fatty acids pass the rabbit placenta from mother to foetus and that these are stored in the foetal liver. This of course raises the problem of the mechanism of fatty acid transfer across the placenta.

The results of our present investigation, taken together with the earlier findings, lead to the inevitable conclusion that all the phospholipins in the foetus (excluding placenta of foetal origin) are synthesized *in situ* from the necessary components and are not derived from the preformed maternal compounds. Nielson (1941-2) has injected pregnant rats with emulsions of ^{32}P -labelled phospholipins and found much less radioactivity in the foetuses than after the injection of inorganic $^{32}\text{PO}_4^{---}$. It was not, however, ascertained whether or not the injected phospholipins were first degraded outside the foetus. Another criticism of Nielson's experiments has been given previously (Popják, 1947).

A further point which emerges from the data shown in Table 3 concerns generally the permeability of foetal cells to PO_4^{---} at different stages of development. On the eighteenth day the specific activity of the inorganic P of the foetal carcass (i.e. the whole foetus without its liver) was nearly the same as that found in the foetal liver, whereas on the twenty-third and twenty-eighth days the former was only about one-fifth of the latter. This difference means that in the younger embryo all cells are uniformly permeable to PO_4^{---} ; with advancing differ-

entiation, however, the extrahepatic tissues become less permeable to PO_4^{---} , a condition approaching more closely that found in the adult animal, in which liver is the tissue most permeable to PO_4^{---} , and muscle and brain the least permeable.

SUMMARY

1. Three rabbits, on the eighteenth, twenty-third and twenty-eighth day of pregnancy, were injected with serum containing phospholipins labelled with ^{32}P , and the radioactivity of phospholipins was compared with that of inorganic phosphorus in the maternal liver, foetal placenta, foetal liver and carcass.

2. The foetal placenta absorbs more phospholipin from the circulation than the maternal liver, but it does not transmit unhydrolysed phospholipin molecules to the foetus. Glycerophosphate, which is the first phosphorus-containing degradation product of lecithin in tissues, does not pass unhydrolysed through the placenta either.

3. The placenta of the eighteenth-day pregnant rabbit is more active than that of the twenty-eighth day pregnant animal in the absorption and degradation of phospholipin.

4. The cells of the 18-day-old rabbit embryo are uniformly permeable to phosphate ions, whereas in the 23- and 28-day-old foetus a condition more approaching that in adult animals is found.

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In Search of a Phospholipin Precursor

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(Received 3 August 1949)

It is well known that soon after the injection of inorganic $^{32}\text{PO}_4^{---}$ to an animal both tissue and plasma phospholipins become labelled with ^{32}P . The biochemical reactions leading to the incorporation of inorganic PO_4^{---} into phospholipin molecules, however, are not known. The various possibilities and researches on this problem have been reviewed by Chaikoff (1942). This investigation aimed at finding

the phosphorus precursor of phospholipins (i.e. of lecithin and kephalin). It is considered that glycerophosphate or a substance with very similar properties is now identified as such a precursor which enters into a reversible reaction in the synthesis of lecithin and kephalin.

In studies of turnover of phospholipins under various experimental conditions with ^{32}P it is not

sufficient merely to determine the total amount of ^{32}P synthesized into phospholipins. Such a course might be very misleading. Since phospholipins are apparently synthesized intracellularly, the amount of ^{32}P which will be found in the phospholipins of the tissues at a given time after the injection of inorganic $^{32}\text{PO}_4^{---}$, will depend first on the permeability of the cells to PO_4^{---} under the particular experimental conditions, and secondly on the velocity of the intracellular reactions. Any condition which would, for example, decrease the permeability of cells to PO_4^{---} , but would leave the intracellular reactions unaltered, would depress the incorporation of ^{32}P into phospholipins, although the turnover rate of the latter had not changed. Such an error can be overcome by relating the specific activity of phospholipin P—at different time intervals after the injection of ^{32}P —to that of tissue inorganic P (Hevesy & Hahn, 1940). Even such a measure would give only the overall reaction rate from inorganic PO_4^{---} to phospholipin, although several intermediate steps may be involved. If the P precursor of phospholipins is known, turnover rates could be determined accurately by studying the rate of conversion of the precursor into product. In addition, the knowledge of the precursor should give an insight into the biochemistry of phospholipin synthesis.

