrations (final dilutions, $0{\cdot}125$–$0{\cdot}5 \times 10^{-5}$ M).

(c) *General remarks.* The solutions taken for the yeast assay were obtained by dilution of the solutions remaining from the chemical method; since they had been passed through ion-adsorbing columns, they were probably free of choline, which has been found to interfere with microbiological assays of inositol using yeast (Taylor & McKibbin, 1952). The inositol content found for any one sample by the yeast assay usually agreed to within 20% with that obtained by the HIO_4 method, except in the analyses of samples containing very small amounts of inositol. This suggests that the chemical method was indeed measuring chiefly free inositol. HIO_4 also reacts with some inositol phosphates (Folch, 1949*b*), and with most 1:2-glycols and related compounds, including carbohydrates (Jackson, 1944); in these experiments, therefore, the inositol assay solutions must have been almost free of carbohydrates and of inositol phosphates, neither of which stimulate the growth of yeast in a solution rich in glucose (Woolley, 1941*b*).

A complete analysis of up to eight samples by both methods took at least a week. It was found essential to store the solutions in intermediate stages at $-20°$ since, if stored at about 0°, they lost inositol, probably by microbial action. The size of each sample taken for analysis was adjusted to contain not more than 2 μmoles of inositol. For the analysis of lipids, 3 mg. samples were used; they were added to the hydrolysis flasks either as aqueous emulsions or as solutions in $CHCl_3$; in the latter case, the HCl (about 5 N) was added before the removal of the $CHCl_3$ by evaporation *in vacuo* since, if the $CHCl_3$ was removed first, recoveries of inositol were low and variable.

Reducing sugar. The solutions used for inositol and glycerol estimations were tested by a drop-scale application of Park & Johnson's (1949) method for estimating glucose. Galactose or glucose could be detected by this method at concentrations down to 10^{-5} M, whereas 10^{-2} M-inositol and glycerol gave negative results.

Detection and measurement of enzymic activity

General. Warburg manometric apparatus was used, the temperature of the bath being 38°. All experiments were carried out under anaerobic conditions in N_2 or 95% N_2 + 5% CO_2 (v/v). Lipids, when added, were put in the side bulbs, brain suspensions in the main vessels; the flasks were shaken in the bath to equilibrate for about 20 min. before tipping. In every experiment, one flask, the 'tissue blank', contained only buffer in the side bulb. Brain suspensions were prepared from fresh whole guinea pig brain, including cerebellum and brain stem, dispersed in the appropriate ice-cold medium just before use; the lipids were added as emulsions in the same salt solutions. The manometers were read every 5 min. for the first 30 min., then at longer intervals until the experiments were stopped, usually 3–4 hr. later.

Experiments with phosphatidylserine. Two samples of fraction III of brain kephalin were used, one containing about 74% and the other about 90% of phosphatidylserine; emulsions were shaken in manometric apparatus under N_2 for up to 3 hr. with fresh brain suspensions in buffers of pH 5–9.

Search for lipolytic enzymes. Brain suspensions were prepared in bicarbonate-saline (Krebs & Henseleit, 1932), so that any formation of acid resulting from the hydrolysis of a phospholipin would be detected by the evolution of CO_2. The medium was gassed before use with N_2 + 5% CO_2, its pH then being about 7·4. Lipid emulsions were freshly prepared by dispersing in ice-cold saline the residues from solutions of the lipids in $CHCl_3$ (taken to dryness *in vacuo* in homogenizer tubes the day before and stored overnight in stoppered tubes over solid CO_2). The lipid added to each flask weighed 20 or 25 mg. in 0·4 ml. of fluid; each main vessel received 2·6 ml. of brain suspension containing 0·65 g. of moist tissue. For some experiments, a portion of each brain suspension was heated for 5 min. in a boiling-water bath, during which time it was gassed with CO_2; tests with saline alone, containing suitable indicators, showed that under these conditions the pH remained between 7 and 8. The initial rate of each reaction was calculated from the rate of evolution of CO_2 over the longest interval (starting at 5 min. after tipping in order to avoid artifacts) for which it appeared to remain constant.

