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ISOTOPES AND THE FORMATION OF MILK AND EGGS

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TER VERKRIJGING VAN DE GRAAD VAN DOCTOR IN DE WIS- EN NATUURKUNDE AAN DE RIJKS-UNIVERSITEIT TE UTRECHT, OP GEZAG VAN DEN RECTOR MAGNIFICUS DR. TH. M. VAN LEEUWEN, HOOGLEERAAR IN DE FACULTEIT DER GENEESKUNDE VOLGENS BESLUIT VAN DE SENAAT DER UNIVERSITEIT TE VERDEDIGEN TEGEN DE BEDENKINGEN VAN DE FACULTEIT DER WIS- EN NATUURKUNDE OP WOENSDAG 5 JULI 1939, DES NAMIDDAGS TE 4 UUR

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AAN MIJN VROUW

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CONTENTS

CHAPTER I.

Isotopes.

1. Discovery and Separation.

About 1910 the fact, that atoms may have the same chemical properties and yet be different in other respects, was definitely proved. First the occurence of radioactive substances, which could not be separated by chemical means, was demonstrated [1]) and soon afterwards it was shown by the well-known "parabola-method", that atmospheric neon contains two kinds of atoms having different masses [2]). The elements had been arranged in the periodic table according to their chemical behaviour and so all atoms having the same chemical properties occupy the same place in the periodic system — therefor substances which were chemically identical, but different in other ways, were called "isotopes".

Soon afterwards it became clear, principally from Bohr's theory in combination with the work of Moseley, that the chemical behaviour of an element is determined by its nuclear charge or "atomic number". Thus isotopes have the same nuclear charge, but differ in some other way. In practically every case they have different atomic weights, though very recently a few examples have been discovered of "iso-

[1]) Soddy, Ann. Rep., **7**, 285 (1910).
[2]) Cf. Aston, Mass Spectra and Isotopes (London 1933), p. 30.
[3]) Moseley, Phil. Mag., **26**, 1024 (1913).

meric radioactive nuclei", having the same atomic number and practically the same atomic weight, while their radioactive properties are different [1]).

That the chemical and physical differences between isotopes are small, if existing at all, had been proved very early by the above mentioned work on the chemical identity of different radio-elements and by ASTON's diffusionexperiments. On the other hand, it was known, that the so-called chemical constant, which constitutes a factor, figuring in all vapour pressures and equilibrium constants, is proportional to the three halves power of the atomic weight (or of the molecular weight, if we consider a compound of the element in question instead of the element itself). This fact leads us to expect, that all vapour pressures and chemical equilibria must be slightly dependent on the isotopic composition of the elements involved. Afterwards it was stated, that the theory of the zero-point energy makes the differences smaller than had been expected before, but they should only disappear entirely at high temperatures and perhaps not even then.

These considerations led to renewed attempts at the separation of isotopes, which were soon successful.

The differences used in these methods were not thermodynamical differences (differences in vapour pressure or in chemical behaviour) but kinetic differences (differences in the velocity of certain processes). So BRÖNSTED and HEVESY [2]) used the method of ideal distillation in separating mercury

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[1]) BOTHE and GENTNER, Z. f. Physik, **106**, 236 (1937).
FLEISCHMANN, Z. f. Physik, **107**, 205 (1937).
SOLTAN and WERTENSTEIN, Nature, **141**, 76 (1938).
PONTECORVO, Nature, **141**, 785 (1938); Phys. Rev. **54**, 542 (1938).
SEGRÉ and SEABURG, Phys. Rev., **54**, 772 (1938).
CRITTENDEN and BACHER, Phys. Rev., **54**, 862 (1938).
THORNTON and CORK, Phys. Rev., **53**, 922 (1938).

[2]) BRÖNSTED and HEVESY, Nature, **106**, 144 (1920); **107**, 619 (1921); Phil. Mag., **43**, 31 (1922).

and chlorine and HEVESY and LÖGSTRUP [1]) fractionated potassium in the same way in their famous study of the radioactivity of the potassium isotopes. Recently this method has been applied to the separation of bromine isotopes by HEVESY and Miss LA CIERVA.

Diffusion methods were successful in the hands of HARKINS [2]) and his collaborators, of HERTZ [3]) and of MC.GILLAVRY [4]).

Separations depending on thermodynamical properties have been much more slowly developed. Vapour pressure differences were the first to be applied. In this way UREY, BRICKWEDDE and MURPHY discovered deuterium [5]). Later KEESOM, VAN DIJK and HAANTJES obtained an appreciable change in the isotopic constitution of neon by distillation in a fractionating column [6]) and PEGRAM, UREY and HUFFMANN prepared water containing quite an appreciable amount of heavy oxygen [7]). Chemical methods were first used in the case of the carbon separation [8]) and later in the concentration of the rarer isotopes of nitrogen [9]), lithium [10]), and sulfur [10]). The electrolyt-

[1]) HEVESY and LÖGSTRUP, Nature, **120**, 838 (1927); Zeitsch. f. anorg. Chemie, **171**, 1 (1928). HEVESY, Naturwiss. **23**, 583 (1935).

[2]) HARKINS and JENKINS, J. A. C. S., **48**, 58 (1926).

[3]) HERTZ, Z. f. Physik, **19**, 35 (1923); **79**, 108 (1932); **91**, 810 (1934).

[4]) MC. GILLAVRY, Diss. Columbia University.

[5]) UREY, BRICKWEDDE and MURPHY, Phys. Rev., **39**, 164, 864 (1932).

[6]) KEESOM, VAN DIJK and HAANTJES, Physica, **1**. 1109 (1934); **2**, 981, 986 (1935).

[7]) UREY, PEGRAM and HUFFMANN, J. Chem. Physics, **4**, 623 (1936).

[8]) UREY, ATEN, JUN. and KESTON, J. chem. Physics, **4**, 622 (1936).

[9]) UREY and ATEN, JUN., Phys. Rev., **50**, 575 (1936).
UREY, FOX, HUFFMANN and THODE, J.A.C.S., **59**, 1407 (1937).
UREY, HUFFMANN, THODE and FOX, J. Chem. Physico, **5**, 857 (1937).
THODE, GORHAM and UREY, Phys. Rev., **53**, 920 (1938).

[10]) G. N. LEWIS and MACDONALD, J.A.C.S., **58**, 2519 (1936).
TAYLOR and UREY, J. Chem. Physics, **5**, 597 (1937).

ical preparation of deuterium compounds by electrolysis is also a chemical process. It is however mostly based on velocity differences [1]).

The experience acquired in these investigations is of special importance for the work with isotopic indicators, because it proves the exceptional inefficiency of all processes for the separation of isotopes. The change in the proportion of the isotopes of an element, which can be obtained, is generally of the order of $\Delta A/A$ of its value, or even much smaller. (A being the atomic weight, ΔA the difference in atomic weight of the isotopes [2]). The high concentrations of heavy isotopes obtained by UREY and his collaborators have therefor been reached by processes which made use of a great many stages. The simplest application of this principle is the distilling column.

From this experience it will be clear that the accidental separation, which might occur during an experiment, will be negligible. If one worked with radio-phosphorus e.g., the proportion of P^{31} and P^{32} would not be changed by more than a few per cents, $\Delta A/A$ being about 3%.

2. ISOTOPES AS INDICATORS.

Soon after the impossibility of separating radioactive atoms from their non-active isotopes had been established, HEVESY and PANETH thought of the possibility of turning this fact to advantage in the study of the movement of some special atoms among their equals. If, to mention a certain case, we add to a lead compound some of the same compound

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[1]) The method by which CLUSIUS and DICKEL prepared chlorine isotopes in a nearly pure state, has been published too late to be included in this survey (Naturwiss. **26**, 546 '38; **27**, 148 '39).

[2]) A great number of separation factors are given in the paper of UREY and GREIFF, J.A.C.S., **57**, 321 (1935).

of *ThB* (which is an active isotope of *Pb*), then, after we have carried out all kinds of processes with this mixture (without introducing fresh radioactive material), we can be certain, that at any moment in any sample the proportion between the number of *Pb* atoms originating from the original sample considered and the number of *ThB* atoms, corrected for radioactive decay, is equal to that in the first preparation. Thus, by measuring the activity of a certain substance, we are able to calculate which part of all the lead atoms, which had been present in our original lead sample, is found in it. It is said, that the *ThB* constituted an "indicator" for the lead. Nowadays we have a large number of radioactive atoms at our disposal, which can be used as indicators. There are also several heavy isotopes known, which may be used in the same way, though their determination usually presents quite formidable difficulties. The measurement of radioactivity, on the other hand, using either an electroscope or a Geigercounter, is a very simple matter.

Before the discovery of artificial radioactivity the only inactive elements of which radioactive isotopes were known were thallium, lead and bismuth. As none of these elements normally occur in organisms, their use as indicators was of little importance for biological purposes. In physical chemistry and analytical chemistry, however, this principle rapidly gained prominence.

It could be shown that a piece of metallic lead, immersed in a solution containing a lead salt, exchanged its atoms with the ions in the solution. It could even be shown that not only the surface layer, but also many layers underneath take part in this process. Probably this behaviour is due to the well-known "local currents" which are supposed to take place on any electrode, dissolving the metal in one spot and depositing it in another. Lead peroxide was shown to exchange too, but

here only the outermost layer comes into play [1]). Later the study of several sparingly soluble lead salts in solutions containing lead ions indicated with *Th B* led to the same conclusion [2]). Lately KOLTHOFF and ROSENBLUM have confirmed these results with the restriction, that the precipitates of the slightly soluble lead salts must have at least a certain minimum age, depending on the circumstances at the beginning of the experiment [3]). If this precaution is not taken, the preparation still contains crystals of very irregular structure, which tend to recrystallise. If the solution already contains radioactive ions at this time, some of these are built into the new parts of the crystals, a process, which is an exact analogue of the uptake of an active isotope from an ionized solution in a metallic electrode. In the next chapter we shall see that an analogous exchange is reponsible for the disappearance of active phosphate from blood into the skeleton.

Next exchange processes in the liquid phase were studied [4]). As was to be expected, two ordinary lead salts exchange their lead ions immediately when dissolved in water. More striking is the fact, that lead chloride and lead nitrate exchange in pyridine, a solvent where electrolytic dissociation is far from complete. The explanation must be found in the very rapid breaking down and rebuilding of the molecules in solution. A very rapid exchange of *Th B* was demonstrated between divalent and quadrivalent lead salts in solution, indicating an electron transfer between the ions of different valency [5]).

[1]) HEVESY, Physik. Z. **16**, 52 (1915).

[2]) PANETH and VORWERK. Z. physik. Chemie **101**. 445, 480 (1922).

[3]) KOLTHOFF and ROSENBLUM J.A.C.S. **56**, 1264; 1658 (1934); **57**, 597, 607, 2573, 2577 (1935); **58**, 116, (1936).

[4]) HEVESY and ZECHMEISTER Ber. **53**, 410 (1920), Z. f. Elektrochemie **26**, 151 (1920).

[5]) It is worth mentioning, that the actual velocity of ionisation of trimethylmethyliodide in liquid sulfurdioxide has been measured in this way.

On the other hand no exchange was found between lead salts and tetraphenyl lead in pyridine or in amyl alcohol nor between diphenyl lead nitrate and lead nitrate in a water-alcohol mixture. The lead in plumbate ions was found unable to exchange with that of plumbite ions.

A very fertile field for the use of isotopic indicators is the measurement of selfdiffusion which cannot be studied in any other way. The mobility of lead ions in liquid lead [1]), in solid lead [2]) and in lead salts [3]) was determined by HEVESY and his collaborators. The selfdiffusion of gold [4]) has been measured too.

A similar though fundamentally different use of radioactive isotopes is the simple determination of elements by measuring the activity of a mixture, if the activity per gram of the element in question is known. In this case there is no question of distinguishing between active and inactive atoms of the same element; the radioactive isotope only provides a simple and sensitive analytical method. It is clear, that this procedure does not offer any possibilities which are essentially new, but it may enable us to carry out researches which are otherwise impossible because of technical difficulties.

The most famous discovery made in this way is that of bismuth hydride [5]). Radioactivity has also been used for the determination of the solubility of very sparingly soluble salts [6]).

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[1]) HEVESY, Z. f. Elektrochemie **26**, 363 (1920). GROH and HEVESY. Ann. d. Physik (4), **63**, 85 (1920).

[2]) GROH and HEVESY Ann. d. Physik (4), **65**, 216 (1921). HEVESY, SEITH and KEIL. Z. f. Physik. **79**, 197 (1932).

[3]) HEVESY and SEITH. Z. f. Physik. **56**, 790 (1929).

[4]) MCKAY, Trans. Far. Soc. **34**, 845 (1938).
SAGRUBSKIJ, Physik. Z. Sovjetunion **12**, 118 (1937).

[5]) PANETH, Z. f. Elektrochemie **24**, 298 (1918), Ber. **51**, 1704 (1918).

[6]) HEVESY and PANETH, Z. f. anorg. Chemie **82**, 322 (1913).
HEVESY and RONA, Z. f. physik. Chemie **89**, 294 (1915).

Our reason for mentioning this work here, though it is not quite analogous to the use of isotopes as indicators, is the fact that the earliest application of radioactive elements in physiological chemistry made use of this principle. CHRISTIANSEN, HEVESY and LOMHOLT measured the rate of excretion of lead and bismuth and the distribution of these elements between different organs [1]). HEVESY also studied the uptake of lead by plants [2]) and the absorption of lead and bismuth by tumors [3]).

An enormous impetus was given to this field of research by the discovery of heavy hydrogen which extended the possibility of using isotopic indicators from very few to a great many compounds. The literature dealing with the use of deuterium for this purpose is so enormous that it cannot be reviewed in its entirety here. The first application of this hydrogen isotope to biological science was the measurement of the uptake and excretion of water by men and gold fish by HEVESY and HOFER [4]). Later the rate of formation of a number of deuterium compounds in living organisms has been investigated by SCHOENHEIMER and his collaborators and by KROGH and USSING [4]). The other heavy isotopes which have been concentrated in UREY's institute are just coming into use as indicators for physiological purposes. SCHOENHEIMER and his collaborators used N^{15} to investigate the digestion of hippuric acid and other nitrogen compounds [5]), and lately

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[1]) CHRISTIANSEN, HEVESY and LOMHOLT, Comptes rend. Carlsberg, **178**, 134 (1924); **179**, 291 (1924).
LOMHOLT, Biochem. J. **18**, 693 (1924).

[2]) HEVESY, Biochem. J. **17**, 439 (1923).

[3]) HEVESY and WAGNER, Arch. f. exp. Path. **149**, 336 (1930).

[4]) HEVESY and HOFER, Nature, **133**, 495 (1934); Z. physiol. Chem., **225**, 28 (1934) Nature, **134**, 879 (1934).
USSING, Nature **142**, 399 (1938).

[5]) SCHOENHEIMER, RITTENBERG, FOX, KESTON and RATNER, J.A.C.S., **59**, 1768 (1937).
SCHOENHEIMER, RITTENBERG, FOSTNER, KESTON and RATNER, Science, **88**, 599 (1938).

oxygen containing a surplus of O^{18} has been used for studying the fate of inhaled oxygen [1]) and of sulphate ions injected into the blood [2]).

The discovery of artificial radioactivity has greatly increased the use of indicators, as several elements which are extremely important in chemistry and biology are now obtainable as radioactive isotopes, the most important of which are: phosphorus, chlorine, bromine, iodine, sodium, potassium, sulfur and arsenic. Not all of these are, however, equally satisfactory. In the first place it must be possible to obtain the radio-isotopes in preparations sufficiently strong and concentrated. We often find that it is quite easy to induce a high activity into an element by neutron bombardment, but then it is usually necessary to separate the active form from the inactive one by some chemical process in order to obtain the activity in a preparation which is sufficiently small to be used for measurement or the performance of an experiment.

If the neutron radiation changes the nuclear charge of the element, an ordinary analytical separation can be used to separate the active element, as e.g. in the isolation of radio-phosphorus according to CHIEVITZ and HEVESY.

If the radioactive atoms are isotopic with those of the starting material it is usually satisfactory to use a compound as starting material. The atoms which absorb a neutron and emit a γ-ray suffer a recoil themselves by which they are entirely knocked out of the surrounding molecule, thus forming atoms or molecules of the free element. So if one adds a trace of this element and separates it out afterwards, it contains practically all the activity formed [3]). This method is generally followed in the preparation of radioactive halogens and is also useful for preparing radio-phosphorus.

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[1]) DAY and SHEEL, Nature, **142**, 917 (1938).
[2]) ATEN, JUN. and HEVESY, Nature, **142**, 952 (1938).
[3]) SZILARD and CHALMERS, Nature, **134**, 462 (1937).

Another property which must be taken into account is the lifetime of the radio element. In artificial radio-elements this has been found to vary between a fraction of a second and several months. If the lifetime of the element is too long, only a very small number of atoms decompose in a second and the activity becomes too small for measurement, unless the neutron source available is strong enough to provide an exceptionally large number of radioactive ions. This is the case with radio-sulfur. If, on the other hand, the half-period of the indicator is much less than an hour, the substance decays so rapidly that nearly all the activity is lost during the preparation of the material and the carrying out of the measurement. Biological work with chlorine (half-period $^1/_2$ hour) is very difficult for this reason.

The most desirable half-period is several days and radiophosphorus (half-period $14^1/_2$ days) is a very satisfactory indicator in physiological chemistry, especially because quite strong preparations can easily be obtained. A neutron source of half a Curie of radium and beryllium will provide a sample of which 10^{-5} part can easily be measured, and with a cyclotron substances several hundred times as strong are made. A special advantage of phosphorus is its occurrence in many different compounds which are formed and found in living organisms.

Other radioactive indicators which have occasionally been used in biochemical work are sodium [1]), potassium [2]) and sulfur [3]).

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[1]) Hamilton, Proc. Nat. Ac. Sci., **23**, 521 (1937).
Griffiths and Maegraith, Nature, **143**, 159 (1939).

[2]) Greenberg, Joseph, Cohn and Tufts, Science, **87**, 438 (1938).

[3]) Borsook, Keighley, Yost and McMillan, Science, **86**, 525 (1937).

CHAPTER II.

Radiophosphorus as an Indicator.

1. Phosphates in Blood and Bones.

In an organism which is in a stationary condition, the amount of any element taken up during a certain time, for example a day, is — on the average — equal to the amount secreted. Part of the atoms absorbed will be lost soon afterwards and another part will be incorporated in the body and will replace atoms which formerly belonged to the organism. Radioactive phosphorus makes a beautiful tool for the investigation of these processes.

Up till now most of the investigations with radio-phosphorus have been carried out in Professor Hevesy's department of the Institute of Theoretical Physics in Copenhague. As my work was part of this program I will begin by giving a survey of the results hitherto obtained.

There is, however, one special point which must be considered first of all. It is often asked whether the radioactivity of an atom is not in itself a property which influences its chemical behaviour. For all practical purposes this is certainly not the case. Up to the moment of its decomposition the radio-atom differs only from other phosphorus atoms in its mass which causes only very small differences in its chemical propertics. Of course at the moment of its decomposition, it turns into a sulfur atom, but the place at which it emits its β-ray — the only effect we can determine — it has reached as a phosphorus

atom. We must keep in mind though, that the mass difference may cause accidental fractionations of the order of a few per cent, but, as the accuracy of our radioactive measurements is about 10%, we need not worry about these effects. On the other hand this consideration shows that, even if we could refine the activity measurements by another power of 10, we would not benefit very much by this fact.

