The Application of Radioactive Indicators in Biochemistry.

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By PROF. G. C. HEVESY, FOR. MEM. R. S.

IT is with a feeling of deep reverence that I am delivering this lecture in commemoration of the great genius and pioneer Michael Faraday.

Throughout the century which saw the birth of Dalton's atomic theory and witnessed Faraday's fundamental discoveries, each chemical element was considered to be built up of one kind of immutable atoms. Radioactivity has completely changed this conception. As shown by Rutherford and his school, we are witnessing here the decay and birth of new atomic species, and it was soon recognized that some of these, seen from the chemist's view-point, were just new editions of well-known chemical elements. To quote a few examples, it became clear that radio-thorium was chemically practically identical with thorium, and radium-D or thorium-B with lead.

The discovery of this extraordinary chemical similarity was due to Soddy, Boltwood, and a few other great chemists, and the new phenomenon was termed " isotopy " by Soddy. Amongst the non-radioactive elements the existence of isotopes was first revealed in the case of neon by Sir J. J. Thomson's study of positive rays, and then much extended by Aston by means of his mass spectrograph. Owing to these discoveries the realm of classical chemistry was enlarged by ever-growing territories the ultimate size of which we cannot foresee. I do not venture to give even a very condensed survey of these fundamental and far-reaching advances, but shall confine myself to the discussion of the application of radioactive isotopes as indicators in biochemical studies, made possible by the above-mentioned great progress.

Radioactive isotopes were first used in the field of inorganic chemistry. The first application of this type was made at the Vienna Institute for Radium Research early in 1913 by my friend Professor Paneth, now Director of the Londonderry Laboratory of Radiochemistry in Durham, and myself. In the years to follow radioactive isotopes were applied by us, and by a small number of other workers, in various studies in the field of inorganic chemistry, and these researches proceeded at a much enhanced rate after the discovery of artificial radioactivity and the construction of the cyclotron and the uranium pile which enlarged immensely the number of radioactive isotopes available as indicators. However, radioactive tracers found their most important application in the field of biochemistry, physiology, and pathology. The intricate chemical processes taking place in the living organism, the numerous competing routes which atoms, molecules, and larger building stones follow in the plant and animal organism, open a most fertile field for the application of radioactive isotopes.

By making use of radioactive indicators we can label atoms (ions), molecules, and even larger units such as erythrocytes, leucocytes, bacteria, and viruses; subsequently, their path and fate in the living organism can be followed. I want to consider some examples of each of these three main types of body constituents, starting with a discussion of the application of radio-sodium in the study of the distribution and circulation of sodium ions.

Application of Radio-sodium in Distribution Studies.

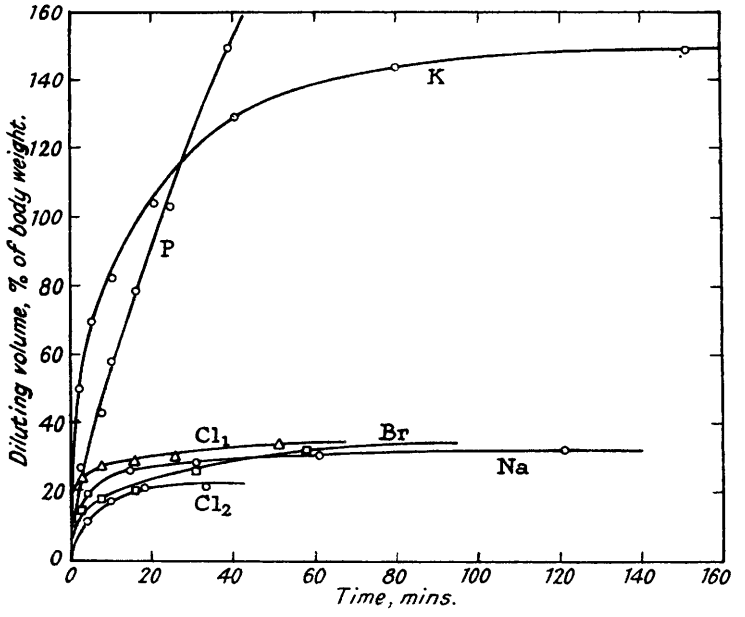
We injected sodium chloride containing some radio-sodium (^{24}Na, half-life = 14 hours) into the circulation of the rabbit. Since the sodium content of the plasma amounts to about 2 g., and the labelled sodium administered amounts to only a few micrograms, the change of the sodium content of the plasma caused by injection of labelled sodium remains within the limits of physiological variations. By introducing ^{24}Na we label, however, the circulating sodium ions. Within the errors of the experiments the radio-active ^{24}Na ions behave in the same manner as do the common ^{23}Na ions. The disappearance of 1% of the injected ^{24}Na from the blood plasma thus indicates the simultaneous disappearance of 1% of all sodium ions present in the circulation at the time of injection and their replacement by sodium ions present in the extravascular space.

When we first carried out such experiments in collaboration with Dr. Hahn, we were amazed at the velocity of disappearance of the individual sodium ions present in the circulation and at the velocity of interchange between intravascular and extravascular sodium, which involves a passage of the capillary wall. After the lapse of one minute almost ⅔ of the sodium ions present at the time of injection were no longer located in the circulation. The rate of disappearance of sodium from the circulation of the rabbit—more correctly the rate of replacement of plasma sodium by extravascular sodium—is seen in Fig. 1.[1] The time following the injection of labelled sodium, the injection taking a few seconds only, is plotted against the volume of diluting fluid necessary to reduce the ^{24}Na concentration of the plasma to the value observed.

Fig. 1 demonstrates the fundamental difference, long known to the physiologist, between the behaviour of sodium, chloride, and bromide, on the one hand, and of potassium and phosphate, on the other. Sodium and chloride ions present at the start of the experiment not only disappear rapidly from the plasma, but an exchange equilibrium between the intravascular and extravascular ions is also obtained in a short time. The same is not true for potassium and phosphate.

FIG. 1.

Rate of disappearance of various labelled ions from blood plasma of the rabbit (reproduced, with permission, from Acta Physiol. Scand., 1941, **1**, 347).



Sodium, chloride, and bromide are found mainly in the body fluid circulating outside the tissue cells, whereas potassium and phosphate appear chiefly inside the tissue cells. The intrusion of potassium or phosphate into the tissue cells requires a much longer time than the passage of the capillary wall which the " extracellular " ions alone have to perform.

