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EXPLANATION OF PLATE 1

Fig. 1. Electron micrograph of a representative field of casein particles (precipitate B_1) from separated milk fraction.

Fig. 2. Electron micrograph of a representative field of lipoprotein particles or 'milk microsomes' (precipitate A_1) from buttermilk fraction.

Fig. 3. Electron micrograph of a representative field of the final supernatant (A_3) from buttermilk fraction. Note an intact fat globule with a protein membrane (upper centre) and several collapsed fat globules.

Studies on the Labelling of Brain Phospholipids with Radioactive Phosphorus

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During the last decade extensive work with radioactive phosphorus (^{32}P) has shown that the phospholipids present in the adult mammalian brain are constantly being broken down and resynthesized. However, little attention has been paid to the relative rates at which the individual phospholipids are metabolized and become labelled with the isotope. This is largely due to the lack of suitable methods for the fractionation of brain phospholipids, especially when only small samples of tissue are available. The use of column and paper chromatography for this purpose has proved somewhat disappointing (Collins, private communication; Zechmeister, 1950; Lea & Rhodes, 1953), while

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countercurrent distribution methods have so far not given adequate separation of the kephalins (Lovern, 1952; Cole, Lathe & Ruthven, 1953). Moreover, it is difficult to apply the brilliant pioneer work of Folch (1942) on the solvent fractionation of phospholipids to this problem, as the method requires an appreciable quantity of tissue, is time-consuming and does not give products which are strictly homogeneous (Clouet, 1953).

An attempt has therefore been made to develop suitable methods for the examination of the phospholipids present in small samples of brain tissue after they have become labelled with ^{32}P. The specific radioactivities of the components of the kephalin fraction, i.e. diphosphoinositide, phosphatidylserine and phosphatidylethanolamine, have been measured by hydrolysing the mixture of phospholipids with various agents and separating identifiable phosphorus-containing and water-soluble breakdown products of the kephalins by paper chromatography.

The methods have been applied to study the incorporation of ^{32}P into the lipids of a guinea pig brain dispersion after incubation with labelled phosphate under conditions which ensured an appreciable uptake of radioactivity (Dawson, 1953*a*). Observations have also been made on the incorporation of ^{32}P into the phospholipids of liver and brain, both in slices and in the living animal.

Preliminary reports of this work have already been published (Dawson, 1953*b*, *c*). In a recent note Streicher (1953) has reported the specific radioactivities of various crude solvent extracts of a ^{32}P-labelled lipid mixture prepared from a rat-brain 'homogenate'.

EXPERIMENTAL

Adult guinea pigs were used throughout the investigation. Dispersions of the whole brain were prepared as described previously (Dawson, 1953*a*). They were incubated at 37° in a medium containing: 0·05 M glycylglycine buffer, pH 7·2; 0·001 M sodium phosphate buffer, pH 7·2; 0·03 M sodium pyruvate; 0·003 M sodium fumarate; 0·001 M potassium adenylate; $2{\cdot}1 \times 10^{-5}$ M cytochrome *c*; 0·02 M-NaF and carrier-free labelled phosphate. In later experiments, cytochrome *c* was omitted because it was found to cause no appreciable stimulation of the incorporation of ^{32}P into the lipids when the concentration of tissue was 80 mg./ml., although at lower tissue concentrations it enhanced the uptake (Dawson, 1953*a*).

Table 1. *Removal of carrier-free labelled inorganic phosphate from a solution of brain lipids in chloroform:methanol by washing with* 0·25 M *magnesium chloride and* N-HCl

Conditions	Radioactivity (%) remaining after washing with N-HCl	Radioactivity (%) remaining after washing with 0·25 M-$MgCl_2$
Shaken for 5 min. with 2 vol. wash liquor	1·2	9·6
Addition of 1 vol. methanol to $CHCl_3$ layer from above; shaken for 5 min. with 1·6 vol. wash liquor	0·8	1·7

After the tissue had been precipitated with trichloroacetic acid (TCA) the lipids were extracted by a technique reported in an earlier paper (Dawson, 1953*a*). Washing the lipid extract with N-HCl, to remove contaminating acid-soluble phosphorus, was avoided because of the possibility that bases would be hydrolysed off the phospholipids (Wadsworth, Maltaner & Maltaner, 1927) which would interfere with the subsequent fractionation. Consequently, the $CHCl_3$:methanol extract was washed twice by shaking with 0·25 M-$MgCl_2$ solution. The aqueous and $CHCl_3$ layers tended to separate far less readily, however, and as can be seen from Table 1 the removal of contaminating inorganic phosphate was not so effective.

