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Incorporation of [^{32}P]Orthophosphate into Brain-Slice Phospholipids and their Precursors

EFFECTS OF ELECTRICAL STIMULATION

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1. The incorporation of [^{32}P]phosphate into phospholipids was measured in slices cut from the pial surface of guinea-pig cerebral cortex; incorporation into the phosphorus of some water-soluble precursors of phospholipid was measured under similar conditions. 2. Slices subjected to overall electrical stimulation at a frequency of 5 pulses/sec. differed from control slices in their pattern of phospholipid labelling. After 1 hr. of stimulation, incorporation of [^{32}P]phosphate into phosphatidylcholine, ethanolamine phospholipid and cardiolipin was respectively 54, 55 and 58% of the control value, and that into phosphatidylinositol was 186% of control. Phosphatidic acid labelling tended to increase with electrical stimulation, but the statistical significance of this change was marginal. Labelling of phosphatidylglycerol and di- and tri-phosphoinositides was not affected significantly by electrical stimulation. 3. Electrical stimulation of the tissue altered the specific radioactivities of water-soluble precursors of phospholipid. 4. The turnover rates of the phosphate groups of phospholipids were estimated approximately from the specific radioactivities of phospholipids and their precursors. Phosphatidylinositol (and its lipid-soluble precursors) showed the largest change in turnover rate in response to electrical stimulation of the tissue; the turnover rates of other lipids were also affected. Changes in the specific radioactivity of phospholipids did not correspond to changes in turnover in these experiments.

When their preganglionic trunks were stimulated *in vitro*, sympathetic ganglia incorporated more [^{32}P]phosphate into phosphatidylinositol than did controls (Larrabee, Klingman & Leicht, 1963). This response to electrical stimulation was not shared by nerves from various sources, nor was it shown by the nodose ganglion of the vagus nerve, which contains bipolar nerve-cell bodies without synaptic connections (Larrabee & Leicht, 1965). Abood, Goldman & Lipman (1957) and Majno, Gasteiger, La Gattuta & Karnovsky (1958) found that the incorporation of [^{32}P]phosphate into the total phospholipid of nerves was slightly diminished by electrical stimulation, and a similar result was found for the total phospholipids of brain by Dawson & Richter (1949). In sympathetic ganglia, the concentration range of tubocurarine that blocked synaptic transmission also blocked the effect of electrical stimulation on phosphatidylinositol labelling. On this basis it was deduced that the faster incorporation of [^{32}P]phosphate into phosphatidylinositol may be associated with post- rather than pre-synaptic activity (Larrabee & Leicht, 1965).

The functional significance of this change in phospholipid metabolism is obscure. Nevertheless, there is intrinsic interest in the fact that an increased synthesis or exchange appears to occur selectively in one phospholipid. The factors determining the lipid composition of cellular membranes are not understood. It is not known how far the differences between the lipid patterns of different types of membrane are necessary to function. The variable rate of incorporation of precursors into phosphatidylinositol provides one starting point from which to approach these questions.

The present work examines comparable phenomena in cerebral-cortex slices. To see if the effects of electrical stimulation on phospholipids were limited to phosphatidylinositol, a survey was made of all phospholipids incorporating readily measurable amounts of [^{32}P]phosphate in 1 hr. The survey included some minor phospholipids that have biosynthetic steps in common with phosphatidylinositol. Electrical stimulation is known to accelerate respiration and glycolysis and to produce associated changes in glycolytic intermediates and other phosphates (McIlwain, 1966). Changes in the

labelling of phospholipids could therefore be due to altered specific radioactivity of water-soluble precursors of phospholipid as well as to altered rates of turnover of the phospholipids themselves. To distinguish between these two possibilities, slices were analysed for water-soluble precursors as well as for phospholipids.

EXPERIMENTAL

Preparation and incubation of tissue

Cutting of tissue slices. Guinea pigs (200–400g. weight) were anaesthetized with Nembutal (60mg./kg.) before decapitation. The cerebral hemispheres were removed to a cutting table. One slice was cut from the upper surface of each hemisphere by using a razor blade and a 0·35mm. recessed guide (McIlwain & Rodnight, 1962, p. 110). Each slice was immediately floated from the razor blade into a dish containing 40ml. of incubation medium at 37° gassed with O_2+CO_2 (95:5). The slice from the first hemisphere was floated 2·5–3·0min. after decapitation, and that from the second 3·3–4·0min. after decapitation. Slices contained 2–4mg. of protein, equivalent to 22–44mg. fresh weight.

To minimize bias from differences between individual animals, one slice from each animal was stimulated and the slice from the opposite hemisphere served as control. First- and second-cut slices were alternated as controls, though there was no indication that they gave different results.

Incubation medium. Slices were incubated in a medium containing (final concentrations) 133mM-NaCl, 5mM-KCl, 1·3mM-$MgCl_2$, 0·75mM-$CaCl_2$, 1·0mM-NaH_2PO_4, 7·5mM-$NaHCO_3$ and 10mM-glucose. The medium was always at 37°, and was gassed with O_2+CO_2 (95:5) before the addition of the slices and throughout the incubation. The pH after equilibration with the gas phase at 37° was 7·3–7·4. Inulin (1%, w/v) was included in the medium in the experiments where intracellular inorganic phosphate was measured. [^{32}P]Phosphate additions were 25–230μc/beaker (see below), the larger additions being required for analysis of some of the minor phospholipids.

Incubation procedure. As soon as the second hemisphere had been sampled, the slices were manoeuvred into quick-transfer slice-holders fitted with silver-wire grid electrodes (McIlwain & Rodnight, 1962, p. 145). The holders were placed in 10ml. beakers containing 3ml. of medium but without [^{32}P]phosphate. After 30min. (to allow recovery of ionic gradients, high-energy-phosphate concentrations etc.), the holders were transferred to a second set of beakers containing 3ml. of ^{32}P-labelled medium. The electrode terminals of one of each pair of slice-holders were connected at once to a stimulator (Electrophysiological Instruments, Edinburgh 1). Alternating condenser pulses (peak voltage 10–11v, time constant 0·3–0·4msec., frequency 5pulses/sec.) were passed across the test slices for 1hr. At the end of 1hr. in ^{32}P-labelled medium, the test and control slices were released from the slice-holders. From this point, different procedures were employed for slices intended for phospholipid analysis and those intended for analysis of water-soluble phosphates.