Zilversmit, Entenman & Fishler (1943) have presented the mathematical basis of the relationship between the specific activity of a precursor and its product in tracer studies. Their method can be used for calculation of the turnover time (and turnover rate) of a compound if its precursor is known. It can also be used to identify the precursor of a substance among a number of suspects. These authors have shown that, when a single injection of the tracer substance is given, the specific activity, or isotope concentration-time curve, of the precursor and its product should be related to one another as shown in Fig. 1. When the specific activity of the product is at the maximum, it is equal to the specific activity of the precursor; before this maximum the precursor is more and after the maximum it is less active than the product. In the derivation of this relationship three assumptions are made: (i) that the compound studied in the tissues is in a state of dynamic equilibrium, i.e. the amount of substance synthesized (and brought to the tissues) is equal to the amount of substance degraded (and leaving the tissues); (ii) that 'the rate of appearance and disappearance of the compound must be constant during the experiment' and (iii) that 'the appearance and disappearance of all molecules of the compound must proceed at random, i.e. the organism does not distinguish between "old" and "newly formed" molecules'. It follows from these assumptions that the specific activity of the molecules formed at any time is equal

to the specific activity of their immediate precursor at that time and that the specific activity of the molecules which are being degraded is equal to the specific activity of the total amount of that substance present in the tissues. Zilversmit *et al.* (1943) on the basis of these assumptions derived the following equation for the relationship between the specific activity of the precursor (A) and its product (B):

$$r/p \left(\frac{d(x/r)}{dt} \right) = f(t) - x/r, \quad (1)$$

where r = the total amount of substance (B) in tissues, constant; p = the rate of conversion of (A) into (B), constant; x = the total amount of radioactive (B) present at time t ; $f(t)$ = the specific activity of the precursor, a function of time. Then x/r = the specific activity of substance (B) at time t , and r/p = the turnover time of substance (B).

By taking the definite integral of equation (1) between any two times, t_1 and t_2 , it can be shown that the turnover time (t_t) of substance (B) is equal to the shaded area divided by m in Fig. 1.

Fig. 1. Relationship between the specific activity of precursor and of product after Zilversmit *et al.* (1943). It can be shown mathematically that the turnover time (t_t) of the product is equal to the shaded area divided by m .

These principles have been applied in this investigation in order to find out whether the immediate P precursor of phospholipins could be identified among the acid-soluble P fractions of liver.

METHODS

Albino rats (200–250 g.) in groups of four to six were injected subcutaneously with Na_2HPO_4 labelled with ^{32}P . The members of a group were killed at different intervals (45 min.–24 hr.) after the injection. The specific activity of the injected PO_4^{---} varied between 0.5 and 1.0 mc. ^{32}P /25 μg . of total P. The total radioactive dose injected per rat was 10 μc . and therefore the weight of injected P was negligible. At the required interval after the injection of ^{32}P , the rats were given intraperitoneally a lethal dose of nembutal and when they were still alive but deeply an-

aesthetized, the liver was cut out, the blood wiped from it and the liver dropped into a freezing mixture of solid CO_2 and acetone. The frozen liver was ground to fine powder in a steel mortar chilled with solid CO_2 -acetone.

Extraction of phospholipins. Approximately 2 g. of the powdered liver were extracted four times with boiling ethanol-ether (3/1, v/v). The filtered and combined extracts were evaporated to dryness under reduced pressure in an atmosphere of N_2 at a temperature not exceeding 40° . The residue was extracted with light petroleum (b.p. 40 – 60°). The petroleum solution was clarified by centrifuging and then concentrated to about 5 ml. The phospholipins were precipitated by 20 ml. of ice-cold acetone and 3 drops of ethanol saturated with MgCl_2 . After centrifuging, the phospholipins were taken up again in light petroleum and reprecipitated; they were then dissolved in ether saturated with water. Only the phospholipins soluble in ether (lecithin and cephalin) were investigated.

Fractionation of acid-soluble phosphates of liver. The extraction and fractionation of the acid-soluble phosphates from 3–5 g. of the powdered liver was carried out as described by Kaplan & Greenberg (1944). This procedure is shown schematically in Fig. 2.

We present in Fig. 3 the specific activity-time curves for inorganic P (precipitated as MgNH_4PO_4 from fraction I), adenylic acid P (Hg precipitate, fraction II), the Pb precipitate (fraction III), the ethanol precipitate (fraction IV), and for phospholipin P.