Quite another matter is that, if we administer very strong preparations of radio-phosphorus (or of another radioactive substance) to an animal, the radiation may damage the organism and in this way cause abnormal processes [1]). But these are secondary effects which have no special influence on the behaviour of radio-phosphorus atoms. It deserves mentioning in this connection that all animal organisms contain potassium, which carries an appreciable radioactivity. It is quite possible however that special organs are more sentive to radiation than others but this question still needs a special study.

In most of our experiments published, radio-phosphorus was administered as a phosphate solution, though at present a number of investigations of the behaviour of other radioactive phosphorus-compounds and of elementary phosphorus in the animal body by the indicator method are in progress in Copenhague. One of these will be mentioned later.

Radioactive phosphate can be administered in two ways: by mouth or by injection into the body. In the latter case the solution may be injected either immediately into the blood or simply into the tissues of the body. If the latter method is used the phosphate ions diffuse into the blood, which transports them to other parts of the body. As the mixing of phosphate ions in tissues seems to be quite rapid, the mode of injection should have little influence. This is not true if other phosphorus compounds are administered, as many tissues are known

[1]) Scott and Cook, Proc. Nat. Ac. Sc., **23**, 265 (1937).

to contain enzymes which decompose some of the more complicated phosphorus compounds. In our experiments we gave the active phosphate as a subcutaneous injection but the hexose monophosphate as an intravenous injection.

A general preliminary study of the fate of radio-phosphorus in the body has been made by CHIEVITZ and HEVESY [1]). Phosphorus in the blood can suffer the following fate:

1. it can remain in the blood;
2. it can be secreted;
3. it can be absorbed by the tissues or organs.

In most animals nearly the entire secretion is taken care of by the urine and the faeces. Saliva and tears are of no account, nor does the secretion through the skin amount to much in the case of phosphate, although it plays quite an important rôle in the water metabolism. If, however, the animal produces milk or eggs, the loss of phosphorus in these products is very important.

In most animals quite an appreciable fraction of the administered radio-phosphorus (about 20%in the case of a human subject) is secreted during the first week. This is a fairly simple process, almost certainly due to a secretion of phosphate ions from the plasma through the kidneys and the bowels into the urine and the faeces. This theory was supported by the proof that the specific activity of the plasma phosphate and urine phosphate of a rabbit two weeks after injection are equal.

Of the rest of the phosphorus nearly the entire activity enters into the bones and the tissues of the body. Taking a goat for an example, we find that after 2 hours the entire blood contains only about 2% of the activity injected. (Cf. Expt. no. 3).

[1]) CHIEVITZ and HEVESY, Nature **136**, 754 (1935).
CHIEVITZ and HEVESY, Kgl. Danske Vid. Selskab Biol. Medd., **13**, 9 (1937).

In the bones practically all the phosphorus is present as phosphate ions, but most tissues and organs contain quantities of different organic phosphorus compounds which are quite comparable to their phosphate content. In Chapter V some figures will be given for the milk glands of goats.

In the higher animals the fraction of the activity absorbed by the bones is several times larger than that taken up by the tissues. Therefor we shall first consider the uptake in the skeleton, which regulates the rate of the decrease of the specific activity of the plasma phosphate.

If we shake a powdered insoluble phosphate with a radioactive solution of sodium phosphate, the P^{32} is soon equally distributed between the phosphate ions in solution and those on the surface of the crystals, thus even providing us with a possibility of calculating the size of the crystalline particles according to the experiments of PANETH mentioned above.

In the case of the equilibrium between plasma and bones the exchange is brought about much more slowly. The figures we obtained in our experiments on goats are shown graphically in Fig. 7 page 81). The fact that a slow decrease in activity takes place continuously is seen very clearly. At the same time one can observe the differences between various experiments; figures obtained with different animals or even with the same animal at different times may vary as much as by a factor 2. The continued drop in the specific activity of the plasma phosphate must be due to a slow absorption of radio-phosphorus by the tissues and by the bones. Of course there is also a constant loss of activity by secretion, but this is not nearly sufficient to account for the rate of disappearance of P^{32} from the blood [1]). In human subjects for example, the loss of radio-phosphorus between the end of the first and the end of the seventh day after injection amounts to less than 10% of

[1]) See in this connection: HAHN, HEVESY and LUNDSGAARD, Biochem. Journ., **31**, 1705 (1937).

the total, on the other hand the specific activity of the plasma phosphate is reduced to $^1/_4$ of its former value. If the skeleton had a constant surface rapidly reaching equilibrium with the blood, then at different times the specific activity of the plasma phosphate should be approximately proportional to the total activity present in the animal.

So there can be no doubt that the phosphate exchange between the plasma and the skeleton is a slow process. Several different causes are to be considered for this fact. It may be that the penetration of plasma into the very thin canals inside the bone is so slow that it takes days to supply sufficient phosphate ions for the exchange. Besides in growing animals there is also a considerable uptake of radio-phosphorus in those parts of the bones which are increasing in weight.

Then there is the possibility that the surface of the crystals reaches equilibrium very rapidly, but that there is slow additional uptake by diffusion of phosphorus from the surface into the body of the crystals. (This diffusion would probably happen most rapidly along the inner surfaces of the crystal and specially over the surface of such cracks as are filled with liquid. In this case one could also speak of a transport through the liquid and the distinction between retardation due to circulation and due to diffusion would become rather vague).

We also have to consider the likelyhood of a process which is intermediate between uptake by growth and by diffusion in adult animals. It is quite probable that all bones are constantly being broken down and rebuilt in the same place, a behaviour which is observable in the case of broken bones being healed. This effect would be comparable to the extra uptake of radio indicators from solutions by fresh precipitates during recrystallisation.

To get some information on this point, Professor HEVESY with his collaborators injected heavy water into a rabbit at a

very slow rate and measured the difference in density between the water which could be distilled out of the plasma and out of the bone [1]). This method has the one fundamental fault that it gives the average rate of renewal of the plasma in the bone, whereas by far the greatest part of the total surface is to be found in the very small canals, while most of the blood of a bone is present in the large vessels.

As this experiment has not been described in extenso before, we may give a few particulars in this place. The solution was injected slowly during the course of three hours. 9 Grams of D_2O were administered in all to a rabbit weighing 2,3 kgrs. After 3 hours the density excess of water isolated from the blood was 562 ppm.; from the femur 476 and from the tibia 498. Thus the concentration of D_2O was about 86% of that in the blood. Assuming the deuterium concentration to rise linearly with time, we find that the bone water at the end of the experiment has the same density as the blood water had $0{,}14 \times 3$ hours $= 25$ minutes before. So we may conclude that the time required for the circulation of the blood contained in a bone (of a rabbit) is about one half hour.

Quite a number of interesting results bearing on the problem of circulation in bones have been obtained in the researches of HEVESY and his different collaborators. The rate of exchange in bones depends to a very high degree on their constitution [2]). Teeth are found to exchange very slowly and hard bones more slowly than soft bones. Therefor the loss of weight on ignition (which is a measure for the water-content and thus for the blood circulation) in different bones is found to run parallel to the uptake of radio-phosphorus as is seen from Table I, taken from the work of HEVESY, HOLST and KROGH.

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[1]) HEVESY, Enzymologia, **5**, 138 (1938).

[2]) HEVESY, HOLST and KROGH. Kgl. Danske Vid. Selskab. Biol. Medd. **13**, 13 (1937).

This is an argument in favour of the circulation being one of the determining factors of the rate of activation of bone-tissue.

TABLE I.

Organ.	Millionth parts of total labelled P found in 1 mgr. ash.	Loss in weight on ignition.
Incisor	6.2	26.0
Molar	3.4	27.4
Jaw	20	36.3
Tibia head	77	68.7
Tibia residue	14	52.7

The real measure of the renewal of phosphorus would of course be the specific activity; i.e. the amount of radio-phosphorus per mgr. *P*. However, the phosphorus content of these various ashes is so little different that the figures in this column are quite sufficient for comparison.

In this connection it is worth mentioning that the rate of activation of the bone-tissue in the legs of chickens was found to be faster than normal in animals suffering from rachitis [1]).

Some very illuminating facts were discovered about the circulation of phosphorus in teeth. The incisors of rats, which grow continuously during the animals' life, are much more active than molars, which are stationary. This difference is certainly due to the strong activation caused by the extra uptake of phosphorus by growing bone tissues. The same difference is found between the rates at which phosphorus enters into the bones of young and adult animals, as shown by CHIEVITZ and HEVESY in their paper mentioned above. Another way of studying the difference in the rate of activa-

[1]) DOLS, JANSEN, SIZOO and VAN DER MAAS, Nature, **142**, 952 (1938).

tion by growth and by displacement was found by measuring the activity of different sections of rats' incisors a few days after the administration of radio-phosphorus. Most of the activity is deposited round the pulpa, where the new bone-tissue is being built, but even the most distant parts of the tooth, which had already been formed some time before the beginning of the experiment, were found to be slightly active. After three days the amount of P^{32} which has entered into 1 mgr. of phosphorus at the top of the tooth is only about $= \frac{1}{300}$ of that at the base. Part of this difference is certainly due to the fact, that circulation through the solid parts of the tooth is very slow, but another part must undoubtedly be caused by the uptake of radio-P in the newly formed part of the tooth at the bottom.

2. Formation of Phosphorus Compounds in Organs.

Whereas the activation of bones and urine has been shown to be relatively simple, as these substances contain practically all phosphorus as phosphates, we must expect the uptake of radio-phosphorus by organs to be much more complicated. In most tissues the amount of organic phosphorus greatly exceeds that found in the form of phosphate ions and therefor we have not only to consider physical processes like circulation and diffusion, but we must also take into account quite a number of chemical phenomena.

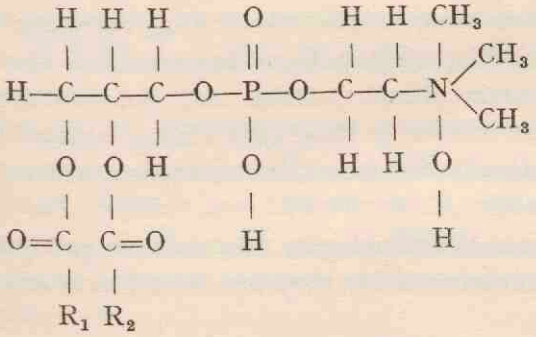
In general the phosphorus compounds occurring in tissues may be devided into four groups: Phosphate ions, organic acid-soluble phosphorus, phosphatides and phosphoproteins. These divisions are not entirely according to chemical composition, but mostly prompted by the present analytical methods.

It is of course impossible to survey the distribution of

phosphorus in all different organs, but we will mention the most important phosphorus compounds which are known in physiological chemistry, paying special attention to their occurence in blood.

1°. Inorganic phosphates are only found as the ions of ortho-phosphoric acid [1]). Pyrophosphate ions do not occur, though organic pyrophosphates exist in muscles. Goat's plasma contains about 4 mgr % (milligrams P per 100 grams) of inorganic phosphorus; the corpuscules somewhat less.

2°. Phospholipids consist mostly of phosphatides of which the most important is lecithin. This is a phosphate of cholin and of glycerin in which the two free hydroxy-groups have reacted with different fatty acids. The formula of lecithin is [2]):



R_1 and R_2 represent alkyl-groups.

Appreciable quantities of cephalin, containing amino-ethanol instead of cholin, occur in the brain, in the blood and probably to some extent in other organs.

[1]) Of late the opinion has been expressed that inorganic pyrophosphates normally occur in plasma.

[2]) PETERS and VAN SLYKE, Quantitative Clinical Chemistry I, 223.

Phosphatides are soluble in ether and in hexane, they are precipitated by trichloroacetic acid. In goats' blood the plasma contains about 4 mgr % of phosphorus as phosphatides, the phosphatides concentration in the corpuscules being about twice as high. As it is exceedingly difficult to differentiate between lecithin and cephalin by chemical methods, I have not attempted to separate these two substances. Therefor all preparations named "lecithin" in the following pages are likely to have consisted partly of cephalin.

Besides the phosphatides the cerebrosides belong to the class of the phospholipids, but their importance is so small that we shall refrain from dwelling further upon them.

3°. Phosphoproteins are large molecules, behaving more or less as proteins and containing phosphorus. A few are relatively well studied like casein, occurring in milk, and vitellin, which is found in eggs. Many tissues seem to contain phosphoproteins and quite an appreciable amount is found in blood cells. Plasma on the other hand is practically free from these substances, at least in most animals.

Compounds belonging to this class are precipitated by trichloroacetic acid; they are insoluble in organic solvents.

4°. Acid-soluble organic phosphorus is found in many different compounds which are exceedingly difficult to distinguish. They are soluble in solutions of trichloroacetic acid and cannot be precipitated by magnesia-mixture. The methods for separation mostly depend on differences in the rates of hydrolysis, either by boiling with acid or by enzymatic action, or on fractionated precipitations with baryum salts.

By far the largest part of the phosphorus found in blood-

cells belongs to this group, in plasma however the total concentration of these substances is quite low, though there can be little doubt about their occurence. The best value seems to be about 0,4 mgr %.

Esters constitute quite an important part of this group. Hexose monophosphates of different constitution, hexosediphosphates and glycerophosphates are known to occur in organs and some of these with even greater certainty in blood. R. ROBINSON holds the opinion that most of the plasma ester is a hexose-monophosphate [1]). The acid-soluble phosphorus of the blood cells, however, has been shown to consist mostly of glycerodiphosphate and adenyl pyrophosphate [2]).

Other acid-soluble organic phosphorus compounds are phosphocreatine, and nucleotides. Whether these substances occur in blood cells too is uncertain; in tissues, specially muscles, they have, however, been found and even studied as far as their formation and disappearance is concerned.

A few figures for the distribution of administered radiophosphorus were published by HEVESY, HOLST and KROGH [3]) and by HAHN, HEVESY and LUNDSGAARD [4]), but as the activity measured could not be connected with any chemical fraction, it was difficult to conclude anything for certain from their results.

A great deal of work on the distribution of radio-phosphorus over different parts of the bodies of rats (normal,

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[1]) The occurrence of esters in plasma is treated in: R. ROBINSON, The significances of phosphoric esters in metabolism (New-York, 1932).

[2]) ROCHE, Bull. Soc. chim. biol., **12**, 636, (1930).

[3]) HEVESY, HOLST and KROGH, Kgl. Danske Vid. Selskab Biol. Medd., **13**, 13 (1937).

[4]) HAHN, HEVESY and LUNDSGAARD, Biochem. Journ., **31**, 1705 (1937).

rachitic, and rachitic rats treated with vitamin B) was published by DOLS, JANSEN, SIZOO and DE VRIES [1]), but as these authors have not determined the phosphorus content of their samples, it is impossible to compare their results with those of HEVESY and his collaborators.

Here we must pause, to consider a point which we will deal with more fully in Chapter III. The problem is, which quantity is most suitable for comparison as a characteristic of radioactive preparations. The activity of a certain sample is of course dependent on the amount of the substance and its purity, and for this reason has a more or less accidental value. The best characteristic quantity for most purposes is the "specific activity", i.e. the activity (measured in some arbitrary unit of activity) per mgr. phosphorus. So one easily sees that if a certain preparation contains 1 mgr. of lecithin, having a specific activity $= x$ and 1 mgr. of phosphate, having a specific activity $= 10x$, the total activity will be $11x$ in 2 mgr. P and the average specific activity $5.5x$, a quantity very different from the specific activities of either of the pure components. Only if we are dealing with a large amount of a very active substance mixed with a small quantity of a less active substance, the average specific activity of the total phosphorus is about equal to that of the principal component.

It stands to reason that much more definite conclusions can be reached from activity determinations on pure phosphorus compounds isolated from different parts of the body. Up till the present most researches have dealt with the activation of lecithin, partly because this is a substance which may easily be isolated and purified, partly because it is supposed to play an exceptionally important rôle in metabolism.

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[1]) DOLS, JANSEN, SIZOO and DE VRIES, Nature, **139**, 1068 (1937). Proc. Kon. Akad. Wet., **40**, 547 (1937).

The first work in this field was published by ARTOM, SARZANA, PERRIER, SANTANGELO and SEGRÉ [1]).

They analysed different organs of a rat which had been injected with radio-phosphorus on four successive days and determined the specific activities of phosphate, total acid-soluble (phosphate and organic acid-soluble) phosphorus, lipidic phosphorus and acid-insoluble *P* (phosphoprotein). Their conclusion is, that the total specific activity in different organs is not very different, only in the brain and medulla it is distinctly lower. The figures which they determined for lipoid activity are collected in Table II:

TABLE II.

Organs.	Specific activity of lipoid *P*.
Liver	17.8
Intestine	14.4
Kidney	13.2
Parenchyme	8.5
Muscle	2.9
Brain and medulla	0.5

The most active lecithin is found in the liver, kidneys and intestine. This means that if we consider how phosphate ions, present in the plasma at a certain moment, will be distributed at some later time, we will find a relatively large fraction of these ions in each milligram of the lecithin of the liver, kidney and intestine and a much lower concentration in the lecithin of the muscles or the brain. There may be two different explanations for this fact, either the rate of formation of lecithin is different in different organs, or the rate of renewal

[1]) ARTOM, SARZANA, PERRIER, SANTANGELO and SEGRÉ, Nature, **139**, 836 (1937).

of the phosphate might not be the same, due to differences in the blood circulation or the diffusion velocity of phosphates. The first explanation assumes an exceptionally rapid lecithin synthesis in the liver, which does not seem unlikely at all, as the liver is known to be the organ with the greatest chemical activity by far. The second possibility cannot be discussed, until the rate of activation of phosphate in different tissues is known, but we know that the rate of renewal of phosphate in organs is many times more rapid than that of lecithin (see also Chapter 5). Also ARTOM and his collaborators mention the fact that, except for the brain, the total phosphorus has about the same specific activity in all organs, which would be quite impossible if the phosphates were not all about equally active. We may even conclude that in their experiments nearly all the organic acid-soluble phosphorus was in radioactive equilibrium with the phosphate. This seems also very likely in view of our results, as most of the radio-phosphorus had been present in the body for days and the time required for exchange in most esters and analogous compounds is much less.

Therefor it seems safe to say that the activity in the table of ARTOM c.s. is approximately a measure for the rate of lecithin formation in different organs. Small differences might however be due to different rates of activation of the phosphates.

It may be mentioned that these conclusions were not put so explicitly at the time, because the different factors influencing the behaviour of phosphorus in the body and specially its modes of disappearance from the blood were not so clearly understood.

A great deal was done to clarify this field by HAHN and HEVESY in three papers, the first of which appeared immediately afterwards [1]). The purpose of their first research was to

[1]) HAHN and HEVESY, Skand. Archiv., **77**, 148 (1937).

show the lecithin synthesis in the brain of adult animals and they were able to prove indeed that even in very old animals there is a constant synthesis and breakdown of phosphatides going on in the cerebrum. Several other highly significant experiments on the appearance of active lecithin in blood were communicated in these studies leading to the following results.

If the specific activity of the phosphate in different parts of the body were constant, it would be possible to calculate the rate of formation of lecithin and other phosphorus compounds from the specific activity of these substances after a certain lapse of time. If, for example, after the lapse of one hour the lecithin in the liver of an animal were found to contain 10% of the activity which the same amount of phosphorus occuring as phosphate ions in the organ has, then we could conclude that 10% of the phosphate ions had been formed during this time. That would mean that during the course of ten hours the liver would form an amount of lecithin equal to its entire lecithin content. This does not mean, of course, that all the lecithin present would be renewed, because some of the active lecithin molecules originating towards the end of the experiment would replace other active molecules formed in the beginning of the experiment, thus even after the lapse of ten hours some of the lecithin present will consist of molecules which were already present in the liver before we started. Now the great difficulty in all researches, in which the total radio-phosphorus is injected at the start, is the very rapid decrease of the specific activity of the blood phosphate. In tissues we have aninitial rise in phosphate activity followed by a continuous decrease, as the radio-phosphorus from the plasma phosphate is taken up by the skeleton.

