Det Kgl. Danske Videnskabernes Selskab.
Biologiske Meddelelser. **XV**, 5.

TURNOVER OF LECITHIN, CEPHALIN, AND SPHINGOMYELIN

BY

G. HEVESY AND L. HAHN

KØBENHAVN
EJNAR MUNKSGAARD
1940

Printed in Denmark
Bianco Lunos Bogtrykkeri A/S

Phosphatide molecules present in the body have been taken up with the food or have been built up in the organism. A spectacular proof of the synthesis of phosphatides in the body is given by the fact that ducks raised on diets containing phosphorus only in inorganic form laid 85—195 eggs during the summer[1]. These eggs contained 200—400 gm. phosphatides (corresponding to 8—16 gm. phosphatide P), and this very appreciable amount was synthesised by the organs of the ducks. On the other hand, phosphatides can enter the circulation from the intestine. The amount of phosphatide which is daily led by the intestinal lymph into the circulation of the rabbit[2] on normal diet was calculated to be about 50 mgm. This is only about 1/5 of the amount daily synthesised in the liver (comp. p. 24); one must further consider that at least an appreciable part of the above mentioned 50 mgm. was synthesised in the mucosa of the small intestine. Thus, the phosphatide molecules of the organs will be only to a small extent obtained directly from the food, the overwhelming majority being built up in the body.

Concept of turnover.

The ultimate aim of the investigation of the origin of the phosphatide molecules present in the body is to be able

[1] G. Fingerling, Biochem. Z. **38**, 448 (1911).

[2] H. Süllmann and W. Wilbrandt, Biochem. Z. **270**, 52 (1934).

to state in which form the hydrogen, carbon, nitrogen, oxygen, and phosphorus atoms present in the phosphatide molecules were taken up by the body and in what steps they were involved until ultimately incorporated into phosphatide molecules. This exacting task can hardly be solved at present, and we must content ourselves with the determination of the place and rate of formation of the phosphatide molecules in the body from glycerol, fatty acid, choline (or another organic base), and phosphate. We will denote, in what follows, as turnover rate the rate of synthesis of phosphatide molecules from inorganic phosphate and other components independent of the actual mechanisms involved, and we shall measure this rate by determining the extent to which labelled phosphate present in the cells of an organ is incorporated into these newly formed phosphatide molecules. As the phosphatide content of an organ is usually constant, we can follow that with the formation of new phosphatide molecules the decomposition of an equal or similar number of old molecules goes hand in hand. The possibility must also be envisaged that new formation and decomposition of phosphatides do not take place in the same organ, but that the newly formed molecules are synthesised in one organ and carried into the other by the circulation. This point will be discussed on p. 25.

The turnover rate can also be measured by following the rate of incorporation of fatty acids or of choline, for example, into the phosphatide molecule. The turnover rates measured by using different indicators need not necessarily be identical. It would be conceivable, for example, that the incorporation of the phosphate radical into the phosphatide molecules would be preceded by the formation of glycero-

phosphate and that this process would be a comparatively slow one in contrast to all other steps involved in the synthesis of the phosphatide molecule. In this case, the turnover rate measured, using labelled P as an indicator, would be slower than that found when using labelled fatty acids or labelled choline. The opposite would be the case if the reorganisation of the phosphate bond were to take place at a faster rate than the corresponding release and incorporation of fatty acids or choline into the phosphatide molecules.

The question if and to what extent the rate of phosphate incorporation into the phosphatide molecule differs, for example, from that of the fatty acid incorporation into the latter cannot be answered at the time being.

Feeding cats with mixed glyceride, the acids of which were composed to 85 per cent of elaidic acid, SINCLAIR[1] found 12 hours later the plasma phosphatide fatty acids to contain 19 per cent of elaidic acid. In our experiments we found (comp. p. 30) that, after the lapse of 16 hours, about 4 per cent of the phosphatides extracted from the plasma of rabbits contained labelled phosphate.

Indicators applied in turnover measurements.

a) Change of the degree of unsaturation of fatty acids.

Since the phosphatides contain both saturated and unsaturated fatty acids, the change of the composition of the fatty acids of the organ phosphatides after ingestion of cod liver oil, for example, can be utilised to get information

[1] R. G. SINCLAIR, J. Biol. Chem. **115**, 215 (1937).

on the rate of the phosphatide turnover in the organ in question. A change in the iodine number of the phospholipids extracted from the liver of dogs[1] and cats[2] after the ingestion of cod liver oil and the disappearance of the changes within 24 hours and 2 to 3 days, respectively, was observed at an early date.

b) Incorporation of iodized fatty acids into the phosphatide molecule.

Iodized fatty acids, whether injected intravenously or given by mouth, enter the phosphatides of the liver, the blood[3], and the milk[4], for example.

c) Incorporation of elaidic acid into the phosphatide molecule.

This method was repeatedly used in the investigation of the turnover of phosphatides. The rate of entrance of elaidic acid into and disappearance from the phosphatides was found to be rapid in the liver and the intestinal mucosa and comparatively slow in the muscle. The process was found to be essentially complete in the liver within a day, but in the muscle only after the period of many days[5].

[1] G. Ioannowics and E. P. Pick, Wien. Klin. Wochenschr. **23**, 573 (1910).

[2] R. G. Sinclair, J. Biol. Chem. **82**, 117 (1929). Comp. also R. G. Sinclair, Phys. Rev. **14**, 351 (1934).

[3] C. A. Artom, Arch. inter. Physiol. **36**, 191 (1933); C. A. Artom and G. Peretti, Arch. inter. Physiol. **36**, 351 (1933).

[4] F. X. Aylward, J. H. Blackwood and J. A. B. Smith, Biochem. J. **31**, 130 (1937).

[5] R. Sinclair, J. Biol. Chem. **111**, 270 (1935), and **121**, 161 (1937). M. F. Kohl, J. Biol. Chem. **126**, 709 (1938).

d) Incorporation of fatty acids, labelled by introduction of heavy hydrogen, into the phosphatide molecule.

Linseed oil was deuterated and the "heavy" fat obtained fed to rats. The investigation of the deuterium content of the phosphatides extracted from different organs gives information on the phosphatide turnover in the organ in question[1].

e) Incorporation of analogues of choline, in which arsenic replaces nitrogen, into the phosphatide molecule.

Arsenic can be detected in the lecithin fraction isolated from the liver and the brain of rats kept for 21 days on a diet containing arsenocholine chloride[2].

f) Incorporation of labelled phosphate into the phosphatide molecule.

This method will be discussed in detail.

Most of the methods outlined above were successfully applied to show that a marked turnover takes place in some of the organs, and the application of the methods a), c), and f) lead to the result that the rate of the phosphatide turnover is much faster in the intestinal mucosa and in the liver than in the other organs. None but the "phosphate method" was applied, however, to arrive at quantitative data as to the rate of rejuvenation of the phosphatide molecules present in the different organs.

[1] B. Cavanagh and H. S. Raper, Biochem. J. **33**, 17 (1939).
[2] A. Welch, Proc. Soc. Expertl. Biol. and Med. **35**, 107 (1937).

Quantitative determination of the turnover rate by using labelled phosphate.

The formation of phosphatide molecules containing ^{32}P inside the tissue cell can only take place when the process of phosphatide formation was preceded by a penetration of ^{32}P into the cell, and the same applies to all indicators used in turnover experiments. This point was hitherto not considered. Its great importance is best seen by the following.

Let us assume that labelled phosphate or elaidic acid cannot penetrate into the cells of an organ. In this case, no turnover could be ascertained, even if a very intense one were actually taking place. To arrive at a proper figure for the turnover rate we have to compare the percentage of ^{32}P in the total inorganic P of the cells with the percentage of ^{32}P in the total phosphatide P extracted from them. If these ratios, which correspond to those of the specific activities of the inorganic P and the phosphatide P, are found to be equal, we can conclude that all phosphatide molecules were renewed during the experiment. In this case, a proportional partition of ^{32}P between the inorganic P and the phosphatide P present in the cells took place. This is only possible if the phosphate radical of all the phosphatide molecules was split off in the course of the experiment, a process which was then followed by a synthesis of phosphatide molecules with incorporation of other phosphate radicals in which $^{32}PO_4$ was represented proportionally to its total number present. If the specific activity of the phosphatide P is found to be, for example, 10 per cent of that of the inorganic P, we can conclude that 10 per cent of the phosphatides were renewed during the experiment.

Due regard must, however, be given to the change of the specific activity of the cellular inorganic P in the course of the experiment. By administering the labelled phosphate in several portions of suitably varying quantities in the course of the experiment, we can maintain a constant specific activity of plasma and interspace phosphate. As to the cellular concentration of ^{32}P, which is nought at the start of the experiment and then gradually increases, we determine the change of concentration with time experimentally and compare the specific activity of the phosphatide P extracted at the end of the experiment with the average value of the specific activity of the inorganic P which prevailed during the experiment.

When determining the specific activity of the cellular inorganic P, due regard must be taken to the fact that a part of the tissue inorganic ^{32}P is of extracellular origin. As the extracellular volume of the tissue is known and the specific activity of the extracellular P does not differ much from that of the plasma P, we can easily correct for the presence of the extracellular P in the tissue inorganic P. Since the extracellular phosphate in the case of the muscle tissue, for example, amounts to only about 1/90 of the cellular inorganic P, the correction mentioned above becomes only significant in experiments of short duration. If the rate of penetration of the inorganic phosphate differs greatly in the cells of different tissues, as it actually does, for example, in the case of the liver and the muscle, we do not get proper information on the relative rate of turnover of the phosphatides in these organs by comparing the specific activity of the liver phosphatide P with that of the muscle phosphatide P. Conclusions based on such a comparison will greatly underestimate the relative rate of phosphatide

turnover going on in the muscle cells into which the inorganic P diffuses at a slow rate, in contrast to its penetration into the liver cells. We will arrive, however, at correct figures by comparing the ratio

$$\frac{\text{specific activity muscle phosphatide P}}{\text{specific activity muscle inorganic P}}$$

with the corresponding ratio of liver products.