Assay of ^{32}P . The various P fractions dissolved in 0.1 N-HCl were made up to 10 or 25 ml. and their radioactivity measured with a cylindrical Geiger-Müller counter surrounded by a fixed sleeve to hold solutions (Veall, 1948). After radioactive assay the P content of the solutions was determined by the method of Allen (1940). The specific activities (radioactive counts/min./mg. P) shown in Figs. 3 and 4 are expressed as the percentage of radioactive counts

Fig. 2. Fractionation of acid-soluble phosphates of liver according to Kaplan & Greenberg (1944).

Purification of phospholipins. In order to remove any contamination by other compounds containing ^{32}P , the ethereal solution of phospholipins (usually about 10 ml.) was shaken with 2 ml. of 0.05 N-HCl. The washing was repeated three times with the same volume of distilled water, the aqueous layer being separated by centrifuging. In the course of this procedure part of the phospholipins separated out as a jelly. This, however, could easily be redissolved by adding acetone to the moist-ether solution (usually $\frac{1}{3}$ – $\frac{1}{2}$ vol. of acetone to 1 vol. of ether). It was ascertained that the above process of purification effectively removed all contaminating ^{32}P from the phospholipins. This procedure was of particular value in *in vitro* experiments with tissue slices.

The purified solution of phospholipins was transferred to digestion flasks and the solvent evaporated off. The phospholipins were digested with 2 ml. of conc. H_2SO_4 and 10 drops of conc. HNO_3 . The digestion was completed by the addition of 5–10 drops of H_2O_2 (100 vol., Microanalytical Reagent, British Drug Houses Ltd.) repeatedly if required. The inorganic PO_4 thus obtained was precipitated as MgNH_4PO_4 .

injected/100 g. body weight. A suitable dilution (2–5 $\mu\text{C}/\text{l.}$) of the injected ^{32}P solution served as standard during radioactive assays.

RESULTS

The results of the first set of experiments, in which the search for a possible P precursor of phospholipin was made, are summarized graphically in Fig. 3. Each point in this figure is the mean of two experiments. The specific activity-time curve for the labile P of adenosinetriphosphate followed closely that of the inorganic P (curve A, Fig. 3) and, like the latter, remained above the specific activity-time curve of phospholipins. Neither of these substances could, therefore, be the immediate P precursor of phospholipins. In fact the only fraction of which the specific activity-time curve fulfils the criteria for the immediate P precursor of phospholipins is the ethanol precipitate (curve C, Fig. 3). When the

specific activity-time curve of phospholipins (curve *E*, Fig. 3) reached its maximum, it cut the curve of the ethanol precipitate; before this maximum had

which resisted hydrolysis by alkali, and that the rate of its hydrolysis by acid was similar to that of glycerophosphate.

Fig. 3. Specific activity-time curves of liver acid-soluble P fractions and of phospholipin P in rats after a single subcutaneous injection of 10 μ c. of $\text{Na}_2\text{H}^{32}\text{PO}_4$. A, inorganic P; B, Pb precipitate; C, ethanol precipitate; D, Hg precipitate; E, phospholipin P. Specific activities (counts/min./mg. P) are expressed as the percentage of radioactive counts injected/100 g. rat.

been reached the specific activity of the phospholipin P was lower and after the maximum higher than that of the ethanol precipitate.

It remained, however, to prove unequivocally that this ethanol-precipitate indeed contained the specific P precursor of phospholipin P, since it was possible that the relationship of the two curves was merely fortuitous and also that the fraction was far from homogeneous.

The immediate problem was to identify this suspected precursor. Kaplan & Greenberg (1944) showed that the ethanol precipitate was an organic ester

Identification of the acid-soluble, ethanol-precipitable P of liver

A rat (300 g.) was injected subcutaneously with 150 μ c. of $\text{Na}_2\text{H}^{32}\text{PO}_4$ and was killed 24 hr. after injection. We prepared from the trichloroacetic acid extract of the whole liver the ethanol-precipitable P after the other fractions, the Ba, Hg and Pb precipitates, had been removed. The ethanol precipitate was dissolved in dilute HCl. The solution, after removal of metals, was made up to 25 ml. The total P and ^{32}P content of the solution was determined on a small

sample. Two 10 ml. portions were then taken and after neutralization with $\text{Ca}(\text{OH})_2$, in one of them there were dissolved 497.2 mg. of pure inactive $\text{Ca-}\alpha$ -glycerophosphate and in the other 371.9 mg. of $\text{Ca-}\beta$ -glycerophosphate. The glycerophosphates from each solution were then crystallized from boiling solutions; the radioactivity of the crystalline materials could not be reduced any further after the second recrystallization. The probable reason for the rapid purification is that the $\text{Ca-}\alpha$ -glycerophosphates have an inverse solubility and hence are crystallized from boiling solution in which the other phosphates are more soluble.