Conditions are greately simplified, if we can work with isolated blood samples or organs. In studying the rate of exchange reactions in blood we can help ourselves by performing

simple vitro-experiments. HAHN and HEVESY [1]) added an infinitesimal amount of active phosphate to blood samples which were shaken in a thermostat at 37° C. They found the rate of renewal of phosphatides in dogs, blood to be extremely slow. In $4\frac{1}{2}$ hrs. 0,007% of the plasma phosphatide was activated and 0,002% of the phosphatide in the corpuscles [2]).

In a preceding paper HEVESY and LUNDSGAARD had dealt with the problem of the origin of the increased lipid content of dogs suffering from artificial lecithinaemia [3]). They fed active phosphate and olive oil to a dog and analysed both the lecithin obtained from its blood and the phosphate present in the intestine. From the specific activity of the latter they calculated the amount of active phosphorus which should have entered the blood lecithin if the additional lecithin in the blood, originated during the lecithaenimia, had been formed from the phosphate in the intestine. The measured activity of the blood lecithin was ten times less than was calculated from the assumption described above. Therefor it was concluded that the excess of blood lecithin was liberated or formed either in the blood or in an organ different from the intestine.

In this special case this way of calculating was made possible by the fact that the active phosphorus was not injected but administered by mouth. Under these circumstances a continuous transport of activity from the bowels into the blood takes place. At the same time the active phosphate ions are taken out of the circulation by the skeleton and these two effects, taking place at the same time, keep the specific activity of the plasma phosphate approximately constant.

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[1]) HAHN and HEVESY, Compt. rend. Carlsberg Série chim., **22**, 188 (1938).

[2]) It is somewhat doubtful whether this formation is real at all. The lecithin had to be separated from a phosphate activity which was about 10 000 times stronger, but great care was taken to make the purification as complete as possible.

[3]) HEVESY and LUNDSGAARD, Nature, **140**, 275 (1937).

To test the possibility of the excess of the lecithin occurring in the blood during lecithinaemia being formed in the blood, HAHN and HEVESY compared the rate of activation of blood lecithin (both in plasma and in corpuscles) in vitro with the corresponding rate in lipaemic blood in vitro. They did not find any difference [1]).

The next point HAHN and HEVESY had to decide in connection with the formation of lecithin in lipaemia was, whether the formation of phosphatides in the liver is accelerated. With this purpose they carried out a series of perfusion experiments, in which blood containing active phosphate was made to circulate through isolated livers. Because of the absence of bone-tissue the specific activity of the plasma phosphate does not change much under these circumstances and one can calculate the rate of renewal of the blood phosphatide and the liver phosphatide with a fair degree of accuracy. During lipaemia the first is increased about 200%, the second about 70%. This makes it seem very probable that the liver is the source of the phosphatide-excess in lipaemic blood as well as the place where this phosphatide has been formed [2]).

From the results mentioned we might expect that the formation of lecithin takes place mostly in the liver and probably to some extent in the kidney and the intestine. In this connection we may not omit, however, to mention some experiments of SINCLAIR [3]) and of ARTOM [4]). The former fed several fats containing unsaturated acids to animals and determined the iodine-number of the fats contained in the phosphatides of different organs after the lapse of a certain period. From the rate at which the unsaturated acids turned up in the dif-

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[1]) HAHN and HEVESY. Compt. rend. Carlsberg, Série chim., **22**, 188 (1938).

[2]) HAHN and HEVESY, Biochem. Journ., **32**, 342 (1938).

[3]) SINCLAIR, Physiol. Rev., **14**, 351 (1934).

[4]) ARTOM, Arch. internat. de physiol., **36**, 101 (1933).

ferent phosphatide fractions, he calculated the rate of renewal of these phosphatides. It must however be kept in mind, that the organism itself possesses the faculty of changing saturated fatty-acids into unsaturated ones and vice versa. Under normal circumstances the fatty-acids contained in the phosphatides of different organs show widely diverging iodine-numbers. This fact makes any conclusions reached by the use of unsaturated fatty-acids as indicators rather uncertain. Later experiments by SINCLAIR, made with fats containing elaidic acid, which is easily determined by chemical means, suffer from the same ambiguity.

ARTOM measured the rate at which iodized fats enter into phosphatides and he came to the startling conclusion that the formation of phosphatides in blood corpuscles was faster than in plasma and that it was slowest in the liver. There can be little doubt that this result is due to a selective uptake of iodized fats by the corpuscle phosphatides as it is known that the organism can differentiate quite well between natural fats and fats containing iodine.

A series of measurements of the activation of different phosphorus compounds in human blood [1]) indicated that the rate of activation of plasma phosphatide was much more rapid than that of corpuscle phosphatide as seen from Table III. We must realize, however, that the phosphatides in blood are not composed of pure lecithin but are really a mixture of different closely related substances, principally of lecithin, cephalin and sphingomyelin. During the usual extraction procedure, which includes dissolving the phosphatides in petrol ether, the sphingomyelin is discarded. Now a long series of analyses by KIRK [2]) have shown that in the case of human blood the ratio of the amounts of lecithin and cephalin is

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[1]) HEVESY and ATEN, JUN., Kgl. Danske Vid. Selskab, Biol. Medd., **14**, 5 (1939).

[2]) KIRK, Journ. biol. Chem., **123**, 637 (1938).

about the same in the plasma and in the corpuscles. As an average KIRK finds that human plasma phosphatide contains 13% lecithin, 47% cephalin and 40% sphingomyelin. The corresponding figures for corpuscles are: 16% lecithin, 60% cephalin and 24% sphingomyelin. This enables us to conclude that the most active component of the phosphatides in the plasma has a greater activity than the same compound in the corpuscles, which proves that at least this fraction of the plasma phosphatides is not formed in the blood cells.

TABLE III.

Specific activity of blood fractions (human blood) in parts per million of the total activity injected per mgr. *P* after 24 hrs.

	Person B.	Person C.
Plasma phosphate . .	105	—
Plasma phosphatide. .	31	11
Corpuscles phosphatide	2.3	1.3

This excludes the possibility that the plasma phosphatide or at least the fraction which is formed most rapidly, originates in the corpuscles and diffuses from there into the plasma. It is not possible to determine as yet which part of the plasma-phosphatide is formed in the liver and which part in the intestine or in the kidney. From the experiments to be described later it can be shown, that the liver is responsible for at least part of the plasma phosphatide. At present we have no proof of a phosphatide-synthesis occurring in the intestinal mucose. In experiments of short duration, in which the activity is administered by injection, we usually find, that the activity of the phosphatide extracted from the intestinal mucose is appreciably lower than that obtained from the liver plasma. This might be due to the fact, that the formation of

phosphatides in the intestinal mucose is a relatively slow process and that active phosphatides are being transported from the liver to the intestine through the plasma. Another, more probable explanation is, that the rate of activation of the phosphate in the intestine is much slower than in the liver. In this case, if the phosphatides of the intestine are renewed with the same velocity as those of the liver, their specific activity will of course be much lower. This supposition agrees with recent results of ARTOM and his collaborators, who showed that the ratio of the specific activities of the phosphatides extracted from the intestine and from the liver is much higher in rats to which radioactive sodium phosphate is adminstered per os than in rats that have received it by subcutaneous injection [1]).

3. ACTIVATION OF THE PHOSPHORUS IN MILK AND EGGS.

Secretion of lecithin occurs in animals laying eggs or giving milk. The activity of the lecithin extracted from different eggs laid by a hen, was measured by HEVESY and HAHN [2]). They found that during the first 7 days the specific activity of the yolk lecithin steadily increased whereas the activity in the white had its maximum in an egg laid one day after the injection. The shell had its maximum activity in the very first egg which was laid 4 hours after the administration of the radioactive sodium phosphate. This last mentioned egg was already in the oviduct at the moment when the hen received the active phosphate. She probably started building the shell about the same time. Thus it is easy to understand that the phosphate incorporated in this shell was very active, while

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[1]) ARTOM, SARZANA and SEGRÉ, Arch. internat. de physiol. **47**, 245 (1938).

[2]) HEVESY and HAHN, Kgl. Danske Vid. Selskab Biol. Medd., **14**, 2 (1938).

the shells of the later eggs were formed from plasma phosphate which had given off most of its activity to the skeleton. The formation of the white takes about 24 hours and therefor it is not surprising that the egg which was laid 24 hours after the beginning of the experiment was more active than the whites of the other eggs. The formation of the yolks is a very slow process and the ovary contains a great number of yolks in different stages of growth. If one assumes, that the amount of lecithin, which the growing yolks receive from the ovary, is large compared to the amount transported the opposite way, we must expect, that after a certain time the highest specific activity is found in the lecithin of those yolks in which the fraction added during the experiment is greatest. Using the curve determined by GERHARTZ for the growth of yolks in the ovary and analyses carried out on yolks of different sizes taken from a hen killed 28 hours after the adminstration of radiophosphorus, HEVESY and HAHN could show that this consideration provides a very satisfactory explanation of the results of their experiments.

The continuation of the work, dealing with the formation of phosphorous compounds in eggs, will be described in the later chapters of this dissertation.

It could be shown, that in an experiment of short duration, where the specific activity of the phosphatides was still rising in the whole body, these activities showed a clear gradient. The phosphatide activity was highest in the liver, lower in the plasma and still lower in the ovary and the little yolks it contained. (Fig. 1 shows the specific activity of the phosphatides in different parts of the body of a hen). Thus one sees, that all the phosphatides present in the eggs come from the blood as such and that no phosphatide-synthesis occurs in the ovary. Another important point in this experiment was the fact, that the specific activity of the plasma phosphatide was higher than that of the phosphatide obtained from the

intestinal mucose. This proves, that the intestinal mucose is not the only source of the phosphatides occurring in the plasma, but that at least an important fraction comes from the liver or other organs, possibly the kidneys.

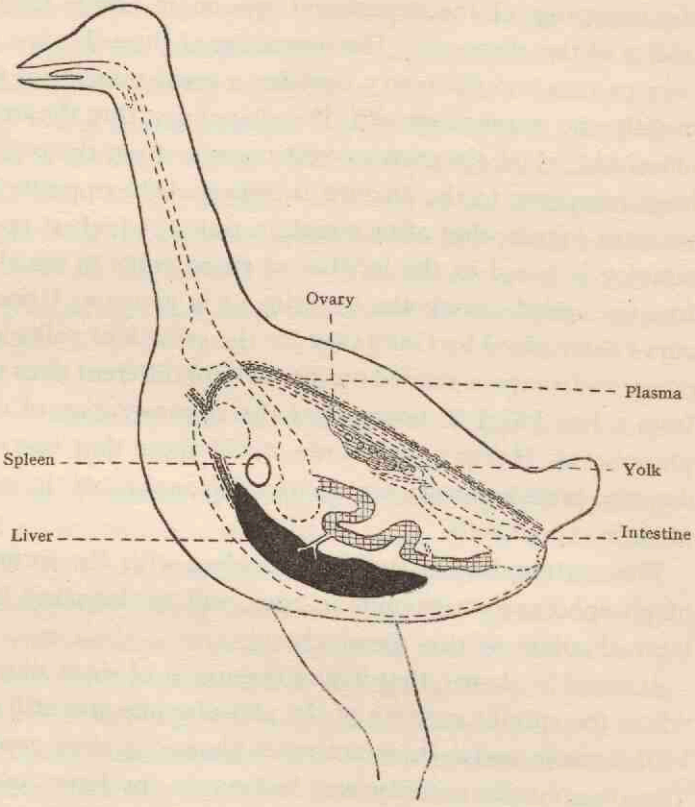

Fig. 1. The heaviness of the shading of the different organs indicates the specific activity of the phosphatide phosphorus in a hen five hours after the injection of radioactive phosphate. (Taken from HEVESY and HAHN Kgl. Danske Vid. Selskab. Biol. Medd. **14**, 2 p. 29).

It seemed worthwhile to ascertain whether the posphatides secreted in milk had the same origin as those found in eggs. The same investigations served to study the formation of the

other phosphorus compounds found in milk. No parallelism was discovered between the processes involved in the secretion of phosphatides in hens and goats. The phosphatide which was extracted from the mammary-gland showed a specific activity a great deal higher than that found for the plasma phosphatide This means, that the milk gland carries out a phosphatide synthesis by itself, and that the milk phosphatides do not originate from the plasma.

Concerning the milk phosphate it was demonstrated, that if one milks several samples of milk, one immediately succeeding another, the inorganic phosphate of these fractions does not have the same activity. This means, that, while the milk is stored in the udder and the milk gland, no mixing occurs.

The formation and secretion of casein takes longer than that of phosphate inorganic. This follows from the fact that the later shows a higher specific activity during the first hours of the experiment. The falling off of the specific activity of the plasma phosphate, the milk phosphate and the casein with time is shown in fig. 2.

The formation and secretion of milk esters takes an even longer time, but here one should consider, that the ester fraction consists of a mixture of different compounds, as can be proved by fractionating hydrolysis followed by a determination of the specific activity of the different samples obtained. One finds, that the substances, which are more easily hydrolysed, are more rapidly activated too. It was shown, that the average specific activity of the milk ester is higher than that of the corpuscles ester, which proves that the former cannot originate from the latter by simple diffusion, unless the differences in diffusion velocity of the esters concerned cause the average value of the specific activity to rise during this process.

To test the possibility of plasma esters diffusing through the milk gland without involving chemical reactions, we in-

jected radioactive hexose phosphate. In the body this substance is changed into inorganic phosphate, which in its turn is taken up by the skeleton. Thus, at least during the beginning, the ester activity must be higher than the phosphate activity. Then, if the plasma ester could diffuse into the milk,

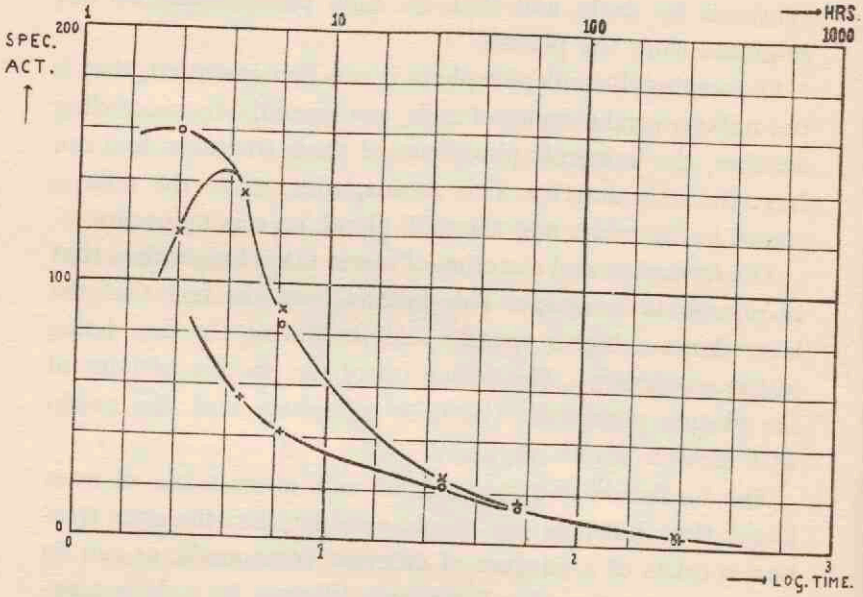

Fig. 2. Change of specific activity (expressed per mgr. phosphorus in parts per million of total activity injected) with time for plasma phosphate (+), milk phosphate (○) and caseine (×). Values taken from experiment no. 2. Time in hours.

one would expect the milk ester, collected during the very first stages of the experiment, to show an abnormally high specific activity, probably even higher than the plasma phosphate. As the opposite is the case, it seems very probable, that the milk esters are, at least for the largest part, formed in the milk gland. So the final conclusion is that, as far as we have been able to prove, all phosphorus compounds in milk

are synthesized in the mammary gland from inorganic phosphate of the plasma.

About the formation of phosphoproteins little is known except the facts discovered about the production of casein which we will describe in this publication. The difficulty we encounter in dealing with phosphorus esters is, that these substances constitute a nearly inseparable mixture of a great many different compounds. The first data obtained about the exchange of organic acid-soluble phosphorus compounds were published by HAHN and HEVESY [1]). In connection with their measurements of the rate of activation of lecithin in dog's blood they determined the corresponding value for the ester phosphorus which they found to be at least 40 times larger than that for the phosphatide phosphorus. Later this exchange process has been investigated in extenso by HEVESY and ATEN, JUN. [2]). They found that there is a very fast reaction going on within the corpuscles between the phosphate and part of the organic acid-soluble phosphorus. Thus in rabbit's blood about 60% of the phosphorus ester has the same specific activity as the phosphate and about 40% does hardly take part in the exchange process at all. The rate of penetration of phosphate ions into the corpuscles was measured too and it was found to be appreciably faster than it is in dog's blood. It could also be shown that the rate at which the activity enters the corpuscles is about the same in vivo and in vitro. Furthermore the rate of decomposition of hexose monophosphate in blood was determined using a radioactive preparation of this substance. It was shown that the reaction is

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[1]) HAHN and HEVESY, Compt. rend. Carlsberg, Série chim., **22**, 178 (1938).

[2]) ATEN, JUN., and HEVESY. Nature **142**, 871 (1938).
HEVESY and ATEN, JUN., Kgl. Danske Vid. Selskab. Biol. Medd., **14**, 5 (1939).

approximately as rapid in blood as in pure plasma, which proves that corpuscles do not exert a catalytic action. It was also found that the disappearance of the radioactivity from the labelled hexose monophosphate is due to a real decomposition and not to an exchange process. Besides the measurements proved that the activation of the phosphorus in the corpuscles is entirely, or at least to an extent of about 90%, due to the diffusion of phosphate ions and only for a very small part to a possible diffusion of phosphorus esters.

Radioactive hexose phosphate injected into a living animal is decomposed at least a thousand times faster than it is in blood in vitro. This must be due to the action of phosphatases contained in the bones or in special organs (possibly the liver).

The rate of the activation of phosphorus compounds in frog's muscles was investigated by HEVESY and REBBE [1]). These authors took great pains to separate and purify the different phosphorus fractions. They investigated creatine phosphorus, adenosin phosphorus, hexose monophosphate (obtained by hydrolysing a 1 n. acid solution at 100° for thirty minutes and afterwards for a hundred minutes) and a non-acid-soluble residual fraction. The rate of activation of the creatin-phosphorus, the adenosin-phosphorus and the hexose phosphate is found to be equal within the limits of the accuracy of the experiment. It can be seen from their values that the formation of all the phosphorus compounds investigated is much faster in an animal living at 21° than in an animal kept at 2° C, the difference being much more pronounced in the case of the organic acid-soluble than in that of the non-acid-soluble phosphorus. We may recall the fact that frogs are cold-blooded animals and that therefor their body-temperature is practically equal to the temperature of their surroundings. This means that HEVESY and REBBE actually

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[1]) HEVESY and REBBE, Nature, **141**, 1097 (1938).

measured the temperature dependence of reactions occurring in the muscle-tissue.

In this connection an experiment, carried out to investigate the decomposition of hexosephosphate in urine, may be mentioned because it has not yet been published elsewhere.

Fresh urine, mixed with radioactive hexosephosphate, was kept at 37° C during 35 minutes. At the end of this period 1.6% of the activity was found in the inorganic phosphate. Quantitative conclusions should not be based on this figure, because it seems possible that some active inorganic phosphate was present in the hexaphosphate preparation before the start of the experiment and a slight decomposition might occur during the chemical work involved in the separation of the fractions. One may however conclude that the decomposition of hexose monophosphate in urine is quite slow and therefor most of the hexosephosphate secreted by the kidneys is not decomposed in the bladder but leaves the body with the urine.