Choice of conditions for the handling of slices. As far as possible, the slices were maintained in conditions found to favour electrophysiological evidence of synaptic transmission *in vitro*. The medium contained a relatively low concentration of Ca^{2+} ions (0·75mM rather than 2–3mM), (*a*) because some features of electrical activity in piriform-cortex slices were suppressed by the higher Ca^{2+} concentrations (Richards & Sercombe, 1968) and (*b*) because 0·75mM-$CaCl_2$ promoted a high intracellular K^+/Na^+ ratio, (Keesey, Wallgren & McIlwain, 1965) and gave Ca^{2+} concentrations in the slice approximating to those found *in vivo* (Lolley, 1963). Nembutal anaesthesia before the killing of the guinea pig did not suppress synaptic function measured electrophysiologically in piriform-cortex slices *in vitro* (C. D. Richards, personal communication).

Stimulation was applied at a frequency that causes only half-maximal changes in respiratory rate and intracellular K^+/Na^+ ratio (McIlwain & Joanny, 1963). This, it was hoped, would ensure that the stimulated slice's energy requirements for recovery processes and other needs would not exceed its capacity for resynthesis of ATP.

Separation of phospholipids

Extraction. Each slice to be analysed for phospholipids was released from the quick-transfer slice-holder into 40ml. of unlabelled incubation medium, lifted out and drained. It was then dispersed in 2ml. of chloroform–methanol (1:1, v/v) at −10° in a filter tube by means of a glass rod within about 45sec. of its release from the slice-holder. [Filter tubes were constructed from lengths (8cm.) of glass tubing (7mm. internal diameter) drawn to a blunt tip at the lower end. The tip was plugged with approximately 15mm.^{2} of Whatman GF/B glass paper, which allowed solvent to drain only slowly into the Quickfit test tube used as a collecting vessel.]

The dispersion was allowed to stand for about 20min. at room temperature. The extract was then driven through the filter under pressure applied from a syringe. The filter tube and the tissue residue were rinsed and re-extracted briefly three times with 1ml. of the same solvent mixture and the washings combined with the filtered extract (I). Extract I contained virtually all the extractable phospholipid except di- and tri-phosphoinositides, which remained with the tissue residue.

The filter tube containing the tissue residue was then transferred to a new collecting tube. Chloroform–methanol–conc. HCl (200:100:0·8, by vol.; 1ml.) was added to extract di- and tri-phosphoinositides, and the tubes were placed in an oven at 40–45° for 15min. (Dittmer & Dawson, 1961). The extract was driven through the filter. The tissue residue was re-extracted twice with 0·5ml. of the same solvent mixture, each time for 15min. at 40–45°. The combined extract and washings (II) contained most of the extractable di- and tri-phosphoinositide in the form of a proteolipid complex.

Removal of non-lipid contaminants. Extract I was washed by mechanical vibration for 1min. with 5·0ml. of 0·1M-HCl. After centrifugation, the upper aqueous phase was withdrawn by suction and discarded. Interfacial scum was left with the chloroform phase. A little water was added without shaking, to rinse the walls of the tube and the surface of the interfacial scum, and was withdrawn at once by suction. Extract II was washed by a similar procedure; 0·4ml. of 0·15M-NaCl was, however, used instead of 5ml. of 0·1M-HCl, and another 0·4ml. of 0·15M-NaCl instead of water to rinse

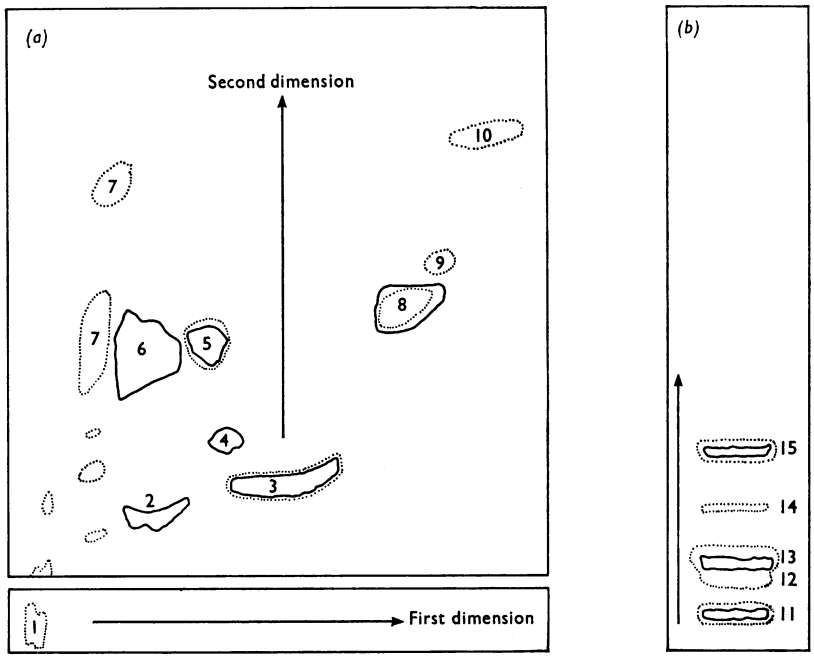

Fig. 1. Fractionation of phospholipids extracted from cerebral-cortex slices. (*a*) Tracing of a two-dimensional chromatogram of extract I. (*b*) Tracing of a one-dimensional chromatogram of extract II. Phospholipid spots revealed by charring with H_2SO_4 are outlined with a solid line and spots revealed by radioautography of ^{32}P label are outlined with a dotted line. Spots that did not contain phosphorus are omitted for the sake of clarity. Phospholipids were identified as follows: 1, origin for extract I containing traces of polyphosphoinositides (plus inorganic phosphate, if present); 2, sphingomyelin; 3, phosphatidylcholine; 4, probably a breakdown product of ethanolamine phospholipid; 5, phosphatidylinositol; 6, phosphatidylserine; 7, phosphatidic acid; 8, ethanolamine phospholipid; 9, phosphatidylglycerol; 10, 'cardiolipin'; 11, origin of extract II (inorganic phosphate, if present); 12, probably lysotriphosphoinositide; 13, triphosphoinositide; 14, probably lysodiphosphoinositide; 15, diphosphoinositide.