Flexner and his colleagues,[2] who made extensive studies on the rate of interchange of plasma and extravascular sodium, found in the guinea-pig 60% of plasma sodium to be replaced each minute by extravascular sodium. In man, the corresponding figure was found to be 78%.* Thus, in the course of each minute, about 7 g. of sodium leave the human circulation and are replaced by sodium circulating in the extravascular fluid of the various organs. This remarkable rate of interchange is made possible by the fact that 1 ml. of blood plasma in the capillaries is exposed to an area of capillary wall of 5600 sq. cm.

By making use of radio-sodium, we can also determine the volume of the extracellular fluid

* These experiments were carried out on pregnant subjects; in normal human subjects 32% is reported (Burch, Reaser, and Cronwick, *J. Lab. Clin. Med.*, 1947, **32**, 1169).

[1] Hevesy and Hahn, *Acta Physiol. Scand.*, 1941, **1**, 347.

[2] *Cold Spring Harbor Symp. Quant. Biol.*, 1948, **13**, 88.

present in the organism,[3] a problem of great interest to the physiologist. From Fig. 1 it is seen that the extracellular volume indicated by the distribution of the injected sodium, the " sodium space," constitutes 23% of the rabbit's weight. By comparing the radio-sodium content of 1 g. of plasma water and 1 g. of fresh tissue, we find in a similar way the values for the sodium space of each organ. We can check these results by injecting into the rabbit a sodium chloride solution containing radio-chloride as an indicator.[4] In both cases very similar values are obtained for the extracellular fluid volume of most organs. The " sodium space " of the skeleton, however, was found to be more than twice as large as its " chloride space," as is seen from Table I. From this result it follows that an appreciable part of the skeleton sodium is not circulating in the cellular interspaces, but is present as an intracellular constituent of the bone tissue, possibly replacing some bone calcium in the apatite-like mineral constituents of the skeleton.

TABLE I.

Percentage extracellular water content of organs of the rabbit, calculated from the radio-sodium : radio-chloride ratio of 1 *g. of fresh tissue and* 1 *g. of plasma water (" sodium space " and " chloride space").*

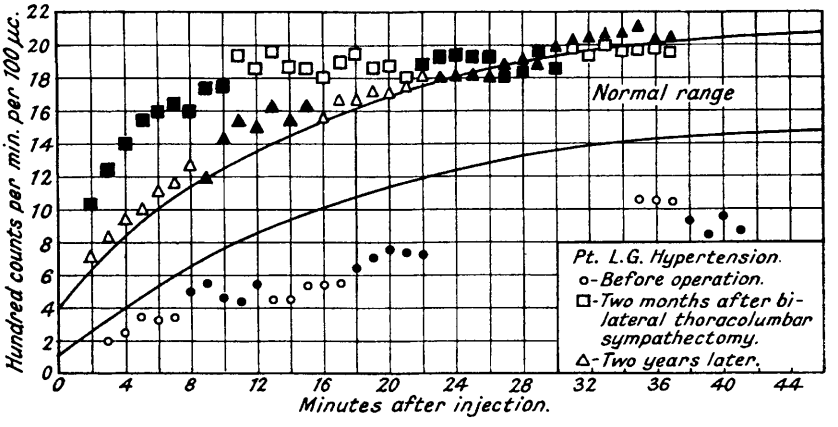
Organ.	Sodium space.	Chloride space.
Liver	22	25
Gastrocnemius muscle	10	9·4
Brain	10	11
Bone femur (dog)	66	24
Bone radius tibia (dog)	66	20
Bone humerus	63	18

Application of Sodium in Circulation Studies.

Radio-sodium can successfully be applied in the study of the circulation velocity of the body fluids. Although, in experiments described earlier, blood-plasma samples were taken at various intervals and their activites were compared with the activity of a known aliquot of the injected

FIG. 2.

Radio-sodium " build-up " curves in a patient with severe hypertension relieved by thoracolumbar sympathectomy (Quimby) (reproduced, with permission, from Nucleonics, 1947, Dec., p. 1).



sodium chloride, yet in circulation studies it may be desirable to measure outside the body the rate of propagation of the activity injected. As radio-sodium emits not only β-rays, but also penetrating γ-rays, we can very conveniently measure the time which the injected sodium takes to reach the foot by injecting radio-sodium into the arm vein and placing a Geiger counter on the foot. The average value for the circulation time is found to be 45 seconds, the values varying between 15 and 90 seconds.

We may also be interested in determining after the injection of radio-sodium into the

[3] Griffiths and Maegraith, *Nature*, 1939, **143**, 159; Kaltreiter *et al.*, *J. Exp. Med.*, 1941, **74**, 569.
[4] Manery and Bale, *Amer. J. Physiol.*, 1939, **126**, 578.

circulation the time necessary to establish equilibrium between the radio-sodium content of the plasma and that of the extracellular fluid. This time, although only a few minutes in the rabbit or guinea-pig, is appreciably longer in human subjects; it is very different in normal subjects from that in patients suffering from peripheral vascular diseases. Such determinations may thus have diagnostic value.

Quimby [5] injected radio-sodium into an anticubital vein and, by holding the window of a portable shielded Geiger counter against the sole of the foot, measured the arm-to-foot circulation As long as a uniform distribution of the injected radio-sodium in the extracellular fluid of the body is not reached, the activity of the foot increases. In individuals with no vascular disturbance this stage is reached after the lapse of 45 minutes. Curves indicating the increase in the activity of the foot with time both in the normal and in the diseased organism are shown in Fig. 2.

For patients with various vascular disorders the curves may be within, above, or below the normal region. In the curves, solid symbols represent counts against the right foot, open ones against the left foot. The chart shows data from a patient suffering from severe hypertension relieved by bilateral thoracolumbar sympathectomy. The lowest curve (circles) represents a test made before the operation. The two upper curves (squares and triangles) show the result of tests made two months and two years, respectively, after operation. They demonstrate the far-reaching change in the circulation of sodium ions produced by the operation.

Information on the effectiveness of blood flow can be obtained by measuring the rapidity of disappearance of radio-sodium following intramuscular injection of labelled sodium chloride.[6] Radio-sodium therefore proves to be an important tool in the study of circulation of body fluids, a process of basic importance.

Further Applications of Radio-sodium.