CHAPTER III.

Technique.

1. MATERIALS.

In their earlier work HEVESY and his collaborators had used radio-phosphorus, obtained from carbon disulphide which had been irradiated with neutrons from radium-beryllium sources. Under those circumstances radio-phosphorus is formed in the following way:

$$S^{32} + n \rightarrow P^{32} + H.$$

This method involves the very tedious separation of small amounts of phosphorus from a larger volume of carbon disulphide.

For the work to be described we could dispose of preparations of a much higher activity. Our radio-phosphorus had been prepared at Berkeley, Cal. by deuteron-bombardment of ordinary red phosphorus with the cyclotron, according to the equation:

$$P^{31} + D \rightarrow P^{32} + H.$$

In this case the activity obtained is so strong that the fact that it is distributed over several tenths of a gram of phosphorus does not cause any difficulties.

Our samples were sent to New York by air and from there to Copenhague by ordinary mail. As a rule the preparations lost somewhat over half of their activity during the transport.

The material was received as red phosphorus and had to be converted into phosphate for our purposes. It is advisable to keep and mail the active phosphorus wrapped in metal foil, as it attacks filter-paper quite strongly, probably through its γ rays.

I used two different methods for the oxidation of the phosphorus. The first one, a dry procedure, has the advantage of being fast though it may possibly cause a loss of part of the material.

About 100 mgrs. of phosphorus are separated as fully as possible from pieces of the metal foil and the consumed paper (which may be mixed with the sample) and wrapped carefully in a small piece of filter paper so as to make a tiny square package. This is thrown into a long-necked round-bottom pyrex flask of about 2 liters capacity. The flask is fitted with a cork. After the introduction of the phosphorus oxygen is led into the flask, until the air is expelled. Then a long thin wooden rod or reed is lighted and rapidly introduced into the flask. As soon as it enters the oxygen atmosphere, it starts burning wildly. One rapidy lights the filter paper and retracts the wooden match as fast as possible. Now the flask is closed immediately to prevent the P_2O_5 formed from escaping. The phosphorus burns with a strong white flame, filling the whole vessel with white fumes. First the flask must be allowed to cool and then a few cc. of destilled water are at once introduced and the flask is corked up again. After several hours all of the phosphor pentoxide has been absorbed . Now the liquid is poured from the flask which is rinsed several times with a few cc. of water.

The liquid, which contains a good deal of charred wood and paper, is filtered and made neutral to litmus paper with a known solution of sodium hydroxide. The volume of the NaOH solution used gives a rough value for the amount of phosphorus present.

If the work can be done slowly, it is just as easy to oxidize the radioactive phosphorus with nitric acid. One carefully adds to the red phosphorus which may contain quite an appreciable amount of paper and metal-foil, some fuming nitric acid and evaporates to dryness on a steam-bath, after the reaction has calmed down. The addition and evaporation of nitric acid is repeated several times. As soon as there is no solid matter left, some hydrogen peroxide is added and the liquid evaporated. Then the residue is extracted with water, the liquid filtered and neutralized with sodium hydroxide. If the complete oxidation of the paper in the mixture presents difficulties, there is no objection to extracting with water containing some nitric acid the solid residue left behind after the treatment with nitric acid and the evaporation of this reagent. Then the filtrate is used for the original preparation in other cases.

If the preparation is used for injections, as it was in our experiments, it is important to make up a solution which is both neutral and approximately isotonic with blood. If one has prepared a neutral solution of radio-phosphorus which contains a very small amount of electrolyte, this can be done by adding the calculated quantity of solid sodium chloride, or even more easily by mixing with several volumes of a physiological salt-solution. If the active phosphorus is administered per os, as in some of the earlier experiments by HEVESY and his collaborators, these precautions are of course unnecessary.

2. MEASUREMENTS.

The quantity, which is characteristic for a radioactive phosphorus compound, is the specific activity, i.e. the activity of a certain amount of phosphorus. We shall always consider one miligram of phosphorus, this being a convenient quantity of

the same order of magnitude as the preparation used for the measurement. Much more complicated is the question of the unit of radio-activity. As mentioned above the halftime of P^{32} is 14,5 days. Therefor direct comparison is only possible between samples which have all been measured the same day. One can of course, correct for the decrease of the activity if one uses measurements, carried out on different days, and this method seems to be used occasionally by biologists, working in this field. It is however necessary to check the apparatus—whether electroscope or Geigercounter—several times a day, by using a preparation of known activity. For this purpose a sample of a uranium compound is usually taken. Now, if one uses a standard preparation of radio-phosphorus instead of uranium as a standard, this falls off the same rate as the samples to be measured. Activities expressed in fractions of a radio-phosphorus standard may be compared without applying any correction for radioactive decay.

Now we are still left to choose the definition of our phosphorus standard. We might of course select any arbitrary sample of radio-phosphorus, but it is practical to choose a certain fraction of the total activity used in the experiment. As a rule we shall use a standard equal to 10^{-6} times the total activity in the work on goats. Therefor the specific activity is expressed in parts per million of total activity per mgr. *P*.

Apart from these units we shall occasionally have use for so-called relative specific activities, obtained from the specific activity by multiplying with a factor which makes a certain rel. spec. act. equal to 1.

The activity determinations were carried out with Geiger counters of a type specially constructed for measurements of this kind, which are in constant use in Copenhague in Professor HEVESY's department. [1]) The sensitive part consists of a

[1]) These instruments were worked by Dr. HILDE LEVI.

window of thin aluminium-foil, having a surface of about 1.1 cm^2. The rest of the counter is made out of heavy brass. Therefor the substance must be put opposite the window, in a way which is quite reproducible. The easiest method is to put the sample into a small aluminium dish which can take about 200 mgrs. of magnesium pyrophosphate. These dishes which are pressed from aluminium foil and can be prepared easily and rapidly, fit into a hole in a flat oblong piece of copper sheet. The latter is made to fit a slit in the lead block, which contains the Geiger counter in such a way, that the small aluminium dish takes up a position just underneath the window of the counter. It will be clear that the counter is found over the end of the slit in the lead block and that the aluminium window is in a horizontal position in the bottom of the Geiger counter. The high-voltage wire which is in the axis of the counter, is horizontal.

It is of some importance to fill the aluminium dishes with an approximately constant weight of substance, as the β–rays from radio-phosphorus are not sufficiently strong to pass through a few hundred milligrams of substance per cm^2, without loosing an appreciable part of their intensity. Besides, one must be careful not to vary the geometrical conditions, as these influence the counting very appreciably. That means that the different dishes should not only have the same shape and contain about the same amount of material, but that this material should also have the same composition and be prepared in the same way, so as to fill the dishes to the same height.

Of course there exist other outfits for using Geiger counters, both such of the kind fitted with a window and ordinary round ones, but as none of those have been used in the work described here, it seems unnecessary to do more than mention the fact of their existence.

Every value of a specific activity obtained is thus the result-

ant of one activity measurement and one phosphorus determination.

In some cases one might of course isolate the phosphorus as magnesium pyrophosphate — the substance which is needed for the radioactive measurement anyway — and from its weight calculate the quantity of the phosphorus. There are however several serious objections to this method. First the amount of phosphorus is often so mall in our experiments (of the order of 1/10 mgr.) that a simple precipitation with magnesia mixture would be very uncertain and incomplete. Then the liquids, we have to deal with, usually contain a certain amount of ions or molecules which disturb the precipitation of ammonium-magnesium phosphate or make the compound impure. Especially calcium is nearly always present. And in the third place, as has already been mentioned, it is desirable to have always a fixed weight of magnesium pyrophosphate in the aluminium dish for activity measurements, so that one would have to mix the precipitate obtained with the right amount of inactive pyrophosphate.

To avoid all these difficulties, the two measurements were carried out on different samples. The phosphate solutions obtained from our different fractions were suitably diluted in a measuring flask. (If one does not need to find the total phosphorus-content of a preparation, any ordinary flask might be used in stead of a measuring flask). A known fraction is then pipetted off into a beaker and inactive sodium phosphate is added to make the total amount of phosphorus present equal to about 16 mgrs. Water is added as well if the volume is less than 50 cc, followed by about 10 or 20 cc. of concentrated ammoniumchloride solution and 10cc. of magnesia-mixture after TREADWELL[1]). Usually a precipitate is formed immediately.

[1]) TREADWELL. Kurzes Lehrbuch der analytischen Chemie II (Leipzig en Vienna. 1927) 11th Ed. p. 369. The solution was made up with dilute ammonia instead of with water.

If this is not the case one adds ammonia till the liquid becomes cloudy. In any case the liquid is left to stand a few minutes and then 10 to 20 cc. of 8% ammonia are added. The solution is left to stand over night and filtered next day. The filter is squeezed into a porcelain crucible, dried over a very small flame and ashed over the hottest flame obtainable with a Bunsen burner. I always checked the weight of the pyrophosphate obtained, but I do not think this quite necessary, as I found only two or three cases in which the weight of the substance was too low. In these cases the solution containing the precipitation of ammonium-magnesium phosphate had been standing longer than usual and the liquid had become some what acid to litmus by the evaporation of ammonia. If one takes the precaution of checking the alkalinity of the liquid before filtration, one can be sure that the precipitation is complete. An other point is, that in case one has a number of crucibles of the same shape in use, checking their weight after the ashing, makes it unnecessary to put on numbers to prevent interchanging them by mistake.

After the crucible has been weighed the contents are pulverized and scratched out as completely as possible with the help of a stainless steel spatula. It is rather important to have a spatula for this purpose which can be cleaned in acid, to prevent infecting non-active samples with activity from very strong preparations. A stainless steel spatula stands being washed in chromic acid or in nitric acid and proves quite satisfactory. The magnesium pyrophosphate is transferred to a small aluminum dish of the type described above and its activity determined in the Geiger-counter.

Another part of the solution is pipetted off for the phosphorus determination which is made in a way adapted from the method of FISKE and SUBBAROW [1]). The principle is the

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[1]) FISKE and SUBBAROW J. biol. Chem. **66**, 375 (1925).

following. Molybdic acid is reduced by reducing agents like stannous ions, sulfite and others. The molybdsenum compounds formed in this process have a strong blue colour. The reduction is quite slow however, but in the presence of phosphate or arsenate ions complex anions are formed which are reduced much more rapidly. Then the blue colour caused by the reduction of a molybdate solution can be used to measure the amount of phosphate ion present.

If possible about 0,07 mgr. *P* was used for each analysis, though even 0,02 mgr. gives satisfactory results. It is even possible to go down to 0,01 mgr. but then the determination became rather inaccurate in our apparatus. If one uses smaller measuring flasks and eventually even smaller cells in the colorimeter it should be possible to use much smaller quantities, but under these circumstances the colorimetric determinations would be more tedious.

The volume of liquid used for the colorimetric measurement was pipetted into a 25 cc. measuring flask with a ground-glass stopper. This liquid must be neutralised accurately as the strength of the colour depend quite appreciably on the P_H. I used a sodium hydroxide solution which was about 5 normal. Some authors advocate adding an indicator which is colorless in acid solution and for this reason should not interfere with the colorimetric measurement. Others who do not think this quite safe, take out a little drop with the help of a glass rod and ascertain its reaction towards litmus paper. This procedure is somewhat laborious, as great care must be taken not to contaminate the drop with acid on the wall of the neck of the flask. I found it quite satisfactory to drop a little strip of litmus paper into each flask and to neutralise the liquid observing its colour. The addition of the reagent should not be too rapid, as it takes a few seconds for the liquid to penetrate the paper. Sometimes, if the organic matter is not entirely destroyed, the alkaline reaction shows itselfs by

the development of a yellow colour in the liquid. When the solution is alkaline, it is made very slightly acid again by the addition of about 1 normal hydrochloric acid, only a few drops at a time.

Then one adds 5 cc. of a solution containing 25 grs of ammonium molybdate and 140 cc. of sulfuric acid per liter [1]) and 1cc. of a solution containing 15 grams sodium sulfixte and 1.5 grs. "Amidol" per liter [2]). The flask is filled to the mark and heated for 5 to 10 minutes in a water bath at 37° to develop the colour and cooled in cold water. Then it is measured as rapidly as possible. I never made readings more than an hour after the development.

The simplest way to measure the intensity of the colour in a Pulfrich-photometer is to compare the solution with plain water. In this case however there is a slow zero-effect, due to the reduction of molybdate in the absence of phosphate. For this reason I compared my solutions with suitable standards, containing about the same amount of phosphorus. I always used two standards at the same time; normally I had ten analyses in each series, making twelve bottles in total. On the left I always had a standard prepared with 0,068 mgr. *P*; this standard giving the best colour for measurement. I started my readings having the same liquid on both sides to get the zero-point. Then I compared this colour with that of my second standard, containing either 0,034 mgr. *P* or no *P* at all. After this the unknowns were put in the right-hand cell

[1]) It takes quite a long time to prepate this solution, as the ammonium-molybdate dissolves very slowly. Therefor it is advisable to mix the components at least one day before use.

[2]) The "sodium sulfite" used by most authors is described as crystalline and is probably hydrated acid sulfite, the same substance which is used as a developer in photography. I used dry powdered Na_2SO_3 which proved quite satisfactory. The "Amidol" is a commercial preparation also used in photography. The samples I used were from AGFA. The solution is stored in the ice box and should not be kept for more than about ten days.

and read one after another. The only cells which I had at my disposal were 20 mm. long.

According to BEER's law the intensity of the light leaving the cell (= I) is connected with the intensity transmitted by a cell containing no absorbing substance (= I_o) and the amount of adsorbing substance present in the solution (= x, expressed in mgr. *P* in 25 cc) by the formula:

$$\log \frac{I_o}{I} = \text{const. } x.$$

I obtained a calibration curve which proved to agree quite well with BEER's law, the constant being equal to 11.0 $mgr.^{-1}$ (using light-filter S 50). This calibration curve was used for all phosphorus determinations.

3. SEPARATIONS

Before measuring the activity of the different phosphorus compounds, the latter must be prepared in a pure state. For our purposes it was not necessary to carry out the separations in a quantitative way; as we were only interested in the specific activity of the different fractions, it was quite sufficient to isolate part of a phosphorus compound in the pure state.

Of the substances with which we had to deal the simplest was blood. The plasma was obtained pure by centrifuging and the blood corpuscles by washing twice with isotonic sodium chloride solution and centrifuging each time. The plasma contains phosphorus as phosphates, as lecithin and a very small amount as ester. In the blood cells are found lecithin, phosphoprotein, so-called acid-soluble phosphorus (which consists mostly of esters, hexose phosphates and glycerophosphates mixed with some other compounds which are difficult to separate from these: adenyl-pyrophosphate etc.) and apparently some phosphate. The amount of simple phosphate ions

in the corpuscles in living organisms is not quite certain however, as some of the acid-soluble substances hydrolyse so very easily, that their decomposition cannot be entirely prevented. Thus some phosphorus enters into this fraction before it is analysed.

Blood samples were always kept in ice and usually even collected in cooled vessels. If a separation of ester phosphorus from phosphate ions was intended, even the centrifuging was carried out in a cooled centrifuge.

Usually I only wanted to obtain the lecithin-phosphorus and the total sum of ester- and phosphate-phosphorus. For the isolation of the lecithin from plasma[1]) the liquid was dropped slowly from a pipette into a mixture of 3 volumes of absolute alcohol and 1 volume of ether, using 75 cc. of this mixture for 4 cc. of plasma, which causes the protein and most of the phosphate and esters to precipitate. This is best done in an erlenmeyer flask. The liquid is heated to boiling in hot water, taking care that there is no fire in the neighbourhood. After the liquid has boiled about 10 seconds the flask is cooled under the running water tap to prevent further loss of ether and filtered immediately into a flat-bottomed or erlenmeyer flask using coarse filter paper. At this stage the work may be discontinued if so desired and the solutions kept till next day. Then one closes the flask with a small watch-glass and evaporates the solution to dryness on an electric hot plate. Great care must be taken that the very inflammable vapours, given off during this process, cannot catch fire. The heating must be discontinued as soon as the last drop of liquid disappears, as prolonged heating will now cause a rapid decomposition of the lecithin. Now forty or fifty cc. of technical hexane ("petrolether") are poured immediately into the flask which start to boil vigorously. The flask is shaken, to loosen

[1]) This method is due to BLOOR: Journ. biol. Chem. **77**, 53 (1928). The use of hexane is a recent improvement.

the solid particles from the walls after which the liquid is filtered hot into a kjeldahl flask. This filtration is not quite simple, because the filterpaper has a strong tendency to jump out of the funnel. For this reason it is advisable to fold it in a special way. It is first folded in the ordinary fashion till the sides make a 90° angle and there are four parallel layers of filter paper. Then it is opened as for any ordinary filtration and flattened out again in a direction perpendicular to the first. If this filter is opened carefully it is found to fit very nicely in a funnel, even when dry.

A glass bead is put into the kjeldahl flask and the contents evaporated to dryness on the hot plate under special care to prevent explosions of the vapour. After the flask has cooled, pure fuming nitric acid is poured on to the residue, initially drop by drop, and followed up with 1 or 2 cc. of sulfuric acid. The flask is heated, while the nitric acid is constantly being renewed, until the liquid remains quite colourless. If the phosphorus compound is heated with sulfuric acid only, while there is still carbon or organic matter present, part of the phosphorus is volatilised. After all the nitric acid is gone, one takes the flask down and allows it to cool. Then a few cc. of water are added and the liquid is boiled about one minute to decompose pyrophosphates. The solution is then transferred to a measuring flask to be used for analysis.

If I wanted to analyse both the phosphate and the ester phosphorus or the phosphate uncontaminated by ester, a sample of the plasma was run slowly into 5 to 10 volumes of 10% trichloroacetic acid, cooled in icewater. This served to precipitate protein and lecithin and after filtration through a coarse filter, the liquid contained most of the phosphate and ester. If it is necessary to isolate these fractions quantitatively, one must wash the precipitate repeatedly with 5% trichloroacetic acid. Immediately after filtration an excess of ammonia is added, because the esters are hydrolysed much more rapidly

in acid than in alkaline solution. After this it is not so necessary to keep the liquid cool, though I usually kept it in the ice box till the separation was completed. Now 10 cc. of magnesia mixture are added and the solution kept till next day. Then the ammonium magnesium phosphate is filtered off and transferred with the filter paper to a kjeldahl flask. The precipitate is treated with nitric and sulfuric acid, as described for the destruction of lecithin. This always causes loss of part of the phosphate which in our case was of no importance. If one wishes to recover all the phosphate, it is necessary to dissolve the precipitate on the filter in hydrochloric acid.

To obtain the ester fraction, the filtrate was evaporated in a porcelain dish on a sand bath and heated red hot over a Bunsen flame. The contents were dissolved in hot hydrochloric acid and filtered into a measuring flask. If I did not aim at a separation of ester phosphorus from phosphate, I often did not carry out any precipitation, but destroyed the organic matter simply by glowing and dissolving the residue in hot hydrochloric acid.

The analysis of blood corpuscles was carried out in a strictly analogous way. Only, the amount of protein being much larger, one must take at least 10 volumes of trichloroacetic acid and it is important to mix the cells with water before adding them to the reagent.

In milk the isolation of lecithin was carried out in exactly the same way as in plasma. The high fat content makes it difficult to concentrate the ether-alcohol extract without loss through bumping. It is best to add a number of glass beads and to shake the flask vigourously during the last stages of the concentration.