If we wish to draw quantitative conclusions from experiments carried out with elaidic acid as an indicator, we have to compare the elaidic acid content of the organ phosphatides with that of the elaidic acid content of the fatty acid mixture present in the corresponding cells in freely disponible state. The latter magnitude is not known and the same consideration applies to the work with deuterated fat as an indicator. We may get some, though very restricted, information by comparing the heavy hydrogen (D) content of the organ phosphatides with that of the organ glycerides. After the lapse of 10 hours, the ratio

$$\frac{\text{liver phosphatide D}}{\text{liver glyceride D}} : \frac{\text{kidney phosphatide D}}{\text{kidney glyceride D}},$$

where D denotes the relative heavy content of the total "non-exchangeable" hydrogen, was found to be 1:2.

Experimental procedure.

The labelled phosphate of negligible weight, dissolved in physiological sodium chloride solution, was injected into the vena jugularis of the rabbit drop by drop during the experiment. Per hour 2,5 cc. were injected, the experi-

ment took usually 4 hours. By taking small samples from the ear vein at different intervals, the change in the activity of the plasma was followed. In several cases, we extracted the inorganic P of the plasma and measured its specific activity (activity per mgm. P), in others we con-

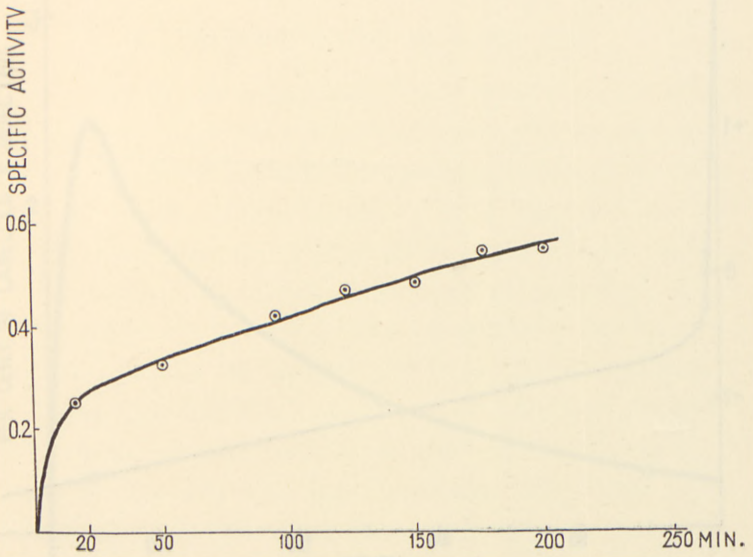

Fig. 1. Change of the specific activity of the plasma inorganic P during continuous intravenous injection of labelled phosphate to a rabbit. (Specific activity = per cent of the labelled P injected, found in 1 mgm. P).

tented ourselves with the measurement of the total activity of the plasma which, in experiments of short duration, is solely due to the inorganic phosphate present.

The labelled P was injected drop by drop into the vena jugularis in order to obtain a comparatively small and easily accountable change in the activity level of the plasma (see Fig. 1). If all the labelled P is injected at the start of the experiment, as in our early experiments and in all experiments carried out by other workers with labelled P,

the activity level of the plasma is very high at the beginning, and it is slow at the end of the experiment (see Fig. 2). If the labelled P is given by subcutaneous injection or by mouth, the activity of the plasma first increases with

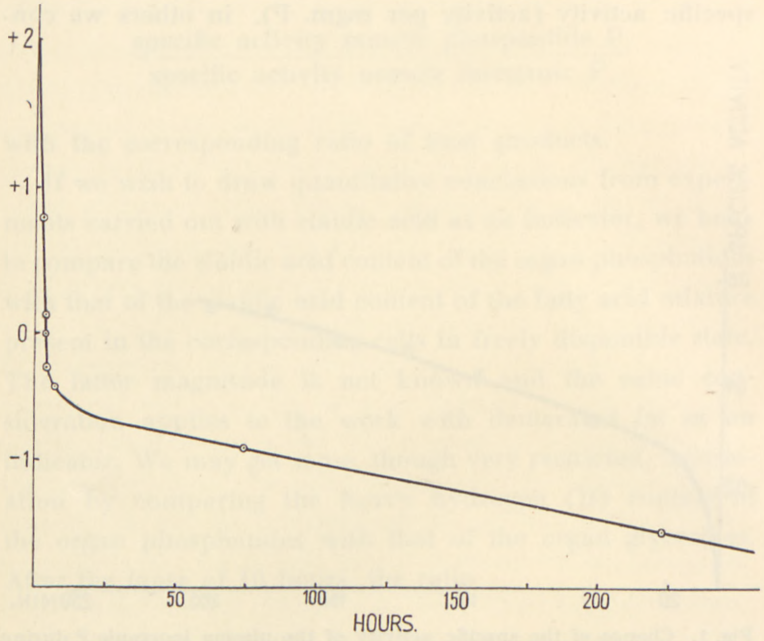

Fig. 2. Change of the logarithm of the labelled P content of the plasma with time after intravenous injection of labelled phosphate.

time and later decreases (see Fig. 3). The sensitiveness of the radioactive indicator, thus, changes very appreciably in the course of the experiment. If we are successful in keeping the activity level of the plasma constant during the experiment, we can eliminate great difficulties otherwise encountered when calculating the turnover rate of organic phosphorus compounds.

The changes in the activity of the plasma, shown in Fig. 1, can be further reduced by injecting amounts de-

creasing with time. In our later experiments we have chosen this procedure and varying amounts of labelled P were administered by subcutaneous injection. In an experiment

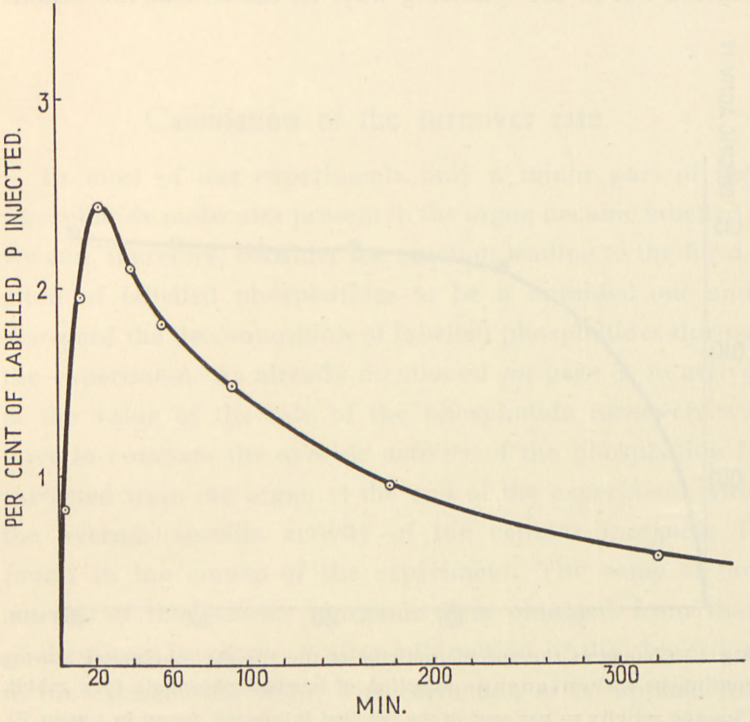

Fig. 3. Change of the specific activity of the plasma inorganic P after subcutaneous injection of labelled phosphate to a rabbit.

taking 12 hours, for example, labelled P was injected every 20 min. In experiments taking several weeks, in the later phases of the experiment injections were made twice a day. The change in the plasma activity in such an experiment taking 4 hours is seen in Fig. 4. In experiments taking several hours or days a constant activity level could be easily obtained.

The determination of the turnover rate of the phospha-

tides present in the different organs necessitates the determination of the specific activity of the inorganic P and phosphatide P extracted from the organ. This determination was carried out in the following way. At the end of the experi-

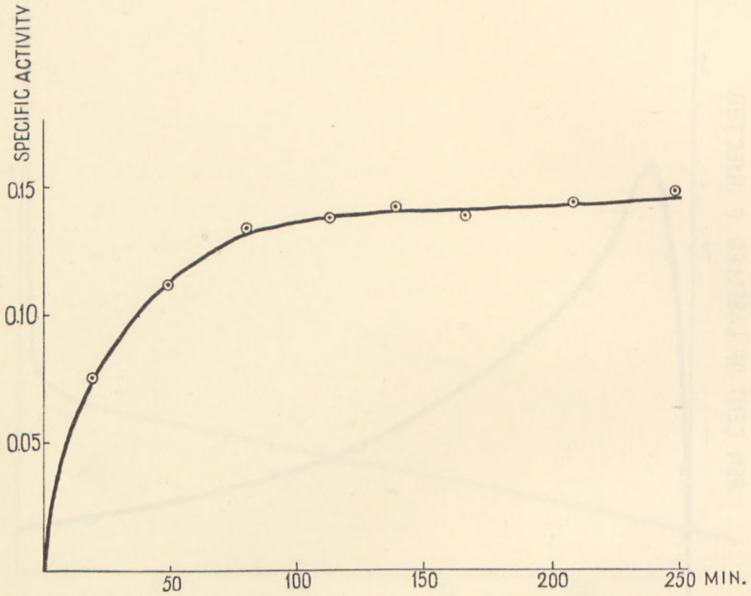

Fig. 4. Change of the specific activity of the plasma inorganic P during continuous subcutaneous injection of labelled phosphate to a rabbit. (Specific activity = per cent of the labelled P injected, found in 1 mgm. P).

ment the animal was killed by bleading. The organs were at once placed in liquid air, minced, and extracted with cold 10 per cent trichloroacetic acid. The inorganic phosphate present was precipitated as ammonium magnesium phosphate at 0°. Muscle samples were taken before death. To secure the phosphatide present in the organs, these were first dried with cold acetone and then treated with ether, later with boiling alcohol. The ether-alcohol extracts were evaporated in vacuo and taken up several times with

petrol-ether; the phosphatides were then converted into phosphate by wet ashing. The procedure applied when isolating lecithin, cephalin, and sphingomyelin will be discussed on page 36.

Calculation of the turnover rate.