The application of radio-sodium as an indicator opens a convenient way to determine the permeability of phase boundaries of different type present in the animal and plant organisms; [7] nevertheless, it has its limitations.[8] Radio-sodium being used as a tracer, the rate was measured at which sodium passes through the placenta from the mother to the offspring, passes from the circulation into the cerebrospinal fluid, penetrates into the aqueous tumour or through the stomach wall, intrudes into the erythrocytes,[11, 12] is distributed between cytoplasm and nucleus,[9] and so on.

Time does not permit me to describe the interesting experiments of Levi and Ussing,[10] which prove that the two sides of the frog's skin have a distinctly different permeability to sodium. In these experiments the Ringer solution facing the inner surface of the excised frog skin was labelled by addition of the short-lived ^{24}Na, while the long-lived ^{22}Na was added to the Ringer solution facing the outer skin surface.

Although the terrestrial and meteoric abundance of sodium is somewhat greater than that of potassium, yet in the living organism with very few exceptions potassium is by far the more abundant element. Mammalia, for example, contain about 3 times as much potassium as sodium. As already mentioned, potassium is located mainly in the tissue cells, sodium in the extracellular body fluid. Before the application of radioactive indicators, the great difference between the sodium content of the muscle cells and the surrounding fluid, or between the red corpuscles and the surrounding plasma, for example, was interpreted as a consequence of the impermeability of the phase boundaries to sodium. The application of radio-sodium in permeability studies revealed, however, that sodium penetrates very easily from the blood plasma into the red corpuscles, and *vice versa*,[11, 12] and the same applies also to potassium.

In spite of the marked permeability of the red corpuscles to sodium and potassium, the latter element is strongly (about 30 times) concentrated in the red corpuscles, while sodium is about 10 times more abundant in the plasma than in the (human) erythrocytes. Such a difference cannot be explained without assuming a distinct difference in the chemical affinity of sodium and potassium to some type of organic cellular constituents, a difference which makes potassium a

[5] *Nucleonics*, 1947, Dec., p. 1.
[6] Elkin, Cooper, Rohrer, Miller, Shea, and Dennis, *Surg. Gynecol. Obstet.*, 1948, **87**, 1.
[7] Cf. Hevesy, " Radioactive Indicators," Interscience Publishers, New York, 1948.
[8] Ussing, *Cold Spring Harbor Symp. Quant. Biol.*, 1948, **13**, 193.
[9] Abelson and Duryee, *Biol. Bull.*, 1949, **96**, 205.
[10] *Nature*, 1949, **164**, 928.
[11] Hahn, Hevesy, and Rebbe, *Biochem. J.*, 1939, **33**, 1549.
[12] Cohn and Cohn, *Proc. Soc. Exp. Biol. Med.*, 1939, **41**, 445.

more effective competitor for the cellular ionic content than sodium. Levi and Ussing [13] brought important arguments for the view, formerly advocated by Lundegardh [14] and by Krogh,[15] that these hypothetical organic complexes are present in the phase boundary. The red corpuscle membrane, though impermeable to sodium ions, contains scattered anions of a substance which forms a stable complex with sodium. Owing to thermal movements these complex molecules will come into contact sometimes with the outside medium and sometimes with the inside medium. If the inside solution contains ^{24}Na ions, these may exchange with ^{23}Na in some of the complex molecules, and when these molecules later touch the outside solution, ^{24}Na will leave the complex in exchange.

The application of radioactive indicators thus revealed the existence of a new type of permeability which may be denoted as interchange permeability. It exhibits some analogy to the phenomenon of self-diffusion. The diffusion process leads usually to changes in the concentration of the diffusing substrate: so does permeability. Self-diffusion leads to a molecular interchange only without any change of concentration. The same applies to interchange permeability. The individual sodium ions are soon found in the corpuscles and soon in the plasma without disturbing the prevailing concentration differences between the two phases.

Although by far most of the sodium of the organism is found in the circulating body fluid, yet it is not only a main constituent of that fluid but fulfils many important tasks besides this. This is obvious, *inter alia*, from the recent results obtained by Buchanan and his colleagues [16] in their investigations of glycogen formation in which radio-carbon was applied as indicator. When liver slices were incubated in the presence of labelled pyruvate, formation of labelled glycogen and other labelled carbohydrates was found to take place. In these experiments the addition of sodium, but not however of potassium, to the incubating medium was found to stimulate 2- and 3-fold the formation of carbohydrates other than glycogen, while addition of potassium promoted the formation of glycogen.

Labelling of Molecular Body Constituents.

Rate of Molecular Renewal.

In the study of the fate of molecular constituents, such as glycerophosphate, nucleic acid, or hæmoglobin, in the organism we frequently do not introduce into the body labelled molecules, but marked precursors of these compounds. Introduction of glycerophosphate containing radio-phosphorus into the circulation, for example, will soon be followed by an enzymatic decomposition of that compound, a small percentage only of the glycerophosphate administered reaching the liver and other organs. It is not the determination of this percentage we are mostly interested in; we wish to know primarily the rate and the main place of formation of glycerophosphate. This information we can obtain by introducing labelled inorganic phosphate, some of which rapidly reaches the liver and is incorporated into newly formed glycerophosphate molecules. By comparing the activity of 1 mg. of liver inorganic P with that of 1 mg. of liver glycerophosphate P, we arrive at a figure characterizing the rate of formation or, as the glycerophosphate content of the liver remains constant during the experiment, the rate of renewal of glycerophosphate molecules or, more correctly, for reasons stated below, the lower limit of that rate. If after the lapse of two hours 1 mg. of glycerophosphate P has an activity corresponding to $\frac{1}{10}$ of the activity of the inorganic P, we may conclude that $\frac{1}{10}$ of the glycerophosphate molecules present in the liver was renewed during two hours.[17] When carrying out such calculations we must taken into account the change with time in the activity of 1 mg. of inorganic phosphate, usually denoted as its " specific activity." The specific activity of glycerophosphate phosphorus, for example, is the percentage of administered ^{32}P present in 1 mg. of that phosphorus. It is the specific activity of the glycerophosphate P measured at the end of the experiment which has to be compared with the mean value of the specific activity of the inorganic P during the experiment. In an experiment lasting two hours the end value and the mean value of the specific activity of the inorganic P in the liver of the rat happen to be about identical. A formula which enables us to calculate for any organ and at any time the rate of turnover from data indicating the change with time of the specific activity of both the precursor and the compound considered is given by Zilversmit and his associates.[18]

[13] *Acta Physiol. Scand.*, 1948, **16**, 232.
[14] *Protoplasma*, 1941, **35**, 548.
[15] *Proc. Roy. Soc.*, 1946, *B*, **133**, 140
[16] Buchanan, Hastings, and Neslett, *J. Biol. Chem.*, 1949, **180**, 435, 447.
[17] Hevesy and Hahn, *Kgl. Danske Videnskab. Selsk. Biol. Medd.*, 1940, **15**, Nr. 5.
[18] Zilversmit, Entenmann, and Fishler, *J. Gen. Physiol.*, 1943, **26**, 325.