Measurement of enzymic activity. For this purpose, more dilute suspensions were used, combined with analyses of the acid-soluble end products; the procedure was modified as follows: (i) KH_2PO_4 was omitted from the saline. (ii) Only dialysed lipids were used. (iii) Lipid emulsions were first prepared as for phosphate estimations (see above) on the day before each experiment, neutralized with 0·1 N-KOH to about pH 7·4 and stored overnight in stoppered tubes at $-20°$; next day, the emulsions were thawed, the necessary salts added, and the emulsions redispersed and gassed with N_2 + 5% CO_2 before use (the addition of the components of the saline to water emulsions of the inositol phosphatide of kephalin converted them into relatively coarse suspensions). (iv) Each experiment included at least four flasks; one (the 'saline blank') containing only saline in both main vessel and side bulb, and another (the 'substrate blank') also having lipid in the side bulb (to allow for CO_2 or residual acidity in, or non-enzymic hydrolysis of, the lipid), besides the usual 'tissue blank' and 'experimental' flasks. The rate of enzymic hydrolysis of the inositol phosphatide could usually then be measured from zero time; but with other lipids, measurements had still to be started from the 5 min. reading.

Preparation of extracts for the estimation of products of enzymic breakdown of lipids. The best reagent for the removal of the remaining intact lipids, the proteins and any fatty acids or glycerides from the mixtures in the manometer flasks after incubation was $HClO_4$, recommended by Neuberg, Strauss & Lipkin (1944); it also had the advantage that about five-sixths of the amount added could be removed from the filtrate by neutralizing with KOH, since the solubility of $KClO_4$ at 0° is only 0·75% (Seidell, 1940). The procedure used was as follows. When the last manometric reading had been taken, all the flasks were cooled to 0°, at which temperature the rest of the preparation of the final extracts was carried out. An equal vol. of approx. 0·9 N-$HClO_4$ was added to the fluid in each flask, the mixture centrifuged and the supernatant fluid filtered. The filtrate was then treated with a succinate buffer of pH 6 and the volume of N-KOH required to neutralize the $KClO_4$, allowed to stand for about 10 min. and filtered; the final filtrate, 'the extract', was stored at $-20°$. Succinate buffer was used because it gave a pH near neutrality and contained neither N nor P; it was prepared by adding the

theoretical amount of NaOH to succinic acid, using the pK_2 of the latter given by Umbreit *et al.* (1949), and its pH checked.

RESULTS

Phosphatidylserine

There was no evidence for rapid decarboxylation of phosphatidylserine in brain suspensions, at any pH between 5 and 9; the highest apparent rate of evolution of carbon dioxide in these experiments was 2·4 μmoles/g. wet wt./hr.

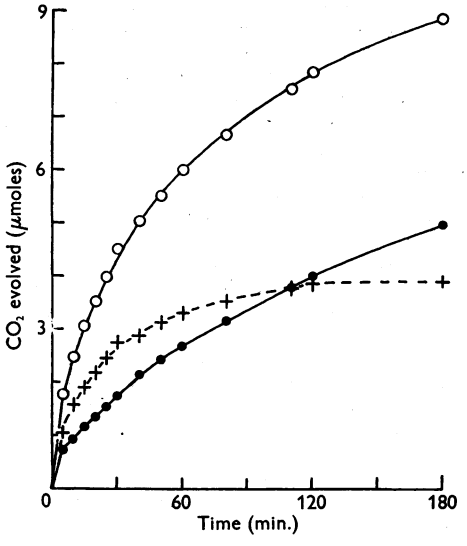

Fig. 1. Evolution of CO_2 under anaerobic conditions by concentrated suspensions of guinea pig brain in bicarbonate-saline with and without added 'inositol phosphatide'. ●—●, brain alone (650 mg. wet wt.); ○—○, brain and lipid (20 mg.); +---+, CO_2 evolution due to addition of lipid (difference between ○—○ and ●—●).

