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RATE OF RENEWAL OF THE ACID SOLUBLE ORGANIC PHOSPHORUS COMPOUNDS IN THE ORGANS AND THE BLOOD OF THE RABBIT

WITH A NOTE

ON THE DURATION OF LIFE OF THE RED BLOOD CORPUSCLES

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In a paper published recently in these Proceedings[1], the rate of renewal of the phosphatide molecules present in various organs of the rabbit and other animals was discussed. In the present publication, data on the rate of new-formation of acid soluble phosphorus compounds are communicated. The acid soluble organic P compounds represent a great variety of chemically very different bodies: esters as, for example, hexosephosphate, nucleotide compounds as adenosintriphosphate, phosphagen, and other compounds. These compounds[2] are renewed at a comparatively fast rate in the organs in contradistinction to the phosphatides and nucleoproteins[3]. Furthermore, while the rate of new-formation of the phosphatides in the circulation is almost negligible, the acid soluble phosphorus compounds are renewed at a remarkable rate in the corpuscles. These facts justify the consideration of the acid soluble phosphorus compounds from our view-point as a definite group of the phosphorus compounds present in the body.

[1] G. Hevesy and L. Hahn, Det Kgl. Danske Vidensk. Selskab, Biol. Medd. XV, 5 (1940).

[2] With the exception of adenylic acid [T. Korzybski and J. K. Parnas, Z. physiol. Chem. **255**, 195 (1938)] and, possibly, of other not yet known minor components of the acid soluble P mixture.

[3] L. Hahn and G. Hevesy, Nature, April 6, 1940.

Experimental method.

Labelled P as sodium phosphate was administered by intravenous or subcutaneous injection to rabbits all through the experiment in order to keep the activity of the plasma inorganic P at a constant level. After the lapse of some hours or days, the animal was killed by bleeding. The fresh organs were placed in liquid air and were extracted immediately with cold 10 per cent trichloroacetic acid. The inorganic phosphate present in the extract was precipitated as ammonium magnesium phosphate. The filtrate obtained was then hydrolysed with 1 n H_2SO_4 for 7 min. at 100° to split off the labile P which was then precipitated as ammonium magnesium salt. The filtrate obtained after the last mentioned operation was hydrolysed 100 min. to split off the phosphate radical of the hexosephosphate present. In order to avoid several consecutive precipitations of ammonium magnesium phosphate which lead to an accumulation of very appreciable amounts of ammonium salt in the soluble fraction, we usually divided the filtrate obtained after precipitation of the inorganic phosphate present as such in the tissue into aliquot parts. One aliquot part was hydrolysed for 7 min., the phosphate split off was precipitated, and the filtrate obtained was hydrolysed for 100 min. Another aliquot part was hydrolysed for 7 min., the filtrate obtained was hydrolysed for 12 hours, and so on. The phosphate of the creatinephosphoric acid was split off by heating the solution for 30 min. to 40°. In some cases, the total acid soluble organic P was converted into phosphate and was investigated in toto. The ammonium magnesium phosphate precipitates obtained were dissolved in diluted hydrochloric acid and an aliquot part was used for a colori-

metric P determination. To another aliquot part about 80 mgm. non-active sodium phosphate was added; the total P present in the solution was then precipitated as ammonium magnesium salt. The radioactivity of these precipitates was determined by the aid of a GEIGER counter.

Though the separation of the different acid soluble P compounds described above is far from being quantitative, it sufficed in most cases for our purpose.

In the experiments with blood, as anti-coagulent, ammonium oxalate was used. The corpuscles were centrifuged off and washed twice with a physiological sodium chloride solution. In experiments in vitro, the blood was kept in a $CO_2 - O_2$ atmosphere and was shaken, after addition of labelled sodium phosphate of negligible weight, for 30—190 min. in a thermostat at 37°.

Rate of new-formation.

As labelled phosphorus atoms can only be incorporated into organic molecules in the course of a synthetic process, the radioactivity of the organic phosphorus compounds isolated from an organ is a measure of the rate of its total or partial resynthesis. It is, however, not permitted to compare the specific activity (activity per mgm. P) of the hexosemonophosphate extracted from the kidney and the muscle, for example, and to conclude from the fact that the hexosemonophosphate extracted from the kidney is much more active than that secured from the muscle, that the rate of new-formation[1] of hexosemonophosphate is

[1] The significance of the notion of the rate of new-formation is discussed in the paper by G. HEVESY and L. HAHN, Det Kgl. Danske Vidensk. Selskab, Biol. Medd. XV, 5 (1940).

correspondingly larger in the kidney. The incorporation of labelled P atoms into the hexosemonophosphate molecules must be preceded by a diffusion of the labelled inorganic P into the cells of the organ. If this diffusion process is slow, the rate of formation of labelled hexosemonophosphate molecules is bound to be comparatively slow, in spite of a possibly very fast rate of new-formation of the hexosemonophosphate molecules inside the cells of the organ in question. In fact, the labelled inorganic phosphate molecules penetrate very much faster into the kidney cells than into the muscle cells. To get proper information on the rate of renewal of an organic compound in an organ, we have to compare the specific activity of the P isolated from the organic compound in question at the end of the experiment with the average value of the specific activity of the cellular inorganic P prevailing during the experiment. The considerations mentioned above are discussed in detail in the publication cited above on the turnover rate of phosphatides. In this paper is described the method which permits us to calculate from the specific activity of the tissue inorganic P, the specific activity of the plasma inorganic P, the extracellular space of the organ, and the inorganic P content of the organ and plasma the average specific activity of the cellular inorganic P during the experiment. From the latter magnitude and the specific activity of the P of the organic phosphorus fraction at the end of the experiment, we can calculate what percentage of the organic compound in question is newly formed during the experiment, if only the extent of new-formation is restricted.