In most of our experiments only a minor part of the phosphatide molecules present in the organ became labelled; we can, therefore, consider the reaction leading to the formation of labelled phosphatides to be a onesided one and disregard the decomposition of labelled phosphatides during the experiment. As already mentioned on page 8, to arrive at the value of the rate of the phosphatide turnover, we have to compare the specific activity of the phosphatide P extracted from the organ at the end of the experiment with the average specific activity of the cellular inorganic P found in the course of the experiment. The value of the activity of the cellular inorganic P is obtained from that of the tissue inorganic P after subtraction of the share due to the extracellular fluid. The correction to be applied for the presence of extracellular P in the tissue inorganic P is, in most cases, a small one. In the liver of the rabbit, for example, out of 30 mgm. inorganic P only about 0.6 mgm. is located in the interspaces. We arrive at this figure by assuming that the interspaces make out[1] 22 per cent of the weight of the liver and the inorganic P content of the interspaces is 3 mgm. per cent. The specific activity of the liver extracellular P is, after 4 hours, 2.5 times higher than the specific activity of the tissue inorganic P; correspondingly,

[1] J. F. Manery and B. Hastings, J. Biol. Chem. **127**, 657 (1939).

5 per cent of the total inorganic P activity of the liver is due to extracellular P.

In the case of the muscle, we arrive by an analogous consideration at the result that 25 per cent of the activity of the tissue inorganic P is of extracellular origin. The extent of the correction to be applied increases with decreasing length of the experiment, since in experiments of short duration only a small amount of labelled P penetrates into the cells.

With regard to the considerations stated above, one must recognise the possibility that some of the phosphorus which one identifies, even after the most careful experimental procedure, as inorganic P, was in fact present in the tissue in the form of very labile, not yet known, organic phosphorus compounds. Very labile P compounds of that kind, if present, would probably be in fast exchange equilibrium with the inorganic P present, and their presence would therefore not much influence the calculation given above. The labile P of adenyltriphosphoric acid comes, for example, very quickly into exchange equilibrium with the inorganic P of the tissues or the corpuscles; it is often permissible to replace the specific activity of the inorganic P by that of the above mentioned labile P. The behaviour of creatinephosphoric acid is discussed on page 28.

When calculating the turnover rate of phosphatides, we must consider the average specific activity of the cellular inorganic P prevailing during the experiment. This value is obtained by determining the specific activity of the tissue inorganic P and the plasma inorganic P at different intervals. The change of the specific activity of the tissue inorganic P is seen in Table 1, that of the plasma inorganic P is discussed on page 11.

Table 1.

Specific activity of the organ inorganic P as percentage of that of the plasma inorganic P.

Organ	100 min.	240 min.
Liver	12.7	42.1
Muscles	0.8[1]	4.6
Intestinal mucosa	14.7	42.8
Brain[2]	0.32	1.4
Kidneys	85	90

[1] In spite of all precaution taken, some creatine P may have been split off before the extraction of the inorganic P. The creatine P being, in experiments of short duration, less active than the inorganic P, such a decomposition may partly be responsible for the low value obtained in the experiment taking 100 min. only.

[2] Comp. p. 45.

It is of interest to remark that, in the case of the kidneys, after the lapse of 100 min. an almost proportional partition of ^{32}P between plasma and cellular P is reached. When investigating, after 4 hours, the inorganic P of the marrow of the kidney, which makes out only a minor part of the total inorganic P of the kidney, the specific activity was found to be only 48 per cent of that of the plasma.

Cellular and non-cellular formation of phosphatides.

The turnover rates recorded in the fourth column of Tables 3 to 9 are calculated on the assumption that the formation of phosphatide molecules takes place inside the cells with participation of cellular inorganic P. Let us assume for a moment that the formation of phosphatide molecules takes place on the cell wall facing the inter-

spaces. Then, not the cellular but the extracellular phosphate radicals[1] would enter the newly formed phosphatide molecules. As the specific activity of the extracellular inorganic P is often much higher than that of the cellular inorganic P, in the last mentioned case more active P atoms would take part in the synthetic process than in the first mentioned one. A high activity of the newly formed phosphatide would then not indicate such a high turnover as it would if the formation of the phosphatide molecules took place with participation of the less active cellular P. It is obvious that the sensitivity of our radioactive indicator will be very different in the two cases mentioned above. Though it is much more probable that the turnover of the phosphatide molecules takes place inside the cells we have also recorded, in the fifth column of the above mentioned tables, the turnover rates calculated on the assumption of an extracellular formation of the phosphatide molecules. The values thus obtained give the lower limit of the turnover rate, while those obtained in column 4 give the upper limit. It is conceivable that some of the phosphatide molecules are renewed inside the cell wall. In that case the inorganic P entering the newly formed phosphatide molecules will have a specific activity being intermediary between that of the extracellular and the cellular P. A continuous drop of the specific activity of the inorganic P in the cell wall may namely take place while the phosphate penetrates from the interspaces into the cells.

In the corpuscles the phosphatides are known to be

[1] From this view-point, it is without any significance whether the phosphate radical is directly incorporated into the phosphatide molecule or through intermediary stages.

practically concentrated in the stroma[1], and the thickness[2] of the latter to correspond to that of very few molecular layers. It is, therefore, quite conceivable that in the outer layer of the stroma a slow rejuvenation of the phosphatide molecules takes place with incorporation of plasma P. Should we find an organic P fraction extracted from the cells or the corpuscles to show a higher specific activity than the cellular, respectively corpuscular inorganic P, in this case we would be justified to conclude that the synthesis of the organic compound in question did not take place inside the cells, respectively the corpuscles. Investigations in the above mentioned direction may bring forward results of histochemical interest.

[1] B. N. Erickson, H. H. Williams, S. S. Bernstein, J. Arvin, R. L. Jones and J. G. Macy, J. Biol. Chem. **122**, 515 (1938).

[2] Danielli, J. Cell. Comp. Physiol. **7**, 393 (1936).

Results of experiments.

PART I

Investigation of the total petrol-ether soluble phosphatide mixture.

Experiments with rabbits.

Table 2.

Specific activity of the inorganic P and phosphatide P extracted from the organs.

Rabbit I. — Weight: 2.4 kg.

Intravenous injection during 4 hours.

Fraction	Specific activity in relative units
Plasma inorganic P	100
Liver tissue inorganic P at the end of the experiment	36.2
Liver tissue inorganic P corrected for the change in plasma activity during the experiment	44
Liver cellular inorganic P at the end of the experiment corrected as above	40.8
Liver cellular inorganic P average value during the experiment	20.4
Liver phosphatide P	3.0
Kidney tissue inorganic P at the end of the experiment	67.7
Kidney tissue inorganic P corrected for the change in plasma activity during the experiment	82.3
Kidney cellular inorganic P at the end of the experiment corrected as above	82.0
Kidney cellular inorganic P average value during the experiment	73.5
Kidney phosphatide P	5.5

Table 3.

Specific activity of the cellular inorganic P and phosphatide P extracted from the organs.

Rabbit II. — Weight: 2.6 kg.

Intravenous injection during 215 min.

Organ	Specific activity		Percentage of phosphatides renewed during the experiment	
	Inorganic P average during the experiment	Phosphatide P at the end of the experiment	A[1]	B[2]
Liver	100	19.0	19.0	3.86
Kidney	382	18.3	4.8	3.7
Small intestine	111	7.9	7.1	1.61
Stomach	58	4.46	7.7	0.91
Heart	57.2	1.53	2.7	0.31
Lungs	66.3	4.04	6.1	0.82
Spleen	70.2	3.65	5.2	0.74
Marrow[3]	40.8	1.63	4.0	0.33
Brain	..	0.06	..	..

[1] Calculated on the assumption that the formation of phosphatides took place with incorporation of cellular inorganic P.

[2] Calculated on the assumption that the formation of phosphatides took place with incorporation of extracellular inorganic P.

[3] In several experiments the specific activity of the marrow inorganic P was found to be surprisingly low, even lower than that of the ester P. These low values were presumably due to the presence of traces of only slightly active bone P in the marrow sample.

Table 4.

Specific activity of the cellular inorganic P and phosphatide P extracted from the organs.

Rabbit III. — Weight: 2.3 kg.

Intravenous injection during 234 min.

Organ	Specific activity		Percentage of phosphatides renewed during the experiment	
	Inorganic P average during the experiment	Phosphatide P at the end of the experiment	A[1]	B[2]
Liver	100	16.3	16.3	3.2
Muscles	7.8	0.56	7.2	0.11

[1] Turnover rate calculated on the assumption that the formation of phosphatides took place with incorporation of cellular inorganic P.

[2] Turnover rate calculated on the assumption that the formation of phosphatides took place with incorporation of extracellular inorganic P.

Table 5.

Specific activity of the cellular inorganic P and phosphatide P extracted from the organs.

Rabbit IV. — Weight: 2.5 kg.

Intravenous injection during 215 min.

Organ	Specific activity		Percentage of phosphatides renewed during the experiment	
	Inorganic P average during the experiment	Phosphatide P at the end of the experiment	A[1]	B[2]
Liver	100	14.8	14.8	2.9
Kidney	374	23.2	6.2	4.6
Small intestine (mucosa)	107	20.0	18.7	3.9
Heart	64.6	3.47	5.37	0.68
Lungs	76.1	7.67	10.1	1.51
Brain	..	0.175	..	..

[1] Turnover rate calculated on the assumption that the formation of phosphatides took place with incorporation of cellular inorganic P.

[2] Turnover rate calculated on the assumption that the formation of phosphatides took place with incorporation of extracellular inorganic P.

Table 6.

Specific activity of the cellular inorganic P and phosphatide P extracted from the organs.

Rabbit V. — Weight: 2.1 kg.

Intravenous injection during 250 min.

Organ	Specific activity		Percentage of phosphatides renewed during the experiment	
	Inorganic P average during the experiment	Phosphatide P at the end of the experiment	A[1]	B[2]
Liver	100	18.6	18.6	2.76
Kidney	364	22.8	6.3	3.58
Small intestine (mucosa)	115	23.6	20.5	3.54
Muscle	12.0	0.87	7.3	0.11

[1] Turnover rate calculated on the assumption that the formation of phosphatides took place with incorporation of cellular inorganic P.

[2] Turnover rate calculated on the assumption that the formation of phosphatides took place with incorporation of extracellular inorganic P.

Table 7.

Specific activity of the cellular inorganic P and phosphatide P extracted from the organs.

Rabbit VI. — Weight: 2.6 kg.

Subcutaneous injection during 255 min.