Table 1 summarizes the results and shows that 75 % of the radioactivity present was attributable to glycerophosphates. Of the glycerophosphate, 70 % was in the α - and 30 % in the β -form. The 25 % of the radioactivity unrecovered from the solution was not identified.

Table 1. *Crystallization of ^{32}P -labelled glycerophosphate with carrier glycerophosphate from acid-soluble, ethanol-precipitable phosphorus of liver*

Total P content in 10 ml. sample (mg.)	0.58
	Counts/min.
Total ^{32}P counts in 10 ml. sample	76,840
Activity recovered in α -glycerophosphate	39,776
Activity recovered in β -glycerophosphate	17,620
Total glycerophosphate activity	57,396
Unidentified	19,444

Since the specific activity of the glycerophosphates and of the unidentified PO_4^{---} present in the original biological material was not known, the 75 % of radioactivity recovered with glycerophosphates could not be said to represent 75 % of the total P in the solution. It seems probable, however, that this is the case, because the specific activities of all acid-soluble P fractions in the liver of an animal are very nearly the same 24 hr. after the injection of inorganic $^{32}\text{PO}_4^{---}$.

It was ascertained that most of the α -glycerophosphate found in the preparation was originally present in the tissues as such, and did not result from a conversion of β - into α -glycerophosphate during the fractionation. A solution of pure $\text{Na-}\beta$ -glycerophosphate in 5 % trichloroacetic acid was treated in exactly the same way as the biological material. A periodate titration at the end of the procedure showed only a 7 % conversion of β - to α -glycerophosphate.

Experiments in vivo with labelled glycerophosphate

It was considered that more definite proof than had so far been obtained was required to establish that glycerophosphate is indeed the P precursor of phospholipins. Glycerophosphate (both α and β) labelled with ^{32}P was therefore synthesized and used for injecting into rats.

The ^{32}P -labelled $\text{Ca-}\alpha$ -glycerophosphate was synthesized by the method of King & Pyman (1914) and $\text{Ca-}\beta$ -glycerophosphate by the method of Poulenc (1911). The PO_4^{---} used in the synthesis was labelled with ^{32}P . The Ca salts were converted to the Na salts before injection.

The results obtained with α - and β -glycerophosphate were similar in every respect, and therefore only one set of experiments with α -glycerophosphate are presented. The α -glycerophosphate synthesized had a specific activity of $0.79 \mu\text{c./mg.}$ glycerophosphate on the day of the experiment. Six rats were injected intraperitoneally at the same time with 12.3 mg. of $\text{Na-}\alpha$ -glycerophosphate containing a total of $10 \mu\text{c.}$ of ^{32}P . The further experimental treatment was the same as for the animals injected with $\text{Na}_2\text{H}^{32}\text{PO}_4$.

The results are shown in Fig. 4. A comparison with Fig. 3 reveals that the relationship between the specific activity-time curves of the various fractions from the liver is the same as after the injection of inorganic $^{32}\text{PO}_4^{---}$. Even at the shortest interval (30 min.) after the injection, the inorganic P had the highest specific activity, indicating that unhydrolysed glycerophosphate did not enter into liver cells. If glycerophosphate had been absorbed unhydrolysed by liver cells, the highest specific activity should have been found in the ethanol precipitate.

Experiments in vitro with liver slices incubated with ^{32}P -labelled substrates

A number of experiments were carried out with liver slices incubated with ^{32}P -labelled substrates. It was hoped that by flooding the slices with large amounts of substrates, penetration of these into liver cells and evidence for a specific phospholipin precursor might be obtained.

The following substances labelled with ^{32}P were tested: α - and β -glycerophosphate, glucose-1-phosphate, glucose-6-phosphate, muscle adenylic acid and adenosinetriphosphate.