Fractionation of the phospholipids

The initial fractionation of the lipid extract was performed to group the phospholipids rather than to separate individual components (Fig. 1). To the washed $CHCl_3$ extract of the lipids obtained from one Warburg vessel (240 mg. brain) was added 0·5 ml. of a 1% solution of beeswax in $CHCl_3$. (This was found to facilitate the separation and centrifugation of an initial methanol-insoluble fraction, although it in no way altered the specific radioactivity or amount of phosphorus in this fraction; Table 2). The $CHCl_3$ solution of lipids and beeswax was taken to dryness under reduced pressure, and the residue completely dissolved in 10 ml. of boiling methanol. The methanol solution was cooled for 30 min. at 0°, and the methanol-insoluble fraction (3·5–6% of total P) centrifuged down and washed twice with 5 ml. portions of methanol. The combined supernatants were filtered through glass wool and treated with 200 mg. of MgO (B.D.H. 'specially pure' grade). The mixture was allowed to stand for 30 min. with occasional stirring, and the MgO-adsorbed fraction (46–57% of total P) centrifuged down and washed twice with 3 ml. portions of methanol. The combined supernatants were taken to dryness under reduced pressure and the residue was incubated for 20 hr. at 37° with 3 ml. N-NaOH. The solution was cooled in ice and 3 ml. N-HCl and 4 ml. 20% (w/v) TCA were added. The unsaponified lipids (6% of total P) were filtered off and thoroughly washed on the paper with 10% (w/v) TCA. The filtrate contained the phosphorus from the saponified lipids (31–42% of total P).

Determination of the specific radioactivities of kephalins

Phosphatidylethanolamine. The specific radioactivity of this component was determined by hydrolysing the original lipid mixture with $HgCl_2$ solution and separating glycerylphosphorylethanolamine from the hydrolysate (Norman & Dawson, 1953).

Phosphatidylserine. The specific radioactivity of phosphatidylserine was obtained by hydrolysing the original lipid extract with alkali and separating a product which is probably glycerylphosphorylserine by paper chromatography. Initial experiments were carried out with phos-

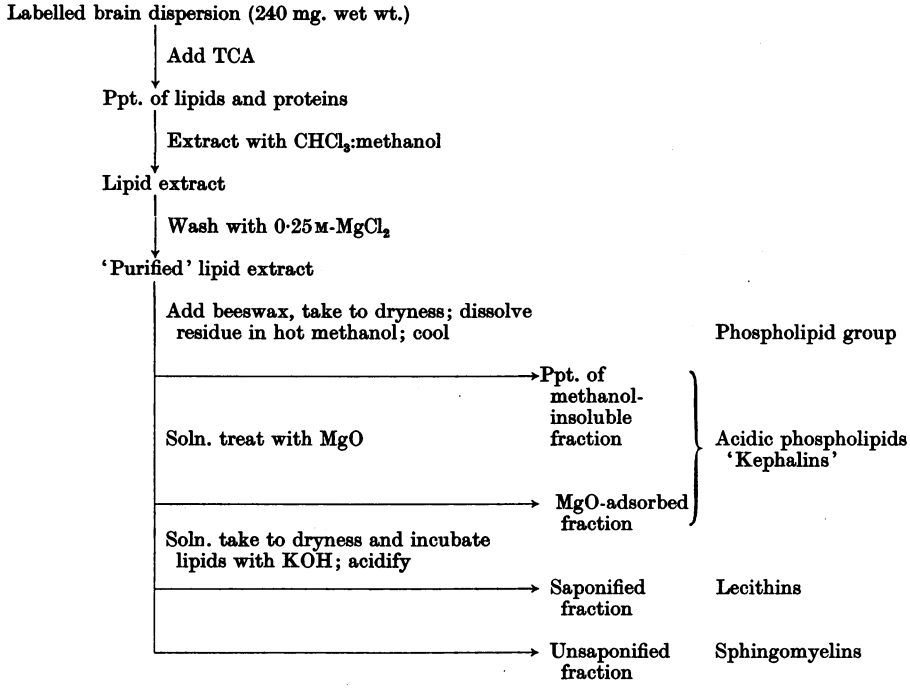

Fig. 1. Fractionation of phospholipids in a labelled brain dispersion.