If a large fraction of the hexosemonophosphate molecules, for example, is newly formed during the experiment, we can no longer disregard the number of hexosemono-

phosphate molecules which were decomposed and resynthesised more than once during the experiment. If such a repeated new-formation takes place, it will have the effect that the active hexosemonophosphate molecules present at the end of the experiment cannot be longer considered as having been formed with participation of inorganic P which had an activity corresponding to the average activity prevailing during the experiment. The inorganic P atoms, which had an activity corresponding to a late stage of the experiment, will clearly be found to a larger extent incorporated in hexosemonophosphate molecules than those P atoms the activity of which corresponds to an early stage of the experiment.

Let us consider active hexosemonophosphate molecules which were formed during the first stage of the experiment and which were again newly formed during the last minute of the experiment. If the second process were not forthcoming, we should find molecules of small activity; if the opposite were the case, we should find the molecules to be strongly active. When calculating the fraction of the hexosemonophosphate molecules which were newly formed (once or several times) during the experiment from the ratio

$$R = \frac{\text{specific activity of hexosemonophosphate P at the end of the experiment}}{\text{average specific activity of inorganic P during the experiment}}$$

we overestimate the percentage of hexosemonophosphate which was renewed during the experiment. This will be especially the case if the ratio

$$\frac{\text{rate of renewal}}{\text{rate of interpenetration}}$$

is large, as, for example, in the case of the corpuscles.

When calculating from the ratio

$$\frac{\text{specific activity of organic P at the end of the experiment}}{\text{average specific activity of inorganic P during the experiment}}$$

the extent of renewal of the acid soluble P mixture in the corpuscles, we arrive at a value of 199 per cent (see Table I). Such a calculation, for reasons stated above, supplies the upper limit of the extent of renewal. The lower limit is given by the ratio

$$\frac{\text{specific activity of organic P at the end of the experiment}}{\text{specific activity of inorganic P at the end of the experiment.}}$$

The actual value clearly lies very much nearer to the lower than to the upper limit.

Table I.

Extent of renewal of the total organic acid soluble P in the organs of the rabbit.

Rabbit II. Weight 2.6 kg.

Intravenous injection during 215 min.

Organ	A Specific activity of the tissue inorganic P at the end of the experiment	B Specific activity of the cellular inorganic P at the end of the experiment	C Average specific activity of the cellular P during the experiment	D Specific activity of the organic P at the end of the experiment	$\frac{D}{C} \times 100$ Upper limit of the percentage renewed	$\frac{D}{B} \times 100$ Lower limit of the percentage renewed
Plasma	100	—	—	—	—	—
Corpuscles	12.7	12.7	6.4	12.7	199	100
Kidney	87.4	87.1	77.8	33.6	43.2	38.6
Small intestine, mucosa	47.4	45.2	22.6	24.0	106	53.1
Liver	44.0	40.6	20.4	14.3	70.2	35.2
Lungs	36.5	26.9	13.4	9.5	71.0	35.3
Spleen	30.8	28.5	14.3	—	—	—
Stomach	25.9	23.6	11.8	6.9	58.5	29.2
Heart	25.5[1]	21.4	10.8	8.6	79.6	40.2
Brain	1.32	—	—	0.56	—	—

[1] The inorganic P extracted from the heart contains partly such inorganic P atoms which were formed through decomposition of creatine-phosphoric acid prior to the extraction. As the specific activity of the creatine P is, after the lapse of 4 hours, lower than that of the inorganic P (comp. the muscle values in Table III!), the specific activity of the cellular inorganic P of the heart is in fact higher than that stated above and, correspondingly, the values of the rate of renewal of the organic acid soluble P compounds in the heart are smaller than those stated in the last and the last but one column of Table I.

Table II.

Extent of renewal of different fractions of the organic acid soluble P.

Rabbits II, III, and IV (average).

Intravenous injection during 4 hours.

Organ	Time of hydrolysis in 1 n H_2SO_4 at 100°	Spec. activity of the organic P at the end of the experiment: Average specific activity of the cellular inorganic P during the experiment. (Upper limit of the percentage renewed)	Spec. activity of the organic P at the end of the experiment: Specific activity of the cellular inorganic P at the end of the experiment. (Lower limit of the percentage renewed)
Liver	0—7 min.	152	76
Liver	non-hydrolysed	66	33
Kidney, cortex...	0—100 min.	64	57
Kidney, cortex...	100 min.—12 hs.	47	42
Kidney, cortex...	non-hydrolysed	29	26
Kidney, cortex..	hydrolysed in 1 n NaOH at 80°	48	43

Table III.

Specific activity of acid soluble P fractions extracted from the organs of the rabbit.

Rabbit VII. Weight 2.4 kg.

Subcutaneous injection during 11.5 hours.

Fraction	Specific activity at the end of the experiment
Plasma inorganic P	100
Corpuscle inorganic P	25
Corpuscle P hydrolysed 15 hours in 1 n H_2SO_4 at 100°	25
Corpuscle P hydrolysed 15—120 hours in 1 n H_2SO_4 at 100°	25
Corpuscle non-hydrolysed residue	13.0
Muscle inorganic P	15.5
Muscle creatine P	8.5
Marrow inorganic P[1]	13.1
Marrow organic P	36.8
Brain inorganic P	3.0
Brain organic P	2.3

[1] The low value is presumably due to the presence of traces of slightly active bone P in the marrow sample.