Hydrolyses of phospholipins

Preliminary experiments. The results of the search for lipolytic enzymes in brain suspensions suggested that all fractions of kephalin, particularly the diphosphoinositide-containing 'inositol phosphatide' (Folch, 1942, 1949*a*, *b*), were hydrolysed; the last-named substance appeared to be attacked about three times as fast as any of the others. A typical progress curve from an experiment with inositol phosphatide is given in Fig. 1; the average initial rate of the reaction in six such experiments was 17·1 μmoles/g. wet wt./hr. (S.E.M. ± 2·5; range 9·8–27·2). The activity of the brain suspensions towards inositol phosphatide appeared to be about equally divided between the particles sedimented by centrifugation at about 3000 rev./min. and the supernatant fluid.

Further experiments were made in order to find whether these results were enzymic, due to a rapid hydrolysis of diphosphoinositide and slower hydrolyses of other lipids. First, it was found that the reaction with inositol phosphatide was almost completely prevented by heating the brain suspensions before use; this suggested that hydrolysis was catalysed by an enzyme. Secondly, attempts were made to find the true enzymic activities of brain suspensions in attacking inositol phosphatide and in the other apparent lipolyses, since the rates of the latter were not negligible; and thirdly, the products were analysed, since the best available samples of these lipids, particularly the inositol phosphatide, were far from pure (e.g. the second batch of inositol phosphatide of Table 3 contained inositol equivalent to only 41·4% of diphosphoinositide, assuming a molecular weight of 750 for the latter).

Measurement of enzymic activities. The apparent enzymic activities of brain suspensions towards inositol phosphatide increased as the amount of

Table 2. *Apparent initial rates of hydrolysis of ox-brain lipids by fresh dilute guinea pig brain suspensions*

(Warburg manometers. Fluid, 3 ml. phosphate-free bicarbonate-saline, pH about 7·4. Gas, 95% N_2 + 5% CO_2 (v/v). Temp., 38°. 52 mg. fresh brain and 15–25 mg. lipid per flask. Results corrected for saline, substrate and tissue blanks.)

Lipid fraction	Probable composition (main component first)	Apparent rate of hydrolysis (μmoles CO_2/g. wet wt./hr.)
Ethanol extract	Lecithin and phosphatidylethanolamine	4·6, 7·1*
Ether-insoluble fraction of light petroleum extract	Sphingomyelin, hydrolecithin and cerebrosides	2·5, 5·9*
'Ethanol-soluble impurity' from preparation of kephalin	Lecithin and phosphatidylethanolamine	7·5, 0, 4·2
'Inositol phosphatide' of kephalin	Diphosphoinositide, phosphatidylserine and phosphatidylethanolamine	60, 84·2, 58·4,† 47·4,* 69·2,* 76·2*
Fraction III of kephalin	Phosphatidylserine (and some phosphatidylethanolamine)	1·1,* 3·1
Fraction V of kephalin	Phosphatidylethanolamine	5·5, 2·0

* End products analysed; see Table 3 and text.
† Progress curve given in Fig. 2. Initial rate with 10 mg. inositol phosphatide: 34·6 μmoles/g. wet wt./hr.

tissue in each manometer flask was reduced to about 50 mg. wet wt.; below this level, the rate of evolution of carbon dioxide was approximately proportional to the amount of tissue present. In all the subsequent experiments reported here, the wet weight of tissue in each flask was 52 mg., i.e. 2·6 ml. of a 2% (w/v) suspension; the amounts of inositol phosphatide used were kept at or near 20 mg. per flask since, on adding only 10 mg. to one flask in one experiment, the apparent initial rate of the reaction was considerably reduced. The results of all the experiments