the interfacial scum. After washing, both extracts were evaporated to dryness in a stream of N_2 at 30–40°. Washed extracts were treated to denature proteolipid protein by the method of Dittmer & Dawson (1961). Extract I from grey matter appeared to contain a negligible amount of proteolipid, but in these experiments it was treated by the same procedure as extract II. After being boiled for a few minutes with acetone and then with ethanol (0·4ml. of each), with evaporation of residual solvent, lipid from the residue of extract I was dissolved in chloroform–methanol (1:1, v/v), and lipid from the residue of extract II in chloroform–methanol–conc. HCl (200:100:0·13, by vol.). The solutions were transferred to clean tubes and the solvent was evaporated. The residues were immediately dissolved in small volumes (about 50μl.) of the same solvent mixtures for transfer to the thin-layer plate.

Thin-layer chromatography. The whole of extract I was transferred in equal portions to two thin-layer strips. Two-dimensional chromatography was carried out on layers of silica gel H (E. Merck A.-G., Darmstadt, Germany) about 0·5mm. thick, a separate plate being clamped to the strips for the second-dimension development (Pumphrey, 1967). The solvent used for the first dimension was chloroform–methanol–aq. 7M-NH_3 (12:7:1, by vol.) and that for the second was chloroform–methanol–acetic acid–water (80:40:7·4:1·2, by vol.). Development proceeded for about 1·5hr. in each dimension, with an interval of 5–6min. to allow the strips to dry at room temperature. Prior equilibration of the freshly activated second-dimension plate with the solvent vapour was found to suppress false-front effects and streaking in the second dimension; the strips used for the first dimension were not equilibrated in this manner after activation.

Extract II lipid was chromatographed in one dimension on layers of silica gel H about 0·25mm. thick. The solvent was propan-1-ol–aq. 6·5M-NH_3 (70:27, v/v). The plates were developed for 2·5–3·0hr.

After being dried at room temperature, the plates were inserted into thin polythene bags for protection of the thin layer during radioautography (12–24hr. exposure of Kodak Blue Brand X-ray film). After radioautography, the plates were sprayed with conc. H_2SO_4 and charred at 160° for 5min. to reveal spots of major lipids (Fig. 1).

Identification of lipids. A preliminary identification of lipid spots was made from published migration rates in similar solvent systems. The identities of labelled spots were

checked by eluting the appropriate areas from thin-layer chromatograms of extracts heavily labelled with ^{32}P; the eluates were mixed with unlabelled extract I or II as carrier, and subjected to mild alkaline hydrolysis (Dawson, Hemington & Davenport, 1962). The hydrolysates were fractionated by one-dimensional paper chromatography with phenol saturated with water–acetic acid–ethanol (50:5:6, by vol.) as the developing solvent. Labelled spots were located by radioautography, and carrier spots by spraying with a ninhydrin or acid molybdate spray (Dawson *et al.* 1962). The migration of labelled spots was compared with data of Dawson *et al.* (1962).

This method of identification of hydrolysis products is ambiguous for the spot marked 'cardiolipin' in Fig. 1, since di(glycerylphosphoryl)glycerol has the same R_F as inorganic phosphate in the chromatographic system used. However, the only phospholipid reported to give inorganic phosphate as a major product after mild alkaline hydrolysis is acyl dihydroxyacetone phosphate (Hajra & Agranoff, 1968). This lipid has a monoesterified phosphate group. It may therefore be expected to resemble phosphatidic acid in its chromatographic behaviour, and to migrate slowly in the ammoniacal solvent used for the first dimension. On this basis, the spot marked 'cardiolipin' was thought unlikely to be acyl dihydroxyacetone phosphate. Lipid in this spot is referred to as cardiolipin throughout the rest of this paper.

The two spots labelled 'phosphatidic acid' both gave glycerophosphate on mild alkaline hydrolysis. The distribution of material between these two positions appeared to depend on slight variations in the conditions of chromatography, such as the order of addition of the plates to the tank.

Separation of water-soluble phosphates

Extraction. Each slice to be analysed for water-soluble phosphates was released from the slice holder into 1 M-$HClO_4$ at 0°, blotted on glass filter paper and then homogenized in 0·45 M-$HClO_4$–1 mM-EDTA (cf. Lowry, Passonneau, Hasselberger & Schulz, 1964). The volume of 0·45 M-$HClO_4$ used for the homogenization varied from 0·25 ml. to 0·4 ml. in different experiments. In subsequent calculations, 1 mg. of protein was taken to be equivalent to 10·7 μl. of slice fluid. After about 10 min., the homogenate was centrifuged, a measured volume of supernatant withdrawn to a clean tube and sufficient 2 M-$KHCO_3$ added to bring the pH into HCO_3^-–CO_2 buffering range. The precipitate of $KClO_4$ was centrifuged down, and samples of the supernatant equivalent to 5–10 mg. of tissue were removed for chromatography within the next hour. During these manipulations, tubes containing the extracts were kept on ice.

Thin-layer chromatography. Neutralized extracts were fractionated in duplicate, by using Bieleski's (1965) method for the two-dimensional t.l.c. of plant extracts. Thin layers were of cellulose MN300 (Macherey–Nagel, Düren, Germany) (washed with 2 M-acetic acid and water and were 0·5 mm. thick). The first solvent was propan-1-ol–aq. 11 M-NH_3 (3:2, v/v) and the second isopropyl acetate–aq. 56% (w/w) formic acid (11:8, v/v). Plates were developed at room temperature, twice (consecutively) in each dimension, for 2 hr. with the first and 1·5 hr. with the second solvent. They were dried for 15 min. between developments in a stream of air.

The above procedure gave a separation adequate for measuring the specific radioactivity of inorganic phosphate, γ-phosphate group of ATP and phosphorylethanolamine, and radioactivity in glycerophosphate. Phosphorylcholine was, however, overlapped by the much larger phosphocreatine spot; also, phosphorylethanolamine appeared to be partially overlapped by a small unidentified spot, possibly phosphoglycerate. To measure phosphorylcholine, extracts were separated on thin layers of cellulose, in the first dimension by chromatographic development with isopropyl acetate–aq. 56% (w/w) formic acid (11:8, v/v) and in the second by electrophoresis in 0·28 M-ammonium acetate buffer, pH 3·6, at 1800 v (about 20 A) for 20 min. This procedure separated phosphorylcholine from phosphocreatine, but caused partial breakdown of phosphocreatine in the first dimension, and in my hands streaking of ATP in the second dimension.