Organ	Specific activity		Percentage of phosphatides renewed during the experiment	
	Inorganic P average during the experiment	Phosphatide P at the end of the experiment	A[1]	B[2]
Liver	100	14.8	14.8	3.2
Corpuscles	29.0	1.51	5.2	0.33

[1] Turnover rate calculated on the assumption that the formation of phosphatides took place with incorporation of cellular inorganic P.

[2] Turnover rate calculated on the assumption that the formation of phosphatides took place with incorporation of extracellular inorganic P.

Table 8.

Specific activity of the cellular inorganic P and phosphatide P extracted from the organs.

Rabbit VII. — Weight: 2.4 kg.

Subcutaneous injection during 11.5 hours.

Organ	Specific activity		Percentage of phosphatides renewed during the experiment	
	Inorganic P average during the experiment	Phosphatide P at the end of the experiment	A[1]	B[2]
Liver	100	25.2	25.2	14.9
Corpuscles	25.5	4.03	15.8	2.39
Muscles	14.7	1.31	8.9	0.78
Brain	—	0.55	—	—
Marrow	36.5[3]	31.8	87.0	18.8

[1] Turnover rate calculated on the assumption that the formation of phosphatides took place with incorporation of cellular inorganic P.

[2] Turnover rate calculated on the assumption that the formation of phosphatides took place with incorporation of extracellular inorganic P.

[3] As the presence of traces only of bone P in the marrow sample investigated lowers the specific activity of the marrow inorganic P, the recorded figure for the inorganic P of the marrow may be too low and that recorded for the rate of renewal of the phosphatide P of the marrow, correspondingly, too high.

Table 9.

Extent of renewal of phosphatides.

Rabbit IX. — Weight: 2.5 kg.

Subcutaneous injection during 50 days.

Organ	Percentage of phosphatides not renewed	
	A[1]	B[2]
Liver	0	0
Muscle	73	64
Marrow	0	0
Corpuscles	3	3

[1] Rate of renewal calculated on the assumption that the formation of phosphatides took place with incorporation of cellular inorganic P.

[2] Rate of renewal calculated on the assumption that the formation of phosphatides took place with incorporation of extracellular inorganic P.

Critical remarks.

In Tables 2—9, data were given for the turnover rate of phosphatides in different organs of the rabbit. When calculating those values we assumed that the labelled phosphatides present in the organs were synthesised in situ. In what follows, we will discuss how far this assumption is justified.

Liver phosphatides.

Let us first consider the liver phosphatides. Apart from the liver, a very intense turnover is going on in the intestinal mucosa, and the possibility must be envisaged that the labelled phosphatides were carried into the liver from the intestine by the plasma. The plasma was found to contain only small amounts of labelled phosphatides, the specific activity of the plasma phosphatide P being, after the lapse of 4 hours, only 1/7 of that of the liver phosphatide P. This fact excludes the possibility that a substantial part of the labelled liver phosphatides was led from the intestine or any other organ into the liver. Large amounts of water can be led from one pond into the other by a narrow channel; salt water, however, (salt corresponding to labelled phosphatides in our case) cannot pass the channel without the water of the channel becoming salt as well. The concept of "specific activity" proves, thus, to be of great use when putting forward considerations such as those discussed above.

One may say, in respect of these considerations, that, while the specific activity of the average plasma phosphatides is low, one of the phosphatide fractions (phosphatides represent a mixture of numerous compounds)

might be synthesised at a very fast rate in the intestinal mucosa, and the labelled molecules formed in this process might have rushed through the plasma at a fast rate into the liver without much raising the specific activity of the average plasma phosphatide P. As shown on page 51, the specific activity of the phosphorus present in different phosphatide fractions can differ substantially, but, in spite of exhaustive fractionation processes no fraction of extremely high or extremely low specific activity was found. Furthermore, the total amount of labelled phosphatides formed in the intestinal mucosa in the course of 4 hours amounts to only 1/5 of that formed in the liver during the same time.

In this connection it is of interest to remark that, according to the results obtained by Süllmann and Wilbrandt which are discussed on page 3, the intestinal lymph carries up to 0.1 mgm. phosphatide P[1] per hour; but, even if this amount of newly formed phosphatides is quantitatively led from the intestine into the liver, it would not suffice to account for the presence of the amount of newly formed phosphatides found in the latter which corresponds to more than 0.5 mgm. phosphatide P per hour.

An entirely different argument against the intestinal origin of the labelled phosphatides found in the liver is the following. The labelled phosphatides present in the plasma

[1] When oil is fed to the rabbit twice that amount was found to be carried by the intestinal lymph. The feeding of oil raises the rate of turnover in the intestinal mucosa and the liver as well, as shown in experiments on rats (C. Artom, G. Sarzana and E. Segrè, Arch. Intern. Physiol. **47**, 245, 1938; B. A. Fries, S. Ruben, J. Perlman and J. L. Chaikoff, J. Biol. Chem. **123**, 587, 1938) and also on isolated perfused cat liver, where the turnover rate was found to be about twice as high as in experiments in which non-lipemic (normal) blood was used (L. Hahn and G. Hevesy, Biochem. J. **32**, 342, 1938).

were not found to leave the blood stream at a very fast rate, half of the labelled phosphatides present leaving the plasma in the course of an hour, 30 per cent of these phosphatides being found in the liver[1]; thus, a rapid rush of labelled phosphatides through the plasma does not take place.

That the labelled phosphatides found in the liver are, at least to a large extent, formed in situ, was also shown in experiments on isolated perfused liver. Such investigations were formerly[2] carried out by us on isolated cat livers in which, after the lapse of 2.5 hours, the specific activity of the liver phosphatide P was found to be about 1.5 per cent of that of the liver inorganic P. A further proof that the phosphatides present in the liver were formed there was brought about by CHAIKOFF and his colleagues[3] who found that, in experiments on rats, the removal of tissues very active in phospholipid turnover, namely the gastrointestinal tract and the kidneys, does not markedly influence the phospholipid turnover in the liver.

Muscle phosphatides.

After discussing the origin of the labelled liver phosphatides we shall put forward similar arguments as to the origin of the labelled muscle phosphatides. The specific activity of the plasma phosphatides is found to be about 3 times higher after the lapse of four hours than that of the muscle phosphatides. Considerations based on the comparison of the specific activity of the plasma phosphatides and the muscle phosphatides do not, therefore, exclude

[1] L. HAHN and G. HEVESY, Nature **164**, 72 (1939).

[2] L. HAHN and G. HEVESY, Biochem. J. **32**, 342 (1938).

[3] B. A. FRIES, S. RUBEN, J. PERLMAN and J. L. CHAIKOFF, J. Biol. Chem. **123**, 567 (1938).

the possibility that the labelled phosphatides present in the muscles were carried into them from other organs. This possibility is, however, excluded by the result of experiments based on the rate of entrance of labelled phosphatides into the muscles[1]. While, in the course of 4 hours, phosphatides showing a relative activity of 0.54 units pass from the plasma into the muscles, phosphatides having an activity of 160 units were found to be present in the muscles after the lapse of the same time.

In experiments of short duration the creatine P of the muscles gets only partly labelled and, therefore, a decomposition of creatinephosphoric acid prior to the extraction of the inorganic P will lead to a "dilution" of the activity of the inorganic P present as such in the muscle tissue. We entertain some doubts as to the possibility of preventing the decomposition of some of the creatinephosphoric acid present even if great precautions are taken. The possibility that in our experiments taking only a few hours too low values are obtained for the specific activity of the muscle inorganic P cannot, therefore, be entirely discarded. As the extent of the newformation of the muscle phosphatides is calculated by comparing the specific activity of the phosphatide P with that of the inorganic P, a too low value of the specific activity of the inorganic P will manifestly lead to a too high value of the rate of newformation of the phosphatides.

Kidney phosphatides.

Kidney phosphatide P is found in experiments of short duration to be more active than the phosphatide P ex-

[1] L. Hahn and G. Hevesy, Nature **144**, 204 (1939); D. Kgl. Danske Vidensk. Selskab, Biol. Medd. XV, 6 (1940).

tracted from all other organs. From this fact we may, however, not follow that the kidney phosphatides are renewed at a faster rate than the phosphatides in the liver or the intestinal mucosa. The labelled inorganic P of the plasma diffuses with a remarkable speed into the kidney cells (see Table 1). This is in no way surprising in view of the role of the kidney cells as to excretion and re-absorption of phosphate. A result of this fast penetration of active phosphate into the kidney cells will be a formation of active phosphatide molecules already in the earliest stages of the experiment. This is not the case in the cells of other organs into which the labelled phosphate diffuses at a slower rate.

Labelled phosphatides of the plasma.

The renewal of phosphatides in the plasma can only be determined in experiments in vitro; in such experiments[1], taking 4.5 hours, the specific activity of the plasma phosphatide P was found to be smaller than 1/1000 of that of the inorganic P.

In experiments in vivo, an exchange between plasma phosphatides and organ phosphatides takes place and, as in some of the organs labelled phosphatides are formed at a fast rate, we will soon after the administration of labelled phosphate find labelled phosphatide molecules in the plasma, which were released from the organs. In fact, almost all phosphatide molecules found in the plasma were synthesised in the organs. The labelled phosphatide content of the plasma, at different times, is seen in Table 10. In this experiment, the labelled inorganic P content of the plasma was kept constant during 9 days.

[1] L. Hahn and G. Hevesy, Mem. Carlsberg **22**, 190 (1937).

Table 10.

Specific activity of phosphatide P and inorganic P of the plasma.

Time	Relative specific activity	
	Inorganic P	Phosphatide P
4 hours	100	0.53
16 hours	100	3.8
25 hours	100	8.1
37 hours	100	15.0
45 hours	100	22.0
55 hours	100	27 5
9 days	100	81.6

Three consecutive processes have to precede the appearance of labelled phosphatides in the plasma. Labelled inorganic P has to diffuse into the cells of the liver and other organs in which the plasma phosphatides are formed. The building up of the labelled phosphatide molecules represents the second process, their release into the plasma the third. In view of the time taken by these processes, it is easy to understand that in the early stages of the experiment the change of the labelled phosphatide content of the plasma has a more rapid than linear dependence with time.