The isolation of the casein was done in a way described by VAN SLYKE [1]). The milk, freed from cream in the centrifuge, is stirred very rapidly in an ice bath. I always used about

[1]) VAN SLYKE and BAKER. J. biol. Chem. **35**, 127 (1918).

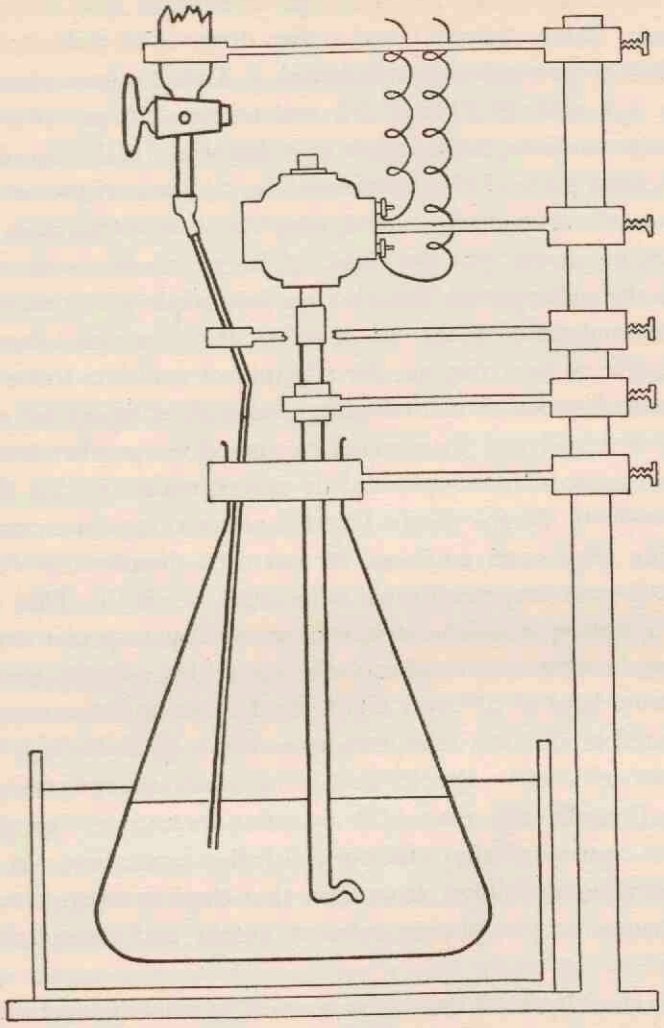

Fig. 3. Apparatus for the isolation of casein from milk after VAN SLYKE.

50 cc. of milk and I had a 250 cc. erlenmeyer flask for a container. Before the start one or two drops of octylalcohol were added, to prevent excessive foaming. Then a solution containing 1/3 mol H Cl and 2/3 mol acetic acid per liter was slowly run into the liquid from a burette. To the tip of this burette a piece of thin rubber tubing (as used on the air-inlet of bicycle tyres) is connected, which leads to a thin glass tube reaching down into the liquid, from which the acid enters into the milk. (comp. Fig. 3). Once in a while a 1 cc. sample is taken and mixed with 1 cc. of water. If this mixture does not separate on centrifuging, the addition of acid has to be continued. As soon as centrifuging gives a clear liquid 1/2 cc. of acid is added and the stirring continued for another minute. The casein is now separated by centrifuging, and the liquid filtered off. To the casein I added water three times, centrifuging after each addition. It was then dissolved in 0,02n. NaOH and reprecipitated with 0,02 n. H.Cl. That this precipitation is satisfactory, was proved by a special experiment, in which active phosphate was added to milk and the mixture kept at 37° over night. Next morning the casein was isolated in this way and decomposed in a kjeldahl-flask with nitric acid and sulfuric acid. The casein-phosphorus was found to be less than 1/30 as active in total as the phosphate, proving the efficiency of the separation. At the same time it follows from this, that there is no appreciable exchange of phosphorus between casein and phosphate in milk.

To the filtrate of the casein some 10% trichloroacetic acid is added, to get rid of proteins and possibly non-precipitated casein. The filtrate of this procedure is made ammoniacal and phosphate and acid-soluble phosphorus are separated as in the case of plasma. It seems, that the danger of esters being hydrolysed in milk is much less than it is in blood and therefor cooling in ice is not so necessary. However, samples that had

to be kept over night, I always stored in the ice-box, as some phosphatases do occur in milk [1]).

Organs which had to be analysed were cut out of the body as soon as possible after death and frozen in liquid air or dry ice immediately. It is practical to put the pieces of the organs in a pyrex reagent tube which is fitted inside a wider one and to immerse the whole in the cooling bath, as the inner one often cracks during freezing. Freezing has two advantages: it prevents the decomposition of unstable compounds and it opens up the cells, which facilitates the extraction of their contents.

To isolate the lecithin the organs are cut very fine and extracted with ether-alcohol in exactly the same way as described for the analysis of plasma. To obtain the acid-soluble phosphorus, the organ, cut to small pieces with scissors, is extracted two or three times with five to ten times its weight of ice-cold 10% trichloroacetic acid. This is done in a small porcelain mortar, which should be cooled in a mixture of ice and salt beforehand. For each extraction the mixture is rubbed energetically for at least ten minutes.

The yolks of eggs were extracted with ether to isolate the lecithin, but before this extraction they were dried by extraction with a small amount of acetone. This does cause a loss of some lecithin, but it seems very unlikely that it will cause an inaccuracy in our results, as it has been shown by HEVESY and HAHN [2]) that the different lecithin fractions, which they obtained by extracting a hen's liver with ether and with ether-alcohol, had the same specific activity [3]).

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[1]) FOLLEY and KAY, Enzymologia (1), 48 '36.

[2]) HEVESY and HAHN. Kgl. Danske Vid. Selskab Biol. Medd. **14**, 2 (1938).

[3]) It must be mentioned however, that results obtained by ARTOM and his collaborators, are not in agreement with these findings.

4. Danger of Contamination

By far the most serious source of error in work of this kind is the contamination of weak preparations by traces of very active phosphorus. Glass vessels and funnels as well as porcelain dishes and crucibles were either boiled in dilute hydrochloric acid, or kept in it for about a week, to extract all traces of activity. The greatest danger of pollution came of course from the radio-phosphorus stock, as I had to carry out quite a number of manipulations with very strong preparations. Even a 10^{-7} part of one of these preparations, mixed accidentally with one of my final samples, would be quite sufficient to destroy its value. The entire preparation and handling of these strongly active materials was carried out in a special room, where I used to wear a special laboratory coat and where I had a special towel to wipe my hands, after washing them, every time before I left the room. I also made it a habit, whenever possible, to handle these radio-phosphorus stocks only late in the afternoon, after I had finished the rest of my work. Next morning, my hands being clean, there was no danger of infecting the fractions I wanted to analyse. Yet there happened one case of very bad radioactive infection, when I was half way through experiment no. 5. All the values obtained in this experiment after the pollution happened, were discarded, through many of them agreed quite well with their duplicates.

5. Experiments with heavy Water.

The heavy water was injected subcutaneously as an isotonic sodium chloride solution. For the preparation of water from milk, I used the apparatus shown in Figure 4. It is made of pyrex glass and can easily be obtained by sealing a piece of glass-tubing (having a diameter = 8 mm.) to a reagent tube and

bending the latter in two places. Both parts must have been cleaned carefully beforehand, as it is very difficult to do this after they have been put together. The milk is introduced into the dry apparatus by a long and thin pipette, which reaches down to C through the long neck D. The apparatus is connected to an oil-pump and the milk, which is lying in C, frozen in a mixture of ether and dry-ice. The apparatus is pumped out and sealed off at D during the pumping. Now the cooling mixture is removed and the substance at C allowed to thaw slowly. (It is important not to warm the liquid with the hand

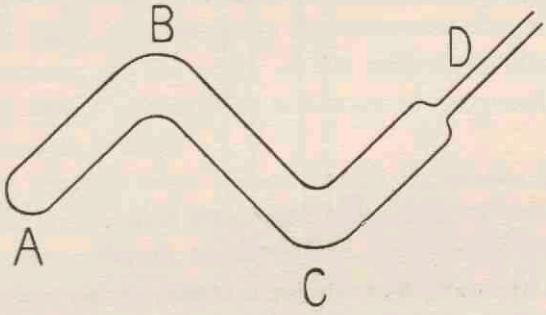

Fig. 4. Apparatus for the destillation of heavy water and its isolation from milk.

or in any other way, as this causes it to sputter). After all the ice has disappeared in C, A is immersed in an ice bath and the whole left to stand, till a sufficient amount of water has distilled over. If there is any liquid left in C, which is usually the case if one has started with more than a few tenths of a cc. of milk, all that is left in C is frozen in a mixture of ether and in dry-ice. Then the apparatus is opened at D and sealed off at B, as rapidly as possible. We always carried out a second distillation in which case all the liquid evaporated from C, so that it was not necessary to cool this part, before opening the apparatus.

From blood the water was obtained in an apparatus which

is shown in figure 5. The sample was put into A with a pipette through B and the apparatus sealed off at B. Now C was connected with two taps, one leading to the oil pump and the other one to the atmosphere trough a tube filled with calcium chloride. D was immersed in a Dewar vessel filled

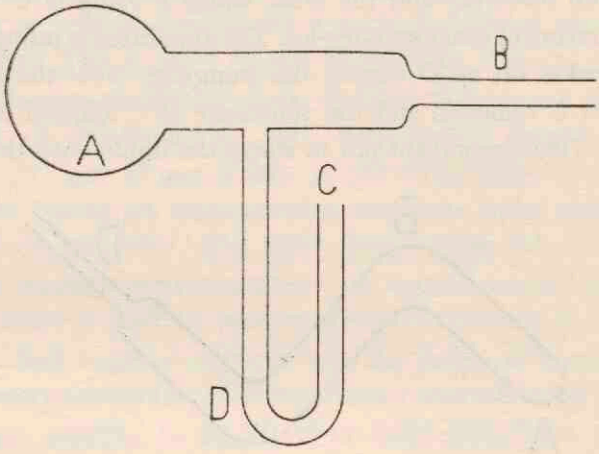

Fig. 5. Apparatus for the isolation of heavy water from blood.

with ether and dry ice. Then the apparatus was pumped out with the oil pump and whenever the liquid in A began to foam in a dangerous way, some air was let in. The water collected in D was redistilled in an apparatus of the type shown in Figure 4.

The water-samples prepared by me in this way were analysed by Mr. OLE JACOBSEN at the Carlsberg Laboratory, using a method invented by Professor LINDERSTRÖM-LANG. This method makes use of small drops of water, floating in a column of liquid having a slight density-gradient. The level at which the drop floats, indicates its density. Before each measurement the water was distilled after the addition of very small amounts of sodium peroxide and potassium permanganate. The entire determination was made using 100 mgrs. of water.

Because of the creeping of the alkaline solution all figures are found somewhat high (on the average 12 parts per million density-excess). This correction was therefor applied to all measurements.

6. Experiments with heavy Fat.

Fat was isolated from milk samples by centrifuging and dried in a dessiccator in vacuo for about two days. As the water in the milk was lighter than that obtained by combustion of the fat, a very small amount of water contained in the latter would not cause great errors in the determination of its deuterium content. (If the water of the milk were many times heavier, it would of course be necessary to carry out a much more thorough drying.)

The fat samples were burned in the apparatus shown in Fig. 6. The combustion tube, with a diameter of about 2 cm., has a narrower U-tube sealed to one end. Special very high-melting glass has to be used for these parts; I used so-called "combustion tubes" of German make. (Ordinary Pyrex glass is unsatisfactory as it devitrifies rapidly at the required temperature.) The tube passes through two furnaces which can be closed on both sides with asbestos sheets having holes for the glass tube. The smaller furnace is movable.

The air used for the combustion is dried in a trap in liquid air, seen on the left. The U-tube in which the water to be analysed is collected can also be cooled in liquid air. To this part can be connected an inverted U-tube which contains a layer of calcium chloride (to dry the air which enters the apparatus while it cools off after a combustion) covered with cotton wool. The outlet of this piece can be connected to a bubbler filled with oil.

Before the start of an experiment the copper oxide to be used is dried by heating it while a current of dried air passes

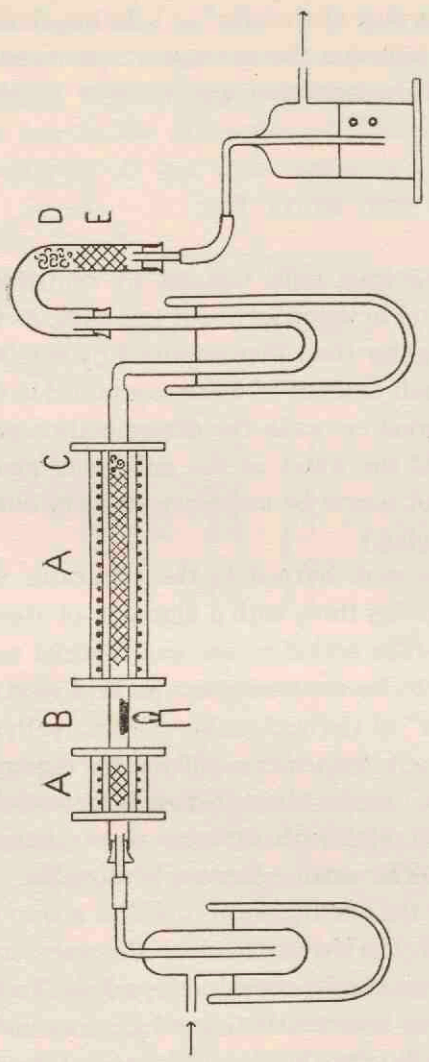

Fig. 6. Apparatus for the combustion of fat containing heavy hydrogen. Copper oxide at A, porcelain boat containing fat at B, asbestos at C, cotton-wool at D, calcium chloride at E.

through the apparatus. The U-tube is not cooled and the tube containing the calcium chloride is disconnected.

To carry out a combustion the fat is filled into a porcelain boat. This is put into the combustion tube, half of which has already been filled with copper oxide. (On the right side the copper oxide should not reach beyond the end of the furnace.) Then some copper oxide is carefully pushed in after the boat. Now the combustion tube, the trap in liquid air on the left, the calcium chloride-filter and the bubbler are connected and the moist air driven out by a slow stream of dried air. After this, liquid air is placed round the U-tube, the air current shut off and the longer furnace heated to a very dull red. Then the smaller furnace on the left is heated and as soon as this has reached the same temperature, a slow current of air is again passed through the apparatus. Now the fat is very slowly and carefully decomposed by heating the glass tube around it with a Bunsen-burner. The decomposition is very apt to get out of hand. Whenever the bubbling becomes too violent the air-current is shut off. Under those circumstances however, the oxidation of the vapours is carried out entirely by the copper oxide. Therefor, after the bubbling has slowed down again, one has to pass air through the apparatus for some time to oxidise the copper formed.

As soon as the fat has been thoroughly charred the heating with the Bunsen-burner is discontinued and the smaller furnace is gradually moved to the right side until it is in contact with the larger one. Then it takes only about ten minutes more to burn all that is left of the fat sample.

When the combustion is complete the oil bubbler is disconnected and the apparatus allowed to cool.

The liquid air is removed from the U-tube and after the water in the latter has reached room temperature it is taken out with a long, thin pipette. The water is distilled in the apparatus shown in Fig. 4 before it is treated with permanganate and analysed in the apparatus of Linderström-Lang.

CHAPTER IV.

Results.

1. Accuracy of the Experiments with Radio-Phosphorus.

The accuracy of the values in my experiments is at best about 10%. Two radioactive measurements performed on preparations obtained from equal volumes of the same solution often differ by that amount and occasionally even a little more. To this must be added the inaccuracy of the phosphorus determination which may amount to 5%. Therefor the difference between two parallel analyses which are carried out separately throughout, can be as high as 20%. Of the measurements performed on samples having an activity which is large compared to the natural effect, only one group shows a divergence which exceeds the limit mentioned above (milk phosphate expt. 6 from 0—1 hr.) [1]). Preparations with very small activity can, of course, only be measured with a smaller accuracy. These cases can be recognised from the large divergence between the duplicate values. In most instances the degree of accuracy is not very important as we usually compare values which differ by a much larger factor. Figures which did not have a sufficient accuracy for the purpose of the comparison desired have been omitted.

2. Experiment no. 1 (Radioactive Sodium Phosphate injected into a Hen.).

To a hen which laid an egg daily, ten mgms. of radiophosphorus were administered by subcutaneous injection. Five hours later the animal was killed, its blood collected and

[1]) The disagreement between the values found for the different ester fractions inexperiment no. 7 is not due to inaccurate measurements but to the incompleteness of the chemical separations involved in the preparation of these samples.

the body rapidly dissected. The ovary contained a great number of yolks in different stages of growth. Two yolks of intermediate size weighing 1.0 and 2.7 grms. were used for analysis, as it had been shown previously that the relative rate of growth is largest in yolks of this size. In the oviduct we found an egg which was nearly full-grown, apparently lacking only its shell. Of this egg we took the white which was destructed to determine the average activity of its total phosphorus content.

The experiment shows that the phosphatides of the yolks are formed in the liver and are carried to their destination by the plasma and the ovary.

TABLE IV.

Experiment no. 1. (Hen treated subcutaneously with radio-active Sodium Phosphate.)
5 hours after the injection of radio-phosphorus.

Organ and substance isolated	Activity per mgr. *P* in parts per thousand of total radio *P*
Liver lecithin	0.82 0.94
Plasma lecithin	0.70 0.70
Ovary lecithin	0.064 0.062
Yolk 1,0 gr. lecithin	0.067 0.053
Yolk 2,7 grs. lecithin	0.042 0.057
Intestine lecithin	0.18 0.18
Spleen lecithin	<0.2
Plasma phosphate	1.6 1.6
Yolk 1,0 gr. total acid soluble	0.36
White of egg in oviduct	0.013
Plasma protein [1]	1.6 1.4

[1]) It is doubtful whether this fraction was sufficiently pure.

3. Experiment no. 2. (Radioactive Sodium Phosphate injected into a Goat.)

Experiment no. 2 was meant to be a preliminary survey. For this reason no notice was taken of the rarer components of milk and blood. The phosphate separations in this case

Table V.

Experiment no. 2 (Goat treated subcutaneously with radioactive Sodium Phosphate).

Time of sample	Activity per mgr. *P* in millionth parts of total activity	
Milk	Phosphate	Casein
Before—$^1/_2$ hr.	17.0	1.8 } Diluted with
	15.8	1.4 } inactive milk.
$^1/_2$—$2^1/_2$ hrs.	163	120
	157	121
$2^1/_2$—$4^1/_2$ hrs.		123
		150
$4^1/_2$—$6^1/_2$ hrs.	87	81
	79	99
$25^1/_2$—29 hrs.	22.4	26.8
	22.4	24.1
$54^1/_2$—73 hrs.	13.4	
	15.7	
223—239 hrs.	4.3	4.9
	3.7	5.8
Plasma		
$4^1/_2$ hrs.	58.8	
	51.0	
$6^1/_2$ hrs.	44	
	38	
73 hrs.	15.3	
	67.1	
243 hrs.	3.7	
	4.2	

were made in a different way. After precipitation of the casein the liquid was boiled with nitric acid and the protein which had been precipitated filtered off. Then a double precipitation with ammonium molybdate was carried out and the precipitate dissolved in hot strong ammonia. From this solution the phosphate was precipitated with magnesia mixture, redissolved in hydrochloric acid and reprecipitated. In this way a very complete removal of the phosphate can be obtained, but it is a disadvantage that during the boiling with concentrated acid part of the esters in the milk will hydrolyse. As the amount of ester-phosphorus is at the utmost 20% of the phosphate-phosphorus, the dilution of the latter is unimportant compared to the inaccuracy of the measurements. If one wants to obtain the ester-phosphorus as an isolated fraction, one has to apply the procedure described in Chapter III.