Table 2. *The specific radioactivity and amount of phosphorus in the methanol-insoluble fraction isolated with and without the addition of beeswax*

	Methanol-insoluble fraction	
	Phosphorus in 1 g. brain dispersion (μg.)	Specific radioactivity (counts/min./mg. P)
Fraction isolated alone	72	28 500
Fraction isolated after the addition of beeswax	70	29 000

phatidylserine prepared from ox brain by the method of Folch (1942). When this was hydrolysed under mildly alkaline conditions over 55% of its phosphorus was converted into a water-soluble phosphorus-containing derivative of serine, while there was little formation of free serine or glycerophosphoric acid. On completely hydrolysing this derivative in N-HCl at 100° for 1 hr., serine and glycerophosphoric acid, but no inorganic phosphate, were found in the hydrolysate. It is therefore likely that the substance is glycerylphosphorylserine. It was found that, by using two-dimensional filter-paper chromatography and employing suitable solvent systems, the 'glycerylphosphorylserine' present in the alkaline hydrolysate of a brain-lipid extract could be adequately separated from the alkaline hydrolysis products of the other phospholipids.

The labelled-lipid extract from 240 mg. of brain was washed with $MgCl_2$ solution and taken to dryness. The residue was dissolved by warming gently with 1·6 ml. of methanol containing 0·2 ml. CCl_4. Water (0·22 ml.) was then added to the solution followed by 0·5 ml. of N-NaOH in methanol. The mixture was incubated for 15 min. at 37° and then cooled and diluted with 8 ml. of ice-cold water. The milky solution was passed through a column of Amberlite I.R.C. 50 resin (7 × 1 cm. diameter) to remove alkali. The effluent and washings (25 ml.) were extracted twice with ether, neutralized to pH 6·8 with ammonia and taken to dryness under reduced pressure, keeping the temperature below 50°; the residue was extracted with water and the extract spotted on to a filter-paper sheet. A two-dimensional chromatogram was developed with the following solvents (*a*) propanol:ammonia (0·880):water (60:30:10, v/v), (*b*) *tert.*-butanol:water (62:38, v/v), TCA (10%, w/v). After drying, the chromatogram was washed twice in ether to remove TCA and sprayed with 0·1% ninhydrin solution in water-saturated butanol containing 0·1% pyridine. Two ninhydrin-reacting spots became visible on heating the chromatogram. The substance which travels the shorter distance in the propanol:ammonia solvent is the phosphorus-containing hydrolysis product of phosphatidylserine (probably glycerylphosphorylserine) and has R_F values of 0·25 and 0·34 in solvents (*a*) and (*b*), respectively. Alkaline hydrolysis products of phosphatidylethanolamine and phosphatidylcholine, and also glycerophosphoric acid and inorganic phosphate were thus separated from the 'glycerylphosphorylserine'. The specific radioactivity of the phosphorus in the spot was then measured by the procedure of Ansell & Dawson (1951). During the cutting out of the spot care was taken to avoid a faint phosphorus-containing spot with high specific activity which ran slightly more slowly in the *tert.*-butanol:TCA solvent. This spot was shown to be an alkaline hydrolysis product of diphosphoinositide.

Diphosphoinositide. The method used for determining the specific radioactivity of diphosphoinositide in the labelled dispersion was based upon the observation of Folch (1949) that the phosphorus of diphosphoinositide is largely converted into inositol-*m*-diphosphate during a short hydrolysis with HCl. When the methanol-insoluble lipid fraction was hydrolysed with HCl, the hydrolysate gave, after chromatography on a *tert.*-butanol:TCA paper chromatogram, a small phosphorus-containing spot which was inseparable from an inositol-*m*-diphosphate marker (kindly supplied by Dr G. H. Sloane-Stanley). Acid-hydrolysates of pure diphosphoinositide showed a spot in the same position, while hydrolysates of phosphatidylserine, phosphatidylethanolamine, phosphatidylcholine and sphingomyelin gave no equivalent spot. This method of acid hydrolysis followed by chromatographic isolation of inositol-*m*-diphosphate has been applied to: (1) the diphosphoinositide, soluble in fat solvents, present in the methanol-insoluble lipid fraction; (2) the diphosphoinositide, insoluble in fat solvents, present in the residue after freeing cerebral tissue from acid-soluble phosphorus, lipids and nucleic acids. The specific radioactivity of the diphosphoinositide insoluble in fat solvents was also determined by the procedure of Strickland (1952). In this method (3) a nucleic-acid- and lipid-free residue from brain is hydrolysed with alkali and the acid-soluble ester phosphorus in the hydrolysate is assumed to arise from diphosphoinositide.