Since a large part of the phosphatide molecules found in the plasma originated from the liver, it is of interest to compare the amount of the active phosphatides found in the plasma with that present in the liver at the end of the experiment.

As seen in column 3 of Table 11, after the lapse of 12 hours, the activity of the plasma phosphatides reached 3/4 of that of the liver phosphatides. A large part of the liver

Table 11.

Active phosphatide content of the liver and the plasma of rabbits.

Duration of the experiment	Ratio of active phosphatide content of liver and plasma	Extent of partition of labelled phosphatides between liver phosphatides and plasma phosphatides
4 hours	94	0.16
12 hours	18	0.76
9 days	14	1.0

phosphatides is, however, not yet renewed and a further substantial increase of the activity of the plasma phosphatides can only be expected by a corresponding increase in the active phosphatide content of the liver and other organs.

Phosphatide turnover in the corpuscles.

Compared with the phosphatide turnover going on in the organs the phosphatide turnover taking place in the corpuscles is but little. This is also shown by results obtained when investigating the origin of the yolk phosphatides[1]. In these experiments, 28 hours after administration of the labelled phosphate, the specific activity of the corpuscle phosphatides was found to be only 1/3 of that of the plasma phosphatides; showing the corpuscles to be, so-to-say, a by-path of the liver and other organ phosphatides on the way through the plasma into the yolk.

The labelled phosphatide molecules of the corpuscles

[1] G. Hevesy and L. Hahn, D. Kgl. Danske Vidensk. Selskab, Biol. Medd. XIV, 2 (1938).

Table 12.

Extent of partition of labelled phosphatides, originally present in the plasma, between the phosphatides of the corpuscles and of the plasma in experiments *in vitro*.

(Plasma of a rabbit containing labelled phosphatides shaken with corpuscles of another rabbit).

Animal	Time in hours	Extent of partition (Percentage)
Rabbit	0.5	1.8
	1.5	3.6
	3.0	4.0
	4.5	5.0
Hen	1.5	1.5
	2.0	2.0
	3.0	1.5

have various origins. Some of them were incorporated in the course of the red cell formation into a tissue containing labelled phosphatides. Some of the labelled phosphatide molecules came into the corpuscles after they reached the circulation. As seen in Table 12, in which the results of some experiments in vitro are recorded, a part of the phosphatide molecules of the corpuscles exchanges easily with those of the plasma. Presumably those situated in the outermost layer of the stroma take part in this exchange process. It is, however, rather difficult to interpret the comparatively high specific activity of the phosphatide P extracted from the corpuscles in experiments in vivo without assuming that a phosphatide turnover takes place in the corpuscles, though the rate of this turnover is small compared with that of most of the acid-soluble P compounds present in the corpuscles (see Table 14).

Table 13.

Extent of partition of labelled phosphatides, originally present in the plasma, between the phosphatides of the corpuscles and of the plasma in experiments *in vivo*.

Animal	Time in hours	Extent of partition (Percentage)
Rabbit (2—2.5 kg.)	24	16
	24	18
	24	17
	25	16
	42	34
Chicken (100—150 gm.)	18	6.0
	22.5	8.1

In experiments in vivo with rabbits (see Table 13), in the course of a day, the activity of the corpuscle phosphatide P was found to be only about 1/6 of that of the plasma phosphatide P. A still greater difference was found when investigating chickens blood.

Table 14.

Specific activity of phosphatide P and acid soluble P of the corpuscles.

Fraction	Relative specific activity after	
	4 hours	12 hours
Phosphatide P[1]	2.6	9.6
Inorganic P	100	100
Pyrophosphate P	99.5	100
Hydrolyzed by 1 n H_2SO_4 in 7 to 100 min.	100	100
Hydrolyzed in 100 min. to 12 hours	100	
Non-hydrolyzed	87	

[1] The active phosphatide molecules are partly such ones which were taken up from the plasma by an exchange process.

Using elaidic acid as an indicator, Sinclair[1] found, 8 hours after ingestion of the elaidic acid, 15 per cent of the fatty acids extracted from the plasma phosphatides to be composed of this distinctive fatty acid, while the corpuscles contained no more than traces of the indicator.

When iodised fatty acid was used as an indicator, it was found[2] not only in the phosphatides of the plasma but also in those of the corpuscles. In the latter, the concentration of iodised fat was even higher (3.3 per cent of the total fatty acids) than in the former (2.0 per cent). The application of iodised fatty acids leads, thus, to a result which is in contradiction to that obtained by using labelled phosphate or elaidic acid as indicators. Phosphatides containing iodised fatty acids are possibly selectively taken up by the corpuscles, another explanation being that the molecules of these compounds present in the plasma were decomposed at a faster rate than those incorporated into the stroma. Phosphatides containing iodized fatty acids represent non-physiological compounds and, as shown by the above example, the results obtained by using such indicators must be interpreted very cautiously.

In this connection, the observation[3] should be also mentioned that in lactating cows during fasting a marked decrease in the concentration of plasma P lipids takes place which persists for several weeks after realimentation, but there is no significant change in the amount of red cell phosphatides. This result also shows the absence of an intense interaction between plasma phosphatides and phosphatides present in the corpuscles.

[1] R. G. Sinclair, J. Biol. Chem. **115**, 211 (1936).
[2] C. Artom, Arch. Intern. Physiol. **36**, 101 (1933).
[3] J. A. Smith, Biochem. J. **32**, 1856 (1938).

PART II

Investigation of lecithin, cephalin, and sphingomyelin.

We discussed above the rate of renewal of the average petrol-ether soluble phosphatide molecules; in what follows, we wish to describe some experiments in which lecithin, cephalin, and sphingomyelin were separately investigated and their turnover rate determined. Chemically, cephalin differs from lecithin by containing aminoethanol instead of choline. The biological consequence of this replacement is very significant[1]. Cephalin is highly active in accelerating blood clothing, whereas lecithin is not. It was even reported[2] that cephalin prepared from cattle blood or cattle brain enhances, while lecithin inhibits the clotting of rabbits blood. The role of the phospholipids as transport agents of fats was much discussed, this role being often ascribed to lecithin alone.

In our first experiments, we determined the turnover rate of lecithin and cephalin in the organs of rabbits 4 hours after intravenous injection of labelled phosphate. We found the turnover rate of cephalin extracted from the liver, the intestinal mucosa and other organs to be pronouncedly faster than that of lecithin. Simultaneously, CHARGAFF[3] found the rate of rejuvenation of cephalin extracted from the liver and the intestinal tract of rats to be slower than that of lecithin. We were first inclined to explain this difference in the findings of CHARGAFF and ourselves by the fact that the former investigated the turnover process, in contradistinction to us, in carnivorous

[1] Comp. E. CHARGAFF, M. ZIFF and B. M. HOGG, J. Biol. Chem. **131**, 35 (1939).

[2] Y. OKARMURA, Mitt. med. Ges. Okoyama **48**, 1585 (1936).

[3] E. CHARGAFF, J. Biol. Chem. **128**, 592 (1939).

animals. We soon found, however, that it is the duration of the experiment which is decisive for the higher or lower rate found for the cephalin turnover. We will, in what follows, first describe the experimental procedure used.

Experimental procedure.

The tissue is dried with cold acetone and extracted first with ether and then with boiling alcohol. The second process is repeated several times. The solutions obtained were evaporated to dryness and taken up by petrol-ether in the presence of pulverised dry sodium phosphate. The latter was added in order to remove traces of active phosphate possibly present. The process was then repeated in the absence of phosphate and the dry residue taken up in ether. The next step was to precipitate the cephalin from the solution by adding 96 per cent alcohol. The filtrate obtained was evaporated and the residue containing lecithin extracted with ice-cold alcohol. This procedure was repeated and the purified lecithin obtained precipitated as chlorocadmium-lecithin. The compound obtained was thoroughly washed with ether in order to remove traces of chlorocadmium-cephalin possibly present.

The cephalin was prepared from the alcoholic precipitate obtained in the early treatment of the phosphatide mixture. To obtain pure cephalin the precipitate was repeatedly dissolved in ether and precipitated with alcohol.

To secure sphingomyelin the fraction insoluble in petrol-ether was collected and treated alternatively with ether and ice-cold alcohol. The last residue thus obtained was dissolved in a mixture of methyl alcohol and chloroform. By adding ether to this solution purified sphingomyelin was

precipitated. A further purification of this product was obtained by repeating the procedure described above. When sufficient amounts were available, the sphingomyelin was recrystallised from pyridin.

Experiments with rabbits.

In the experiments, the results of which are given in Tables 15 and 16, all the labelled phosphate was administered at the start of the experiment. In all later experiments, labelled phosphate was administered all through the

Table 15.

Specific activity of inorganic P and P of different phosphatide fractions.

Rabbit X. — Weight: 2.9 kg.

All labelled phosphate was administered at the start of the experiment by stomach tube. — The animal was killed after 19 hours.

Fraction	Specific activity relative to the	
	Plasma inorg. P	Inorg. P of the organ
	found at the end of the experiment	
Plasma lecithin P	39.1	..
Liver inorganic P	89.7	100
Liver lecithin P	46.3	51.6
Liver cephalin P	35.4	39.5
Liver sphingomyelin P	28.2	31.2
Brain inorganic P	4.67	100
Brain lecithin P	0.40	8.6
Brain cephalin P	1.04	22.4

Table 16.

Specific activity of inorganic P and P of different phosphatide fractions.

Rabbit XI. — Weight: 2.2 kg.

Labelled phosphate administered to the rabbit by subcutaneous injection at the start of the experiment. — The animal was killed after the lapse of 7 days.

Fraction	Relative specific activity
Plasma lecithin P	100
Plasma cephalin P	48.1
Plasma sphingomyelin P	74.5
Corpuscles lecithin P	88.5
Corpuscles cephalin P	73.1
Brain inorganic P	26.6
Brain lecithin P	14.1
Brain cephalin P	20.9

experiment to keep the specific activity of the plasma inorganic P at a constant level. In the experiments of short duration taking only 4 hours, the cephalin extracted from all the organs investigated was found to be much more active than the lecithin. While the sphingomyelin extracted from the liver did not much differ in its specific activity from that of the lecithin of this organ, in the muscle the sphingomyelin was found to be much more active than the lecithin but less active than the cephalin.