Table IV.

Specific activity of acid soluble P fractions extracted from the organs of the rabbit.

Rabbit VIII. Weight 2.0 kg.

Subcutaneous injection during 9 days.

Fraction	Specific activity at the end of the experiment
Plasma inorganic P	100
Corpuscle total acid soluble P	94
Muscle inorganic + creatine P	40
Muscle ester P	18.7
Brain inorganic + creatine P	18.8
Brain ester P	17.3

Table V.

Specific activity of acid soluble P fractions extracted from the organs of the rabbit.

Rabbit IX. Weight 2.5 kg.

Subcutaneous injection during 50 days.

Fraction	Specific activity at the end of the experiment
Plasma inorganic P	100
Corpuscle total acid soluble P	100
Muscle inorganic + creatine P	88
Muscle ester P	77
Brain inorganic + creatine P	56
Brain ester P	68

Table VI.

Specific activity of acid soluble P fractions extracted from the corpuscles of the rabbit.

Rabbits II, III, and IV (average).

Intravenous injection during 4 hours.

Time of hydrolysis in 1 n H_2SO_4 at 100°	Specific activity at the end of the experiment
Inorganic P (present as such in the corpuscles)...	100
0—7 min.	100
7—100 min.	100
7 min.—12 hours	100
Non-hydrolysed in the course of 12 hours (residue)	87
Non-hydrolysed in the course of 24 hours (residue)	77

Table VII.

Experiments in vitro with rabbits blood.

Corpuscle fraction	Duration of the experiment	Specific activity at the end of the experiment
Inorganic P	30 min.	100
Hydrolysed 7 min.	30 -	77
Hydrolysed 7 min.—12 hours	30 -	16
Non-hydrolysed	30 -	13
Inorganic P	60 -	100
Hydrolysed 7 min.	60 -	90
Hydrolysed 7 min.—12 hours	60 -	41
Non-hydrolysed	60 -	28
Inorganic P	90 -	100
Hydrolysed 7 min.	90 -	82
Hydrolysed 7 min.—12 hours	90 -	57
Non-hydrolysed	90 -	46
Inorganic P	190 -	100
Organic acid soluble P	190 -	57

Table VIIIa.

Effect of temperature on the distribution of ^{32}P between plasma and corpuscles.

Rabbits blood after addition of labelled phosphate of negligible weight is shaken for 90 min.

Fraction	Temperature	
	37°	5°
Plasma inorganic P	78	96.7
Corpuscle inorganic P	3.8	0.62
Corpuscle organic P	18.2	2.64

Table VIIIb.

Effect of temperature on the distribution of ^{32}P between plasma and corpuscles.

Relative specific activity of the P fractions of the blood.

Fraction	Temperature	
	37°	5°
Plasma inorganic P	100	100
Corpuscle inorganic P	13.8	0.37
Corpuscle pyrophosphate P	11.3	0.36
Corpuscle non-hydrolysed P	6 9	0.087

Table VIIIc.

Effect of temperature on the ^{32}P fractions of the corpuscles.

Relative specific activity of the P fractions of the corpuscles.

Fraction	Temperature	
	37°	5°
Corpuscle inorganic P	100	100
Corpuscle pyrophosphate P	82	84
Corpuscle non-hydrolysed P	50	23.5

Discussion.

A. Renewal of the acid soluble P compounds in the organs.

B. Renewal of the acid soluble P compounds in the corpuscles.

A. Renewal of the acid soluble P compounds present in the organs.

As seen in Table I, in the course of 4 hours a very appreciable part of the average acid soluble P compounds present in many of the organs was renewed. A very active turnover takes place in the mucosa of the small intestine. One half or more of the molecules of the organic acid soluble P compounds present in this organ became renewed in the course of 215 min. This very marked rate of new-formation of the organic acid soluble P compounds is of interest in connection with the view put forward by VERZÁR and others on the role of intermediary phosphorylation processes in the resorption of sugar from the intestine[1]. The highest value for the specific activity of the acid soluble organic P was found in the kidneys. The labelled inorganic P diffuses faster into the cells of the kidneys than into those of any other organ. The high value of the specific activity of the acid soluble kidney P is, to some extent, due to the fact that the cellular inorganic P within 215 min. acquires a higher value in the kidneys than in other organs. If due regard is taken to this phenomenon we find that, in spite of the fact that the specific activity of the intestinal acid soluble P is lower than that of the corresponding fraction extracted from the kidneys,

[1] F. VERZÁR and E. J. MCDOUGALL, Absorption from the intestine. London 1936. Comp. also E. LUNDSGAARD, Z. physiol. Chem. **261**, 19 (1939).

the rate of renewal in the intestinal mucosa is greater than in the kidneys.