Preparation of labelled compounds. α - and β -Glycerophosphate were synthesized by the methods already cited.

Glucose-1-phosphate was prepared by incubation of starch with PO_4^{---} and potato phosphorylase according to the method of Sumner & Somers (1944). The PO_4^{---} in the incubation mixture was labelled by the addition of carrier-free $^{32}\text{PO}_4^{---}$. The specific activity of the glucose-1- PO_4 preparations varied from 6 to $25 \mu\text{c.}$ $^{32}\text{P}/100 \text{ mg.}$ of compound.

Glucose-6-phosphate was obtained by the enzymic conversion of glucose-1- $^{32}\text{PO}_4$ as described by Colowick & Sutherland (1942).

Adenosinetriphosphate and muscle adenylic acid were prepared according to the method of Lohmann (1931) from the muscle of one rabbit which was injected daily for 1 week with $200 \mu\text{c.}$ $^{32}\text{PO}_4^{---}$. The

specific activity of the labile P of adenosinetriphosphate was 29×10^3 counts/min./mg. P, and that of adenylic acid 2000 counts/min./mg. P.

The incubation medium for the liver slices was a bicarbonate-Ringer solution (Krebs & Henseleit, 1932), pH 7.4, with 0.5% added glucose. Slices of approx. 1 g. obtained from the same liver were used

in excess of what is described by Krebs & Henseleit (1932), especially when organic substrates of low specific activity were used in the experimental vessels. The slices were incubated for 1–4 hr.

The results of two typical experiments (one with glycerophosphate and one with glucose-1- PO_4) are shown in Figs. 5 and 6; but all of them can be sum-

Fig. 4. Specific activity-time curves of liver acid-soluble P fractions and of phospholipin P in rats after a single intra-peritoneal injection of 12.3 mg. of ^{32}P -labelled Na- α -glycerophosphate containing a total of $10 \mu\text{c.}$ of ^{32}P . A, inorganic P; B, Pb precipitate; C, ethanol precipitate; D, Hg precipitate; E, phospholipin P. The specific activities (counts/min./mg. P) are expressed as the percentage of radioactive counts injected/100 g. rat.

for controls and for the experiment. The chemical composition and the total ^{32}P content of the control and the experimental mixtures were the same, but in the controls all ^{32}P was initially present as inorganic PO_4^{---} and in the experiments the ^{32}P was contained as labelled organic substrate. In some cases, the specific activity of the P (counts/min./mg. P) were identical in both. This latter condition, however, often necessitated the addition of inorganic- PO_4^{---}

marized briefly. No evidence was obtained that any of the substrates tested might be used preferentially in phospholipin synthesis. All these substances were rapidly hydrolysed by liver slices and the results could be explained satisfactorily by assuming that the liberated inorganic $^{32}\text{PO}_4^{---}$ entered the liver cells and started its cycle in the internal P metabolism of the cell, in the course of which it also became synthesized into phospholipins.

Fig. 5. Synthesis of phospholipins by liver slices *in vitro* with ^{32}P -labelled substrates. Composition of incubation medium in control vessels: Krebs-Henseleit solution, pH 7.4, 100 ml. + 0.5% glucose + 50 mg. inactive $\text{Na-}\alpha$ -glycerophosphate + 0.22 ml. saline containing 20 μc . carrier-free $\text{Na}_2\text{H}^{32}\text{PO}_4$; in experimental vessels: Krebs-Henseleit solution 100 ml. + 0.5% glucose + 50 mg. (= 20 μc .) ^{32}P -labelled $\text{Na-}\alpha$ -glycerophosphate + 0.22 ml. saline. The mixtures were saturated with 95% O_2 + 5% CO_2 . Volume of incubation mixture in each vessel was 25 ml. to which 2 g. (moist wt.) of liver slices were added. (a) Specific activity of phospholipin P shown as observed radioactive counts/min./mg. P; $\odot$ control; $\bullet$ experimental. (b) Specific activity of phospholipin P expressed as percentage of average specific activity of inorganic P in the control vessels ($\odot$), and as percentage of specific activity of the added glycerophosphate P in the experimental vessels ($\bullet$). (c) Specific activities of inorganic P in the control ($\odot$) and in the experimental vessels ($\bullet$) and of glycerophosphate P in the experimental vessels (----). The falling specific activity of inorganic P in the control vessels was due chiefly to hydrolysis of added glycerophosphate. (d) Rate of hydrolysis of α -glycerophosphate by liver slices in control and experimental vessels.