(1) The combined crude lipid extract from five Warburg vessels (1·2 g. brain) was washed with N-HCl and the methanol-insoluble fraction prepared. This was hydrolysed for 25 min. with 25 ml. 6N-HCl under reflux. The hydrolysate after cooling was washed with 50 ml. of $CHCl_3$ and taken to dryness under reduced pressure. The residue was taken up in 25 ml. of water and passed through a column of Amberlite I.R. 100H resin to remove cations. Before the treatment with Amberlite the hydrolysate contained appreciable quantities of cations, even though the lipids had been precipitated with TCA and washed with HCl. The removal of these cations was essential for the subsequent chromatography of the inositol diphosphate in the hydrolysate, as otherwise this stuck tenaciously to its point of application on the paper. The hydrolysate was concentrated by evaporation and applied to a paper which had been well washed with 2N acetic acid and 8-hydroxyquinoline. A one-dimensional chromatogram was prepared using the *tert.*-butanol:TCA solvent described previously. After drying, the paper was sprayed with the acid-molybdate phosphorus-detecting reagent of Hanes & Isherwood (1949), the blue spots being developed with ultraviolet irradiation (Bandurski & Axelrod, 1951). The inositol diphosphate spot whose R_F value varied between 0·22 and 0·28 was cut out and assayed for radioactivity and phosphorus content (Ansell & Dawson, 1951).

(2) Labelled brain tissue (0·6 g.) was precipitated with TCA and the lipids were extracted according to the procedure of Strickland (1952); nucleic acids were removed from the 'lipid-free' residue by the method of Schneider (1945). The residue was then hydrolysed with 6N-HCl and the inositol diphosphate separated by the procedure described for method (1).

(3) This followed the procedure of Strickland (1952). The TCA precipitate from 0·15 g. brain was freed from lipids and nucleic acids and the residue hydrolysed with alkali according to Schmidt & Thannhauser (1945). After acidification and filtration, the ester phosphorus in the filtrate, which had been derived from diphosphoinositide, was freed from inorganic phosphate by the method of Friedkin & Lehninger (1949) and assayed for radioactivity and phosphorus.

Phosphorus estimations

The procedures of Fiske & Subbarow (1925) and Berenblum & Chain (1938) were used to estimate phosphorus. Organic phosphorus was converted into the inorganic form by oxidation with $HClO_4$ and H_2O_2. In agreement with the observations of Börgstrom (1952) it proved difficult to elute the phospholipids adsorbed on MgO. The MgO was therefore dissolved in $HClO_4$ and the oxidation of the lipids performed as usual. It was shown that in the presence of considerable quantities of $Mg(ClO_4)_2$ the colour development of phosphate in the Fiske & Subbarow method still obeyed Beer's Law.

The spraying of chromatograms with acid-molybdate solution to detect phosphorus was observed to catalyse the subsequent oxidation of the paper spot by $HClO_4$. However, a slight water-insoluble residue was usually found adhering to the walls of the oxidation vessel, but this did not seem to affect the quantitative recovery of phosphorus. The Berenblum & Chain procedure gave quantitative recoveries from inorganic phosphate spots (1 and 10 μg.) which had been sprayed with acid-molybdate and irradiated with ultraviolet light.

RESULTS

In initial experiments fractionations of the phospholipids were performed after brain dispersions had been incubated for 1 hr. in the presence of labelled phosphate. It was found that the distribution of radioactivity was by no means uniform (Table 3), the major part of the ^{32}P incorporated being found in the methanol-insoluble phospholipids and the phospholipids which were adsorbed on magnesium oxide. The specific radioactivity of the methanol-insoluble fraction varied between 344 and 790% of that of the whole phospholipid fraction in six independent experiments. The composition of this small fraction is complicated and no complete analysis was undertaken. However, chromatographic experiments showed it to contain about 32% of phosphatidylserine and less than 5% of phosphatidylethanolamine, while nearly 20% of its phosphorus was stable to mild alkaline hydrolysis.

It has been shown that when magnesium oxide is added to a methanolic solution of liver phospholipids it completely adsorbs the 'kephalins', leaving the choline-containing lecithins and sphingomyelins in solution (Entenman, Taurog & Chaikoff, 1944). In the present experiments it was confirmed that none of the brain choline-containing phospholipids were adsorbed from a methanolic solution by magnesium oxide. It was apparent therefore that in brain dispersions the incorporation of ^{32}P into the phospholipids was largely confined to the 'kephalin' fraction, while the synthesis of the choline-containing phospholipids was comparatively slow.

Table 3. *Fractionation of the lipids of a guinea pig brain dispersion after incubation with labelled phosphate*

Fresh brain (240 mg.) incubated for 1 hr. at 37° in air with ^{32}P (10^6 counts/min.) and reaction mixture defined in the Experimental section. Figures in brackets indicate that the counting rate was too low for accurate measurement.