The rate of renewal of the organic acid soluble P molecules in the liver and in the lungs (see Table I) is also quite appreciable. The comparatively high value found for the ratio of the specific activities of the organic P and inorganic P in the case of the brain tissue is, at least to some extent, due to an extremely low activity of the average inorganic P of the brain. It is a puzzling result that the total activity found in the brain tissue, due to the presence of active inorganic and organic P, is smaller than that we should expect to find in the interspaces of the brain alone when assuming a proportional distribution of the active inorganic P between the plasma and the extracellular space of the brain tissue. In this calculation, the extracellular space is taken to be 30 per cent of the weight of the brain, as found from the distribution figures of chlorine and sodium[1] between the plasma and the brain tissue. Our results suggest the assumption that the labelled phosphate ions penetrate at a very slow rate through the capillaries of the brain or, alternatively, that the figures obtained by determining the distribution of chlorine or sodium between the plasma and the brain do not represent the proper extracellular space of the brain. It is for these reasons that we did not state in Table I any figures for the rate of renewal of the acid soluble P compounds present in the brain.

Table II contains data on the activity of different organic P fractions extracted from the kidneys and the liver. The phosphate obtained after 7 min. hydrolysis contains, as well known, besides P split off from creatinephosphoric acid, the labile P of the adenosintriphosphate molecules.

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

That the adenosintriphosphate molecules present in the muscles are reorganised at a fast rate was found in our previous experiments[1]. MEYERHOF and his collaborators[2] studied the rate of reorganisation of the adenosintriphosphate molecule with incorporation of active inorganic P in experiments in vitro and found this process to take place at a very fast rate. Data on the activity of the phosphorus obtained by hydrolysing the organic acid soluble phosphorus extracted from perfused cat liver for 7 min. are given by LUNDSGAARD[3].

Our experiments lead to the result that at least 76 per cent of the 7 min. product extracted from the liver of the rabbit became renewed in the course of 215 min. In LUNDSGAARD's perfusion experiment, the specific activity of the 7 min. fraction was found, after 90 min., to amount to 60 per cent of that of the inorganic P extracted from the plasma at the end of the experiment.

As seen in Tables II-VII the more readily hydrolysable compound is renewed at a faster rate than the less readily hydrolysable one. That even those compounds which resist treatment with 1 n H_2SO_4 at 100° for 12 hours or more are renewed, however, at a very appreciable rate is seen in Tables II and III. More than 1/4 of the non-hydrolysable residue of the organic acid soluble P fraction secured from the kidneys was, for example, found to be renewed in the course of 215 min. (see Table II.)

After the lapse of so long a time as 9 and 50 days (see Tables IV and V), the muscle inorganic + creatine P has

[1] G. HEVESY and O. REBBE, Nature **141**, 1097 (1938). G. HEVESY, Enzymologia **5**, 138 (1938).

[2] O. MEYERHOF, P. OHLMEYER, W. GENTNER and H. MAIER-LEIBNITZ, Biochem. Z. **298**, 398 (1938).

[3] E. LUNDSGAARD, Skand. Arch. f. Physiol. **80**, 291 (1938).

only reached 40 and 88 per cent, respectively, of the specific activity of the plasma inorganic P. After the lapse of 50 days, the specific activity of the ester P of the muscles was found to be 77 per cent of that of the plasma inorganic P. A detailed investigation of the rate of renewal of the acid soluble P compounds present in the muscles of the frog will be published shortly.

B. Renewal of the acid soluble P compounds present in the corpuscles.

1. Phosphorylation processes going on inside the corpuscles.

In our early investigations[1] on the circulation of phosphorus, using radioactive P as an indicator, we found that the organic acid soluble P compounds of the red blood corpuscles are normally in a state of flux, being continuously decomposed and resynthesised. Labelled phosphate ions were found to penetrate into the corpuscles at a fairly slow rate and to take part in very rapid phosphorylation processes inside the corpuscles. Labelled hexosemonophosphate introduced into the plasma was found not to penetrate at any significant rate into the corpuscles. However, the labelled phosphate present in such hexosemonophosphate molecules after being split off diffuses as inorganic phosphate into the corpuscles and is incorporated inside the erythrocytes partly into hexosemonophosphate molecules. Presumably, the P atoms of the plasma diffuse exclusively or almost exclusively as phosphate ions into the corpuscles.

That phosphorus compounds, as hexosephosphoric acid,

[1] L. Hahn and G. Hevesy, C. R. Lab. Carlsberg **22**, 188 (1938). G. Hevesy and A. H. W. Aten, Det Kgl. Danske Vidensk. Selskab, Biol. Medd. XIV, 5 (1939).

triosephosphoric acid, phosphopyruvic acid, phosphoglyceric acid, and so on, take an important part in glycolytic processes going on in the corpuscles was emphasised by v. EULER and BRANDT[1], and others. According to the views of MEYERHOF, PARNAS, and others, in the course of the glycolytic cycle, hexosediphosphate, for example, is found to be formed through the interaction of dextrose with adenosintriphosphate. Hexosediphosphate is maintained in enzymatic equilibrium with two molecules of triosephosphate. The last mentioned compound reacting with pyruvic acid forms phosphoglyceric acid which is converted into phosphopyruvic acid and this, in turn, reacts with adenylic acid in the resynthesis of adenosintriphosphate. The last mentioned compound is also formed by direct phosphorylation of adenylic acid from inorganic phosphate or by transfer of the phosphate radical of glycerophosphate to adenylic acid. The synthesis of adenosintriphosphate is a very rapid process and the active inorganic phosphate ions which penetrate into the corpuscles will soon be found to be incorporated in adenosintriphosphate molecules. The participation of the active adenosintriphosphate molecules in the synthesis of various organic P compounds will lead to the formation of active hexosephosphate, active phosphoglyceric acid, and so on, in the corpuscles. In this connection, the result obtained by DISCHE[2] is of interest: he found that the total phosphate transferred

[1] H. v. EULER and K. M. BRANDT, Z. physiol. Chem. **240**, 215 (1936). Comp. also H. LAWACZECK, Biochem. Z. **145**, 351 (1924); NEGELEIN, Biochem. Z. **158**, 121 (1925); M. MARTLAND, Biochem. J. **19**, 117 (1925); P. RONA and K. IWASAKI, Biochem. Z. **184**, 318 (1917); H. K. BARRENSCHEEN and B. VASARHELYI, Biochem. Z. **230**, 330 (1931); H. K. BARRENSCHEEN and K. BRAUN, Biochem. Z. **231**, 144 (1931).