This experiment shows the falling off of the specific activity of plasma phosphate, milk phosphate and casein (comp. Fig. 2). It is used for the calculation of the time needed for the formation of milk phosphate and casein from blood phosphate.

4. Experiment no. 3. (Radioactive Sodium Phosphate injected into a Goat).

The separations for experiment no. 3 were carried out in the way which was definitely adopted and has been described on page 49. It shows the change of the specific activity of plasma phosphate, of milk phosphate, milk ester and casein. The results are seen in Figure 9. This experiment provides data for the calculation of the rates of formation of the three constituents of milk which have just been mentioned.

TABLE VI.

Experiment no. 3. (Goat treated subcutaneously with radioactive Sodium Phosphate.)

Time of sample	Activity per mgr. *P* in millionth parts of total activity		
Milk	Phosphate	Casein	Ester
0—2 hrs. .	66.5	57	33.5
	72	53	30
2—4¼ hrs. .	186	165	117
	176	181	
4¼—6⅓ hrs. .	186	165	128
	160	181	144
23¾—25¾ hrs. .	49.5	55	51
	50		48.5
Plasma			
2 hrs.	277		
	288		
4¼ hrs.	117		
	85		

5. EXPERIMENT NO. 4. (RADIOACTIVE SODIUM PHOSPHATE INJECTED INTO A GOAT.)

Experiment no. 4, performed on another goat, shows that the difference in specific activity of the various milk fractions is very striking at the beginning.

TABLE VII.

Experiment no. 4 (Goat treated subcutaneously with radioactive Sodium Phosphate).

Time of sample	Activity per mgr. *P* in millionth parts of total activity		
Milk	Phosphate	Casein	Ester
2 hrs. before—1 hr. after	9.4 9.4	4.4 3.5	1.6 2.6
Plasma			
$1^1/_4$ hr.	309 367		

6. EXPERIMENT NO. 5. (RADIOACTIVE SODIUM PHOSPHATE INJECTED INTO A GOAT.)

Experiment no. 5 was meant to provide material for the calculation of the rate of diffusion of phosphate ions from the plasma into the milk.

TABLE VIII.

Experiment no. 5. (Goat treated with radioactive Sodium Phosphate).

Time of sample	Activity per mgr. *P* in millionth parts of total activity
Milk	Phosphate
$^3/_4$—$2^1/_2$ hrs.	66 60,5
Plasma	
$1^1/_3$ hr.	266 324

7. Experiment no. 6. (Radioactive Sodium Phosphate injected into a Goat.)

Experiment no. 6 was carried out with our third goat.

When half the measurements of this experiment were finished, a very bad case of radioactive contamination happened as I had to prepare of a stock solution of very high activity just at that time. Therefor only the values obtained before this day are given in the table. As most of the figures were now single, a parallel experiment no. 7 was carried out afterwards, which checked the other one quite satisfactorily.

Table IX.

Experiment no. 6. (Goat treated subcutaneously with radioactive Sodium Phosphate).

Time		Activity per mgr. *P* in millionth parts of total activity
0—1 hr.	Milk phosphate	64
		70
		81
$1^1/_6$ hr.	Plasma phosphate . . .	610
3—$4^1/_3$ hrs.	Milk phosphate	178
	Milk ester	130
	Milk lecithin	10
$4^1/_2$ hrs.	Plasma phosphate . . .	106
	Plasma lecithin	1
		3
		2
	Corpuscles ester	44
	Corpuscles lecithin . . .	1
$4^3/_4$ hrs.	Milk gland phosphate . .	97
	Milk gland lecithin . . .	14
	Liver lecithin	11
		9
	Kidney lecithin.	12

Experiments 6 and 7 show that an independant synthesis of phosphatides occurs in the milk gland. They also show the milk ester to be more active than the ester in the corpuscles.

8. Experiment no. 7. (Radioactive Sodium Phosphate injected into a Goat.)

The seventh experiment, made with another goat, was used for a trial to carry out a fractionated hydrolysis of the milk ester. For this purpose I used a milk sample collected during the first three hours. After the precipitation of the phosphate with magnesia mixture the liquid was made 1-normal with hydrochloric acid and hydrolysed for 7 minutes at 100° C. The hydrolysed fraction was precipitated by adding ammonia and after filtration the hydrolysis with acid was continued for another 60 minutes. Now a precipitation with ammonium molybdate followed. The difficulty was that all precipitations except the first one were incomplete. The separation with magnesia mixture is unreliable in the presence of large quantities of electrolytes (which are added in making the solution alternatively acid and ammoniacal) while the precipitation with molybdate is seriously disturbed by chlorides. This is the reason of the bad agreement between the duplicates in table 10.

The values measured for the activity of milk lecithin are somewhat uncertain because the total activity of the milk phosphate was about 1000 times larger than that of the lecithin in this case. Therefor the lowest values are likely to be the best. This consideration does not hold for the lecithin found in the milk gland, where the total activity of the phosphate is only about 20 times that of the lecithin.

TABLE X.

Experiment no. 7. (Goat treated subcutaneously with radioactive Sodium Phosphate.)

Time of sample		Activity per mgr. *P* in millionth parts of total activity
0—3 hrs.	Milk phosphate (first 33 grs.)	102
		110
	(middle 76 grs.) . .	169
		188
	(last 66 grs.) . . .	138
		143
	Milk 7 Min. hydrolysis (middle fraction) .	89
		89
	Milk 60 Min. hydrolysis (middle fraction) .	99
		64
	Milk non-hydrolysable (middle fraction). .	37
		49
	Milk lecithin (first fraction)	0
		—3
	Milk lecithin (last fraction) .	3
		4
3—4¼ hrs.	Milk phosphate	185
		192
	Milk ester.	117
		117
	Milk lecithin	4
		10
4¼ hrs.	Plasma phosphate	136
		124
	Plasma lecithin	1
		0
		—4
	Corpuscles	49
	total acid-soluble . . .	43
	Corpuscles lecithin	1
	Corpuscles protein	about 20

Experiment no. 7.

Time of sample	Activity per mgr. *P* in millionth parts of total activity
$4^1/_2$ hrs. Milk gland phosphate . . .	121
	129
Milk gland ester	83
	101
Milk gland lecithin	11
	11
Liver lecithin	13
	15
Kidney lecithin	10
Epiphysis (total phosphorus) .	0.43
Diaphysis (total phosphorus) .	0.064

9. Experiment no. 8. (Radioactive Hexose Phosphate injected into a Goat.)

One experiment was made with hexose monophosphate which was administered by intravenous injection as it was thought that, if this compound should be injected in another way, there might be danger of it being decomposed by phosphatases which are known to be quite generally present in the body. Barium hexose phosphate, prepared in the institute of Professor Parnass at Lemberg, was dissolved in an isotonic solution of sodium chloride. This was mixed with a slightly hypertonic solution of sodium sulfate containing a little more than the quantity of sulphate required for the precipitation of the barium present. The liquid was filtered and kept in ice till the moment it was used.

This experiment showed that no appreciable amount of hexose monophosphate diffused through the milk gland from the plasma into the milk without exchanging with the phosphate ions.

TABLE XI.

Experiment no. 8. (Goat treated intravenously with radioactive Hexose Phosphate.)

Time of sample		Activity per mgr. *P* in arbitrary units
0—1¼ hr.	Milk phosphate	1.87
	Milk ester	0.73
	Casein	0.8
1¼—2¾ hrs.	Milk phosphate	1.46
	Milk ester	1.03

The activities to be determined were too small to allow division of the milk samples for the purpose of carrying out the separations and measurements in duplicate.

10. EXPERIMENT NO. 9. (HEAVY WATER INJECTED INTO A GOAT.)

To compare the rate of secretion of water to that of phosphate ions, an isotonic solution of sodium chloride in heavy water was injected and milk collected over different periods. Different water samples obtained from milk and blood all showed equal density within the limits of the accuracy of the determination. The figures found also agreed with the density calculated from the assumption that the D_2O injected had been distributed equally over all the water contained in the body.

The values given in Table 12 represent averages of two or more density determinations, performed on the same water sample.

TABLE XII.

Experiment no. 9. Heavy water experiment. (Goat treated subcutaneously with 20 grs. of heavy Water.)

Time of sample	Excess of density in parts per million
Milk	
0 —1½ hr.	86*
	77
1¼—2¾ hrs.	74
2¾—4 hrs.	84*
4 —5½ hrs.	77
Blood	
1⅓ hr.	86*

The figures in this table are averages obtained from two or more density determinations carried out on the same water sample, except for those marked with an asterisk, which are the results of single determinations.

If the heavy water injected is supposed to be equally divided over all the water present in the body, it can be calculated that the density-excess would amount to 91 ppm. It is assumed that on the average 75% of the body tissues and organs consist of water. The animal's weight was 32½ kgrs.

11. EXPERIMENT NO. 10. (HEAVY FAT GIVEN TO A GOAT PER OS.)

The secretion of heavy fat in milk was studied with the purpose of demonstrating a possible difference in the composition of fat samples isolated from milk portions taken in immediate succession. It had already been found that the inorganic phosphate shows different specific activities in such a series, but I had not been able to observe an analogous variation in the specific activities of the lecithin fractions because of the very

low activity of the latter. It was hoped however that these differences might show themselves in the deuterium content of the fat in a series of milk samples taken one directly after the other. Such differences were not observed (the differences seen in table 13 being within the limits of error); it is hard to say whether they might have been found if the accuracy of the experiment had been greater. As it was, the inaccuracy was quite appreciable (30 parts per million in the density seems to be a good estimate), but no better results could be obtained with the small samples we had to work with. The significance of our figures would of course have been enhanced if larger quantities of heavy fat had been given to the animal. This,

TABLE XIII.

Experiment no. 10. Heavy Fat Experiment. (Goat treated with heavy Fat per Os.)

Time of milk sample	Density excess of water obtained from milk fat in parts per million
0—1 hr.	37
	−13*
1—4 hrs. First sample	52*
Second sample	1*
Third sample	−4*
7—24 hrs. First sample	229
Second sample	227
Third sample	236
48—50 hrs.	88
105—120 hrs.	−10*

The figures in this table are averages obtained from two density determinations carried out on the same water sample, except those marked with an asterisk, which are the results of single determinations.

however, did not seem advisable, considering the great value of the material involved.

Although the original aim was not reached, the results of this experiment are published none the less, as some very important conclusions about the rate of secretion of fat in milk can be obtained from them.

The material used was obtained by reducing unsaturated fats with deuterium. The deuterium content of the hydrogen in this preparation amounted to 4.5%.

CHAPTER V.

Discussion

1. Origin of Phosphatides in Yolks.

The results of the chicken experiment may be explained in a simple way. The only fraction which has a special interest is the lecithin and the phosphorus occuring in this compound in different organs shows appreciable differences in its specific activity. Its value is highest in the liver, indicating beyond doubt that here the lecithin is formed in situ and does not originate from other parts of the body. Though we did not investigate all the different organs of the chicken, we think this conclusion to be justified because Artom and his co-workers have shown that lecithin synthesis is much slower in most other parts of the body. Only in the kidneys and in the intestine lecithin is built up at a rate comparable to that of the liver. The kidney however, is much smaller than the liver and its total lecithin content does not amount to more than a small fraction of the quantity found in the latter organ. Therefor it is quite out of question that the liver phosphatide can come from the kidney. That the liver phosphatide is not synthesised in the intestinal mucose (or at least that part of the liver lecithin is not) follows directly from the fact that the lecithin of the intestine has a specific activity several times lower than that of the liver, as is seen in table 4. This conclusion is by no means surprising as the data obtained by Artom and his collaborators had already led to the assumption of a fast phosphatide turnover in the latter organ.

The problem of the origin of the plasma phosphatide is a much more intriguing one however. The possibility has to be considered that phosphatides are formed in the cells of the intestinal wall from fats during the digestion of the latter. That at least part of the plasma phosphate does not originate from this organ but from elsewhere, follows from the fact that it has a higher specific activity than the corresponding fraction extracted from the intestine. One is led to look for the source of at least some of the plasma phosphatide in the liver and the kidneys.

The specific activity of the ovary phosphatide is about ten times smaller than that of the plasma phosphatide. This gives strong reason to believe that no phosphatide synthesis takes place in this organ or possibly a very slow one which is of no importance for the metabolism as a whole. One might consider the possibility of a very slow activation of the phosphate in the ovary which would cause the lecithin to have a low activity even in the case of a rapid phosphatide synthesis. This alternative is excluded however by the high value found for the specific activity of the acid-soluble phosphorus in the growing yolk.

This being the case we must assume that the lecithin in the ovary is formed in the liver, transported from there by the plasma to the ovary, finally entering the yolks which are being built. From table 4 it can be seen that in the small yolk it has a specific activity amounting only to 8,6% of that of the plasma lecithin. If we assume for the sake of simplicity that the plasma lecithin has a constant activity, we calculate that in the course of one hour 1,7% of the lecithin found in the yolk has entered from the plasma, as the total experiment lasted five hours. We may recall that the yolks in question are those which are growing most rapidly according to GERHARTZ's experiments [1]). In fact the situation is much

[1]) Gerhartz Arch. f.d. gesamt. Physiol. *156*, 215 (1914).

more complicated because the specific activity of the plasma lecithin is not constant but increases with time. If the rate of increase of this quantity during the experiment were known it would enable us to calculate the rate of absorption of phosphatides by growing yolks quite accurately. Unfortunately this is not the case and the determination of the rate of activation of the plasma phosphatide by experiment would involve a great deal of labour. None the less an approximate value for the lecithin absorption which whould be accurate to about 25%, may be calculated in the following way. If we assume that the specific activity of the plasma phosphatide rises linearly with time its average value during the experiment would be equal to one half of its final value. Under this assumption 3.4% of the yolk's phosphatide would be absorbed in one hour. Now the real value must lie between the two figures we have calculated: 1,7 and 3.4% per hour, for the following reason. The rate of activation of the liver phosphatid and therefor presumably that of the plasma phosphatide too [1]), is proportional to the difference between the specific activities of the inorganic phosphate and the phosphatide in the liver. As this difference decreases during the experiment (except for a short period at the beginning when the active phosphate still has to spread from the plasma to the liver) the rate of activation of the plasma lecithin decreases too. This causes the specific activity to change in a way which is intermediate between the two extremes mentioned above. It will probably be a good approximation if one puts the specific activity of the plasma lecithin proportional to the square root of the time Then the average value of this quant-

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[1]) The assumption that the specific activity of the lecithin in the plasma varies in the same way as that of the lecithin in the liver seems to be justified by the fact that the difference between the two quantities was found to be relatively small in expt. no. 1.

ity would amount to two thirds of its final value. From this assumption we may calculate that 2.5% of the lecithin contained in a small growing yolk of one gram has been taken up during the last hour. For the sake of comparison it may be mentioned that from GERHARTZ's curve it can be concluded that the growth of this yolk during one hour amounts to 4.6%. The agreement between these two figures is as good as may be expected. It shows that the phosphatide is transported in practically only one way from the blood to the yolk. The transport in the opposite direction which might be termed a phosphatide exchange does not seem to be very important.

The only point which is not quite clear about the lecithin distribution in laying hens, is the rate of activation in the ovary. It was found that the average specific activity of the phosphatide in the ovary was about equal to the corresponding quantity in the yolks, which amounts to about $^1/_{10}$ of the specific activity of the phosphatide in the plasma. As we have seen that the lecithin transport from the plasma to the yolk goes in one direction only, we would be inclined to expect that the specific activity of the phosphatide in the former should be about the same as in the ovary. Otherwise the lecithin entering the yolk would not have the same specific activity as that of the plasma and the preceding calculation would not be justified. Considering the good results it gave, it seems that we have to look for an explanation elsewhere. Probably the lecithin in different parts of the ovary has a varying activity, strong in those places where it is given off to the growing yolks and weak in other parts where the lecithin circulates much more slowly or not at all. The equality of the average phosphatide activities in the ovary and in the yolks would then be accidental. This explanation is quite hypothetical however and at present no experimental evidence exists for it.

The specific activity of the total acid-soluble phosphorus

extracted from the smaller yolk is about $^1/_4$ of that of the plasma phosphate. Though the composition of this acid-soluble fraction is not known it seems likely that it consists mostly of inorganic phosphate. If this is true, we may calculate that 5% of the inorganic phosphate in the yolk has been taken up from the plasma during the last hour, if we assume the specific activity of the latter fraction to be constant. But the plasma phosphate has (as is explained in Chapter II.,) a constantly decreasing activity. This causes the average value during the experiment to be higher than the final value, meaning that if our assumptions are justified, the amount of phosphate taken up by the growing yolk during one hour is somewhat less than 5%. This would be in excellent agreement with the rate of growth deduced from the rate of activation of the lecithin in yolks and with the result of GERHARTZ's experiments, if we may assume that the movement of inorganic phosphate from the yolks to the plasma is small compared to the transport the opposite way. Only in case the largest part of the yolks' acid soluble phosphorus should not be inorganic this figure should be much higher.

It may be of interest to point out that the specific activity of the phosphorus in the white of the egg found in the oviduct is as low as 2% of the specific activity of the plasma phosphate. This leads to the conclusion that the rate of formation of these phosphorus compounds (probably mostly proteins) from phosphate is quite slow. We do not regard this fact as definitely proved however as the figure on which it is based has been obtained by a single measurement.

2. PHOSPHORUS COMPOUNDS OCCURRING IN MILK.

Milk contains the same four classes of phosphorus compounds which are found in blood. Whereas in the latter liquid most of the phosphorus occurs as an organic acid-

soluble compound, by far the largest part of the phosphorus in milk occurs in the inorganic phosphate. The next largest quantity of phosphorus is usually found as casein. This is a protein and by far the most thoroughly studied of the phosphoproteins. At present it is not known for certain whether the natural casein is a mixture, but there are indications that it is. The normal amount of casein *P* in goats' milk amounts to 10 to 20% of the inorganic *P*. Organic acid-soluble phosphorus occurs in quantities of approximately the same magnitude. The composition of this fraction has not been studied successfully until now, though it is known that its compounds are slowly decomposed by phosphatases occurring naturally in milk and that the same phosphatases can decompose hexose monophosphate. Thus it seems possible, though by no means certain, that part of this fraction consists of hexose monophosphate.

TABLE XIII.

Quantities of Phosphorus Compounds occurring in different Body Liquids and Organs of Goats.

	Per 100 grs. plasma	Per 100 grs. milk	Per 100 grs. milk gland tissue	Per 100 grs. liver tissue	Per 100 grs. kidney tissue
Phosphate .	6	50	42	?	?
Casein . . .	—	17	?	—	—
Ester	0	17	23	?	?
Lecithin . .	3	2	19	27	14

The figures in his table are not very accurate. They have been obtained by me from purified preparations during the isolation of which some material was undoubtedly lost. Also the figures found are quite different for different individual animals, and to a lesser degree even for the same animal at different times.

Phosphatides are also found in milk but in much smaller quantities. The total amount of phosphorus found in this fraction does not exceed 2 or 3% of the amount of inorganic *P*. It is not known whether the composition of these phosphatides is the same as that of the phosphatides found in blood. (To be sure the composition of the blood phosphatides is only known in the case of human blood and not for any of the animals used for milk production). Table XXIII shows the concentration of the different phosphorus fractions in the blood resp. in the milk of goats. These figures indicate averages and the individual deviations may be as high as 50%.