In experiments taking 12 hours, lecithin and cephalin were renewed in the liver to about the same rate while sphingomyelin was found to show a slower turnover rate. The relative activity of lecithin and cephalin was, thus, very materially different in the experiment taking 12 hours

Table 17.

Renewal of lecithin and cephalin.

Rabbit III. — Weight: 2.3 kg.

Intravenous injection during 234 min.

Fraction	Percentage of phosphatide renewed during the experiment	
	A[1]	B[2]
Liver lecithin	10.9	2.1
Liver cephalin	27.9	5.5

[1] Turnover rate calculated on the assumption that the formation of phosphatides took place with incorporation of cellular inorganic P.

[2] Turnover rate calculated on the assumption that the formation of phosphatides took place with incorporation of extracellular inorganic P.

Table 18.

Renewal of lecithin, cephalin and sphingomyelin.

Rabbit IV. — Weight: 2.5 kg.

Intravenous injection during 215 min.

Organ	Percentage of phosphatides renewed during the experiment					
	A[1]			B[2]		
	Lecithin	Cephalin	Sphingomyelin	Lecithin	Cephalin	Sphingomyelin
Liver	4.38[3] / 3.67[4]	26.5	4.4	0.86[3] / 0.72[4]	5.2	0.86
Small intestine (mucosa)	17.0	40.6	—	3.55	8.47	—

[1] Turnover rate calculated on the assumption that the formation of phosphatides took place with incorporation of cellular inorganic P.

[2] Turnover rate calculated on the assumption that the formation of phosphatides took place with incorporation of extracellular inorganic P.

[3] Fraction extracted with cold ether (not protein-bound lecithin?).

[4] Fraction extracted, after removal of the ether-soluble lecithin, with hot alcohol (protein-bound?).

from that found in experiments of only 4 hours duration. This is not the case with the different phosphatide fractions secured from the muscles. In these fractions, both after 4 and after 12 hours cephalin and sphingomyelin are more active than lecithin.

When looking at the results of the experiments taking one day or more (see Tables 15, 16 and 22) we notice that the lecithin extracted from the liver is more active than the cephalin, while the opposite was found to be the case for the fractions secured from the brain.

Table 19.

Renewal of lecithin, cephalin and sphingomyelin.

Rabbit V. — Weight: 2.1 kg.

Intravenous injection during 250 min.

Organ	Percentage of phosphatides renewed during the experiment					
	A[1]			B[2]		
	Lecithin	Cephalin	Sphingomyelin	Lecithin	Cephalin	Sphingomyelin
Liver	12.4	24.6	8.95	1.86	3.68	1.34
Kidney	3.7	13.5	—	2.10	7.7	—
Muscle	2.9[3] / 1.6[4]	21.7	15.1	0.044[3] / 0.024[4]	0.33	0.23
Small intestine (mucosa) ...	15.6	33.4	—	2.69	5.77	—

[1] Turnover rate calculated on the assumption that the formation of phosphatides took place with incorporation of cellular inorganic P.

[2] Turnover rate calculated on the assumption that the formation of phosphatides took place with incorporation of extracellular inorganic P.

[3] Fraction extracted with cold ether.

[4] Fraction extracted, after removal of the ether-soluble lecithin, with hot alcohol.

Table 20.

Renewal of lecithin and cephalin.

Rabbit VI. — Weight: 2.6 kg.

Subcutaneous injection during 255 min.

Fraction	Percentage of phosphatides renewed during the experiment	
	A[1]	B[2]
Liver lecithin	9.9	2.2
Liver cephalin	35.4	7.9

[1] Turnover rate calculated on the assumption that the formation of phosphatides took place with incorporation of cellular inorganic P.

[2] Turnover rate calculated on the assumption that the formation of phosphatides took place with incorporation of extracellular inorganic P.

Table 21.

Renewal of lecithin, cephalin and sphingomyelin.

Rabbit VII. — Weight: 2.4 kg.

Subcutaneous injection during 11.5 hours.

Organ	Percentage of phosphatides renewed during the experiment					
	A[1]			B[2]		
	Lecithin	Cephalin	Sphingomyelin	Lecithin	Cephalin	Sphingomyelin
Liver	27.5[3] / 22.0[4]	25.9	14.8	16.3[3] / 13.0[4]	15.3	8.8
Muscle	5.6	20.6	17.2	0.49	1.81	1.51

[1] Turnover rate calculated on the assumption that the formation of phosphatides took place with incorporation of cellular inorganic P.

[2] Turnover rate calculated on the assumption that the formation of phosphatides took place with incorporation of extracellular inorganic P.

[3] Fraction extracted with cold ether.

[4] Fraction extracted, after removal of the ether-soluble lecithin, with hot alcohol.

Table 22.

Renewal of lecithin, cephalin and sphingomyelin.

Rabbit VIII. — Weight: 2.0 kg.

Subcutaneous injection during 9 days.

Fraction	Specific activity at the end of the experiment	Lower limit of percentage renewed
Liver inorganic P	100	..
Liver lecithin P	84.0	84.0
Liver cephalin P	84.8	84.8
Muscle inorganic + creatine P	40.3	..
Muscle ester P	18.7	46.4
Muscle lecithin P	12.5	31.0
Muscle cephalin P	11	27
Muscle sphingomyelin P	16.1	40.0
Brain inorganic P	18.8	..
Brain ester P	17.3	92
Brain lecithin P	5.3	28
Brain cephalin P	5.6	30
Plasma inorganic P	100	..
Plasma phosphatide P	82	..
Corpuscle acid soluble P	94	..
Corpuscle phosphatide P	61.7	65.6

Table 23.

Renewal of lecithin and cephalin.

Rabbit IX. — Weight: 2.5 kg.

Subcutaneous injection during 50 days.

Organ	Percentage of phosphatides renewed during the experiment	
	Lecithin	Cephalin
Liver	100	100
Marrow	100	100
Brain	75[1] (42[2])	81[1] (46[2])
Muscle	74[1] (65[2])	71[1] (62[2])

[1] Calculated on the assumption of formation inside the cells (with incorporation of plasma inorganic P).

[2] Calculated on the assumption of formation outside the cells (with incorporation of plasma inorganic P).

Discussion.

The fact illustrated by the results described above — that in experiments taking only a few hours, the cephalin shows a higher extent of renewal than the lecithin, while, in experiments taking one day or more, the opposite is the case — suggests that not all cephalin present in the organs is renewed at the same rate, some fractions showing a much faster turnover rate than others. These fractions could differ either in their chemical composition or in their location in the cells. Numerous chemically different cephalins and lecithins exist differing, for example, in the type of fatty acids they contain. It is, however, not probable that the difference in the chemical constitution is responsible for the remarkable difference in the turnover rate of the different cephalin fractions. The specific activity of successive fractions of cephalin crystallised repeatedly from alcoholic or other solutions does not vary appreciably (comp. p. 52). A much more probable explanation of the difference mentioned above is that in some parts of the cell a decidedly more pronounced enzymatic breakdown and building up of cephalin takes place than in others. In experiments of short duration, we mainly measure the rejuvenation taking place in these favoured districts. The behaviour of lecithin is different in that we do not encounter such a pronounced variation in the rate of turnover of different fractions. The average lecithin molecule is, however, renewed at a similar rate as the average cephalin molecule. This explanation is suggested by the fact that, while in experiments of short duration the cephalin P extracted from the liver, for example, is found to be more active than the lecithin P, in experiments of long duration both fractions are found to

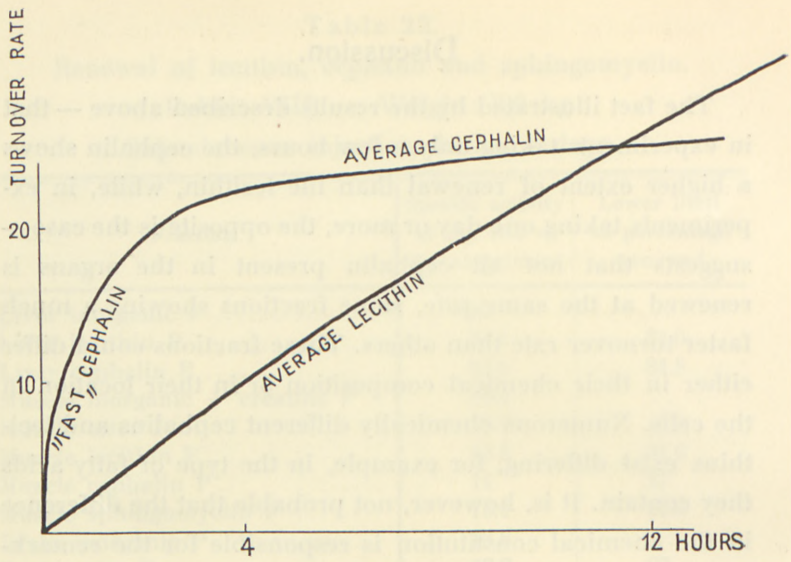

Fig. 5. Turnover of lecithin and cephalin in the liver.

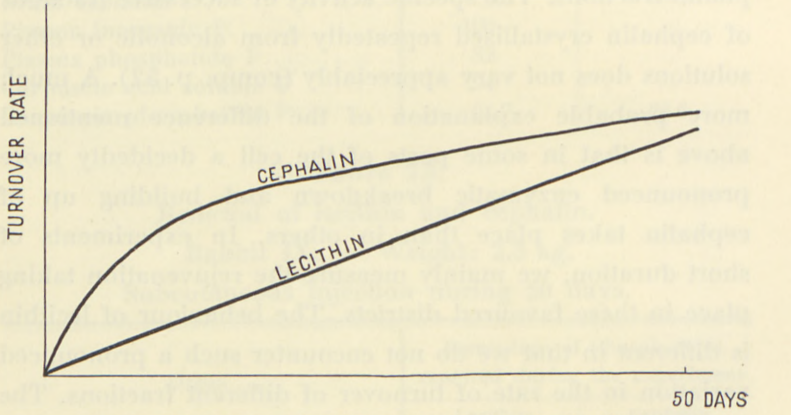

Fig. 6. Turnover of lecithin and cephalin in the brain.

have about the same activity. Not only cephalin and lecithin extracted from the organs of the rabbit show this behaviour, but also the phosphatide fractions secured from the organs of rats, frogs, hens, and of isolated cat liver.