The greatest difficulty we encounter when determining the renewal rates of molecular body constituents is often the lack of knowledge of the pertinent precursor of the compound. We can probably assume inorganic P to be the pertinent precursor of glycerophosphate P. In the case of lecithin, however, the labelled inorganic P may, before its incorporation into the lecithin molecules, have to be converted into glycerophosphate P or another intermediary compound. If the formation of glycerophosphate takes an appreciable time, in the first phase of the experiment, then after administration of labelled inorganic phosphate, the newly formed lecithin molecules do not become labelled as they were synthesized with the participation of yet inactive glycerophosphate molecules. When calculating the renewal rate of glycerophosphate by comparing the specific activities of the liver inorganic P and liver lecithin P we thus underestimate the turnover rate of lecithin. This would not be the case if we compared the specific activities of the glycerophosphate P and the lecithin P of the liver.

In the liver of the dog, for example, the half-life of the lecithin molecules was calculated, inorganic P being assumed to be the pertinent precursor, to be 12·5 hours, while with the assumption that glycerophosphate P is incorporated into the lecithin molecule, the much lower value of 3·6 hours was obtained.[19] These figures indicate the half-life of the average lecithin molecule of the liver. When, however, investigating the renewal rate of lecithin present in different cellular fractions of the liver, pronounced differences are found. As seen in Table II, the rate of renewal of the phosphatide molecules present in the cell nuclei, and to a minor extent also those present in the mitochondria, is markedly lower than the corresponding figure for the average tissue phosphatides. The figures were obtained two hours after injection of labelled phosphate.[20]

TABLE II.

Renewal rate of phosphatide P *of the liver fractions of the rat.*

Phosphatide fraction.	Activity of 1 mg. of phosphatide P in percentage of the activity of 1 mg. of :		
	Plasma phosphate.	Liver phosphate.	Liver glycerophosphate.
Total tissue	13·4	12·8	18·8
Mitochondria	9·4	9·0	13·1
Cell nuclei	5·6	5·4	7·9

Ample evidence is available to support the assumption that the incorporation of labelled phosphate into phosphatide or other organic phosphorus compounds involves enzymic processes, no " physical " interchange taking place. In the study of phosphatides in surviving liver slices incubated in a Ringer solution containing labelled phosphate, for example, the formation of " marked " phosphatides takes place under aerobic conditions only.[21] Absence of oxygen or presence of respiratory inhibitors should clearly not interfere with a non-enzymic type of interchange.

It was the introduction of the method of isotopic indicators which has drawn the attention to the notion of the lifetime of the molecules building up the organism, a magnitude formerly not considered. The first lifetime determination, performed almost immediately after the discovery of deuterium, was that of the water molecules of the goldfish.[22] The water molecules present in the *living* goldfish were found to interchange rapidly with those of the surrounding water, and some of the hydrogen atoms (the " labile " hydrogen) of the organic tissue components were found to be replaced at a remarkable rate by the hydrogen atoms of the body water.

Shortly afterwards, the lifetime of the water molecules present in the human body was measured.[22a] Dilute heavy water being used as an indicator, following the intake of a known volume of dilute (2%) heavy water, the heavy-water content of the urine was determined; from these and other excretion figures the average lifetime of water molecules in a test person was found to be 14 days. This value clearly depends not only on the total water content but also on the water intake of the subject.

In the same investigation the total water content was calculated from the heavy-water content of the water drunk and that of the body water after exchange equilibrium between the water molecules taken in and those already present in the organism was reached. The heavy-water content of the body water was obtained by determining the heavy-water content of urine

[19] Zilversmit, Entenmann, and Chaikoff, *J. Biol. Chem.*, 1948, **176**, 193; Popják and Muir, *Biochem. J.*, 1950, **46**, 103.
[20] de Elliott and Hevesy, *Acta Physiol. Scand.*, 1950, **19**, 370; cf. Ada, *Biochem. J.*, 1949, **45**, 422.
[21] Taurog, Chaikoff, and Perlman, *J. Biol. Chem.*, 1942, **145**, 281.
[22] Hevesy and Hofer, *Z. physiol. Chem.*, 1933, **225**, 28.
[22a] *Idem, Nature*, 1934, **134**, 879.

water. The total water content of the test person was found to form 64% of the body weight. For abnormally adipose individuals figures as low as 40%, for lean ones as high as 70%, have recently been obtained.[23]

The interest of the biochemist in the lifetime of the molecules building up the organism was soon much enhanced by the classical work of Schoenheimer and Rittenberg,[24] who determined first the lifetime of fat molecules and later many other types of molecules in the animal body and, moreover, by the investigation of radio-phosphorus in similar investigations, the first of which was the renewal rate of the mineral constituents of the bone.[25]

Place of Formation of Molecular Constituents.

Having discussed the rate of renewal of phosphatide molecules, I want now to say a few words about the place of formation of phosphatide molecules in the yolk of the hen's egg and in the blood plasma. The determination of the place of formation of body constituents is another important field of application for isotopic indicators.

Only a few hours after administration of labelled phosphate to the hen, the presence of radioactive lecithin and other phosphatides can be detected in the yolks of the ovary. These phosphatide molecules may have been synthesized in the yolk or carried into the yolk by the circulating plasma, an alternative explanation being the incorporation into the yolk of phosphatide molecules built up in the ovary. Now, it can be shown by experiments *in vitro* that penetration of labelled phosphates into the egg followed by intrusion into the yolk does not lead to the formation of labelled phosphatide molecules.[26]

In view of the fact that the specific activity of the phosphatides of the ovary is lower than that of the yolk phosphatides, as seen in Table III, the former cannot be the source of the latter.[26]

TABLE III.