3. Change of Phosphate Activity in the Plasma.

As has been mentioned in Chapter 2 the specific activity of the plasma phosphate shows a continuous decrease, different in different species and even — to a smaller extent — in different individuals. The results of all experiments I have performed on goats, are seen in Figure 5.

Artom, Sarzana and Segré expressed the opinion that the only important process in this connection should be the uptake of phosphate ions by the skeleton, which would occur according to a monomolecular scheme. [1]). In case this were true the points in Figure 5 should lie on a straight line, which is far from being the case [2]). Therefor we distinguish at least

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[1]) Artom, Sarzana and Segré. Arch. int. de physiologie. **47**, 245 (1938).

[2]) Of course only points obtained in a single experiment are strictly comparable. However in the steep part of the curve on the left side of the figure an inaccuracy in the specific activity amounting to a factor 2 is of no account and this is the only part where measurements from different experiments are combined. In the lower part of the curve, where the accuracy of the activity measurements is much more important, all data are taken from experiment 2.

two different processes. It seems likely that during the first period, when the decrease is very fast, the radioactive phosphate is taken up predominantly by the organs and tissues,

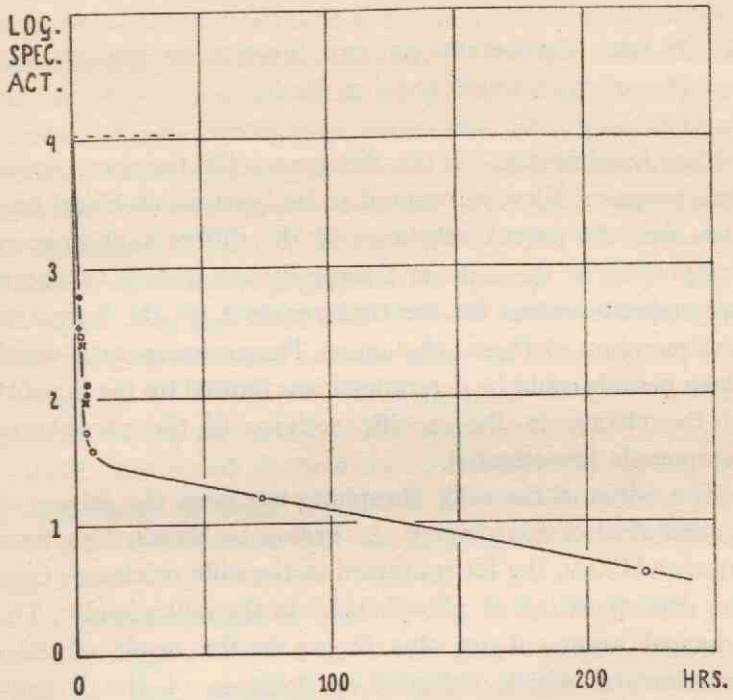

Figure 7. Change of specific activity of plasma phosphate (expressed in parts per million of total activity injected) with time. The values used were taken from experiment 2 (○), experiment 3 (×), experiment 4 (+), experiment 5 (□), experiment 6 (●) and experiment 7 (■). A simple first-order decrease of the specific activity would be indicated by a straight line. The dotted line indicates the value the specific activity would have, if the entire activity injected were present in the plasma.

while during the second stage it moves into the bones. This explanation is supported by a determination of the distribu-

tion of labelled phosphate in a rat [1]). After 4 hours which is about the duration of the first and faster diffusion process, it was found that only 40% of the injected phosphate had gone into the skeleton, while 59% had been transported by the blood to other parts of the body.

4. Plasma Phosphate as the Source of the organic Phosphorus Compounds in Milk.

Our investigation of the different milk fractions served two purposes. First we wanted to find out which blood fraction was the parent substance of the different phosphorus compounds in the milk and secondly we wished to obtain approximate values for the time required for the formation and secretion of these substances. The accuracy with which these periods could be determined was limited by the rapidity of the change in the specific activity of the phosphorus compounds investigated.

The origin of the milk phosphate has been the subject of a great deal of controversy. According to Meigs, Blatherwick and Cary, the fat contained in the milk originates from the decomposition of phosphatides in the milk gland [2]). The principal argument for this theory is the result of older experiments, which indicated a decrease of the lecithin content of the plasma as it passed the mammary gland. Recent experiments however contradict these results. These investigations which were carried out with an improved technique, showed that the only phosphorus compound which disappears from the blood as it circulates through the milk gland is inorganic phosphate [3]).

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[1]) Unpublished measurements by Professor Hevesy and Mr. Rebbe.

[2]) Meigs, Blatherwick and Cary, J. biol. Chem. **37**, 1 (1919).

[3]) Graham, Jones and Kay, Proc. Royal Soc. B **120**, 330 (1936).

From our experiments we may gather conclusive arguments to settle this point. As the fat formed from the phosphatide should enter the milk according to the opinion of MEIGS, BLATHERWICK and CARY, one would expect the phosphate to go the same way. As the proportion of fat and phosphorus as they occur in lecithin is about 25 and the fat concentration in goats' milk is approximately 4%, milk should contain 160 mgr.% of inorganic phosphorus originating from plasma lecithin, if none of this phosphorus went back into the blood stream. This is two or three times as much as the quantity actually found. So in any case the larger part must return to the blood. If the milk phosphate were formed by decomposition of plasma phosphatide its specific activity should be equal to or less than that of the latter substance. (This difference between the specific activities is to be expected because of the increase of the activity of the plasma phosphatide during the course of the experiment).

Tables no. 9 and 10 show the specific activity of the milk phosphate to be many times as large as that of the plasma lecithin. It is evident that the latter substance cannot be the source of the former.

It might be supposed however that after the lecithin has been decomposed in the mammary gland, its fat enters the milk but that the phosphate formed returns to the blood. It is easy to show that there are other objections which invalidate this explanation too. The amount of phosphatide present in the total blood of a goat would be just sufficient to provide the amount of fat secreted in the milk during 2 or 3 hours. Therefor the theory of MEIGS, BLATHERWICK and CARY would require the plasma phosphatide to be practically entirely renewed in the course of three hours. According to the knowledge obtained from previous experiments of ARTOM, SARZANA and SEGRÉ and ourselves these phosphatides originate in the liver. Even though the specific activity of

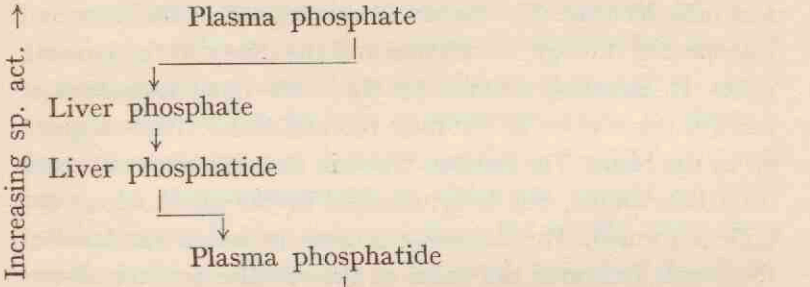
the phosphatide in the liver rises continually, we should expect the plasma lecithin after $4^1/_2$ hours to have a specific activity equal to at least half the specific activity of the liver phosphatide. Experiments no. 5 and 6 show this proportion to be much lower than would be expected. Thus it is seen that the rate of formation of the phosphatides in the plasma is too slow to account for all the fat secreted in the milk.

Another argument which seems to be just as strong is the following. If a decomposition of plasma phosphatides would take place in the milk gland there would be a steady current of phosphatide from the plasma into the tissue of the mammary gland. The phosphatide in the latter would thus be older than that in the former and have a lower specific activity — at least so long as the activity of the plasma phosphatide increases, which is the case during several days after the injection of radioactive sodium phosphate. Again it may be seen from experiment no. 5 and 6 that on the contrary the milk gland phosphatide has a much higher specific activity, proving definitely that it has not been absorbed from the plasma, but has been formed in situ. So we conclude that the phosphate (phosphatide) in the milk originates from the plasma phosphate as supposed by GRAHAM, JONES and KAY.

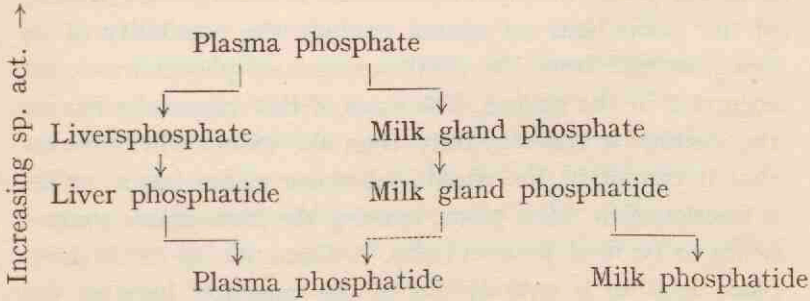
A question of great importance however which we are at present unable to answer is, whether the phosphorus secreted in the milk lecithin, has entered the gland from the plasma in the form of phosphate or of organic acid-soluble phosphorus. In the latter case it would probably do so as glycero phosphoric acid. It seems most likely that the blood phosphate is the parent substance, as the amount of ester phosphorus in the plasma is extremely small and probably consists mostly of easily hydrolysable substances and not of glycero phosphates. One might imagine the phosphorus

SCHEME FOR THE SPECIFIC ACTIVITY OF PHOSPHATIDES.

Phosphatide transport in laying hens.



Phosphatide transport in lactating goats.



esters in the corpuscules to be the parent substance, but this would require either a rapid breakdown of corpuscles in the milk gland which does not seem to agree with the present ideas about the function of this organ, or it would involve a rapid diffusion of esters out of the corpuscles which would be in contradiction to the conclusions obtained from experiments described above.

Here it may be worth while to point out once more the lack of parallelism between the formation of egg lecithin and milk lecithin. The former is synthesised in the liver and transported through the plasma and the ovary to the growing yolks. In lactating animals on the other hand formation of lecithin takes place in the liver too and this lecithin is given off to the blood. The lecithin found in the milk does not come from this source, but is synthesised in the gland where the milk is formed. The following scheme in which the level of the words indicates the value of the specific activity, shows the direction of the lecithin diffusion in both cases, as concluded from the activity of the different fractions.

About the origin of the phosphorus in the casein there has been some difference of opinion, In this case again it seems most likely that the plasma phosphate is used for the synthesis of the casein, but we cannot exclude the possibility of its being formed from the small amount of phosphorus ester occurring in the plasma. The ester of the corpuscles has on the average a lower activity than the casein which proves that it cannot be the parent substance of the latter, unless a fractionation takes place, causing the more active components to be used preferentially. A formation of casein from blood lecithin is entirely out of the question because the specific activity of this substance is many times lower than that of the casein. So we have very good reason to assume that the phosphorus in the casein is derived from the phos-

phate ions in the plasma. GRIMMER [1]) considered it likely that the casein phosphorus is taken from nucleoproteides occurring in the milk gland, which substances would probably be formed from inorganic phosphate. The time required by the active phosphate in the blood to enter the casein molecule is however so short that it seems very unlikely that so large a molecule could be formed and decomposed during this period.

Concerning the organic acid-soluble compounds in milk the first question we have to consider is whether this fraction is homogeneous or not. To settle this point we carried out a fractionation by hydrolysis in experiment no. 7. Though the separation of the different fractions was not complete, owing to the impossibility of obtaining several complete precipitations in the same solution, the results show quite conclusively the existence of at least two ester fractions. Here too, as in the experiments on blood esters, we find the fraction which is hydrolysed fastest to be the most active.

The milk ester phosphorus probably comes from the plasma phosphate too. It cannot originate from the plasma lecithin for the same reason which excludes it being the origin of the milk phosphate and the casein.

That the milk ester would be identical with the ester of the blood corpuscles seems unlikely because of the fact that we found the activity of the former to be 2—3 times as high as that of the latter in our experiments no. 5 and no. 6. Thus, if the milk ester should be due to a diffusion of the organic acid-soluble phosphorus out of the corpuscles, this process should be accompanied by a fractionation causing an increase in the specific activity.

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[1]) GRIMMER, Lehrbuch der Chemie und Physiologie der Milch. 2nd Ed. 1926, p. 31.

We carried out a special experiment to find out whether hexose monophosphate occurring in the plasma enters the milk as such. Radioactive hexose monophosphate (EMDEN ester) was injected into the jugular vein of a goat and the milk analysed after appropriate periods of time. If hexose monophosphate were actually able to enter the milk from the plasma by simple diffusion without being involved in a chemical process, we would expect the ester to be the most active fraction in the milk. Of course the active hexose monophosphate will steadily disappear from the blood as it is broken down by the phosphatases present in different organs, specially in the liver. It is to be expected however, that the inorganic phosphate in the plasma will always remain much weaker than the ester, as the former goes on loosing its activity to the skeleton. Table XI shows that the inorganic phosphate in the milk was more active than the organic acid-soluble fraction, which constitutes a strong support for the assumption that the ester secreted in milk is built up in the mammary gland, instead of diffusing into the milk out of the plasma. It is interesting however to know that in this experiment the ester concentration in the milk was found to be much higher than usual, as may be seen by comparing Tables XIII and XIV. Beyond doubt this curious fact was due to an abnormally large amount of sugar or sugar compound present in the blood after the injection of the hexose monophosphate. This sugar was available and used for the synthesis of ester molecules in the milk gland. Here we see that part of the milk ester may be and probably is hexose phosphate. That the ester fraction also contains other compounds has already been mentioned. Considering the evidence given here, we consider it exceedingly likely that the milk ester too is synthesised from inorganic phosphate in the milk gland.

5. Different specific Activity of Milk Samples obtained in immediate Succession. Rates of Secretion.

For a time it was thought that milk production was a slow continuous process and that milking had no other effect than the extraction of the milk already secreted. Later it was shown that the different fractions of the milk extracted immediately one after the other do not have an identical composition. The concentration of fat continually increases during milking but the amount of fat free residue remains the same, as is shown in Table XV) [1]. At first it was supposed that this was due to a mechanical separation of the fat from the milk in the canals of the milk gland. Such a separation could not possibly influence the substances present in real non-colloidal solution like electrolytes and sugars. Recently the opinion has become prevalent that these differences have another reason. It is probable that the milk is secreted in two different stages, the first part being formed slowly and stored in the canals of the milk gland, the second being formed during the milking after the liquid stored has been taken out. If the second assumption is right, there would be no contact between these two milk fractions and one would have to conclude that under different circumstances the gland secretes milk having a variable fat content but a constant concentration of other dissolved substances. This hypothesis may be tested, if we investigate the specific activity of the phosphate in milk samples taken immediately after each other, making use of the fact that the specific activity of the phosphate in the milk changed during the period of its formation. If the liquid secured at the end of this period had been mixed thoroughly the specific activity of the phosphate should be the same in the different samples.

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[1]) Grimmer l.c. p. 27.

In this case the eventual differences in the concentration of the fat would be due to a kind of churning action or to a sort of filtration of the fat globules in the thin capillary canals of the mammary gland. In the other case, if there is no contact between the different milk fractions obtained in one series, there is no reason why the phosphate activity should be constant. From experiment no. 7 one sees that this quantity shows very appreciable variations, proving the second assumption to be right and the first one to be wrong.

After having got some information about the origin of the substances occurring in milk we want to consider the velocity of the processes concerned. Any conclusions of this kind involve the use of a model for the working of a gland which must necessarily be of a highly simplified nature. In reality the secretion of milk starts immediately after the udder has been emptied. Some milk is soon collected in the canals of this organ, whereas another part stays in the milk gland

TABLE XV.

Composition of different Milk Samples taken immediately after each other. The Volume of all Samples was the same.

No. of sample	% fat	% dry substance without fat
1	1.35	8,55
2	1,50	8,82
3	1,60	8,49
4	2,40	8,85
5	3,40	8,90
6	4,45	8,80
7	5,20	8,70
8	5,65	8,65
9	6,40	8,62
10	8,60	8,35

from which it is only expelled during the act of milking. A consequence of these different modes of secretion is the lack of constancy of the specific activity of the inorganic phosphate in several milk samples taken in immediate succession, as has been mentioned above.

For the sake of simplification we shall be obliged to disregard the inhomogeneity of the milk in the body. We shall consider three schemes for the secretion of milk, none of which is accurate. They will be sufficient however to give us a rough estimate of the time required for the various processes.

In the first place we may imagine, that the inorganic phosphate which enters the milk gland, slowly diffuses through the different cells — eventually taking part in chemical reactions — without being mixed with phosphorus which entered the milk gland at an earlier or at a later moment. According to this model a certain phosphorus atom is always found in a phosphorus fraction of a constant specific activity, while this phosphorus fraction moves through the milk gland as a unit, eventually undergoing chemical changes. The time needed for the formation of a certain milk fraction from plasma phosphate is thus given by the interval between the moments when the same activity occurs in the plasma phosphate and in the milk phosphorus compound considered.

This hypothesis involves that all phosphate ions which have entered the milk gland from the blood are secreted through the milk without the possibility of a further exchange with the plasma phosphate, a restriction which is certainly not true.

Figures 8 and 9 show the change of the activities of plasma phosphate and milk phosphate with time as obtained from experiment no. 00. Though the shape of theses curves is rather uncertain owing to the scarcety of the points, we may conclude that according to the first scheme phosphate ions need about

4 hours, if we consider experiment no. 2, and 2 hours if we use experiment no. 3, or 3 hours on the average, to pass from the plasma into the milk.

The formation of the other phosphorus compounds in milk constitutes a process even more complicated than that of the phosphate. Here we have to distinguish at least three fundamentally different stages, first the diffusion of phosphate into the gland cells, next the chemical reaction and then the diffusion to the place, where the formed milk is stored. The first process is the same in all cases, but the others are different. Therefor, it is not astonishing that the formation of casein, esters or phosphatides, involving a chemical process, takes longer than the diffusion of phosphate ions into the milk. That this is actually the case is seen immediately from the fact that at the beginning of the experiment the phosphate in the milk has a higher specific activity than the other fractions.

Unfortunately our measurements have not been sufficiently numerous to make possible an estimation of the time required for the formation of other phosphorus compounds in milk according to scheme no. 1. Therefor we shall make use of the following considerations. The specific activity of the plasma phosphate is highest immediately after the injection. The moment of highest radioactivity comes later for the substances in the milk, but with them the specific activity rises gradually instead of jumping suddenly to their highest value, as should be the case according to our first model. Besides the activity at the maximum is hundreds of times lower than that of the plasma phosphate at its highest point. In the milk these maxima are flattened out, some of the active phosphorus atoms being secreted too early, and some too late. So if we assume that the maximum is approximately in the same place where it should be if it were not rounded off, the time needed for the secretion of the different phosphorus com-

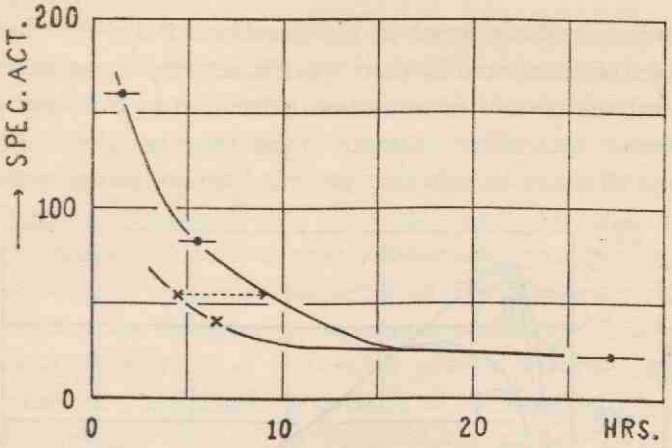

Fig. 8. Change of specific activity (expressed in parts per million of total activity injected, found per mgr. phosphorus) of plasma phosphate (×) and milk phosphate (-••-) with time. The horizontal distance between the two lines, indicated by the dotted line, shows the time needed for the phosphate ions to move from the plasma to the milk according to scheme 1. The horizontal lines indicating the activity of the milk phosphate show the time during which the milk-sample was accumulated. Values taken from experiment 2. (Cf. also fig. 2).