Radioautography of these plates was carried out as in the phospholipid procedure. Usually no spray was used; spots were located by the radioautograph.

Identification of spots. A preliminary identification of water-soluble phosphate spots was made by comparison with Bieleski's (1965) separation pattern. The chromatographic behaviour of authentic compounds was used to check identification. In case of doubt, ^{32}P-labelled extract was co-chromatographed with a sufficient quantity of authentic sample to be revealed by a stain for phosphates (Wade & Morgan, 1953). This method of identification does not exclude the possibility of unrecognized minor phosphates overlapping the compounds analysed.

Determination of specific radioactivity

Digestion of organic phosphorus. The spots scraped off from silica-gel and cellulose plates were each mixed with 0·08 ml. of conc. H_2SO_4 in a stoppered tube. The tubes were heated in an oven at 160–170° for 2 hr. Water (1·5 ml.) was added and the mixture was heated at 100° for 5 min., cooled and centrifuged. Samples of the supernatant were withdrawn for the measurement of phosphate and radioactivity.

The cellulose was incompletely digested by this procedure, leaving a charred residue. This did not appear to interfere with the release of phosphate from stable esters. Neither cellulose nor silica gel affected the extinction obtained with standard inorganic phosphate. Blank areas of both cellulose and silica gel made a small contribution to the reagent blank. Correction for this was made in all assays

Phosphate assay. Phosphate was determined by a modification of the methods of Chen, Toribara & Warner (1956) and Bartlett (1959).

Measurement of radioactivity. [^{32}P]Phosphate samples on 1·25 in.-diameter planchets were counted in a gas-flow counter (Nuclear–Chicago Corp., model 4338). The efficiency of counting was approximately 35%, and the background was about 8 c.p.m.

Calculation of relative specific radioactivity. At the end of each experiment, samples of the ^{32}P-labelled medium were taken from each beaker for the determination of specific radioactivity. The relative specific radioactivities of compounds in Tables 4 and 5 are the ratios c.p.m./μg.atom of P in compound/c.p.m./μg.atom of P in external inorganic phosphate.

Specific radioactivity of the γ-phosphate group of ATP. ATP spots were scraped from thin-layer plates immediately

after radioautography, and transferred to glass-stoppered tubes. The γ-phosphate group was liberated by the addition of 0·1 mg. of protein of a washed ox brain-microsome preparation (Rodnight, Hems & Lavin, 1966) to a suspension of the cellulose spot in a medium containing (final concentrations) 30 mM-tris–HCl buffer, pH 7·6, 100 mM-NaCl, 5 mM-KCl and 25 μM-$MgCl_2$ in a final volume of 1 ml. After 5 min. at 37° the reaction was terminated with 1 ml. of cold 1 M-$HClO_4$. Liberated inorganic phosphate was determined in the presence of silicotungstate (Lindberg & Ernster, 1956). After extraction of the phosphomolybdate with 1·25 ml. of 2-methylpropan-1-ol–benzene (1:1, v/v), samples of the organic phase were withdrawn for measurement of phosphomolybdate and radioactivity.

Trial measurement of the γ-phosphate of 26 μmoles of authentic ATP by this method showed that 90–100% of the calculated γ-phosphate was liberated as inorganic phosphate during the 5 min. incubation. When ADP was added, 5% of the calculated β-phosphate was liberated under the conditions of the ATP assay (cf. Table 6 of Rodnight *et al.* 1966).

Enzymic measurement of glycerophosphate. Glycerophosphate was present in amounts too small for measurement by phosphate assay of the thin-layer spot. Enzymic assays were therefore carried out on a separate series of slices. These slices were homogenized in 0·45 M-$HClO_4$–1 mM-EDTA, as for the chromatography of water-soluble phosphates, and the homogenates were centrifuged. The supernatants from individual slice homogenates were assayed for α-glycerophosphate by the method of Hohorst (1963), scaled down to a final reaction volume of 0·45 ml.

Other analytical procedures

Protein. Protein (excluding proteolipid) was determined by the biuret method (Gornall, Bardawill & David, 1949). Residues of slices extracted for phospholipids were dissolved directly in the biuret reagent; $HClO_4$ precipitates of slices extracted for water-soluble phosphates were extracted with chloroform–methanol (2:1, v/v) before being dissolved. Trials with slices weighed before incubation showed that the same yield of protein was obtained from the two sets of slices: 1 g. of fresh tissue gave 89·6 ± 1·1 mg. of protein (mean ± S.D. of six experiments).

Inulin and non-inulin space. Inulin was determined by the method of Varon & McIlwain (1961). The concentration and specific radioactivity of 'intracellular' inorganic phosphate were calculated on the assumptions: (1) that all inulin in the slice was extracellular; (2) that inulin in the extracellular space of the slice had the same concentration as inulin in the medium; (3) that inorganic phosphate in the extracellular space of the slice had the same concentration and specific radioactivity as inorganic phosphate in the medium. These assumptions are not necessarily valid. For the purpose of comparison with other workers' data on total inorganic phosphate of the slice, it should be stated that the calculated 'extracellular' inorganic phosphate averaged 23% of the total inorganic phosphate in control slices and 11% in stimulated slices.

RESULTS

Concentrations of phospholipids. Phospholipid concentrations found in five pairs of control and stimulated slices are shown in Table 1. No significant changes in concentration resulted from stimulation. Phosphatidylglycerol and phosphatidic acid were, however, present in concentrations too low for accurate measurements under the conditions employed here. Some change in these two lipids cannot be excluded.