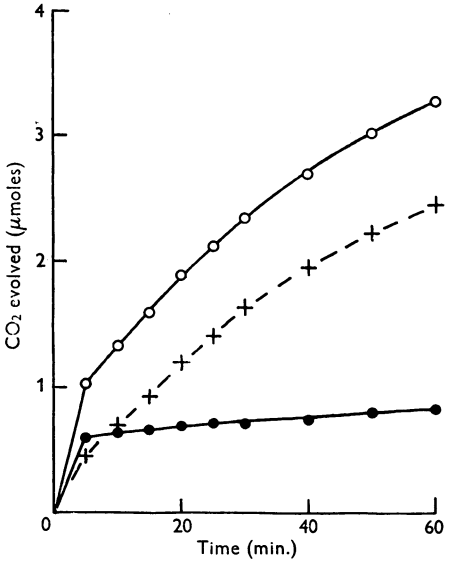

Fig. 2. Evolution of CO_2 under anaerobic conditions by 'inositol phosphatide' in bicarbonate-saline, with and without dilute suspension of guinea pig brain (no CO_2 evolved by brain alone). ●—●, lipid (20 mg.) alone; ○—○, lipid + brain (52 mg. wet wt.); +---+, CO_2 evolution due to addition of brain (difference between ○—○ and ●—●).

done under these conditions are given in Table 2; Fig. 2 gives part of a typical progress curve of the apparent enzymic hydrolysis of inositol phosphatide (initial rate, 58·4 μmoles/g. wet wt./hr.), together with that of the substrate blank. The mean initial rate of the enzymic reaction of the inositol phosphatide, for all the experiments of Table 2, was 65·9 μmoles/g. wet wt./hr. (S.E.M. ± 5·4), the mean rate after 4 hr. being 9·1 μmoles/g. wet wt./hr. (S.E.M. ± 1·7; range 3·2–14·6). There was often an apparent continuous slow evolution of carbon dioxide in the substrate blanks (Fig. 2) at a maximum rate of 0·67 μmole/hr., the absolute rates of the enzymic reaction being 2·46–4·38 μmoles/hr.; as, however, there was sometimes an apparent uptake of carbon dioxide in the substrate blanks, the non-enzymic hydrolysis of the lipid was probably negligible. The rates of evolution of carbon dioxide in the tissue blanks ranged from 0 to 13·8 μmoles/g. wet wt./hr.

The apparent enzymic hydrolyses of the nitrogenous lipids, including phosphatidylserine and phosphatidylethanolamine, which occur in the inositol phosphatide (Folch, 1949*a*), were much slower than that of the latter, and frequently slower than the evolution of carbon dioxide from the blanks.

Analysis of reaction products. The enzymic hydrolysis of inositol phosphatide was accompanied by the release of acid-soluble phosphate and inositol, in amounts similar to those of the carbon dioxide evolved, probably together with smaller quantities of glycerol and nitrogen. Average values for the enzymic hydrolysis (corrected for tissue, substrate and saline blanks), and for the tissue blanks, in three experiments are given in Table 3, together with the analyses of the lipid samples used. It will be seen that the molecular proportion of acid-soluble phosphate to inositol was nearly 2:1, about half the total phosphate being inorganic. Most of the inositol was in the combined form, the difference between the total and free inositol values being almost exactly equal to the difference between the figures for the total and inorganic phosphate.

Acid-soluble phosphate and nitrogen were also estimated in three of the experiments with the other lipids; the amounts released were small, being similar to or less than those liberated in the blanks.

All these results suggest that the main reaction taking place in these experiments was the rapid hydrolysis of diphosphoinositide, with the release of inorganic phosphate and inositol monophosphate in approximately equimolecular amounts; the residual parts of the hydrolysed molecules of diphosphoinositide probably remained intact as monoglycerides or phosphatidic acids and were precipitated as such; this would agree with previous findings of very low lipase activity in brain (Edlbacher, Goldschmidt & Schläppi, 1934; Gomori, 1945, Copenhaver, Stafford & McShan, 1950). The hydrolyses of the nitrogenous lipids, both as contaminants of the inositol phosphatide and independently, must have been slight.