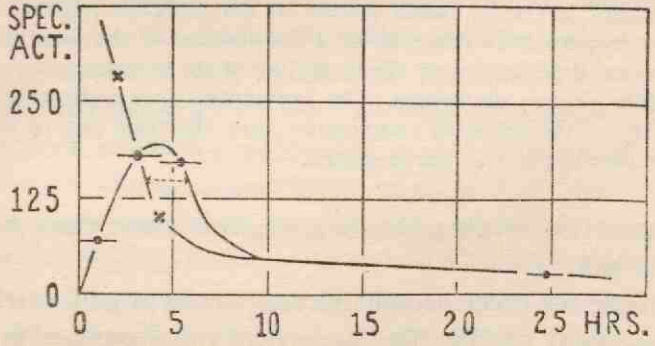

Fig. 9. Change of specific activity (expressed in parts per million of total activity injected, found per mgr. phosphorus) of plasmaphosphate (×) and milk phosphate (-••-) with time. The horizontal distance between the two lines, indicated by the dotted line, shows the time needed for the phosphate ions to move from the plasma to the milk according to scheme 1. The horizontal lines indicating the activity of the milk phosphate show the time during which the milk-sample was accumulated. Values taken from experiment 3.

pounds in milk is equal to the duration of the experiment before the maximum in their specific activity is reached. In general the periods of formation calculated in this way will be longer than those evaluated from the first model. From Figure 10 it may be seen that according to our second scheme

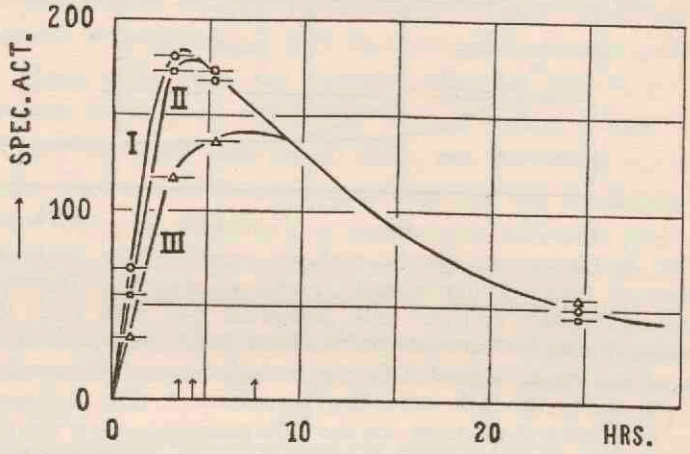

Fig. 10. Change of specific activity (expressed in parts per million of total activity injected, found per mgr. phosphorus) of milk-phosphate (curve I), casein (curve II) and milk-ester (curve III) with time; According to scheme 2 the abscissa of the maxima indicate the time needed for the formation of the substance considered from plasma-phosphate. The horizontal lines indicating the activity of the different compounds show the time during which the milk-sample was accumulated.

the secretion of phosphate-ions requires about three hours and a half.

Among the other phosphorus compounds in milk casein is formed most rapidly. The location of the maximum in the specific activity obtained in experiment no. 3 (comp. Fig. 10) shows the difference of the times needed for the secretion of inorganic phosphate and casein to be of the order of one half hour.

We have already seen that the milk esters constitute a

mixture of different compounds. So we can only consider the average rate of formation as no data are available for the activity of separate fractions in a pure state. In Figure 10 the ester activity, as determined in experiment no. 3, is compared to the phosphate activity, which leads to the conclusions that it takes the phosphate ions from the plasma about 4 hours longer to enter the milk as ester molecules than to diffuse into it as phosphate ions. This difference we have ascribed, at least in part, to the slowness of the chemical reaction. That the latter actually is not a very rapid process is borne out by the difference in the specific activity of the milk gland phosphate and the milk gland ester which, though small, is probably real.

For a third simplified model we shall imagine that a certain amount of milk is present and that its constituents are being renewed from the plasma phosphate. In this case we therefore assume that the rate, at which the substances occurring in the milk are formed, is much faster than that at which they leave the body, or in other words that most of the phosphorus atoms present in the milk gland in different compounds, exchange with the plasma phosphate and that only a small fraction leaves the body by the milk. It seems likely that the reality presents an intermediate between the simplified cases considered, but such a mechanism would be too complicated to be dealt with in a quantitative way.

It is important to note that according to the last scheme it takes an infinetely long time to replace all phosphorus atoms in the milk by atoms originating from the plasma phosphate. It will be clear that when the milk phosphorus begins to get labelled, not all of the phosphorus which leaves the milk will be inactive any more, but part of it consists of labelled phosphorus which has come from the plasma during an earlier stage of the experiment. Thus the part of the milk phosphate which was present already before the start of the experiment

decreased continually without reaching zero during a finite time. Therefor in this case we have to consider another quantity, being the time during which as many molecules of a certain substance enter the milk as are contained in it. These period we will evaluate for phosphorus compounds in milk and the results are to be compared to the times estimated for the formation of theses substances according to our first or second scheme. Probably the real values will lie between the two periods calculated.

The difficulties connected with this question have already been considered. The activity of the plasma phosphate decreases quite rapidly and there is no possibility of obtaining accurate limits between which this change can be confined, as was done with the change of the specific activity of the phosphatide in the hen's plasma. It was known from experiment no. 2 that the activation of the milk phosphate during the first half hour amounts to only 10% of the activity which appears during the next 2 hours. As it is this very first half hour during which the activity of the plasma phosphate changes in a way which is difficult to evaluate it was thought advisable to leave this period out of consideration during the experiment to be described. The goat was milked three quarters of an hour after the injection of the labelled sodium phosphate and the milk obtained discarded. The sample to be analysed was collected from this moment till $2^1/_2$ hours after the beginning of the experiment. It appears from experiment no. 3 that equilibrium between milk and plasma phosphate is far from being reached within this period. In the mean time the activity of the plasma phosphate is continuously decreasing. If the decrease were linear with time, the specific activity would have an average value equal to the value at the middle of the period considered which would be 100 minutes after the injection. In reality, the average value of the specific activity will probably be somewhat higher

and was therefore supposed to be equal to the actual value 80 minutes after the administration of the radioactivity. It is not easy to estimate the size of the error introduced by this assumption. From the values given in experiment no. 7 we may conclude that at this stage the specific activity of the plasma phosphate drops approximately 30% in the course of twenty minutes.

As the moment at which the specific activity reaches its average value is not likely to come before the experiment has lasted 60 minutes (that is only quarter of an hour after the collecting of the milk sample had begun which was to continue for another hour and a half) and not to be later than 100 minutes after the start, it may be considered probable that the value taken for the average specific activity of the plasma phosphate between 45 and 150 minutes after the injection is not in error by more than 25%. Supposing that 10% of the activity found after $2^1/_2$ hours was present in the milk phosphate at the beginning of the collecting of the sample we find that the specific activity of the milk phosphate increased by 57 ppm. during 7 quarters of an hour. This is equal to 19% of the average value of the specific activity of the plasma phosphate. (295 ppm.). Thus we see that during 105 minutes as many phosphate ions moved from the plasma into the milk stored in the canals and cells of the milk gland as make up 19%. Thus it would take approximately 5 times as long or 9 hours before the amount of phosphate ions diffused into the milk from the plasma would be equal to the total amount present in the milk. Or, expressing the same thing in different words it takes a phosphate ion approximately 9 hours to move from the plasma into the milk. Most of the phosphate ions contained in the cells of the milk gland exchange at very fast rate with the ions of the plasma (cf. experiments no. 6 and 7) whereas the diffusion from the milk gland into the milk is the slow process which accounts for the

difference in specific activity between the phosphates in the plasma and in the milk.

To compare the diffusion of phosphate ions through the milk gland with that of water we performed one experiment in which we injected deuterium oxyde subcutaneously. Different milk samples were collected and the density of the water determined. It was found that even the first sample which was collected during the first five quarters of an hour showed the same concentration of heavy water as the blood at the end of this period and as the later milk samples.

The circumstances of this diffusion experiment were comparatively simple, because the concentration of the injected deuterium oxide remains approximately constant in the different tissues and liquids of the body while the heavy water diffuses into the milk (comp. table XII). The complicating effect of the absorption by the bone which is so troublesome in the work with phosphate, does not play a rôle in this case. On the other hand the kinetics of the process under consideration are complicated by its rapidity. If we consider a period during which as many water molecules, as are present in it, diffuse out of the milk contained in the milk gland into the blood, and vice versa, we do not find the two liquids in equilibrium at the end of this time. The reason is easily found. At the beginning of the exchange process some heavy water enters the milk and, after this has occurred, part of the water molecules which move into the blood are heavy molecules which have entered the milk by the same exchange process at an earlier moment in the experiment. Thus a number of molecules are left in the milk at the end of the experiment, which were present in it at the beginning. The equilibrium in a process of this kind is reached at the rate of a monomolecular reaction. Let N denote the number of heavy water molecules present in the blood and other body liquids and n the number of heavy molecules

which have diffused into the milk from the blood both per gram of water. The time, measured in hours, is indicated by t. This leads to:

$$\frac{N-n}{N} = e^{-kt}.$$

Thus, as after five quarters of an hour the concentration of heavy water in the milk is equal to at least 90% of that in the blood, we find for $t = {}^5/_4$ that $kt > 2{,}3$ and therefor k has a value 2 or higher. This means that it takes half an hour or less for a number of water molecules equal to the total number present in the milk to be replaced by water from the blood.

If t is sufficiently small we may write

$$n = Nkt.$$

Combining this with the conclusion reached about the phosphate diffusion we may say that the time which must pass before a certain small fraction — say 1% — of the milk phosphate is renewed from the blood, is at least about 10 times as long as that required for the renewal of the same fraction of the water, possibly longer.

The reason for this difference seems to be twofold; in the first place there are indications that the diffusion of water through membranes in living organisms is appreciably faster than that of many ions and besides the volume of the blood required to provide the amount of phosphate occurring in a certain amount of milk is about 20 times as large as the amount of blood required to provide the water. Thus, if in parts of the milk gland the blood supply should not be ample, the possibility exists that the phosphate supply becomes exhausted before the water supply does.

In an analogous way the length of the period involved in

the renewal of the casein may be compared to that of the milk phosphate. It must be kept in mind however that the kinetics of this process are much more complicated than those of the phosphate secretion, and accordingly the value obtained will be even less accurate. Experiment 2 shows that during the first half hour the casein reaches an activity equal to $^1/_{10}$ of that of the milk phosphate at the end of this period. Assuming the phosphate activity in the milk-gland to rise with time in a linear way, which cannot be strictly true, the average value of this quantity is equal to $^1/_2$ of the value it has at the end of the half-hour, and so $^1/_5$ of the casein has been renewed during this time. Thus a phosphorus atom from the plasma would require roughly $2^1/_2$ hours more to enter into the milk casein than into the milk phosphate.

The secretion of the phosphatides is so slow that its duration cannot be estimated, as none of the fractions obtained from milk showed an activity which was definitely positive. We know however that milk collected between 3 and $4^1/_2$ hours had a lecithin activity less than 8% of the activity of the plasma phosphate at the end of this period. Thus as the latter is decreasing, less than 4% of the phosphatide-phosphorus is replaced by phosphorus from the plasma phospate. Therefor a lower limit for the time required by the inorganic phosphorus of the plasma to enter the milk phosphatide is about 2 days.

The milk gland lecithin actually has a measurable specific activity after $4^1/_2$ hours. This is about the same as the specific activity of the phosphatides found in the liver and in the kidneys. It is only due to the impossibility of obtaining reliable average values of the specific activity of the plasma phosphate, that we are unable to calculate how long the phosphorus in the latter needs to enter the phosphatide of the mammary gland. The slow rate of this reaction is un-

doubtedly connected with the fact that although the quantity of phosphatides excreted by the mammary gland amounts to only $^1/_8$ of the quantity of ester phosphorus and to about $^1/_{50}$ of the amount of inorganic phosphorus, the gland contains equal amounts of ester and phosphatide *P* and only twice as much inorganic *P*. Therefor it is clear that to produce the quantities required, the relative rate of renewal of the phosphatide in the gland can be much slower than that of the other phosphorus compounds.

Considering the relatively long time involved in the secretion of phosphatides it is worth noticing that the time required by a molecule of fat to pass from the stomach to the milk is of the order of 1 day, as can be seen from the figures in table XIII.

Finally a rough estimate may be made of the fraction of the fat given per os which was secreted during the first week. It amounts to approximately 10%.

TIME REQUIRED FOR THE FORMATION OF MILK CONSTITUENTS AFTER DIFFERENT SCHEMES

Change of plasma-phosphate into	Scheme 1	Scheme 2	Scheme 3
Milk phosphate .	3 hrs.	$3^1/_2$ hrs.	9 hrs.
Milk casein . . .	—	4 hrs.	$11^1/_2$ hrs.
Milk esters . . .	—	$7^1/_2$ hrs.	—
Milk phosphatide	—	—	>2 days

SUMMARY

Chapter I deals briefly with the discovery of the first radio active and non-radioactive isotopes. The principles used in the different methods for separating isotopes are discussed.

The application of isotopes as indicators is explained, using radio-lead as an example. Then follows a short survey of the most important investigations that have been carried out with natural radioactive elements as indicators. In this connection the biochemical applications of radio-lead and radio-bismuth by HEVESY and his collaborators are mentioned. The great importance of the discovery of heavy hydrogen for the work with isotopic indicators is pointed out.

In this field the discovery of artificial radioactivity has provided a number of new possibilities and of technical improvements. The factors that limit the usefullness of radio-elements as indicators are mentioned and the many advantages which radio-phosphorus has in this respect are pointed out. Finally the other artificial radioactive elements, which have so far been used in physiological research, are mentioned.

Chapter II contains a survey of the work already published about applications of radio-phosphorus in biochemistry. First of all it is pointed out that radioactivity is not a property which influences the chemical properties of atoms to an appreciable degree.

Next the fate of phosphateions after entering the body is dealt with. By far the largest part is taken up by the skeleton, but several percents are already secreted during the first

week. Several investigations about the growth of bones are reviewed in this connection. The rate of activation of the phosphorus atoms proves to have very divergent values in different bones. The reasons for this fact are mentioned.

The various groups of phosphorus compounds occurring in organs and in blood are enumerated. In a number of cases the rate of formation of these substances has been measured by radioactive methods. Phosphatides have received special attention. Reasons are given for the assumption that the phosphatides in the blood have not been formed in this liquid but that they originate from certain organs (probably the liver.).

The results obtained by Hahn and Hevesy in their work on the activity of lecithin in eggs had already made it seem very probable that the lecithin found in eggs has been built up in the liver. Their work was continued by the author of the present publication. The conclusions, reached in the investigations described in this dissertation, are found at the end of this chapter.

In this chapter results are mentioned of two experiments which have not yet been described extensively elsewhere; i.e. a determination of the rate of perfusion of bones (p. 16) and a measurement of the rate of breakdown of hexose phosphate in urine, which was found to be very slow. (p. 37).

Chapter III starts by describing how solutions of radioactive sodium phosphate for physiological use are prepared from red phosphorus. Next the making of samples for the activity determination with a Geiger counter is treated. Then the method used for the determination of the phosphorus content of various fractions is dealt with. For this purpose a colorimetric method was used.

The separation of erythrocytes from plasma is described. It is told how the different phosphorus compounds in blood

plasma, blood corpuscles, milk, organs and yolks were isolated and the further purification of casein is described.

The great danger of radioactive contamination is pointed out, which exists if one is working with other — very strong — preparations at the same time.

In a separate paragraph the technique of the isolation of water from milk and blood is described, which is used in the study of the secretion of heavy water in milk. In this connection experiments are treated which deal with the occurrence in milk of heavy fat administered per os. The apparatus is described which serves to burn fat samples.

Chapter IV shows a collection of tables containing the results of the author's measurements.

First the accurracy of the values obtained is discussed. Then the results are given of experiments with radioactive sodium phosphate on a hen and with radioactive sodium phosphate, radioactive hexose phosphate, heavy water and heavy fat on goats. It is also described how hexose phosphate and heavy fat were administered to the animals.

Chapter V brings the conclusions which can be reached from the data mentioned in the preceding chapters.

Concerning the formation of eggs, the assumption of HEVESY and HAHN that the phosphatides are carried to the growing yolks by the blood is supported by proving the absence of a phosphatide synthesis in the ovary. It is shown that the rate of activation of the phosphatides is a measure for the rate of growth of the yolks in the ovary.

In the experiments on goats the first conclusion is that the decrease in the activity per mgr. of organic phosphorus in the plasma cannot be represented as a single first-order reaction. At least two processes, with greatly different velocities, play a rôle.

The investigation of the activity of different milk fractions proved that no mixing occurs in the milk while it is stored in the udder. Besides it was found that a few hours after the start of the experiment the specific activity of the phosphorus in the casein and in the acid-soluble organic phosphorus compounds is but slightly lower than that in the inorganic phosphate in the milk. This makes it seem very probable that these substances are formed in the milk gland from inorganic phosphate. That phosphatides are formed in the milk gland too was demonstrated by special experiments.

The time required for a water molecule to pass from the blood into the milk was found to be one half hour or less; for phosphate ions this period amounts to a few hours. A fat molecule, given per os, needs about a day before it is secreted by the milk gland.

AUTHOR INDEX

STELLINGEN

I.

Het schema voor de afleiding van detonatie-snelheden volgens Bernard Lewis is niet aanvaardbaar.

J.A.C.S. *52*, 3120 (1930)

II.

De photographische methode van Dols, Jansen, Sizoo en van der Maas voor de vergelijking van de radioactiviteit van verschillende gedeelten van beenderen behoeft verbetering.

Nature, *142*, 953 (1938).

III.

De bruikbaarheid van de door Wefelmeier voorgestelde kernmodellen is niet beperkt tot kernen, die uit α-deeltjes zijn opgebouwd.

Z.f.Physik, *107*, 332 (1937).

IV.

De waarde der door Flügge en von Droste voorgestelde verklaring van het voorkomen van twee beperkte groepen van atoomgewichten onder de brokstukken, die ontstaan bij het splitsen van uraankernen, is twijfelachtig.

Z.f. Physik. Chemie B, *42*, 274 (1939).

V.

De meening van Hammet, dat zijn metingen der overspanning aan werkende waterstof-electroden een argument vormen voor de geldigheid der theorie van Tafel bij lage stroomdichtheden, is onjuist.

J.A.C.S. *46*, 7 (1924).

VI.

De berekening der energie van een „gat" in een ionen-kristal door Jost verdient weinig vertrouwen.

Trans.Far.Soc. *34*, 860 (1938).

VII.

De door Antweiler gegeven verklaring van het polarografisch maximum is te verkiezen boven die van Heyrovski.

Z.f Elektrochem. *44*, 719 (1938).

VIII.

Bij chromatografische analyses zullen somtijds radioactieve indicatoren van nut kunnen zijn.

IX.

Bij het bepalen van omzettingssnelheden van zouten zal men in sommige gevallen met voordeel gebruik kunnen maken van metingen van oploswarmten.

X.

Metingen van racemisatie-snelheden kunnen belangrijke aanwijzingen geven over het voorkomen van vrije radicalen in vloeistoffen en gassen.