That, in the case of the muscle and brain tissue, cephalin is found in experiments of long duration as well to have a faster turnover rate than lecithin is in no way in contradiction to the conclusion arrived at when investigating the liver fractions. All phosphatide fractions present in the muscle, and especially those in the brain, are renewed at a comparatively slow rate. This remark applies also to the "fast" cephalin fraction present which, though "fast" relative to the average cephalin or lecithin, is in fact "slow". This slowness has the effect that seven days do not suffice to reach the point where the amount of newly formed lecithin is larger than that of the newly formed cephalin. The considerations made above are illustrated by Figs. 5 and 6.

Brain phosphatide.

While, in the case of other tissues, the penetration of phosphate from the plasma into the interspaces can be considered as an almost momentary process and, accordingly, the specific activity of the plasma inorganic P can be taken to be equal to that of the extracellular inorganic P, it cannot in brain tissue. We find that after 4 hours only 1/3 of the amount of labelled phosphorus (incl. organic P) is present in the brain tissue, which we should expect to be present in the extracellular space alone in case of a prompt distribution of the labelled phosphate between the plasma and the interspaces. The extracellular space of the brain tissue was calculated from the chlorine or sodium distribution to amount to 30 per cent of the weight of the tissue[1].

In view of the foregoing statements, we cannot give

[1] J. F. Manery and B. Hastings, J. Biol. Chem. **127**, 657 (1939).

Table 24.

Formation of labelled phosphatides in the brain of the rabbit.

Duration of the experiment	Spec. activity of brain inorganic P / Spec. activity of plasma inorganic P	Spec. activity of brain phosphatide P / Spec. activity of plasma inorganic P	Spec. activity of brain phosphatide P / Spec. activity of brain inorganic P	Spec. activity of brain phosphatide P / Spec. activity of liver phosphatide P
215 min.	0.013	0.0001	0.0093	0.0032
250 min.	0.015	0.0002	0.016	0.005
11.5 hours	0.030	0.0033	0.11	0.022
9 days	0.19	0.054	0.29	0.063
50 days	0.56	0.43	0.77	0.43

exact figures for the specific activity of the extracellular and cellular inorganic P of the brain. Since these figures enter the calculation of the turnover rate of the brain phosphatides the calculation cannot be carried out. A further complication arises from the fact that the decomposition of the brain creatinephosphoric acid prior to the extraction of the inorganic P could not be avoided in our experiments. The brain creatine P may be appreciably less active than the brain inorganic P. This fact would lead to a dilution of the labelled inorganic P by non-labelled inorganic P. We record, therefore, in Table 24, a) the specific activity of the brain phosphatide P relative to the plasma inorganic P, b) relative to the brain total inorganic P, and c) relative to the liver phosphatide P. While the brain phosphatides are found to be much less active than the liver phosphatides, high values are obtained for the ratio of the specific activity of the brain phosphatide P and the brain inorganic P. Even if we divide these values by 2, to account

Table 25.

Formation of labelled lecithin and cephalin in the brain of the rabbit.

Duration of the experiment	Relative specific activity		
	Plasma inorganic P	Brain lecithin P	Brain cephalin P
250 min	100	0.0092	—
11.5 hours	100	0.25	1.08
19 hours[1]	100	0.40	1.04
50 days	100	42	46

[1] In this case, the total activity was injected at the start of the experiment.

for the diluting effect of the creatine[2] P, the resulting figures will still be high.

CHANGUS, CHAIKOFF and RUBEN[2] observed a progressive increase in the content of radioactive phosphatides in the brain on rats for about 200 hours after the administration of labelled phosphorus and it is of interest to note that, in a recent investigation, CHAIKOFF and his colleagues[3] found that the specific activity of the phosphatide P is not uniform throughout the central nervous system.

Experiments with rats.

The specific activity of lecithin P and cephalin P extracted from the rat's liver is given in Tables 26 and 27.

While the ratio of the specific activity of cephalin and lecithin P was found, after 3 hours, to be 1.33, after 24

[2] G. W. CHANGUS, J. L. CHAIKOFF and S. RUBEN, J. Biol. Chem. **126**, 493 (1938).

[3] B. A. FRIES, G. W. CHANGUS and J. L. CHAIKOFF, J. Biol. Chem. **132**, 24 (1940).

Table 26.

Specific activity of lecithin and cephalin in the rat's liver.
Weight of the rat: 200 gm.
All labelled phosphate was injected subcutaneously
at the start of the experiment;
190 min. later, the rat was killed.

Fraction	Percent of activity injected, found in 1 mgm. phosphatide P
Liver lecithin	0.21
Liver cephalin	0.28

Table 27.

Specific activity of different P fractions in the rat's liver.
Weight of the rat: 222 gm.
All labelled phosphate was injected subcutaneously
at the start of the experiment;
24 hours later, the rat was killed.

Liver fraction	Relative specific activity[1]
Labile P	100
Lecithin P	117
Cephalin P	82
Non-labile acid-soluble P	103
Protein P	31

[1] The figures are given relative to the labile acid-soluble P, the value of which, after 24 hours, closely corresponds to that of the inorganic P.

hours we find the value 0.7. Similarly, Chargaff[2] found, in experiments taking 24 hours, greater turnover figures for lecithin than for cephalin. He found the above ratio to be 0.8. It is also of interest to note that an early paper

[2] E. Chargaff, J. Biol. Chem. **128**, 592 (1939).

of ARTOM[1] and his colleagues contains data on the relative activity of lecithin and cephalin extracted from the liver of rats to which olive oil and labelled sodium phosphate was administered 9 hours previously. They state the above ratio to be about 0.6.

Experiment with frog.

The turnover figures of cephalin and lecithin extracted from the frog's liver were found, as would be expected, to be lower than the corresponding figures found in experiments with mammalia. The specific activity of the cephalin P was found to be much higher than that of the lecithin P.

Table 28.

Specific activity of different P fractions in the liver of a frog.

Labelled phosphate was injected into the lymph-sack of a frog kept at 20° all through the experiment (4 hours).

Liver fraction	Relative specific activity
Inorganic P	100
Cephalin P	7.8
Lecithin P	1.3

Ratio of specific activity of cephalin and lecithin = 6.

Experiments with laying hens.

That, in a laying hen, the specific activity of cephalin P of the liver is found, after the lapse of 5 hours, to be slightly

[1] C. ARTOM, C. PERRIER, M. SANTANGELO, G. SARZANA and E. SEGRÈ, Archiv Intern. Physiol. **45**, 35 (1937).

Table 29.

Specific activity of P fractions in the organs of a hen weighing 900 gm.

Labelled phosphate was administered to a laying hen by subcutaneous injection at the start of the experiment; the hen was killed 5 hours later.

Fraction	Relative specific activity
Plasma lecithin P	1.00
Plasma cephalin P	0.98
Liver lecithin P	2.76
Liver cephalin P	2.93
Liver sphingomyelin P	1.38
Liver protein P	0.15
Kidney lecithin P	1.15
Kidney cephalin P	1.69
Intestinal mucosa lecithin P	0.90
Intestinal mucosa cephalin P	1.05
Intestinal mucosa sphingomyelin P	1.10

higher than that of lecithin P, while in experiments with rabbits a very great difference was found, is just the result which we have to expect in view of the arguments discussed on p. 43. In the course of 5 hours, the phosphatide molecules present in the liver of the hen are renewed to an extent which in the case of the rabbit is first reached after the lapse of many hours. It is, therefore, not surprising that the fractions obtained from the hen's liver are similar to those secured from the rabbit's liver in experiments of much longer duration. In the kidneys of the laying hen the phosphatide molecules are renewed at a slower rate than in the liver and, in this organ, as was to be expected, cephalin is found to be markedly more active than lecithin.

The liver sphingomyelin of the laying hen which does

not enter the yolk to any appreciable extent is renewed at a decidedly lower rate than the petrol-ether soluble phosphatides. It is also of interest to note that the rate of rejuvenation of the protein P in the liver of the laying hen is about 20 times slower than that of the phosphatide P.

In the intestinal mucosa, cephalin and sphingomyelin are formed at a somewhat higher rate than lecithin. In the kidneys cephalin was found more active than lecithin. That the rate of renewal of phosphatides in the liver of laying hens is decidedly higher than in the intestinal mucosa or other organs was also found in our earlier researches[1].

Experiment with perfused cat liver.

The experiment on cat liver which was carried out with the kind help of Professor LUNDSGAARD also indicates the faster cephalin turnover in experiments of short duration. The fasting cat used in this experiment weighed 3.3 kg. Blood circulated for 70 min. through the isolated liver.

Table 30.

Specific activity of P fractions in the liver of a cat.

Duration of experiment: 70 min.

Fraction	Relative specific activity
Plasma inorganic P	100
Plasma lecithin P	0.18
Liver lecithin P	2.43
Liver cephalin P	4.05

Ratio of the activity of cephalin P and lecithin P = 1.67.

[1] G. HEVESY and L. HAHN, D. Kgl. Danske Vidensk. Selskab, Biol. Medd. XIV, 2 (1938).

Besides labelled phosphate of negligible weight, 500 mgm. alcohol was added to the blood at the start of the experiment and 500 mgm. glycocoll after 30 min.

When fractionating the alcoholic solution of the liver cephalin, the less soluble fraction was found to show the higher specific activity amounting to 4.47. The low lecithin activity of the plasma is, in view of the short duration of the experiment, not surprising. The labelled phosphate requires some time to penetrate into the liver cells, the formation of labelled lecithin takes some time as well and, finally, the release of the phosphatides into the plasma is far from being a momentary process.

Survey of the results.

In the course of 4 hours, an appreciable part of the petrol-ether soluble phosphatides present in the intestinal mucosa and the liver were found to be renewed. This result is in conformity with that found by Artom and his colleagues[1], by Chaikoff and his collaborators[2], and in this laboratory[3]. In Tables 31 and 32, a summary of the data obtained on the renewal rate of lecithin, cephalin and sphingomyelin fractions is given. In Table 32, the very different behaviour of lecithin from cephalin is clearly seen. While, in the case of lecithin, the labelled percentage increases more or less linearly with time, this is far from being the case with cephalin. We find an almost linear increase with time in the amount of labelled lecithin formed in the liver and the

[1] C. Artom, C. A. Perrier, M. Santangelo, G. Sarzana and E. Segrè, Archiv Internat. Physiol. **45**, 32 (1937).