Table 1. *Phospholipid concentrations in resting and stimulated slices*

Phospholipids were determined as described in the Experimental section. The values for each slice were obtained by averaging the results from two thin-layer plates. Usually duplicates of the major phospholipid spots (> 10 μmoles/g. of protein) differed by less than 10%. In two experiments, however, one of the duplicate phosphatidylcholine or ethanolamine phospholipid spots gave about half the expected phosphate yield, probably due to incomplete digestion of organic phosphate: these values were omitted. One high value for phosphatidylglycerol (5·5 μmole/g. of protein) was attributable to incomplete separation of the spot from ethanolamine phospholipid, and was also omitted. Results are expressed as means ± S.D., with the numbers of slices analysed in parentheses.

Phospholipid	Concentration (μmoles/g. of protein) Control slices	Stimulated slices
Sphingomyelin	31·5 ± 5·8 (5)	32·5 ± 4·6 (5)
Phosphatidylcholine	150 ± 6·5 (4)	157 ± 17 (3)
Ethanolamine phospholipid	146 ± 6·0 (3)	140 ± 13 (5)
Phosphatidylserine	55 ± 3·7 (5)	56·0 ± 6·3 (5)
Phosphatidylinositol	16·0 ± 1·5 (5)	15·1 ± 1·4 (5)
Phosphatidylglycerol	1·2 ± 0·7 (4)	1·5 ± 1·0 (5)
Cardiolipin	4·6 ± 0·4 (5)	4·7 ± 0·4 (5)
Phosphatidic acid	1·7 ± 0·5 (5)	1·8 ± 0·6 (5)
Diphosphoinositide	1·8 ± 0·3 (5)	2·1 ± 0·4 (5)
Triphosphoinositide	2·4 ± 0·6 (5)	2·3 ± 0·4 (5)

There are no comparable published data for phospholipids in the upper layers of guinea-pig cerebral cortex, but it is worth comparing the present results with values obtained for whole brain by different analytical methods (Table 2). The correspondence in phospholipid pattern is unexpected: the slices included no white matter, but nevertheless appeared to resemble whole brain in their content of some of the lipids (e.g. triphosphoinositide; Sheltawy & Dawson, 1966) thought to be characteristic of myelin. Eichberg, Whittaker & Dawson (1964) and Wells & Dittmer (1967) used hydrolytic methods of analysis, which (unlike the present method) distinguish between diacyl phosphatides and their alkyl or alkenyl ether analogues, but do not distinguish between diacyl phosphatides and lysophosphatides. In the thin-layer radioautograph, some very faint spots were often visible diagonally between the main labelled diacyl

Table 2. *Comparison of phospholipid percentages obtained by different analytical methods*

The guinea-pig cerebral-cortex phospholipids analysed in the present work (mean of all control and stimulated slices) are compared with the phospholipids in guinea-pig brain homogenate (Eichberg *et al.* 1964) and adult rat whole brain (Wells & Dittmer, 1967). All phospholipid values have been calculated as percentages of the total phospholipid phosphorus recovered after chromatography. The alkyl ether and alkenyl ether phospholipids determined separately by Eichberg *et al.* (1964) and Wells & Dittmer (1967) have been included with the corresponding diacyl phospholipids. The thin-layer spot that appeared to be a breakdown product of ethanolamine phospholipid in this study has similarly been included with the parent compound. —, Not determined.

	Percentage of total phospholipid phosphorus recovered in each phospholipid		
Phospholipid	Present work	Eichberg *et al.* (1964)	Wells & Dittmer (1967)
Sphingomyelin	7·52	9·0	5·85
Phosphatidylcholine (plus corresponding alkenyl ether lipid)	36·0	35·2	36·0
Ethanolamine phospholipid (including breakdown product)	34·2	36·7	35·4
Serine phospholipid	13·1	13·3	12·8
Phosphatidylinositol (plus corresponding alkenyl ether lipid)	3·67	3·1	3·44
Phosphatidylglycerol	0·33	—	0·41
Cardiolipin	2·18	1·4	1·57
Phosphatidic acid (plus corresponding alkenyl ether lipid)	0·41	1·0	2·11
Phosphatidylglycerol phosphate	—	—	0·11
Diphosphoinositide	0·92	—	0·57
Triphosphoinositide	1·65	—	1·67
Total phospholipid recovered (μg.atom of P/g. fresh weight)	38·1	58·5	70·1

Table 3. *Concentrations of some water-soluble phosphates*

Water-soluble phosphates were determined as described in the Experimental section. In the experiments of series 1, extracts from four pairs of slices were fractionated by two-dimensional t.l.c. In series 2, extracts from four pairs of slices were fractionated by t.l.c. and thin-layer electrophoresis. In series 3, glycerophosphate was measured enzymically in unfractionated extracts from six pairs of slices. Results are expressed as means ± S.D., with the numbers of slices analysed in parentheses.

		Concentration (μmoles/g. of protein)	
Series	Compound assayed	Control slices	Stimulated slices
1	Inorganic phosphate in intracellular space	20·6 ± 3·0 (4)	37·4 ± 7·3 (4)
	Phosphocreatine (plus phosphorylcholine)	42·5 ± 2·4 (4)	18·7 ± 2·0 (4)
	ATP	17·9 ± 1·2 (3)	15·6 ± 1·1 (3)
	Phosphorylethanolamine (plus unknown materials)	23·2 ± 6·4 (4)	19·5 ± 4·1 (4)
2	Phosphorylethanolamine	21·7 ± 1·0 (4)	20·8 ± 1·7 (4)
	Phosphorylcholine	4·0 ± 0·8 (4)	3·3 ± 1·3 (4)
3	Glycerophosphate	0·66 ± 0·20 (6)	1·84 ± 0·39 (6)

Table 4. *Relative specific radioactivities of some water-soluble phosphates*

Details of measurement are given in the Experimental section. Series 1 and series 2 have the same connotation as in Table 3. Compounds found in concentrations too low for accurate measurement of phosphorus are expressed as $10^3 \times$ relative specific radioactivity × concentration of lipid (μmoles/g. of protein): these values are in square brackets. Results are expressed as means ± S.D., with the number of slices analysed in parentheses.