Specific activity of phosphatide P *in organs of the hen.*

Organ.	Relative specific activity.
Liver	100
Plasma	79
Ovary	7·2
Yolk	9·2
Intestinal mucosa	18

The specific activity of the precursor of a product obviously cannot be lower than that of the product itself, at least at that phase of the experiment in which the specific activity of the precursor increases with time. Thus we have to conclude that the yolk phosphatides originate from the plasma phosphatides. The source of the plasma phosphatides, or at least their main source, must be the liver phosphatides as alone the liver phosphatides have a higher specific activity than the plasma phosphatides. Even the specific activity of the phosphatides of the intestinal mucosa in which phosphatides are turned over at a remarkable rate as seen in Table III is lower than that of the plasma phosphatides.

While the plasma phosphatides contain mainly lecithin, appreciable amounts of cephalin are also present in the liver. In view of the small differences in the turnover rate of lecithin and cephalin in the liver, the difference in the composition of liver and plasma phosphatides does not influence the above conclusion.

The most direct approach to the solution of the problem of the origin of plasma phosphatides is a comparison of the specific activity of plasma phosphatides in the intact and in the hepatectomized animal. In their experiments Fishler *et al.*[27] found that, after the administration of labelled phosphate in contrast to the phosphatides of the plasma of the intact dog, the plasma phosphatides of the liver-less dog contained only a negligible amount of ^{32}P. As in both cases the same amount of labelled phosphate was administered to the dog, this result fully confirms the above conclusion that the liver is almost the sole place of formation of plasma phosphatides.[28]

[23] Drabkin, *Fed. Proc.*, 1950, **9**, 182.
[24] *J. Biol. Chem.*, 1935, **111**, 175; Schoenheimer, "The Dynamic State of Body Constituents," Cambridge, Mass., 1942.
[25] Chievitz and Hevesy, *Nature*, 1935, **136**, 753.
[26] Hevesy and Hahn, *Kgl. Danske Videnskab. Selsk. Biol. Medd.*, 1938, **14**, Nr. 2.
[27] Fishler, Entenmann, Montgomery, and Chaikoff, *J. Biol. Chem.*, 1943, **150**, 47; Entenman, Chaikoff, and Zilversmit, *ibid.*, 1946, **166**, 15.
[28] Cf. also Popják and Beeckmans, *Biochem. J.*, 1950, **46**, 99.

The above-mentioned lack of extra-ovarian formation of labelled phosphatide in the yolk is also brought out in experiments with incubated fertilized eggs into which labelled phosphate was injected.[29] While the fœtal phosphatides present in such eggs have a high ^{32}P content, the remainders of the yolk still present in such eggs do not contain significant amounts of labelled phosphatides, as seen in Table IV. The slight activity of yolk phosphatides, which increases with age of the embryo, is possibly caused by influx from the embryo into the yolk of a small amount of labelled phosphatides or of the enzymes responsible for resynthesis of phosphatides.

TABLE IV.

Specific activity of phosphatides extracted from embryo and residual yolk of the hen's egg.

Time of incubation days.	Phosphatides extracted.	Specific activity.
6	Yolk	0·032
	Embryo	100
11	Yolk	0·10
	Embryo	100
18	Yolk	0·92
	Embryo	100

Similar considerations were applied by Aten [30] to the study of the origin of the milk phosphatides in the goat. Table V demonstrates that at least most of the phosphatide molecules of the milk are not those which passed from the blood plasma into the milk, but those which had their origin in the milk gland, the phosphatides of the milk being much more active than those of the plasma, but less active than those of the milk gland.

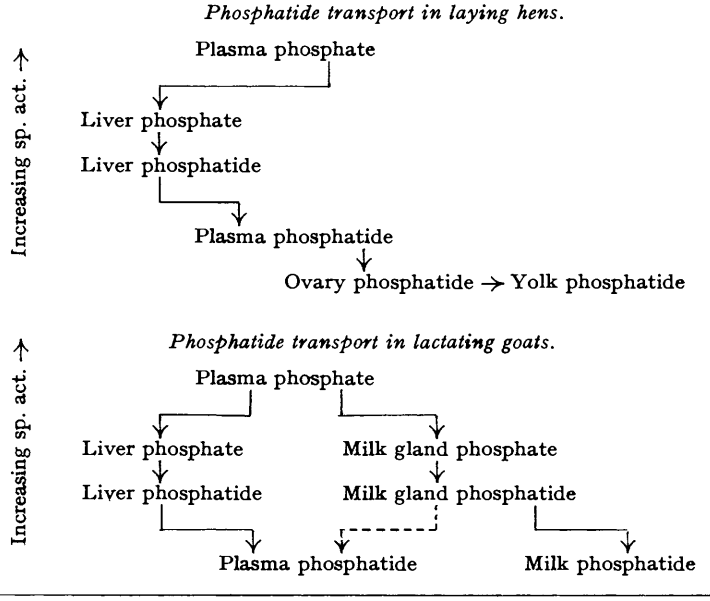
TABLE V.

Activity of phosphatide phosphorus of milk and organs of a goat.[30]

Organ.	Specific activity.	Organ.	Specific activity.
Milk	1	Liver	1
Plasma	0·02	Kidneys	1·2
Milk gland	1·4		

The following scheme represents the course of phosphatide transport in laying hens and in lactating goats.[30]

Scheme for the specific activity of phosphatides.[30]



[29] Hevesy, Levi, and Rebbe, *ibid.*, 1938, **32**, 2147.

[30] Aten, "Isotopes and Formation of Milk and Egg," 1939, Diss., Utrecht; Aten and Hevesy, *Nature*, 1938, **142**, 111.

Effect of Irradiation with X-Rays on the Formation of Nucleic Acids.

The application of radioactive indicators proved to be an efficient tool for the measurement of the change in the rate of formation of molecular body constituents caused by administration of chemicals inducing such interference or by irradiation with ionizing radiation which produces such interfering chemicals *in situ*. Such radiation may diminish the growth rate, and we can expect that it might interfere with the rate of formation of some cellular constituents already in the early or earliest phase of the experiment and with that of deoxyribonucleic acid, a main constituent of the cell nucleus.

In a growing tumour, growth, and correspondingly, additional formation of deoxyribonucleic acid, may amount to about 1% in the course of 2 hours. If this formation is supressed by irradiation, the irradiated tumour after the lapse of 2 hours can be expected to contain 1% less deoxyribonucleic acid than do the controls. The distinction between 100 and 99 deoxyribonucleic acid molecules present in a tumour by means of chemical analyses is a most difficult task. However, when applying radioactive indicators such a difference can easily be measured. Radioactive indicators permit us to distinguish between old and new molecules, between those present before the start of the experiment and those formed afterwards. The old molecules are not radioactive, but the new ones contain ^{32}P.