[2] Z. DISCHE, Die Naturwiss. **24**, 462 (1936).

to glucose added to human erythrocytes originates from adenosintriphosphate.

Important evidence that the organic acid soluble phosphorus compounds and, primarily, diphosphoglycerate of the red blood corpuscles constitute a labile phosphorus reserve of considerable consequence serving various functions was presented in recent years by Guest and his colleagues[1]. Some of their findings are described in what follows.

The development of ricketts in rats is associated with decreases in all fractions of the acid soluble phosphorus. During the first five days, the concentration of inorganic phosphorus and adenosintriphosphate phosphorus drops abruptly to a low level and then remains constant for 25 days and longer. The decrease in the organic acid soluble phosphorus is accounted for almost entirely, after the first few days, in the diphosphoglycerate fraction. Guest and Rappaport state that diphosphoglycerate makes out about half of the acid soluble phosphorus present in the corpuscles.

In experiments carried out on dogs after nephrectomy, it was found that, due to the failure of excretion of the vaste endogenous P, a large increase in the inorganic P content of the blood takes place, which is followed by a corresponding increase in the acid soluble organic P content of the corpuscles. The increase is mainly due to the rise of the diphosphoglycerate content of the corpuscles, the increase in organic acid soluble P and in diphosphoglycerate P being 47 and 43 mgm., respectively, per hundred cc.

They found, furthermore, that the increase of phos-

[1] A summary of many of their results is to be found in the paper by G. M. Guest and S. Rappaport, American J. of Diseases of Children 58, 1072 (1939).

phorus excretion in the urine during acidosis comes partly from mobilised diphosphoglycerate of the corpuscles. As an effect of pyloric obstruction, an increase of the acid soluble P content amounting to 37 mgm. Eq per kg. corpuscle water of the dog was found to take place. From this increase, 32 mgm. Eq were due to the rise in the glycerophosphate content.

These and numerous other findings clearly show that the acid soluble phosphorus compounds of the red corpuscles are readily synthesised and decomposed in the blood through reactions of the glycolytic cycle. That these processes take place in the corpuscles at a remarkable speed was shown by us when making use of radioactive phosphorus as an indicator. We have, thus, two independent lines of evidence as to the remarkably high rate of turnover of phosphoglycerate and some other phosphorus compounds present in the corpuscles.

By comparing the specific activity of the inorganic P of the corpuscles with that of the P extracted from various organic compounds present in the corpuscles we get information on the rate of resynthesis of these compounds. The comparison of the specific activity of the inorganic P present in the corpuscles with that of the inorganic P present in the plasma informs us, on the other hand, on the rate of penetration of phosphate ions from the plasma into the corpuscles.

2. *Rate of new-formation of the acid soluble P compounds present in the corpuscles.*

As seen in Table VII which gives the result of experiments in vitro, the product of 7 min. hydrolysis has, after the lapse of 30 min., a specific activity amounting to 77 per

cent of that of the corpuscle inorganic P. The product hydrolysed between 7 min. and 12 hours, which contains besides hexosephosphate P and other fractions appreciable amounts of diphosphoglycerate P as well, is markedly less active than the readily hydrolysed fraction, while the specific activity of the P of the non-hydrolysed residue is only 1/8 of that of the corpuscle inorganic P. This fraction[1] consists mainly of 2, 3-diphosphoglyceric acid P though it contains also P of the adenylic acid which amounts, in the corpuscles of the rabbit, to about 5—10 mgm. per cent, thus to about 1/10—1/20 of the total acid soluble P of the corpuscles.

In experiments in vivo taking about four hours, all but the non-hydrolysed fraction were found to be entirely renewed; only about 1/5 of the last mentioned fraction, presumably mainly its adenylic acid content[2], was found to be unchanged. Diphosphoglyceric acid is, thus, renewed at a high rate as well.

Rate of penetration of plasma inorganic P into the corpuscles.

To obtain information on the rate of penetration of the inorganic phosphate of the plasma into the corpuscles, we have to compare the specific activity of the plasma inorganic

[1] E. Greenwald, J. Biol. Chem. **63**, 339 (1925); H. Jost, Z. physiol. Chem. **116**, 171 (1927); S. E. Kerr and A. Autaki, J. Biol. Chem. **121**, 531 (1927); E. Warweg and G. Stearns, J. Biol. Chem. **115**, 567 (1936); S. Rappaport and G. M. Guest, J. Biol. Chem. **129**, 781 (1939); A. Lennerstrand and M. Lennerstrand, Arkiv f. Kemi, Miner. og Geol. 13 B, No. 15 (1939).

[2] S. E. Kerr and L. Daoud, J. Biol. Chem. **109**, 304 (1937) state that, out of 88 mgm. per cent organic acid soluble P found in the corpuscles of the rabbit, 16 mgm. per cent are pyrophosphate P and 8 mgm. per cent adenylic acid P.