Fig. 6. Synthesis of phospholipins by rat-liver slices *in vitro* with ^{32}P -labelled substrates. Composition of incubation medium in control vessels: Krebs-Henseleit solution, pH 7.4, 50 ml. + 0.5% glucose + 0.5 ml. of 0.9% NaCl containing 10 μ c. of carrier-free $\text{Na}_2\text{H}^{32}\text{PO}_4$ + 38.6 mg. unlabelled glucose-1- PO_4 ; in experimental vessels: Krebs-Henseleit solution, 50 ml. + 0.5% glucose + 0.5 ml. 0.9% NaCl + 38.6 mg. (= 10 μ c.) glucose-1- ^{32}P . The mixtures were saturated with 95% O_2 + 5% CO_2 . Volume of medium in each vessel was 10 ml. with 0.7 g. of liver slices added. Incubation period 2–4 hr. at 37°, two vessels being used for each period. (a) Observed specific activities of phospholipin P in the control (○) and in the experimental vessels (●). (b) Micrograms of newly synthesized phospholipin P/mg. of phospholipin P present in the slices. It has been assumed in the calculation of these figures that in the control slices (○) the synthesis took place with inorganic ^{32}P and in the experimental vessels (●) with the ^{32}P of glucose phosphate. (c) Specific activity of inorganic P in the control (○) and experimental (●) vessels. (---) shows the specific activity of the P of added glucose-1- ^{32}P . Since the conversion of the 1- to 6-ester does not involve the inorganic- PO_4 of the medium, the specific activity of the latter was the same as that of the former. (d) P-content (μ g. P/ml.) of incubation medium. ○ glucose-1- PO_4 ; ● glucose-6- PO_4 resulting from conversion of the 1-ester; △ inorganic P obtained from hydrolysis of glucose phosphate.

Evidence for a reversible reaction between phospholipins (lecithin and kephalin) and glycerophosphate

A short interval (up to 10–15 hr.) after the injection of inorganic $^{32}\text{P}\text{O}_4^{--}$, the specific activities of

Table 2. *Specific activity of phospholipin, glycerophosphate and inorganic phosphorus in placenta and liver of pregnant rabbit after intravenous injection of serum containing ^{32}P -labelled phospholipins.*

(Serum (20 ml.) injected on twenty-eighth day of pregnancy. This serum contained 5.96 mg. phospholipin P/100 ml.; of the total ^{32}P content (approximately 2×10^4 counts/min./ml.), 67.3% was associated with phospholipins, 25.4% with phosphoproteins and 7.3% with unidentified acid-soluble P. (For the preparation of this serum see Popják & Beeckmans, 1950.) Specific activities determined 2 hr. after injection and expressed as radioactive counts/min./mg. P.)

	Specific activity of		
	Phospholipin P	Glycerophosphate P	Inorganic P
Foetal placenta	2880	2090	1395
Maternal liver	1009	137	184

inorganic P (I), glycerophosphate P (II) and phospholipin P (III) of tissues in descending order are: $\text{I} > \text{II} > \text{III}$. It was thought, therefore, that if the reaction between phospholipin and its precursor is a

reversible one, and if this precursor is really glycerophosphate, then, after the intravenous injection of ^{32}P -labelled phospholipins, the specific activities of the P fraction mentioned should be in the following order: $\text{III} > \text{II} > \text{I}$. This argument of course should apply only to tissues which absorb phospholipins from the blood and also metabolize them.

It was found (Popják & Beeckmans, 1950) that the rabbit placenta absorbs large amounts of phospholipins from the maternal circulation and that it also degrades them rapidly. Table 2 shows that 2 hr. after the injection of a serum containing ^{32}P -labelled phospholipins to a 28-day-pregnant rabbit, the specific activity of the phospholipin P was 2880 counts/min./mg., of the glycerophosphate P 2090 counts/min./mg. and of the inorganic P 1395 counts/min./mg. In the liver of this animal, however, the inorganic P was more active than the glycerophosphate P, although the phospholipin P had the highest specific activity. In view of the fact, however, that the injected serum contained an appreciable amount of ^{32}P -labelled phosphoprotein and a little acid-soluble ^{32}P beside phospholipin ^{32}P it seemed very likely that the higher activity of the liver inorganic P was attributable to rapid degradation of absorbed phosphoprotein in addition to that of glycerophosphate. To eliminate this unwanted factor we prepared emulsions of lecithin and kephalin labelled with ^{32}P and used them for intravenous injection of rabbits.