	$10^3 \times$ Relative specific radioactivity			
	Control slices		Stimulated slices	
Compound Series ...	1	2	1	2
Inorganic phosphate in 'intracellular' space	321 ± 28 (3)		214 ± 10 (3)	
γ-Phosphate group of ATP	266 ± 28 (3)		241 ± 29 (3)	
α-Glycerophosphate	[121 ± 33 (3)]		[189 ± 34 (3)]	
Phosphorylethanolamine	21·9 ± 4·0 (3)	20·6 ± 0·2 (4)	9·1 ± 2·8 (3)	8·1 ± 0·6 (4)
Phosphorylcholine		56 ± 11 (4)		39 ± 16 (4)

phosphatides and the origin. These were tentatively identified as lysophosphatides. The most prominent of these faint spots was also visible by sulphuric acid charring of the thin-layer plate, and was ninhydrin-positive. When lipid extracts were left overnight before chromatography, this spot increased in amount with a corresponding loss of phosphorus from the main ethanolamine phospholipid spot. It was therefore probably derived from ethanolamine phospholipid. In the series of analyses shown in Table 1, this ninhydrin-positive spot amounted to $3{\cdot}3 \pm 0{\cdot}7$ μmoles/g. of protein. It was not thought worth while attempting to analyse the other smaller spots probably composed of lysophosphatides.

Concentrations of water-soluble phosphates. Concentrations of some water-soluble phosphates in control and electrically stimulated slices are shown in Table 3. ATP and α-glycerophosphate were included because they are precursors of the phosphorus in phosphatidic acid and phospholipids synthesized by the CDP–diglyceride pathway. CDP-choline and -ethanolamine, the immediate precursors of the phosphorus in choline and ethanolamine phospholipids, were not located on the thin-layer plate. This was expected from their low concentrations *in vivo* (Ansell & Spanner, 1967). Phosphoryl-choline and -ethanolamine, the next nearest precursors, were measured instead. The other two large water-soluble-phosphate fractions, inorganic phosphate and phosphocreatine, were also measured for an index of the effect of the low-frequency electrical stimulation on the high-energy-phosphate reserves in the slice.

As demonstrated with a higher frequency of electrical pulses by McIlwain & Gore (1951), stimulation by 5 pulses/sec. caused a marked fall in phosphocreatine concentration. The rise in inorganic phosphate after stimulation for 1 hr. was rather less than equivalent to the fall in phosphocreatine. Glycerophosphate was increased by stimulation. The other compounds listed in Table 3 did not show significant changes in concentration.

The sum of phosphocreatine plus inorganic phosphate in control slices is close to the values reported by Lowry *et al.* (1964) for whole brain of young rats, fixed in liquid nitrogen. The phosphocreatine/phosphate ratio is higher than that reported for conscious rats: it is closer to the ratio in anaesthetized or cooled (18°) animals (Lowry *et al.* 1964). Glycerophosphate concentration in control slices is not significantly different from the normal concentration *in vivo* found by Lowry *et al.* (1964) in young rats. The change occurring with electrical stimulation is in the same direction as the change occurring in ischaemia in rat whole brain (Lowry *et al.* 1964). The control-slice ATP concentrations are substantially lower than values *in vivo*, but are

Table 5. *Relative specific radioactivities of phospholipids in resting and stimulated slices*

Details of measurement are given in the Experimental section; see also the legend to Table 1. The results for phospholipids present in concentrations too low for accurate measurement of phosphorus are expressed as $10^3 \times$ relative specific activity $\times$ concentration of lipid (μmoles/g. of protein); these values are given in square brackets.

	$10^3 \times$ Relative specific radioactivity												
	Control slices						Stimulated slices						*P*
Expt. no.	1	2	3	4	5	Mean ± S.D.	Expt. no. ..	1	2	3	4	5	Mean ± S.D.	(Student's *t* test)
Phosphatidylcholine	0·71	0·84	0·66	—	1·05	0·82 ± 0·17		0·39	0·58	0·36	—	—	0·44 ± 0·12	< 0·025
Ethanolamine phospholipid	0·156	0·161	0·145	—	—	0·154 ± 0·008		0·062	0·096	0·087	0·070	0·109	0·085 ± 0·019	< 0·005
Phosphatidylinositol	12·4	15·0	12·4	14·5	13·6	13·6 ± 1·2		31·0	23·1	27·3	20·2	24·9	25·3 ± 4·1	< 0·001
Phosphatidylglycerol	[2·7]	[1·8]	[1·9]	[2·8]	[2·6]	[2·4 ± 0·5]		[2·6]	[3·0]	[2·5]	[2·5]	[4·5]	[3·0 ± 0·8]	< 0·3
Cardiolipin	0·118	0·056	0·095	0·105	0·071	0·089 ± 0·025		0·058	0·040	0·050	0·052	0·062	0·052 ± 0·007	< 0·02
Phosphatidic acid	[94]	[95]	[69]	[187]	[135]	[116 ± 46]		[138]	[148]	[137]	[176]	[197]	[159 ± 26]	< 0·2
Diphosphoinositide	121	88	74	113	96	98 ± 19		102	67	84	70	79	80 ± 14	< 0·2
Triphosphoinositide	147	117	103	115	107	118 ± 17		140	126	110	101	101	116 ± 17	> 0·8

similar to concentrations found in slices elsewhere (see, e.g., Thomas, 1957; Rolleston & Newsholme, 1967). Phosphorylethanolamine concentrations appear to be rather higher than is usual in adult rat whole brain (Ansell & Spanner, 1959). Phosphorylcholine concentrations are not significantly different from those found for adult rats by Ansell & Spanner (1959).

Specific radioactivities. Table 4 shows the relative specific radioactivities of 'intracellular' inorganic phosphate and some precursors of phospholipid phosphorus. Electrical stimulation caused a fall in the relative specific radioactivity of inorganic phosphate in the slice. Under different conditions, a similar effect of electrical stimulation was noticed by Heald (1956); he attributed it to faster equilibration of phosphorus in the trichloroacetic acid-insoluble fraction with that in the acid-soluble phosphates. The relative specific radioactivity of the γ-phosphate group of ATP was decreased slightly but not significantly by electrical stimulation. It was closer to that of inorganic phosphate in stimulated than in resting tissue; this is consistent with the increase in ATP turnover expected to occur during increased tissue activity. The relative specific radioactivity of glyceroposphate can be estimated approximately by using the radioactivity from the thin-layer plate (Table 4) and the enzymic assay of concentration (Table 3); from these the control value for $10^3 \times$ relative specific radioactivity is 183 and the stimulated is 103.