	No incubation	Incubated 1 hr.	Incubated 1 hr. with 0·001M azide
Oxygen uptake (μl.)	—	155	190
Specific radioactivities of lipid fractions (counts/min./mg. P):			
Methanol-insoluble	(780)	11 400	7 900
Adsorbed on MgO	450	6 350	2 450
Unadsorbed, saponified	(90)	286	(131)
Unadsorbed, unsaponified	(0)	(0)	(0)
Total radioactivity of lipid fractions (counts/min.):			
Methanol-insoluble	(3)	142	54
Adsorbed on MgO	45	641	260
Unadsorbed, saponified	(14)	36	(17)
Unadsorbed, unsaponified	(0)	(0)	(0)

The residual phospholipids which were not adsorbed on magnesium oxide had a low specific radioactivity, the whole of which was confined to the saponified or 'lecithin' fraction. Although magnesium oxide adsorbed none of the choline-containing phospholipids, it was found that the choline:phosphorus ratio of the unadsorbed phospholipids was just below unity. As the extracts were free from non-lipid phosphorus this indicated that with brain phospholipids a little of the non-choline containing phospholipids remained unadsorbed by magnesium oxide. This was also suggested by the detection of ninhydrin-reacting bases other than sphingosine in hydrolysates of the unadsorbed phospholipids. Although, therefore, it is clear that the phosphorus turnover of lecithin must be very slow, it may even be negligible as the turnover of the 'lecithin' fraction could be accounted for by traces of non-choline containing phospholipids which were unadsorbed by magnesium oxide. The specific radioactivity of the 'lecithin' fraction was not appreciably diminished by repeating the magnesium oxide adsorption step several times with fresh samples of magnesium oxide.

The unsaponified fraction, consisting largely of sphingomyelins (Schmidt, Benotti, Hershman & Thannhauser, 1946), contained no detectable radioactivity. The amount of sphingomyelin phosphorus recovered was apparently low and the reason for this will be dealt with in a subsequent communication.

When 0·001M azide, an agent which 'uncouples' oxidative phosphorylation (Case & McIlwain, 1951), was added to the reaction medium, the respiration of the brain dispersion was stimulated, while the incorporation of ^{32}P into all the phospholipid fractions was lowered (Table 3). This suggests that the uptake of ^{32}P into each fraction is dependent upon an active oxidative phosphorylation (Dawson, 1953*a*).

The incorporation of ^{32}P into phosphatidylethanolamine

Previous results had shown that when rat-brain dispersions were incubated with labelled phosphate there was a comparatively slow incorporation into phosphatidylethanolamine (Norman & Dawson, 1953). When the same method for measuring the specific radioactivity of phosphatidylethanolamine was applied to the labelled guinea pig brain dispersions used in the present work, a similar result was obtained. After incubation of the dispersions for 1 hr. in a medium which produced an active incorporation of ^{32}P into the lipid fraction, radioactivity could scarcely be detected in the phosphatidylethanolamine. This means that with the counting apparatus used, the specific radioactivity of the latter was less than 5% of the specific radioactivity of the whole phospholipid fraction. Moreover, the synthesis of phosphatidylethanolamine appeared to be no greater in slices of guinea pig brain incubated in a medium containing both 0·003M ethanolamine and glycerol.

The incorporation of ^{32}P into phosphatidylserine

When the method for determining the specific radioactivity of phosphatidylserine was applied to a labelled guinea pig brain dispersion, the phospholipid was found to have no detectable radioactivity. This means that the specific radioactivity of phosphatidylserine was below 5% of that of the whole phospholipid fraction.

The incorporation of ^{32}P into diphosphoinositide

The specific radioactivity of diphosphoinositide in labelled brain dispersions was considerably higher than that of the total phospholipid fraction (Table 4). This applied also to the diphosphoinositide which was not extracted from brain

Table 4. *The relative specific radioactivities of diphosphoinositide and the whole phospholipid fraction in labelled dispersions of guinea pig brain*

Methods (1), (2) and (3) are described fully in the Experimental section.

Expt. no.	Method used for determining specific radioactivity of diphosphoinositide	Ratio of specific radioactivities of diphosphoinositide P / total phospholipid P
1	(1) Separation of inositol-*m*-diphosphate from hydrolysate of methanol-insoluble phospholipids	9·5
2	(1) As above	9·6
3	(2) Separation of inositol-*m*-diphosphate from hydrolysate of 'lipid-free' and nucleic acid-free brain residue	7·8
3	(3) Method of Strickland (1952)	6·1

dispersions with lipid solvents and which may be in combination with neurokeratin (Folch, 1952). The specific radioactivity of this solvent-insoluble diphosphoinositide, determined by the method of Strickland (1952), is also included in Table 4 for comparison with other values.