[2] B. A. Fries, S. Ruben, J. Perlman and J. L. Chaikoff, J. Biol. Chem. **123**, 587 (1938).

[3] L. Hahn and G. Hevesy, Nature **144**, 204 (1939).

Table 31.

Extent of renewal of the petrol-ether soluble phosphatide mixture extracted from the organs of the rabbit in the course of 4 hours.

The results are computed from the figures of Tables 2—7.

Organ	Percentage of phosphatides renewed during the experiment	
	A[1]	B[2]
Small intestine (mucosa)	19.6	3.7
Liver	16.7	3.1
Lungs	8.1	1.2
Stomach	7.7	0.9
Muscle	7.3	0.11
Kidney	6.2	4.3
Spleen	5.2	0.74
Corpuscles	5.2	0.33
Heart	4.0	0.50

[1] Turnover rate calculated on the assumption that the formation of phosphatides took place with incorporation of cellular inorganic P.

[2] Turnover rate calculated on the assumption that the formation of phosphatides took place with incorporation of extracellular inorganic P.

muscles, assuming that the formation of this compound takes place inside the cells. This linearity does not hold if we assume the formation of phosphatides to take place with incorporation of extracellular P. The bulk of the labelled liver lecithin could not be formed in the last mentioned way, since in that case (see column 5 of Table 32) nine times as much labelled lecithin should have been formed in the course of 12 hours than was found after 4 hours. Similar considerations apply to the muscle lecithin where in the course of 12 hours seventeen times as much labelled lecithin should have been formed as after 4 hours. Such an increase with time is highly improbable.

Table 32.

Extent of renewal of lecithin, cephalin and sphingomyelin in the organs of the rabbit in the course of experiments lasting 4 hours and 12 hours, respectively.

The results are computed from the figures in Tables 17—21.

Organ	Percentage of phosphatides renewed					
	A[1]			B[2]		
	Lecithin	Cephalin	Sphingomyelin	Lecithin	Cephalin	Sphingomyelin
	after 4 hours					
Small intestine (mucosa)	16.3	37	—	3.1	7.1	—
Liver	9.3	28	6.7	1.7	5.6	1.1
Muscle	2.3	21	15.1	0.03	0.33	0.23
Kidney	3.7	13	—	2.1	7.7	—
	after 12 hours					
Liver	25	26	15	14.6	15.3	8.8
Muscle	5.6	21	17	0.5	1.8	1.5

[1] Turnover rate calculated on the assumption that the formation of phosphatides took place with incorporation of cellular inorganic P.

[2] Turnover rate calculated on the assumption that the formation of phosphatides took place with incorporation of extracellular inorganic P.

If we consider the two possibilities of the formation of cephalin, i. e. incorporation either of cellular or of extracellular labelled inorganic P, we arrive at the following result. If the labelled cephalin is formed inside the liver cells, as much as 1/4 became labelled within 4 hours; thus, 1/4 of the total cephalin present undergoes a rapid renewal, the remaining 3/4 being comparatively inert. In the course of the following 8 hours, hardly any further increase of the amount of newly formed cephalin can be noticed. That

also the remaining part of the cephalin is renewed, though at a very slow rate, is, however, shown by the fact that, after 9 days, most of the cephalin present at the start of the experiment was found to be labelled. Muscle cephalin beheaves in an analogous way.

If we now consider the possibility that the labelled cephalin is formed with incorporation of extracellular P, we arrive at an entirely different interpretation of the results. The amount of labelled liver cephalin formed in the course of 12 hours then works out to be about three times that formed during 4 hours. This result is quite plausible. The result obtained in the case of the muscle cephalin, where as much as five times more labelled cephalin should have been formed in the course of 12 than in 4 hours, seems less plausible. While it is unprobable that the cephalin present in the liver should have been formed with incorporation of extracellular inorganic P, we must envisage the possibility that a part of the cephalin located in the cell membranes is renewed with incorporation of inorganic P located inside the membrane. We discussed above the two extreme cases, formation of phosphatides with incorporation of cellular and of extracellular P. While penetrating through the cell wall, the inorganic P may experience a more or less continuous drop in its activity and the renewal of phosphatide molecules located in the cell membranes could take place by incorporation of "intermediary" labelled phosphate radicals.

It is of interest to remark that in a laying hen, where the liver has to supply large amounts daily of both lecithin and cephalin, the "slow" cephalin fraction is also renewed at a remarkable rate and the rejuvenation of the average lecithin and cephalin in the course of 5 hours hardly differs.

Difference between "fast" and "slow" cephalin.

That the organs contain a small cephalin fraction which is renewed at a fast rate and a larger one which is slowly renewed may possibly be due to a difference in the chemical composition of these fractions. Since different cephalin fractions obtained by fractional crystallisation of the total cephalin extracted from the organ in question did not show large variations, it is not probable that the above result can be explained as due to different rates of newformation of cephalins of different chemical composition.

In fractional crystallisation of alcoholic cephalin solutions, only minor differences in the specific activity of the fractions were noticed. The least soluble fraction extracted from the liver showed, for example, a turnover rate of 4.47, while the value found for the average fraction was 4.05. When organs were extracted first with ether and then with hot alcohol, the lecithin prepared from the first extract was found to be somewhat more active than was the lecithin prepared from the alcohol extract (see Table 19).

Since the renewal of cephalin is an enzymatic process, its velocity should be determined by the effectivity of the enzymes present. It is probable that that part of the cephalin which is located in such a region of the cells, where the enzymatic action is very pronounced, is renewed at a very fast rate. It is also probable that this "fast" fraction has a different biological significance from the "slow" fraction. The fact that the phosphatides have a much larger turnover in some organs than in others induced Sinclair[1] to distinguish between metabolic and non-metabolic phosphatides. The former ones found in the liver, for example, should

[1] R. G. Sinclair, Physiol. Rev. **14**, 357 (1934).

be involved in fat metabolism; the latter ones, found for example, in the muscle, should play an important role in building up cell membranes. Our results suggest the interpretation that we have in all the organs investigated a "fast" and a "slow" cephalin fraction as well. The "fast" fraction is the smaller one. To what extent the "fast" cephalin and other phosphatide fractions are involved in fat metabolism is under investigation.

It is of interest to remark that, if we want to calculate the amount of fatty acid which passed through the phosphatide stage in the liver, for example, we are not sure to arrive at a correct result by calculating from the known phosphatide turnover in the liver the amount of fatty acid or that of inorganic phosphate which passed through the phosphatide stage during the experiment. A minor percentage of the phosphatides present may, as discussed above, have a very high turnover rate, i. e. a minor percentage of the phosphatides may have been decomposed and built up several times during the experiment. It is obvious that such a process would make it possible that much larger amounts of fatty acids or inorganic P pass through the phosphatide stage than those calculated from the known turnover rate of the average phosphatide molecules. The possibility must, therefore, be envisaged that, in an organ, an appreciable amount of fatty acids may pass through the phosphatide stages in spite of the fact that the rejuvenation of the total phosphatide content of the organ in question is a slow process.

Summary.

Labelled sodium phosphate was administered to rabbits, rats, frogs and laying hens. In order to keep the concentration of the labelled phosphate in the plasma constant, labelled phosphate was injected from time to time throughout the experiments.

The specific activity of the inorganic P extracted from the plasma and the organs was measured at intervals. From these data the average specific activity of the cellular inorganic P prevailing during the experiment was calculated.

The phosphatides present in various organs were extracted as well, and the specific activity of the phosphatide P and also of the lecithin, cephalin and sphingomyelin P determined.

The knowledge of the average specific activity of the cellular inorganic P during the experiment and that of the phosphatide P at the end of the experiment permits us to calculate the extent of newformation (turnover) of the phosphatides on the assumption that this process takes place inside the cells. In case the phosphatide molecules are renewed with incorporation of extracellular inorganic phosphate, the specific activity of the latter enters the calculation.

The specific activity of cephalin P extracted from different organs was found in experiments of short duration (4 hours) to be much higher (up to 10 times) than that of lecithin P. With increasing time of experiment this difference was found to diminish. In the fractions obtained from the rabbits liver, after the lapse of 12 hours, both fractions showed the same activity. In organs like muscle

and brain, in which a slow phosphatide turnover takes place, an equal activity of lecithin and cephalin is only reached after the lapse of several days.

Sphingomyelin is renewed in the liver at a slower rate than the ethersoluble phosphatides. In the muscles, in experiments taking not longer than 12 hours, sphingomyelin was found to be appreciably more active than lecithin, but less active than cephalin; after the lapse of 9 days, sphingomyelin was found to be the most active fraction.

Two alternative explanations are put forward to explain the difference in the behaviour of cephalin and lecithin: (a) A part (about 1/4) of the cephalin present inside the cells is renewed at an appreciably higher rate than the average cephalin present, while the bulk of the cephalin showed a similar turnover rate as the average lecithin; or (b) a part of the cephalin located in the cell walls is renewed in situ with incorporation of inorganic phosphate which has a higher specific activity than the inorganic P located inside the cells.

In the course of 50 days, all phosphatide molecules present in the liver and the skeleton were found to be renewed. However, only 74 per cent of the lecithin and 71 per cent of the cephalin extracted from the muscles were newly formed in the course of the experiment. In the brain tissue, 1/4 or more of the lecithin and 1/5 or more of the cephalin molecules remained unchanged.

The amount of active lecithin and cephalin present in the plasma and corpuscles was determined. The active plasma phosphatide molecules are not formed in the circulation but in the organs and are led into the blood. Most of the individual phosphatide molecules present in the corpuscles were incorporated during the formation of

the erythrocytes, but some turnover also took place inside the corpuscles.

We wish to express our hearty thanks to Professor Niels Bohr and to Professor J. N. Brønsted for kindly putting numerous facilities at our disposal, to Professor E. O. Lawrence for the gift of the active phosphorus and to Professor E. Lundsgaard for valuable suggestions in connection with this work.

København. Universitetets Institut for teoretisk Fysik og Universitetets Institut for fysisk Kemi.

Indleveret til Selskabet den 29. Marts 1940.
Færdig fra Trykkeriet den 6. Juli 1940.