Fig. 7. Preparation of lecithin and kephalin.

*Preparation of ^{32}P -labelled lecithin and
kephalin from liver*

A 2.5 kg. rabbit was injected intravenously with 1.7 mc. of carrier-free $\text{Na}_2\text{H}^{32}\text{PO}_4$ in isotonic saline and was killed 24 hr. later. The liver of the animal was cut into slices, blotted with filter paper and frozen in solid CO_2 -acetone mixture. It was then ground to fine powder in a chilled steel mortar. First the crude phospholipins were obtained as described under 'Methods'. The separation of lecithin and kephalin was carried out by differential precipitations based on methods described by Maclean & Smedley-Maclean (1927). This is shown diagrammatically in Fig. 7. The final lecithin and kephalin preparations were emulsified with 0.9% NaCl by grinding; the crude emulsions were then subjected to ultrasonic waves of 300 kcyc./sec. frequency for 15 sec. Although these lecithin and kephalin preparations may still have been impure, nevertheless the kephalin preparation was free from choline as tested by the method of Entenman, Taurog & Chaikoff (1944), and gave a N/P ratio of 1/1; the lecithin preparation gave a choline/P ratio (molar) of 0.9, but the N/P ratio was 1.59 indicating the presence of nitrogenous impurities. The specific activity of lecithin P was 6.8×10^5 counts/min./mg. and that of kephalin P 4.56×10^5 counts/min./mg.

Treatment of experimental animals. One rabbit (no. 33, 2.04 kg.) was injected intravenously with 2 ml. of lecithin emulsion containing a total of 177 μg . of lecithin P and 1.2×10^5 counts/min. One hour after the injection the rabbit was anaesthetized with nembutal and ether, and a lobe of liver removed for extraction of lecithin and kephalin and for fractionation of acid-soluble P. The animal was allowed to survive under the anaesthetic for a further 30 min., when the rest of the liver was removed.

Table 3. *Specific activity of liver phosphorus fractions after the injection of emulsions of ^{32}P -labelled lecithin and kephalin*

(Rabbit 33 was injected with a lecithin emulsion containing a total of 12×10^4 ^{32}P counts/min. Rabbit 34 was injected with a kephalin emulsion containing a total of 7.5×10^4 ^{32}P counts/min.)

Time after injection (min.)	Rabbit 33		Rabbit 34
	72	97	72
	(^{32}P counts/min./mg. P)		
Lecithin P	676	652	310
Kephalin P	360	348	692
Glycerophosphate P	115	144	129
Inorganic P	61	101	74

Another rabbit (no. 34, 2.28 kg., the litter mate of no. 33) was injected with 3 ml. of the kephalin

emulsion containing a total of 75×10^3 ^{32}P counts/min. and 165 μg . of kephalin P. This experiment was concluded 1 hr. after the injection.

For the fractionation of lecithin and kephalin from the liver of these two rabbits the same methods were used as for the preparation of phospholipins from the donor animal.

The results of these two experiments are shown in Table 3 and confirm those obtained with the placenta (cf. Table 2), and further show that the first P-containing degradation product of both lecithin and kephalin is glycerophosphate.

DISCUSSION

It has been shown by *in vivo* experiments, using the technique of Zilversmit *et al.* (1943), that after the injection of inorganic $^{32}\text{PO}_4^{---}$ into rats the only part of the acid-soluble P fraction, which fulfilled the mathematical criteria for an immediate P precursor of lecithin and kephalin, was the ethanol precipitable P. This fraction was identified as consisting mainly of α - and β -glycerophosphate.