Table 5 shows the relative specific radioactivities of the phospholipids themselves. Sphingomyelin and phosphatidylserine did not incorporate detectable radioactivity in the 1 hr. incubation with [^{32}P]phosphate and were therefore omitted from the table. Electrical stimulation decreased incorporation of ^{32}P label into phosphatidylcholine, ethanolamine phospholipid and cardiolipin, and caused a highly significant increase in incorporation into phosphatidylinositol. It tended to increase incorporation into phosphatidic acid; this increase was not statistically significant if the slices were treated as a homogeneous group (cf. Larrabee *et al.* 1963; Larrabee & Leicht, 1965), but was ($P < 0{\cdot}05$) if they were treated as individual pairs, which may differ in their basal level of phosphatidic acid labelling.

DISCUSSION

The aim of this investigation was to test how far phospholipid turnover in cerebral cortex is affected by the state of activity of the tissue.

If turnover conformed to the idealized steady-state system:

$$\xrightarrow{k} \text{Precursor } A \text{ (Sp.radioact. } \alpha \text{ c/mole)} \xrightarrow{k \text{ (mole/unit time/unit wt. of tissue)}} \text{Product } B \text{ (Sp.radioact. } \beta \text{ c/mole, pool size } [B] \text{ mole/unit wt. of tissue)} \xrightarrow{k}$$

it would follow that:

$$\frac{d\beta}{dt} = \frac{k\,(\alpha-\beta)}{[B]} \text{ (c/mole/unit wt. of tissue)} \qquad (1)$$

or using integrals, for time interval t_1 to t_2:

$$\frac{k}{[B]} = \frac{\beta_{t_2} - \beta_{t_1}}{\int_{t_1}^{t_2} \alpha \,.\, dt - \int_{t_1}^{t_2} \beta \,.\, dt} \text{ [(unit time)}^{-1}\text{]} \qquad (2)$$

[Eqn. (2) is illustrated graphically in Fig. 5.3 of Rescigno & Segre (1966).] Table 6 shows turnover rates estimated for the phosphate groups of phospholipids and precursors on the basis of this simplified model. Relative specific radioactivities (p) of inorganic phosphate (or of ATP where this appeared to be greater; see Table 4) were described by $p = 1 - e^{-K_p t}$, where K_p is a constant fixed by the experimental value of p at $t = 1$ hr. Relative specific radioactivities of phosphate compounds derived in series from intracellular inorganic phosphate and ATP were described by eqns. (1) and (2). The approximate value of $k/[B]$ for each product in the series was determined graphically by trial, to fit the experimental values of β at $t = 1$ hr.

Cerebral cortex is not an ideal tissue for analysis of turnover along these lines. One inevitable complication arises from the multiplicity of cell types. This implies the possibility of compartmentation of lipid and precursor. Glial and neuronal metabolism, for example, may react in different ways to electrical stimulation. If, in addition, the different cell types contain different molar precursor/product ratios, the average specific radioactivity in the whole tissue will have rather doubtful significance.

Apart from the problem of compartmentation, the simplified treatment used for Table 6 diverges from conditions in the tissue in at least two ways. First, some low-concentration intermediates known to exist in the tissue (CDP-ethanolamine, -choline, and -diglyceride, and phosphatidylglycerophosphate) are ignored in the model. Secondly, each phosphate in the model is treated as though it had only one immediate precursor. The first simplification leads to underestimation of phospholipid turnover. The magnitude of this error can be predicted in some cases where the probable pool size of the ignored intermediate is known, provided that each phosphate involved has only one immediate precursor. Thus, omission of CDP-

Table 6. *Turnover rates estimated for phosphate groups of phospholipids and precursors*

The rates were estimated on the basis described in the text. The values for concentration and specific radioactivity used in these estimations were taken from Tables 1, 3, 4 and 5, except that: (*a*) values used for the phosphorylcholine and phosphatidylcholine rates given in parentheses excluded phosphorylcholine values from two analyses considered to be less reliable; (*b*) values used for phosphorylethanolamine and ethanolamine phospholipid excluded phosphorylethanolamine values obtained by two-dimensional chromatography. Where compounds had 'concentrations too low for accurate measurement' (see the legends to Tables 4 and 5), an approximation to their mean specific radioactivity was obtained by dividing the mean radioactivity/g. of protein (Table 5) by the mean concentration (Table 1). The range of rates given for di- and tri-phosphoinositides (monoesterified phosphates) allows for variation in the specific radioactivity of the diesterified phosphate, from the values found for phosphatidylinositol to zero (see Brockerhoff & Ballou, 1962). Cardiolipin was omitted because of uncertainty about its precursors and the extent to which they are compartmented inside mitochondria.

Compound	'Precursor' for purpose of calculation	Fractional turnover rate (fraction of pool replaced/hr.)		Turnover rate (μmoles replaced/g. of protein/hr.)	
		Control	Stimulated	Control	Stimulated
Phosphorylcholine	γ-Phosphate of ATP	0·55 (0·47)	0·35 (0·23)	2·2 (1·7)	1·3 (0·8)
Phosphatidylcholine	Phosphorylcholine	0·048 (0·055)	0·032 (0·048)	7·2 (8·3)	5·1 (7·5)
Phosphorylethanolamine	γ-Phosphate of ATP	0·185	0·066	4·0	1·4
Ethanolamine phospholipid	Phosphorylethanolamine	0·025	0·032	3·6	4·5
Phosphatidic acid	γ-Phosphate of ATP	0·70	0·92	1·2	1·7
Phosphatidylinositol	Phosphatidic acid	0·72	1·02	11·5	15·4
Phosphatidylglycerol	Phosphatidic acid	0·10	0·065	0·13	0·10
Diphosphoinositide (monoesterified phosphate)	γ-Phosphate of ATP	3·5–4·3	1·8–2·6	6·3–7·7	3·8–5·5
Triphosphoinositide (mean of monoesterified phosphates)	γ-Phosphate of ATP	2·9–3·1	2·6–3·2	7·1–7·5	6·1–7·3