Further observations on the incorporation of ^{32}P into the lipids

The results already described have shown that there was an almost negligible incorporation of ^{32}P into the phosphatidylserine, phosphatidylethanolamine, phosphatidylcholine and sphingomyelin of brain dispersions. On the other hand, the diphosphoinositide phosphorus in such dispersions possessed a high specific radioactivity. The specific radioactivity of diphosphoinositide is approximately 10 times that of the whole lipid phosphorus. If it were therefore the sole active component its phosphorus would have to be about 10% of the total lipid P. All available results suggest that the actual percentage of diphosphoinositide in brain tissue is much less than this (Brante, 1949; Taylor & McKibbin, 1953), and calculation showed that it was unlikely to account for all the radioactivity found in the whole lipid fraction. An experiment was therefore made in which a heavily labelled lipid extract was hydrolysed for 1 hr. in 6N hydrochloric acid at 100°, whereby practically all the lipid phosphorus and radioactivity in the extract were converted into acid-soluble form. On two-dimensional paper chromatography of these acid-soluble phosphorus compounds using propanol:ammonia and *tert.*-butanol:trichloroacetic acid solvents, it was found that the spot corresponding to glycerophosphoric acid had an appreciable specific radioactivity (12900 counts/min./mg. P) and contained approximately 61% of the total radioactivity found in the hydrolysate. This activity could not be accounted for by the low specific activity glycerophosphate (<1150 counts/min./mg. P) arising from the hydrolysis of phosphatidylserine, phosphatidylcholine or phosphatidylethanolamine, which comprise the bulk of the phosphoglycerides found in brain tissue. Moreover, it is known that inositol-*m*-diphosphate rather than glycerophosphate is the initial product formed during the acid hydrolysis of diphosphoinositide (Folch, 1949). Consequently it is clear that the major part of the incorporation of ^{32}P into the lipid fraction of a brain dispersion must occur during the synthesis of some other fat-solvent soluble substance containing glycerophosphate as an integral part of its molecule.

It has recently been shown by Kennedy (1953) that when a ^{32}P-labelled lipid extract prepared from liver mitochondria was passed through a column of Amberlite I.R.A. 400 resin saturated with formate, most of the activity was adsorbed. The active fraction could then be readily eluted from the column with ethanolic HCl. When a similar experiment was performed with the labelled lipids extracted from a brain dispersion, the resin was found to adsorb nearly all the radioactivity, the specific radioactivity of the effluent lipids being only 4% of that of the original lipid extract. However, the adsorbed lipid(s) proved very difficult to elute and only about half of the activity was recovered on prolonged treatment of the column with ethanolic HCl. Most of the activity in this eluted fraction was combined in the lipid(s) as glycerophosphoric acid.

Labelling of cerebral phospholipids in slices and in the whole animal

The previous results had shown that a respiring brain dispersion did not synthesize its phospholipids at identical rates, and it thus became important to know whether this was so in tissue slices or in the whole animal. Experiments were also performed with liver tissue, so that the results could be compared with those obtained on the brain.

When the phospholipids present in slices of brain tissue which had been incubated with labelled phosphate were fractionated it was found that the distribution of specific radioactivities in the fractions was very similar to that found in labelled brain dispersions (Table 5). Essentially similar results were obtained with liver slices, although the synthesis of the choline-containing lipids appeared to be slightly more active. The addition of 0·003M choline and glycerol to the incubation medium in which brain slices were being incubated did not appreciably alter the distribution of radioactivity.

Table 5. *Incorporation of ^{32}P into the lipids of guinea pig brain and liver slices*

Specific radioactivities are expressed as % of the specific radioactivity of the total lipid phosphorus. Figures in brackets indicate that the counting rate was too low for accurate measurement.

Lipid fraction	Expt. 1: Brain slices incubated 3·25 hr. in O_2	Expt. 1: Liver slices incubated 3·25 hr. in O_2	Expt. 2: Liver slices incubated 1 hr. in air
Methanol-insoluble	631	250	631
Adsorbed on MgO	127	170	131
Unadsorbed, saponified	5	15	4·5
Unadsorbed, unsaponified	(0)	(4)	4

Table 6. *Fractionation of the lipids present in the brain and liver of the guinea pig after ^{32}P exchange* in vivo

Specific radioactivities calculated for 1 mc ^{32}P injected at zero time.