Zilversmit, Entenman & Chaikoff (1948), in a recent article, also concluded that glycerophosphate is the immediate P precursor of phospholipins. They injected dogs with inorganic $^{32}\text{PO}_4^{---}$, took liver biopsies at intervals up to 16 hr. after the injection and hydrolysed the acid-soluble P compounds for 3 hr. with NaOH. They found that by its specific activity-time curve the alkali-stable P compound fulfilled the criteria for a precursor of phospholipins. The relationship between the specific activity-time curve of this alkali-stable organic P compound and of lecithin P was the same as the relationship in our experiments between the ethanol-precipitable P and phospholipin P. Zilversmit *et al.* (1948), by using the periodate method, concluded that 70% of their alkali-stable P was glycerophosphate. They have further shown that in choline-treated animals in which the specific activity-time curves of lecithin P and glycerophosphate P were significantly altered, the latter still retained the characteristics of a precursor of the former.

It has further been shown by the injection of ^{32}P -labelled phospholipins that in the liver and placenta of rabbits the first P-containing degradation product of lecithin and kephalin is glycerophosphate. It is inferred, therefore, that the reaction between glycerophosphate and phospholipins is reversible.

In neither *in vivo* nor *in vitro* experiments could it be demonstrated that there was a higher incorporation of ^{32}P from labelled glycerophosphate than from labelled inorganic PO_4^{---} . This negative result, however, does not exclude glycerophosphate as the P precursor of phospholipins, since the *in vivo* experiments showed that unhydrolysed glycerophosphate did not enter liver cells. *In vitro* experiments with

labelled glucose phosphate, adenylic acid and adenosinetriphosphate were likewise negative. Choline-phosphate (Riley, 1944), and aminoethylphosphoric acid (Chargaff & Keston, 1940) have also been tested *in vivo* as possible phospholipin precursors with negative results.

It is thought that the evidence presented is sufficiently strong to justify the conclusion that glycerophosphate, or a substance closely similar, is the P precursor of lecithin and kephalin. By applying the integrated form of equation (1) to the data shown in Fig. 3 we obtained 6.75 hr. for the turnover time of liver phospholipins. This period for the rat is very close to the values (5–7 hr.) found by Zilversmit *et al.* (1948) for the dog.

From the point of view of the biochemistry of phospholipin synthesis more definite mechanisms may now be suggested. If glycerophosphate is the immediate phosphorus precursor of phospholipins, it follows that the phrase 'phosphorylation of fats' which is often used, is misleading and inaccurate, because the phosphorylating energy has been spent in the course of reactions leading to the formation of glycerophosphate. Formation of phospholipins through phosphorylation of di- or tri-glycerides would require an energy-rich phosphorus compound (cf. Chaikoff, 1942). Instead, it is suggested that lecithin and kephalin are formed by the esterification of glycerophosphate with fatty acids and by the formation of the choline- or ethanolamine-phosphoric acid ester linkage. It has been pointed out (Chaikoff, 1942, and others) that the rates of renewal of the various molecular components of phospholipins may not be identical, for example, the rate of renewal of fatty acids in phospholipins might be different from the rate of renewal of phosphoric acid. However, if glycerophosphate is the P precursor, it follows that the rates of renewal of glycerophosphate and fatty acids in lecithin and kephalin must be equal. The turnover of choline or ethanolamine, however, may still differ from that of the other components. The results shown in Table 3 suggest interconversion of lecithin and kephalin. It has been shown by Stetten (1941*a, b*) that ethanolamine is converted to choline, but not choline to ethanolamine. The interconversion of lecithin and kephalin could then happen only by the scission of the bases followed by re-esterification. Although the latter results are suggestive, further work is required for their confirmation.

SUMMARY

1. It has been shown that the specific activity-time curve of the ethanol-precipitable phosphorus, obtained from the acid-soluble phosphates of liver, fulfils the criteria for this fraction being the phosphorus precursor of lecithin and kephalin.

2. This acid-soluble phosphorus fraction, labelled with ^{32}P , has been identified by crystallization with carrier as consisting mainly of α - and β -glycerophosphate.

3. Lecithin and kephalin labelled with ^{32}P were injected into rabbits. The glycerophosphate from placenta and liver had the highest specific activity after the phospholipins. The results are considered to indicate that glycerophosphate is the P precursor of phospholipins and that the reaction is reversible.

4. Although neither *in vivo* nor *in vitro* experiments with ^{32}P -labelled glycerophosphate provided supporting evidence for the above thesis, the negative results are attributed to failure of penetration of liver cells by glycerophosphate.

5. The turnover time of phospholipins in the rat liver is calculated to be 6.75 hr.

6. Possible mechanisms for the biochemical synthesis of lecithin and kephalin are discussed.

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