P with that of the corpuscle inorganic P. After the lapse of 11.5 hours (see Table III), this ratio is found to be 4, showing that the rate of penetration of the phosphate ions from the plasma into the corpuscles and vice versa is slow, a much slower process than the reorganisation of most of the acid soluble organic P compounds present in the corpuscles. After the lapse of nine days, the ratio of the specific activity of the plasma inorganic P and the corpuscle average acid soluble P is only slightly larger than 1 (1.06) (after so long a time, the activity of the average corpuscle acid soluble P acquired almost the same value as shown by the inorganic P of the corpuscles); and after the lapse of fifty days, a completely proportional distribution of the labelled P atoms between the plasma P and the P of the acid soluble P compounds present in the corpuscles is attained. While, after the lapse of 11.5 hours, the chance of a normal distribution of a P atom which diffused into the corpuscles between organic and inorganic P is almost = 1, the corresponding figure for the distribution of an inorganic P atom between plasma and corpuscles is only of the order of magnitude of 1/4.

The interesting phenomenon that an individual phosphate ion, while penetrating fairly slowly into the corpuscles, is incorporated at a remarkably fast rate into organic molecules present in the corpuscles finds many analoga in the processes going on in various organs. It is especially conspicuously shown in the study of the penetration of labelled phosphate into the muscle cells and of that of the rate of renewal of the acid soluble P compounds present in these cells; the former process being slow, the latter process being, in the case of some of the compounds, very fast.

There can hardly be any doubt that the large majority of the P atoms present in the molecules of most of the acid soluble organic P compounds of the corpuscles were incorporated into these molecules inside the corpuscles and reached the erythrocytes as inorganic phosphate ions which passed from the plasma into the corpuscles. The possibility that hand in hand with the process mentioned above a slow exchange of, for example, organic phosphoglycerate between plasma and corpuscles takes place cannot be disregarded. In view of the very low content of organic acid soluble P compounds of the plasma, if a migration of such compounds between corpuscles and plasma would take place, it should be mainly directed from the corpuscles into the plasma. In view of the fast rate of renewal going on in the corpuscles and the fast turnover of the acid soluble P compounds in the plasma, the investigation of a migration of organic acid soluble P molecules from the corpuscles into the plasma or vice versa encounters great difficulties.

In the above connection it is of interest to remark that Solomon, Hald and Peters[1] found, in a recent investigation, that phosphate esters present in the corpuscles are restrained from escaping by some force in addition to the membrane of the corpuscles. The restraining force is presumably a chemical aggregation or combination with substances of large molecular size. They arrived at the result mentioned above by the following observation. When filtering blood which was hemolysed by freezing, the ultrafiltrate obtained at 7° did not contain any appreciable amount of organic P, while the opposite was the case when

[1] R. Z. Solomon, P. M. Hald and J. P. Peters, J. Biol. Chem. **132**, 721 (1940).

saponin was used to obtain hemolysis. Frozen blood acts much as does intact blood so far as phosphates are concerned. The organic esters remain intact as long as the blood is kept cold and their combination with substances of high molecular size remains unpaired. This is not the case when saponin is added. Under the action of this agency the binding forces break down, and the organic phosphate esters can enter the ultrafiltrate. At 37° the phosphate esters can be ultrafiltrated even if the blood was hemolysed by freezing. In experiments in vitro with intact blood at 37°, during 18 hours no appreciable amount of organic phosphate ester was found to escape from the corpuscles into the plasma. These results support the view that the P atoms present in the phosphate ester molecules of the corpuscles reach the plasma, and vice versa, after being converted into constituents of inorganic phosphate ions.

As seen in Tables VIII a, b and c, with decreasing temperature the rate of penetration of ^{32}P from the plasma into the corpuscles and also the rate of its incorporation into organic P compounds strongly decreases. While at 37°, in the course of 90 min., 22 per cent of the ^{32}P originally present in the plasma diffused into the corpuscles, at 5° only 3.3 per cent of the ^{32}P originally present in the plasma found their way into the corpuscles. The comparison of the specific activity of the inorganic P present in the corpuscles at 37° and 5°, respectively, leads to the result that this activity is 37 times larger at 37° than at 5°. A similar comparison of the specific activity of the organic P of the corpuscles (exclusive the labile P of adenosintriphosphate) leads to a ratio of 80. It is of interest to note that a decrease of the temperature hardly affects the very fast rate of new-formation of adenosintriphosphate molecules, since more

than 80 per cent of the labile P of the adenosintriphosphate present in the corpuscles became renewed during the experiment both at high and at low temperatures.

Summary.

Labelled phosphate was administered to rabbits all through the experiments in order to keep the activity of the inorganic phosphate of the plasma at a constant level. The experiments took 215 min. to 50 days. The comparison of the specific activities of the organic P and the cellular inorganic P extracted from the organs leads to the result that in the course of 215 min. more than one half of the acid soluble P compounds present in the mucosa of the small intestine became renewed. Next the intestinal mucosa, the fastest rate of turnover was found to take place in the kidneys, liver and lungs.

From the various organic acid soluble phosphorus compounds the most readily hydrolysable ones were found to be renewed at the fastest rate. Fractions containing mainly phosphoglycerate were found to be renewed at an appreciable rate as well.