Wt. of animal (g.)	500			505			470		
Time of ^{32}P exchange (hr.)	3·4			23·0			162·3		
Tissue examined	Brain (grey matter)	Brain (white matter)	Liver	Brain (grey matter)	Brain (white matter)	Liver	Brain (grey matter)	Brain (white matter)	Liver
Specific radioactivity of total lipid P (counts/min./mg. P)	428	400	48500	1340	990	51800	10500	6420	39000
Specific radioactivities of lipid fractions (expressed as % of specific radioactivity of total lipid P)									
Methanol-insoluble	254	580	209	185	385	172	81	144	98
Adsorbed on MgO	144	111	105	114	95	112	80	88	103
Unadsorbed, saponified	64	51	97	112	125	99	105	118	107
Unadsorbed, unsaponified	<80	<41	53	<77	<47	79	74	59	83

Table 6 expresses the results of similar fractionation studies performed on cerebral grey and white matter, and also on liver of guinea pigs killed at various times after an intraperitoneal injection of labelled phosphate. With the shortest time allowed for ^{32}P exchange (3·4 hr.) there was again a predominance of deposition in the phospholipids which were insoluble in methanol and adsorbed on magnesium oxide both in the brain and to a lesser extent in the liver. However, the synthesis of the 'lecithin fraction' (unadsorbed, saponified) was more rapid than in the experiments with isolated tissues. The specific radioactivity of the methanol-insoluble fraction was about twice as high in white matter as in grey matter, and in the white matter it remained at a higher specific radioactivity than the whole phospholipid fraction, even after 162 hr. of ^{32}P exchange. The synthesis of sphingomyelin both in the brain and liver seems to be at a comparatively slow rate.

DISCUSSION

It was shown by Fries, Schachner & Chaikoff (1942) that the lipids present in slices and 'homogenates' of rat brain became radioactive when they were incubated with labelled phosphate. Up to now, however, no attempt has been made to determine how this activity is distributed between the individual phospholipids. Among the phospholipids of brain tissue which have been examined in the present investigation only diphosphoinositide was found to be synthesized at an appreciable rate in actively respiring guinea pig brain dispersions. Although the ideal rule when measuring the specific radioactivity of any compound is to isolate and purify it to a constant value such a course was not possible with diphosphoinositide in the present circumstances. Consequently the possibility must exist that small amounts of uncharacterized lipids with a high turnover rate are interfering with the determination. Diphosphoinositide is known to be broken down at a rapid rate in guinea pig brain dispersions (Sloane-Stanley, 1953) and if such a breakdown occurs *in vivo* an active synthetic system must be required. Strickland (1952) has recently shown that 4 hr. after the intracisternal injection of ^{32}P in the cat, the cerebral diphosphoinositide has a specific radioactivity which is appreciably above that of the whole lipid phosphorus.

It is apparent, however, that in brain dispersions the synthesis of diphosphoinositide can account for only a small proportion of the total incorporation of ^{32}P into the lipid fraction, and other material soluble in fat solvents is also synthesized from the labelled phosphate. This material although

containing glycerophosphate in its molecule, cannot be either of the other kephalins or lecithin. As it is removed from a lipid extract by an anion-exchange resin saturated with formate it is probable that it is acidic in nature.

It has not been possible to detect any appreciable synthesis of phosphatidylethanolamine, phosphatidylserine or sphingomyelin in brain dispersions, while the synthesis of lecithin must be very slow or even negligible. Although these phospholipids show a considerable turnover in the brain of the intact animal it is very unlikely that they are synthesized in other tissues and carried to the brain, because of the impermeability of the blood-brain barrier. However, the mere demonstration that ^{32}P is incorporated into the lipids of isolated brain preparations cannot now be considered as valid evidence in assuming that the bulk of the brain phospholipids are synthesized within the tissue from inorganic phosphate.

Little evidence has previously been available to indicate the relative rates of synthesis of various phospholipids present in the intact brain. Using solvent fractionation methods which must now be considered obsolete, Chargaff, Olson & Partington (1940) reported that after 24 hr. ^{32}P exchange in the intact rabbit, the brain sphingomyelin and kephalin had higher specific radioactivities than had the lecithin fraction. Hevesy & Hahn (1940), employing similar methods, also reported that in the rabbit the specific radioactivity of the 'kephalin' in the brain was initially higher than that of the lecithin. While the present results are consistent with these findings in that initially the specific radioactivity of the composite 'kephalin' fraction is greater than that of the 'lecithin', they indicate that the rate of synthesis of sphingomyelins is comparatively low. This latter result is more in keeping with the postulated role of sphingomyelins as structural elements in the nerve myelin sheath (Johnson, McNabb & Rossiter, 1950).