Let us assume that out of 100 deoxyribonucleic acid molecules present in the non-irradiated tumour two are found to contain ^{32}P two hours after administration of labelled phosphate while in the irradiated tumour, in which deoxyribonucleic acid formation is suppressed, the corresponding figure is only 1. Such a difference is readily ascertained. In the first case, the Geiger counter or another suitable measuring instrument will indicate an activity of, say, 200 counts per minute, in the second case only 100. Radioactive measurements can very easily be made, a fact which contributes materially to the wide application of the method of radioactive indicators.

In Table VI the effect of irradiation with *X*-ray doses ranging from 335 to 1500 r. on the Jensen sarcoma of the rat is shown.[30a] A few minutes after the irradiation, labelled sodium phosphate was injected into 28 rats and into the same number of controls. The effect of irradiation on the formation of deoxyribonucleic acid molecules is seen to be most effective shortly after irradiation.

TABLE VI.

Effect of X-rays on the formation of labelled deoxyribonucleic acid after administration of labelled sodium phosphate.

Dose, in r. units.	Duration of experiment, hours.	Ratio of labelled nucleic acid formation in non-irradiated and irradiated sarcoma.
750—1500	½	3·2
350—1500	1	2·4
650—1500	2	2·2

In another set of experiments [31] with 32 rats the mean ratio of the labelled nucleic acid formation in the non-irradiated and irradiated rats after the lapse of 2 hours was found to be 2·05, and in a third set [32] (40 animals) 2·2. Similar results were obtained by Barbara Holmes,[33] who, injecting labelled phosphate into 14 rats, found a mean value of 2·1 for the above ratio ½—2 hours after irradiation with 2·000 r.

In organs such as liver, kidney, spleen, and intestinal mucosa, the formation of deoxyribonucleic acid formation was found to be reduced to a similar extent to that in the tumour [34] under the effect of ionizing radiation. Hardin Jones's results [35] in this field are instructive. He compared the depressing effect of whole-body radiation on blood-cell counts with the reduction in the turnover rate of formation of labelled deoxyribonucleic acid in tumour and liver. Some of his results are seen in Table VII. They show similar figures for percentage depression of blood corpuscles, which is at least partly caused by inhibition of deoxyribonucleic acid formation in the bone marrow, and deoxyribonucleic acid formation in the tumour and liver.

[30a] Von Euler and Hevesy, *Arkiv Kemi*, 1944, **17**, *A*, Nr. 30; Hevesy, *Rev. Mod. Physics*, 1950, **17**, 102.
[31] Von Euler and Hevesy, *Kgl. Danske Videnskab. Selsk. Biol. Medd.*, 1942, **17**, Nr. 8.
[32] Ahlström, v. Euler, and Hevesy, *Arkiv Kemi*, 1945, **19**, *A*, Nr. 13.
[33] *Brit. J. Rad.*, 1947, **20**, 45; 1949, **22**, 487.
[34] Ahlström, v. Euler, and Hevesy, *Arkiv Kemi*, 1944, **19**, *A*, Nr. 9.
[35] Proc. Oberlin College Radiation Biology Meeting, in the press.

TABLE VII.

Depressing effect of irradiation with a dose of 1 *r. of* X-*rays* (Hardin Jones).

System investigated.	Percentage depression due to irradiation.
White corpuscle count or lymphocyte count	0·23
Red corpuscle count	0·3
Formation of labelled deoxyribonucleic acid in tumour ...	0·18
Formation of labelled deoxyribonucleic acid in liver	0·28

Indirect Radiation Effects.

The work of Dale [36] and others on the effect of *X*-rays on enzyme suspensions led to the result that part of the effect of irradiation on enzymes is caused by the production of noxious agents in the solvent. Inactivation of the enzymes is to a large extent caused by the interaction of these noxious agents produced in the water with the enzyme. Dale found that with a given dose inactivation is obtained of a considerably smaller fraction of a concentrated than of a dilute carboxy-peptidase solution. This observation suggests the explanation, and the only feasible one, that the number of inactivating atoms or molecules produced under the effect of weak doses of *X*-rays in the solvent suffices only to inactivate a restricted number of enzyme particles. The primary process in the action of radiation on water is detachment of an electron and its subsequent transfer from the ion to one of the neighbouring molecules or ions.[37] For pure water the primary process is $(HO)^-H^+ +$ radiation $\rightleftarrows HO + H$.

Most of these decomposition products have an exceedingly short life and may recombine to form water or react with protective molecules present before they reach the enzyme surface. Besides the existence of this indirect radiation effect due to more or less short-lived radicals, the application of labelled phosphate in radiation studies revealed the existence of another type of indirect effect [38] due to a radiation product of an appreciably long life.

In animals bearing two distant tumours, one of which was irradiated while the other was effectively shielded with lead, incorporation of ^{32}P into the deoxyribonucleic acid of not only the irradiated but also the shielded sarcoma was observed. Irradiation with a dose of 280—2000 r. had an effect on the deoxyribonucleic acid formation of the shielded tumour which lagged behind with only 20—40% of that observed in the irradiated sarcoma.

Kelly and Hardin Jones [39] extended these studies and found that even local irradiation of the liver of the rat with a dose of 170 r. leads to a 34% reduction of labelled deoxyribonucleic acid formation in the tumour, while local irradiation of the muscles with 230 r. reduces the labelled deoxyribonucleic acid formation in the liver by 26%.

On introduction of the blood of a strongly irradiated rabbit into the circulation of another rabbit, the rate of incorporation of ^{32}P into deoxyribonucleic acid of the kidneys of the second rabbit was found to be reduced, an observation which suggests the presence of a substance in the circulation of the irradiated rabbit which has a blocking effect on nucleic acid formation.[40] In view of more recent results obtained when using ^{14}C as an indicator in radiation studies, which I shall discuss later, such indirect effects appear less puzzling now than at the time of their observation.