While the rate of formation of labelled acid soluble organic P compounds inside the corpuscles is rapid, the diffusion of labelled phosphate ions from the plasma into the corpuscles is a slow process. In the course of 12 hours, only about 1/4 of the P atoms of the acid soluble phosphorus compounds of the corpuscles entered into exchange equilibrium with the P atoms of the plasma phosphate, while most of the molecules of the compounds mentioned above were renewed during this time inside the erythrocytes. The contrast between the rate of interpenetration of labelled

phosphate and that of its incorporation into several of the acid soluble phosphorus compounds inside the cells is also found in the case of the muscles.

Labelled phosphate was found to penetrate into the brain tissue at an exceedingly low rate.

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ON THE DURATION OF LIFE OF THE RED BLOOD CORPUSCLES

Several methods have been applied to determine the lifetime of the red blood corpuscles[1].

1. The number of the corpuscles is artificially increased and the time observed after the lapse of which the normal level is re-established.

2. The number of the corpuscles is artificially diminished and the time necessary to obtain the normal level is determined.

3. Blood of another species or another type of blood is introduced into the circulation and the rate of disappearance of the "foreign" corpuscles is observed by using microscopic or serologic methods.

4. Determination of the time of excretion of the products of destruction of the blood corpuscles.

5. Observation of the rate of increase of the reticulocyte figure which follows the recovery after haemorrhage.

In what follows, we shall outline a method which much differs from those enumerated above. This method makes use of the application of isotopic indicators.

a) We administer to a rabbit, for example, a compound of an element which is normally to be found in the cor-

[1] A detailed survey of these methods was recently given by E. SCHIØDT, Acta Med. Scand. XCV, fasc. I (1938).

puscles. This element contains an isotopic indicator. We can then expect that the corpuscles formed after the start of the experiment will contain some of the labelled element administered. After the administration af radioactive iron, for example, we will find the haemoglobin of the newly formed corpuscles of the rabbit to contain labelled iron. We introduce some of these labelled corpuscles into the circulation of another rabbit. Hand in hand with the destruction of these corpuscles in the circulation of the second rabbit, there takes place a decrease of the activity of the corpuscles of this rabbit which decrease is a measure of the duration of life of the erythrocytes introduced.

b) We introduce labelled iron, for example, into the circulation, keep the specific activity (activity per mgm. Fe) of the plasma at a constant level, and follow the change of the ratio

$$\frac{\text{activity of haemoglobin Fe}}{\text{activity of plasma Fe}}$$

with time. The rate of increase of this ratio will inform us on the rate of formation of new corpuscles.

In the preliminary experiments to be described in this note we used labelled phosphorus as an indicator and followed both a) the rate of decay of the radioactive organic P compounds extracted from the corpuscles and b) the rate of activation of organic phosphorus molecules found in the corpuscles. We are describing our preliminary experiments in this note, since we have no more hope in a near future to come into possession of the strong ^{32}P preparations which would enable us to continue our experiments.

a I. Rate of disappearance of labelled phosphatides incorporated into the corpuscles.

The method denoted above as a) can be applied only if the labelled P is unable to leave the intact corpuscles. If this is not the case, the decrease in the activity of the corpuscles is no longer a measure of the rate of destruction of the erythrocytes.

Table 1.

Change in the activity of the corpuscle phosphatides with time.

The corpuscles were formed in rabbit A and introduced into the circulation of rabbit B.

Time	Activity
7 min.	100
5 hours	91
3 days	43
7 —	27
13 —	19.8
25 —	11.4
39 —	5.27

In a former investigation, we found that a significant part of the labelled phosphatides present in the corpuscles can exchange with the phosphatide molecules of the plasma[1]. Presumably, the phosphatides in the surface layer of the stroma can take part in such an exchange process. Furthermore, some replacement of the labelled phosphatide P by non-labelled inorganic P takes place inside the corpuscles giving the phosphatide P atoms a possibility to escape as labelled inorganic P from the intact corpuscles into the

[1] L. Hahn and G. Hevesy, Nature **144**, 72 (1939).

plasma. A part of the labelled phosphatides present in the corpuscles can, thus, be lost without the decay of the erythrocytes in which they are located. The fall in the activity of the corpuscle phosphatides in the earlier stages of the experiment (see Table 1) will, thus, to a large extent be due to other effects than the disintegration of the corpuscles containing these molecules.

These facts explain the rapid decrease of the activity of the phosphatide fraction in the early stages of the experiment. With increasing time, the rate of decay of the corpuscle phosphatide activity becomes slower and slower. In the last stage of the experiment, between the 25th and the 39th day, 54 per cent of the activity present the 25th day was found to be lost. Should this loss be solely due to the decay of "old" corpuscles containing labelled phosphatides, then the figure mentioned above would indicate the percentage of about 29 days old corpuscles[1] destroyed in the circulation in the course of 14 days to be 54. The figure given above represents thus the upper limit of the percentage of the 29 days old corpuscles destroyed in the course of 14 days.

a II. Rate of disappearance of labelled organic acid soluble compounds incorporated into the corpuscles.

The rate of renewal of the acid soluble P compounds present in the corpuscles is, with the exception of that of adenylic acid and possibly of some minor constituents, a fast one. Correspondingly, the active acid soluble P molecules present in the corpuscles which are suspended in an inactive plasma will soon lose most of their activity. These

[1] The active corpuscles were formed in rabbit A in the course of 7 days.

acid soluble P compounds can, thus, not be used as indicators in the determination of the lifetime of the corpuscles. Adenylic acid can, however, be presumably applied to that purpose. We found that, after the lapse of 39 days, the activity of the acid soluble P of the corpuscles amounted to half of its value measured after the lapse of 25 days. This activity was presumably due to that of adenylic acid, since the other acid soluble P compounds lost their activity in the early stages of the experiment. By considering the activity of the acid soluble residual fraction we arrive thus at about the same value for the decay rate of corpuscles as we found by using labelled phosphatides. As the next step, we intended to isolate the adenylic acid P by making use of the method of Fiske[1] and to follow the rate of its decay in the corpuscles. For reasons stated above (p. 29), these experiments could not be carried out.