DISCUSSION

As a result of the experiments with fraction III (Folch, 1942) of kephalin, it seems unlikely that there is any system in brain which can decarboxylate phosphatidylserine at all rapidly. The results of the search for lipolytic processes in brain suspensions agreed with previous findings in that none of the nitrogenous lipids was rapidly hydrolysed; but it did seem, and further study supported this view, that there is an active enzyme system in brain, able to hydrolyse the most recently discovered of the

Table 3. *Acid-soluble products of enzymic hydrolysis of ox-brain 'inositol phosphatide' by fresh guinea pig brain suspensions*

(Averaged results (±S.E.M.) of three experiments (corrected for all blanks), together with tissue blanks (corrected for saline blanks) and analyses of lipid samples used. Conditions as in Table 2. Reaction stopped after 4 hr. by addition of 3 ml. approx. 0·9 N-$HClO_4$; filtrates from duplicate flasks pooled for analysis.)

	Products of enzymic hydrolysis of inositol phosphatide (μmoles) Mean	Products from tissue blanks (μmoles) Mean	Contents of lipid samples* (μmoles) Mean
CO_2	3·86±0·15	0·2 ±0·3	0·10†±0·07†
Total P	5·58±0·44	1·2 ±0·2	34·9±5·4
Inorganic P	2·90±0·42	1·16±0·10	1·84,† 0·92±0·80,† 0·30
Total inositol (HIO_4)	2·98±0·12	0·70±0·06	9·4±1·6
Total inositol (yeast)	3·23±0·39	0·58±0·13	9·9±1·2
Free inositol (HIO_4)	0·36±0·05	0·77±0·07	0·27†±0·09†
Free inositol (yeast)	0·35±0·13	0·60±0·04	0·20†±0·02†
Total glycerol	0·69±0·16	0·19±0·12	13·8±1·2
Free glycerol	0·65±0·06	0·34±0·12	0·11†±0·01†
Total reducing sugar	0·2 ±0·1	0 ±0·1	<0·9±0·4
Free reducing sugar	0·07±0·07	0·1 ±0·2	0†±0†
Total N	1·93±0·61	7·8 ±1·4	12·2±2·0

* First sample used for one experiment (15·5 mg./flask), second sample used for two experiments (20 mg./flask).
† Values obtained from filtrates from 'substrate blank' flasks of manometric experiments, corrected for 'saline blanks'. Other values obtained by direct analysis of the lipids.

brain kephalins, diphosphoinositide. It appears probable, from the nature of the products and from what is known of the structure of the substrate (Folch, 1949*b*), that this hydrolysis of diphosphoinositide involves at least two enzymes. Quantitative study of this system is complicated by the likelihood that the substrate is attacked in the form of solid particles; it appears insoluble in water, and there is evidence, from the effects both of increasing the concentration of brain and of decreasing the quantity of inositol phosphatide added, that the rate of the reaction can be limited by the amount of the solid substrate present.

The evidence of *in vivo* metabolism of diphosphoinositide in the central nervous system, and possible functions therein of this and other acidic lipids, and of enzymes attacking them, have been discussed elsewhere (Sloane-Stanley, 1952); but there is no reason to believe that either the enzyme system or its substrate is confined to nervous tissues, although neither has yet been reported to occur elsewhere. Inositol-containing phospholipins have been found in mammalian liver (MacPherson & Lucas, 1947; MacPherson, 1950; McKibbin & Taylor, 1952), tubercle bacilli (Anderson, 1930; Sütö-Nagy & Anderson, 1947) and soya beans (Klenk & Sakai, 1939; Woolley, 1943); while the metabolism of phospholipins in some tissues other than brain is well known to be rapid (Wittcoff, 1951). Whatever the physiological significance of the enzyme system hydrolysing diphosphoinositide, it has two outstanding features: first, the physicochemical properties of the substrate are unusual (Folch, 1949*a*, *b*; Folch, Ascoli, Lees, Meath & LeBaron, 1951), presumably due to its polar nature; and secondly, the enzyme system is of high activity, and thus similar to other rapid enzymic processes occurring in brain preparations, such as the breakdown of diphosphopyridine nucleotide and respiration (McIlwain & Rodnight, 1949).