The distribution of radioactivity between the labelled phospholipids of liver slices is remarkably similar to that found in slices of brain tissue, with a comparatively small synthesis of the 'lecithin' fraction. In the intact liver, however, the renewal of 'lecithin' phosphorus appears to be much more rapid and even in 3·4 hr. its specific radioactivity is nearly equal to that of the whole phospholipid fraction (Table 6). In early experiments Hevesy & Hahn (1940) showed that rabbit-liver 'kephalin' had initially a much higher specific radioactivity than had the lecithin, but using more recent solvent-fractionation methods Hahn & Tyrén (1946) could find little difference between the specific radioactivities of liver lecithin and three kephalin fractions, 4 hr. after ^{32}P injection.

The present finding that liver sphingomyelin phosphorus is renewed at a comparatively slow rate is in agreement with the results of Hevesy & Hahn (1940) and Hunter (1941) who used a different method for isolating sphingomyelin.

SUMMARY

1. Methods have been developed for determining the specific radioactivities of some of the phospholipids present in small samples of tissue labelled with ^{32}P.

2. When a guinea pig brain dispersion is actively incorporating labelled phosphate into its lipids, there is no appreciable incorporation of ^{32}P into phosphatidylethanolamine, phosphatidylserine or sphingomyelin, while the renewal of lecithin phosphorus is very slow.

3. Measurements suggest that diphosphoinositide phosphorus is renewed at an appreciable rate in actively respiring brain dispersions.

4. Brain dispersions also synthesize uncharacterized material, soluble in fat solvents, which is probably acidic in nature and contains glycerophosphate as part of its molecule. The synthesis of this material is responsible for most of the incorporation of ^{32}P into the lipids of a brain dispersion.

5. The distribution of ^{32}P in the lipids of a labelled brain dispersion has been compared with that in the lipids of brain and liver slices and in the same tissues of the intact guinea pig.

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The Incorporation of ^{65}Zn into Avian Eggs

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Zinc is fairly widely distributed in animal tissues, and small amounts can be detected in the pancreas, liver, intestine, skin, erythrocytes, leucocytes and blood plasma and practically all the tissues of the animal body. In a few cases only, however, is information available about the nature of the compounds with which the metal is closely associated. For example, erythrocyte zinc is largely, if not entirely, present in the zinc protein carbonic anhydrase, and the zinc in the pancreas may be loosely associated with insulin, or in such a form that it can readily act in association with this hormone.

The possibility that zinc in other tissues may also be combined or associated with proteins has received a certain amount of attention in recent years. Using ^{65}Zn, Heath (1949) obtained evidence that some of the zinc in tumour tissue is loosely bound to nucleoprotein in the cell nuclei (cf. also Heath & Liquier-Milward, 1950), but Rosenfeld & Tobias (1951) were unable to find any appreciable amount of this metal in the nuclear fractions of liver, kidney, lung and transplanted mammary tumour tissues of mice after the intravenous injection of ^{65}Zn as zinc chloride. Leucocytes contain considerably more zinc than do erythrocytes (Vallee & Gibson, 1948*b*), and Hoch & Vallee (1952) have obtained evidence that human leucocytes contain two zinc-containing protein complexes.

There is much evidence that zinc can readily combine *in vitro* with many proteins; for example, insulin (Scott, 1934; Scott & Fisher, 1935) and the β_1-metal-binding globulin of human plasma (Surgenor, Koechlin & Strong, 1949). Furthermore, Cohn and his colleagues (see Cohn *et al.* 1950) have utilized the specific interaction between zinc ions and proteins in the methods which they have developed for fractionating the proteins of human plasma; very small amounts of zinc salts are required in these separations, and it is believed that the metal combines stoicheiometrically with specific protein groups, possibly the imidazole groups (cf. also Gurd & Goodman, 1952, and the review by Edsall, 1953).

There are other proteins which may have a special affinity for zinc, for evidence has been presented that this metal activates several enzymes, sometimes specifically; e.g. carnosinase (Hanson & Smith, 1949), some peptidases (Johnson, 1941; Yudkin & Fruton, 1947; Smith, 1948), yeast zymohexase (Warburg, 1949), enolase (Warburg & Christian, 1941*a*, *b*) and phosphatases (Cloetens, 1941*a*, *b*, 1942). Removal of the zinc in these cases usually resulted in considerable loss of enzymic activity which could be restored by adding zinc salts. This evidence alone is, however, insufficient to justify the assumption that this stimulating effect of zinc necessarily takes place *in vivo*, and furthermore a similar activation can often be effected by other metals. Thus there is no unequivocal evidence that any enzyme other than carbonic anhydrase contains zinc as an integral part of the molecule or that it requires zinc as a coenzyme or activator.