It is of interest to remark that the method outlined in this chapter shows some resemblance to that method of determining of the duration of life of the corpuscles in which blood of another species or another type of blood is introduced into the circulation and the rate of disappearance of the "foreign" corpuscles observed. While, however, in the last mentioned method corpuscles of another species are used, which may decay with a different speed as do the corpuscles under physiological conditions, the indicator method makes use of corpuscles of the same species. The replacement of a minute percentage of the ^{31}P atoms present in the P compounds of the corpuscles by ^{32}P atoms can hardly have any influence on the biochemical behaviour of these corpuscles. The same applies to the weak β-radiation emitted by the active P compound

[1] C. H. Fiske, Proc. Nat. Acad. Sc. **20**, 25 (1934).

of the corpuscles. The remark made on p. 29—that in such experiments phosphorus preparations of very appreciable activities have to be applied— does not contradict the above statement. The corpuscles injected into rabbit B will, at the start of the experiment, contain less than 1/1000 of the activity administered to rabbit A and, in the later phases of the experiment, still less.

b. Rate of increase in the labelled phosphatide content of the corpuscles.

The corpuscles formed in a medium containing active phosphatide molecules will necessarily contain such molecules. If only the active phosphatide content of the organs producing corpuscles is kept at a constant level, the rate at which the activity of the phosphatides present in the corpuscles reaches this level is a measure of the rate of formation of the corpuscles. As in the case discussed under a I, in the earlier phases of the experiment, some of the phosphatide molecules of the corpuscles are replaced by phosphatide molecules previously located in the plasma. Therefore, and also for other reasons mentioned on p. 30, only the figures obtained in the late stage of the experiment permit us to draw conclusions on the rate of formation of corpuscles. The increase in the activity of the corpuscle phosphatides is seen in Table 2. In these experiments, the level of the activity of the plasma inorganic phosphate was kept constant.

We found the marrow phosphatides to have reached the activity level of the inorganic P of the plasma and, thus, the maximum value obtainable after the lapse of nine days.

Table 2.

Ratio of the specific activity of the corpuscle phosphatide P and the marrow phosphatide P.

(The specific activity of the marrow phosphatide P reached its maximum value after the lapse of 9 days).

Rabbit	Time in days	Percentage of marrow activity reached
B	9	66
D	25	77
D	31	99
D	50	97

We have, therefore, to compare the activity values of the corpuscle phosphatides obtained after that date. As seen in Table 2, after the lapse of nine days, the specific activity of the corpuscle phosphatides reached 66 per cent of the maximum activity. If half of the corpuscles present after the lapse of nine days is replaced by newly formed corpuscles in the course of the following 14 days, $66 + 1/2\ (100 - 66) = 83$ per cent of the maximum activity should have been obtained. After the lapse of 16 days, 77 per cent were obtained.

As the corpuscle activity approaches the maximum value the method becomes less and less sensitive. It is, therefore, practically only applicable in a fairly narrow range. The investigation of the rate of activation of the corpuscle phosphatides or the corpuscle adenylic acid is, therefore, a less suitable method of determination of the lifetime of corpuscles than is the measurement of the decay rate of transfused active corpuscles. The former me-

thod has, however, the advantage that the transfusion of corpuscles from one rabbit to another one can be avoided and, consequently, the possible errors inherent in the introduction of corpuscles of a rabbit into the circulation of another rabbit can be eliminated.

We mentioned on p. 29 the determination of the change in the activity of the haemoglobin iron as an example for the application of isotopic indicators in the determination of the time of life of the corpuscles. HAHN, BALE, LAWRENCE and WHIPPLE[1] have administered labelled iron to dogs and found that some of the active iron rapidly entered the corpuscles. This observation by no means excludes the possibility of applying labelled iron in the determination of the lifetime of the corpuscles. The penetration of similar amounts of labelled Fe into the corpuscles as are present in the plasma, as found by them, cannot be interpreted as a sign of a swift renewal of haemoglobin molecules with incorporation of active Fe inside the corpuscles. This would only be the case if the specific activity of the haemoglobin Fe of the corpuscles reached a value similar to that shown by the plasma iron. We can infer from the figures published by the authors just mentioned that this was not the case. It is, therefore, quite possible that labelled iron or any other labelled component of the haemoglobin could be used as an indicator in the determination of the lifetime of the corpuscles. Should the renewal of haemoglobin molecules with incorporation of other iron atoms take place inside the corpuscles at a rate which is faster than the rate of production of the corpuscles, this

[1] P. F. HAHN, W. F. BALE, E. O. LAWRENCE and G. H. WHIPPLE, J. Am. Med. Assoc. **111**, 2285 (1938); J. Exptl. Med. **69**, 739 (1939).

might frustrate the application of the method. In this case, just as when applying labelled P as an indicator, the observation of the rate of decay of the activity of the haemoglobin iron in another animal is more likely to be successful than the observation of the formation of corpuscles containing labelled haemoglobin which, from a physiological point of view, might be preferable.

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