SUMMARY

1. Lipid samples rich in phosphatidylserine were incubated under nitrogen with suspensions of guinea pig brain in media of pH 5–9. There was no conclusive evidence of decarboxylation of the phosphatidylserine to phosphatidylethanolamine; if such a reaction did occur, its maximum rate was 2·4 μmoles/g. wet wt./hr.

2. Brain lipid samples of varied composition were incubated under 95% nitrogen + 5% carbon dioxide (v/v) with suspensions of guinea pig brain in bicarbonate-saline at a pH of about 7·4. The evolution of carbon dioxide, in excess of that from the brain homogenates alone, suggested that some of these lipids were being hydrolysed with the formation of free acid; the 'inositol phosphatide' fraction of kephalin (containing about 40% of diphosphoinositide) reacted about ten times as rapidly (average rate, corrected for blanks, 66 μmoles/g. wet wt./hr.) as any of the other lipid samples.

3. The evolution of carbon dioxide from mixtures of brain suspensions with 'inositol phosphatide' was accompanied by the release of about two molecular proportions of phosphate (about half

organic) and one of combined inositol. Smaller amounts of nitrogen and of free inositol and glycerol were also found; but the main reaction appeared to have been the partial hydrolysis of diphosphoinositide.

I am most grateful to Dr H. McIlwain for suggesting the investigation of lipid metabolism in brain, and for his most valuable advice. I also wish to thank Miss S. D. Birchenough and Mr M. P. Patel for most capable technical assistance; Mr R. Rodnight for help in the preparation of some lipid samples, and other members of this Department and Dr J. Folch-Pi for many useful suggestions; Dr E. R. Dawson and Dr H. Weil-Malherbe for giving details of their methods in advance of publication; and Messrs Ciba Laboratories Ltd. for samples of their products. Some of the apparatus used in this work was purchased with a grant kindly supplied by the Board of Governors of the Bethlem Royal Hospital and The Maudsley Hospital from the Research Fund.