The availability of the long-lived (half-life = 5700 years) ^{14}C for tracer research was an event of great importance. It immensely increased the stimulus already provided by the applicability of the short-lived ^{11}C (half-life = 20 minutes) and the stable ^{13}C. To mention only a single example, it elucidated to a remarkable extent many steps involved in glycogen synthesis. ^{14}C was also applied in the study of the biochemical effects of *X*-rays. Into a great number of rapidly growing rats, ^{14}C incorporated with the carboxyl group of sodium acetate was injected. Before the injection, half of the rats were irradiated with a dose of 900 r. The silver purines of deoxyribonucleic acid isolated from some of the organs were then obtained, and their radioactivity was compared.[41] On irradiating new-born mice with about 900 r., the incorporation of ^{14}C into the purines of the deoxyribonucleic acid was found to be depressed to an extent similar to that of ^{32}P incorporation into deoxyribonucleic acid in the above-mentioned

[36] *Biochem. J.*, 1940, **34**, 1367.
[37] Weiss, *Nature*, 1944, **53**, 748.
[38] Ahlström, v. Euler, and Hevesy, *Arkiv Kemi*, 1945, **19**, *A*, Nr. 13; Holmes, *Brit. J. Radiol.*, 1949, **22**, 487.
[39] *Proc. Soc. Exp. Biol. Med.*, 1950, **74**, 493.
[40] Ahlström, v. Euler, Hevesy, and K. Zerahn, *Arkiv Kemi*, 1946, **23**, *A*, Nr. 11.
[41] Hevesy, *Nature*, 1949, **163**, 869.

experiments. Furthermore, experiments carried out in Chicago * brought out a depressing effect of irradiation on the incorporation of ^{14}C into the purines of ribonucleic acid similar to that observed in the case of the deoxyribo-compound. The incorporation of ^{32}P into ribonucleic acid [33] was found, however, to be markedly less susceptible to the effect of irradiation than its incorporation into the deoxy-compound.

Renewal of Ribonucleic Acid.

A very appreciable part of the incorporation of ^{32}P into ribonucleic acid molecules may be due to rephosphorylation of such molecules. If, in contrast to the synthesis of the total molecule the rephosphorylation process is not radiosensitive, we may find an explanation in the difference of the effect of radiation on the incorporation of ^{32}P and ^{14}C into the ribonucleic acid molecules. This explanation is very hypothetical, and I mention it mainly because it offers a welcome opportunity to emphasize the fact that isotopic tracers may indicate fundamentally different processes, such as renewal of one of the molecular constituents only or synthesis of the whole molecule from primitive precursors and, furthermore, intermediates between these two extreme cases.

Let us consider a compound such as adenosine triphosphoric acid. This molecule contains two labile, easily removed phosphate groups, and a third one incorporated into the adenylic acid moiety. In the animal organism, intrusion of the administered labelled inorganic phosphate into tissue cells is followed by a strikingly rapid interchange between the labelled phosphate ions and the labile phosphate groups of the adenosine triphosphoric acid. An interchange may even take place during the passage of the labelled inorganic phosphate through the boundary of the intra- and extra-cellular spaces, as pointed out by Sachs.[42] The adenylic acid moiety is not involved in this very rapid renewal process. The rate of incorporation of ^{32}P into the adenosine triphosphate is thus a measure of the rate of rephosphorylation only of that molecule and not of its formation from early precursors. Even in a rapidly growing mammalian organ, in which the adenosine triphosphoric acid content may increase by as much as 1% per hour, such an increase involving 1% additional formation of adenosine triphosphoric acid from early precursors, the incorporation of ^{32}P is negligible compared to incorporation of ^{32}P by rephosphorylation.

Incorporation of ^{32}P into deoxyribonucleic acid of a growing organ, *e.g.*, a tumour, is of a very different type. Even if some rephosphorylation in such molecules cannot be excluded, the incorporation of ^{32}P into deoxyribonucleic acid of a growing organ takes place mainly in the course of mitotic processes in which necessarily the whole molecule is involved. This appears from the parallelism often found between the mitotic figure and the rate of ^{32}P incorporation into deoxyribonucleic acid of the organ. In organs in which appreciable cell division takes place, such as in the bone marrow, the thymus gland, the intestinal mucosa, or the spleen, administration of labelled phosphate to the rat is promptly followed by a remarkable formation of labelled deoxyribonucleic acid molecules. This is not the case in the liver or kidney of the fully-grown animal in contrast to the corresponding organs of the newly-born rat. We shall see later, when discussing the life-cycle determination of blood corpuscles, that the deoxyribonucleic acid phosphorus of the circulating avian red corpuscle and of the mammalian white corpuscle in which mitotic processes do not occur is entirely stable during the lifetime of such particles, and that incorporation of ^{32}P into the deoxyribonucleic acid of such particles takes place only during their formation.

The Jensen sarcoma of the rat grows by about 1% per hour, and its deoxyribonucleic acid content increases correspondingly by about 1%. When calculating the growth rate from ^{32}P data, assuming inorganic ^{32}P to be the pertinent precursor of deoxyribonucleic acid ^{32}P and also the absence of all rephosphorylation of the deoxyribonucleic acid molecules, we arrive at about twice the above value.

When calculating the formation rate of deoxyribonucleic acid molecules from ^{32}P data, we assume that the pertinent precursor of deoxyribonucleic acid P is the intracellular inorganic P or labile adenosine triphosphate P, and compare the value of the specific activity of deoxyribonucleic acid P at the end of a 2-hour experiment with the mean value of the specific activity of inorganic P during the experiment. If the incorporation of phosphate into a deoxyribonucleic acid molecule was preceded by a comparatively much slower formation of a phosphate-containing precursor, for example of phosphorylated sugar, the above method of calculation would not be

* Personal communication by Dr. Guzman Barron.

[42] *Cold Spring Harbor Symp. Quant. Biol.*, 1948, **13**, 180.

justified. We should then compare the specific activity of the P of the hypothetical precursor with that of the P of deoxyribonucleic acid. Ottesen, however (cf. p. 1631), succeeded in showing that in the case of the avian red corpuscles the existence of such a precursor is incompatible with the results obtained in his life-cycle determination.

In the Jensen sarcoma the probability of the incorporation of a ^{32}P atom into a ribonucleic acid molecule was found to be 2—3 times higher than that of its incorporation into a deoxyribonucleic acid molecule.[43] As during the 2-hour experiment the percentage additional formation of ribonucleic acid in the growing tumour cannot differ much from that of deoxyribonucleic acid, the above figures indicate that out of three ^{32}P atoms at least 1—2 are incorporated into ribonucleic acid by a process which does not involve synthesis of ribonucleic acid molecules from early precursors.