ethanolamine (concentration 0·025 μmole/g. of rat brain; Ansell & Spanner, 1967) from the model would not in theory make much difference to the estimated turnover of ethanolamine phospholipid; but the omission of phosphatidylglycerophosphate (concentration about 30% of the concentration of phosphatidylglycerol, in rat brain; Wells & Dittmer, 1967) could cause the estimated turnover of phosphatidylglycerol in Table 6 to be as little as 20% of the real value. The second simplification is obviously inappropriate in the case of phosphatidic acid, which is synthesized by the acylation of glycerophosphate as well as by the phosphorylation of diglyceride (see, e.g., Hokin & Hokin, 1959). It is also inappropriate where recycling occurs, i.e. where a biosynthetic-precursor pool is fed by products of a degradative reaction (as happens to inorganic phosphate itself), or where a biosynthetic reaction is reversible to an appreciable extent under the conditions prevailing in the slice. For example, replacement of phosphatidylcholine phosphorus appears faster than phosphorylation of choline in Table 6. This strongly suggests a relatively rapid exchange of the phosphorylcholine radical between the lipid and its water-soluble precursors (cf. Bjørnstad & Bremer, 1966). Similarly, other precursor pools may be fed from low-specific-radioactivity sources as well as from ATP. Thus the apparent rate of replacement of phosphatidic acid seems rather low in relation to that of phosphatidylinositol, even when an approximate allowance is made for the alternative synthetic route from glycerophosphate.

These considerations must be borne in mind in assessing Table 6. Nevertheless, it seems reasonable to conclude that slight to moderate differences in phospholipid turnover exist between the resting and activated tissue slices. In no case does the estimated turnover rate in the stimulated slices differ from control by more than 50%. Possibly larger changes could be demonstrated by using higher frequencies of stimulation or shorter periods of incubation. The biggest effect of electrical pulses (an increase of about 4 μmoles/g. of protein/hr.) is with phosphatidylinositol. It is not clear from the results which enzymic reactions are responsible for this change. But since the concentration of phosphatidylinositol is not significantly altered after stimulation for 1 hr., both synthetic and degradative directions must be affected to about the same extent.

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REFERENCES

Abood, L. G., Goldman, E. & Lipman, V. (1957). *J. Neurochem.* **2**, 318.

Ansell, G. B. & Spanner, S. (1959). *J. Neurochem.* **4**, 325.

Ansell, G. B. & Spanner, S. (1967). *J. Neurochem.* **14**, 873.

Bartlett, G. R. (1959). *J. biol. Chem.* **234**, 466.

Bieleski, R. L. (1965). *Analyt. Biochem.* **12**, 230.

Bjørnstad, P. & Bremer, J. (1966). *J. Lipid Res.* **7**, 38.

Brockerhoff, H. & Ballou, C. E. (1962). *J. biol. Chem.* **237**, 49.

Chen, P. S., Toribara, T. Y. & Warner, H. (1956). *Analyt. Chem.* **28**, 1756.

Dawson, R. M. C., Hemington, N. & Davenport, J. B. (1962). *Biochem. J.* **84**, 497.

Dawson, R. M. C. & Richter, D. (1949). *J. Physiol.* **109**, 21*P*.

Dittmer, J. C. & Dawson, R. M. C. (1961). *Biochem. J.* **81**, 535.

Eichberg, J., Whittaker, V. P. & Dawson, R. M. C. (1964). *Biochem. J.* **92**, 91.

Gornall, A. G., Bardawill, C. S. & David, M. M. (1949). *J. biol. Chem.* **177**, 751.

Hajra, A. K. & Agranoff, B. W. (1968). *J. biol. Chem.* **243**, 1617.

Heald, P. J. (1956). *Biochem. J.* **63**, 242.

Hohorst, H.-J. (1963). In *Methods of Enzymatic Analysis*, p. 215. Ed. by Bergmeyer, H. U. New York: Academic Press Inc.

Hokin, L. E. & Hokin, M. R. (1959). *J. biol. Chem.* **234**, 1387.

Keesey, J. C., Wallgren, H. & McIlwain, H. (1965). *Biochem. J.* **95**, 289.

Larrabee, M. G., Klingman, J. D. & Leicht, W. S. (1963). *J. Neurochem.* **10**, 549.

Larrabee, M. G. & Leicht, W. S. (1965). *J. Neurochem.* **12**, 1.

Lindberg, O. & Ernster, L. (1956). *Meth. biochem. Anal.* **3**, 1.

Lolley, R. N. (1963). *J. Neurochem.* **10**, 665.

Lowry, O. H., Passonneau, J. V., Hasselberger, F. X. & Schulz, D. W. (1964). *J. biol. Chem.* **239**, 18.

McIlwain, H. (1966). *Biochemistry and the Central Nervous System*, 3rd ed., p. 55. London: J. and A. Churchill Ltd.

McIlwain, H. & Gore, M. B. R. (1951). *Biochem. J.* **50**, 24.

McIlwain, H. & Joanny, P. (1963). *J. Neurochem.* **10**, 313.

McIlwain, H. & Rodnight, R. (1962). *Practical Neurochemistry*. London: J. and A. Churchill Ltd.

Majno, G., Gasteiger, E. L., La Gattuta, M. & Karnovsky, M. L. (1958). *J. Neurochem.* **3**, 127.

Pumphrey, A. M. (1967). *Biochem. J.* **102**, 30P.

Rescigno, A. & Segre, G. (1966). *Drug and Tracer Kinetics*, 1st American ed., p. 160. Waltham, Mass.: Blaisdell Publishing Co.

Richards, C. D. & Sercombe, R. (1968). *J. Physiol.* **196**, 94*P*.

Rodnight, R., Hems, D. A. & Lavin, B. E. (1966). *Biochem. J.* **101**, 502.

Rolleston, F. S. & Newsholme, E. A. (1967). *Biochem. J.* **104**, 524.

Sheltawy, A. & Dawson, R. M. C. (1966). *Biochem. J.* **100**, 12.

Thomas, J. (1957). *Biochem. J.* **66**, 665.

Varon, S. & McIlwain, H. (1961). *J. Neurochem.* **8**, 262.

Wade, H. E. & Morgan, D. M. (1953). *Nature, Lond.*, **171**, 529.

Wells, M. A. & Dittmer, J. C. (1967). *Biochemistry*, **6**, 3169.