REFERENCES

Anderson, R. J. (1930). *J. Amer. chem. Soc.* **52**, 1607.
Artom, C. (1945). *J. biol. Chem.* **157**, 585.
Atkin, L., Schultz, A. S., Williams, W. L. & Frey, C. N. (1943). *Industr. Engng Chem. (Anal.)*, **15**, 141.
Borsook, H. & Dubnoff, J. W. (1939). *J. biol. Chem.* **131**, 163.
Chargaff, E., Olson, K. B. & Partington, P. F. (1940). *J. biol. Chem.* **134**, 505.
Conway, E. J. (1947). *Microdiffusion Analysis and Volumetric Error*, 2nd ed. London: Crosby Lockwood.
Copenhaver, J. H., Stafford, R. O. & McShan, W. H. (1950). *Arch. Biochem.* **26**, 260.
Dawson, R. M. C. & Richter, D. (1950). *Proc. Roy. Soc.* B, **137**, 252,
Edlbacher, S., Goldschmidt, E. & Schläppi, V. (1934). *Hoppe-Seyl. Z.* **227**, 118.
Fishler, M. C., Taurog, A., Perlman, I. & Chaikoff, I. L. (1941). *J. biol. Chem.* **141**, 809.
Folch, J. (1942). *J. biol. Chem.* **146**, 35.
Folch, J. (1947). In *Psychiatric Research*, ch. 2. Harvard University Monographs in Public Health, no. 9. Cambridge, Mass.: Harvard University Press.
Folch, J. (1948). *J. biol. Chem.* **174**, 439.
Folch, J. (1949*a*). *J. biol. Chem.* **177**, 497.
Folch, J. (1949*b*). *J. biol. Chem.* **177**, 505.
Folch, J., Ascoli, I., Lees, M., Meath, J. A. & LeBaron, F. N. (1951). *J. biol. Chem.* **191**, 833.
Fries, B. A., Schachner, H. & Chaikoff, I. L. (1942). *J. biol. Chem.* **144**, 59.
Goebel, A. & Seckfort, H. (1948). *Biochem. Z.* **319**, 203.
Gomori, G. (1945). *Proc. Soc. exp. Biol., N.Y.*, **58**, 362.
Hevesy, G. & Hahn, L. (1940). *Biol. Medd., Kbh.*, **15**, no. 5.
Hiller, A., Plazin, J. & Van Slyke, D. D. (1948). *J. biol. Chem.* **176**, 1401.
Hopkins, R. H. & Pennington, R. J. (1947). *Biochem. J.* **41**, 110.
Jackson, E. L. (1944). *Org. React.* **2**, 341.
King, E. J. (1931). *Biochem. J.* **25**, 799.
King, E. J. (1934). *Biochem. J.* **28**, 476.
Klenk, E. & Sakai, R. (1939). *Hoppe-Seyl. Z.* **258**, 33.
Krebs, H. A. & Henseleit, K. (1932). *Hoppe-Seyl. Z.* **210**, 33.
Lowry, O. H. & Lopez, J. A. (1946). *J. biol. Chem.* **162**, 421.
McIlwain, H. & Rodnight, R. (1949). *Biochem. J.* **44**, 470.
McKibbin, J. M. & Taylor, W. E. (1952). *J. biol. Chem.* **196**, 427.
MacPherson, L. B. (1950). *Rev. canad. Biol.* **9**, 84.
MacPherson, L. B. & Lucas, C. C. (1947). *Fed. Proc.* **6**, 273.
Markham, R. (1942). *Biochem. J.* **36**, 790.
Martin, J. B. & Doty, D. M. (1949). *Analyt. Chem.* **21**, 965.
Neuberg, C., Strauss, E. & Lipkin, L. E. (1944). *Arch. Biochem.* **4**, 101.
Park, J. T. & Johnson, M. J. (1949). *J. biol. Chem.* **181**, 149.
Perlman, I., Ruben, S. & Chaikoff, I. L. (1937). *J. biol. Chem.* **122**, 169.
Platt, B. S. & Glock, G. E. (1943). *Biochem. J.* **37**, 709.
Posternak, S. (1921). *Helv. chim. acta*, **4**, 150.
Rossi, A. (1935). *Hoppe-Seyl. Z.* **231**, 115.
Seidell, A. (1940). *Solubilities of Inorganic and Metal Organic Compounds*, 3rd ed. vol. **1**. New York: Van Nostrand.
Shaw, J. & Beadle, L. C. (1949). *J. exp. Biol.* **26**, 15.
Sloane-Stanley, G. H. (1951). *Biochem. J.* **49**, iv.
Sloane-Stanley, G. H. (1952). *Symp. biochem. Soc.* no. 8, 44.
Sperry, W. M. (1947). *J. biol. Chem.* **170**, 675.
Sütö-Nagy, G. I. de & Anderson, R. J. (1947). *J. biol. Chem.* **171**, 749.
Taylor, W. E. & McKibbin, J. M. (1952). *Proc. Soc. exp. Biol., N.Y.*, **79**, 95.
Thannhauser, S. J. & Reichel, M. (1936). *J. biol. Chem.* **113**, 311.
Tyrrell, L. W. (1950). *Nature, Lond.*, **166**, 310.
Umbreit, W. W., Burris, R. H. & Stauffer, J. F. (1949). *Manometric Techniques and Tissue Metabolism*, 2nd ed. Minneapolis: Burgess.
Weil-Malherbe, H. & Green, R. H. (1951). *Biochem. J.* **49**, 286.
Wittcoff, H. (1951). *The Phosphatides*. New York: Reinhold.
Woolley, D. W. (1941*a*). *J. biol. Chem.* **140**, 453.
Woolley, D. W. (1941*b*). *J. biol. Chem.* **140**, 461.
Woolley, D. W. (1943). *J. biol. Chem.* **147**, 581.