I have here discussed the effect of irradiation on nucleic acid formation, as the application of radioactive indicators in the study of biological radiation effects is a good example of the usefulness of the possibility of distinguishing between " old " and " new " molecules. Since only the deoxyribonucleic acid molecules formed during the experiment, and therefore a minor percentage of those present, contain ^{32}P or ^{14}C, we can, from the fact that irradiation with ionizing radiation depresses ^{32}P or ^{14}C incorporation with such molecules, conclude that a main effect of restricted doses of irradiation as far as cell division is concerned is the obstruction of the *formation* of nucleic acid which is presumably caused by inactivation of enzymes involved in the synthesis of these compounds. A chemical analysis could only disclose changes in the deoxyribonucleic acid content, leaving the question open how far these changes are caused by enhanced degradation or obstructed formation.

In experiments in which the effect of irradiation on the incorporation of ^{14}C into the deoxyribonucleic acid was studied, acetate labelled with ^{14}C in the carboxyl group was injected into mice. In Table VIII, in addition to the effect of irradiation on ^{14}C incorporation into purine carbon of deoxyribonucleic acid, the corresponding effect on ^{14}C incorporation into protein of the liver is seen as well. Whereas incorporation into purines is markedly depressed, that into proteins is enhanced under the effect of irradiation. It is not probable that irradiation promotes protein formation. Correspondingly, the enhanced ^{14}C incorporation is interpreted as the consequence of a change in the sensitivity of the ^{14}C indicator, a change in the activity level of the protein precursor or precursors by irradiation. The activity level being higher, more ^{14}C but no more, possibly even less, protein ^{12}C is turned over. Sensitivity of the indicator plays a very important part in the application of isotopic tracers and this fact will now be considered.

TABLE VIII.

Effect of irradiation with an X*-ray dose of* 880 *r. on the incorporation of* ^{14}C *into deoxyribonucleic acid purines and into tissue proteins prepared from the livers of* 55 *new-born fed rats (mean values).*

Fraction.	Percentage change in the incorporation of ^{14}C due to the effect of irradiation.
Purines	−44
Proteins	+27

Change of Sensitivity of the Radioactive Indicator in the Course of the Experiment.

When applying isotopic indicators in the study of animal metabolism, two main lines of technique can be followed. We can keep the tracer in the body at a constant level. Deuterium for example, being used as an indicator, dilute heavy water is given to the animal throughout an experiment taking days or weeks. The body fluids soon reach a constant heavy-water content and, by comparing the deuterium content of the body water with that of fatty acids extracted from an organ, we can state what percentage of the fatty acid deuterium of that organ reached the body-water deuterium level and thus find the percentage fatty acid renewal during the experiment or, more correctly, the lower limit of that percentage. This method was much applied by Schoenheimer and Rittenberg in their classical experiments and also by Stetten, Bloch, Bernhard, and others, who obtained important information.[24]

When studying acetate metabolism, acetate labelled, for example, in the carboxyl group with ^{13}C or ^{14}C can be added to the daily food and an almost constant ^{13}C or ^{14}C content of the acetate of the body fluid will thus be obtained. As shown by Rittenberg and Bloch, and also by others,[43a] here again a comparison of the ^{13}C or ^{14}C content of the ingested acetate with that of fatty acid or cholesterol, etc., yields the lower limit of percentage renewal of these compounds.

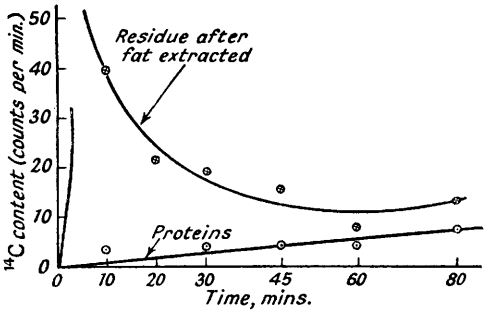
[43] Von Euler, Hevesy, and Solodkowska, *Arkiv Kemi*, 1948, **26**, *A*, Nr. 4.
[43a] Rittenberg and Bloch, *J. Biol. Chem.*, 1946, **160**, 417; Pihl, Bloch, and Anker, *ibid.*, 1950, **183**, 441.

In the work with radioactive indicators we mostly choose, however, a different technique. We administer the labelled precursor at the start of the experiment only, so the specific activity of the indicator decreases with time. On injection of labelled phosphate of negligible weight, having an activity of 1 mc., into the circulation of a human subject containing 130 mg. of plasma inorganic P this mc. will first indicate the presence of 130 mg. of inorganic phosphorus. Soon, however, as the ^{32}P of the plasma inorganic P rapidly passes the capillary wall, it interchanges with the extra-vascular inorganic ^{31}P. Owing to these and other interchanges, the ^{32}P content of the blood plasma rapidly decreases. After the lapse of 60 minutes, the ^{32}P content is reduced to about one-tenth of its original value, the presence of 130 mg. of inorganic P in the blood plasma being now indicated by an activity of 0·1 mc. only. This sensitivity of the radioactive indicator is thus increased to 10 times its initial value. A similar behaviour is shown by many other radioactive indicators.

The sensitivity of acetate ^{14}C as an indicator of body acetate increases at a remarkable rate, as seen in Fig. 3.[56] In this figure are plotted the results of experiments in which groups of mice were killed at different times after interperitoneal injection of acetate labelled in the carboxyl group with ^{14}C. The radioactivity of the fat-free brain tissue and also that of the proteins of the brain was then determined, and the figures obtained plotted against time. After only

FIG. 3.

Change with time in the ^{14}C content of brain fractions of mice after intraperitoneal injection of labelled acetate (reproduced, with permission, from Arch. internat. Pharm. Therap., 1951, **86**, 33).



30 minutes, the activity of the fat-free (acid-soluble + protein) fractions is less than half of the value observed after 10 minutes, the protein fractions showing a slow increase in their ^{14}C content with time.

The change of sensitivity of the radioactive indicator with time may be disturbing and can make the interpretation of the results obtained very difficult. On the other hand, it may also have great advantages. We shall describe some examples showing both disadvantages and advantages which result from the marked time-dependency of the sensitivity of radioactive indicators.

Renewal of the Mineral Constituents of the Skeleton.

The great inconvenience inherent in the change of the sensitivity of radioactive indicators with time becomes clear from an attempt to determine the rate of renewal of the mineral constituents of the skeleton by making use of radio-phosphorus.[44] Here we refer to the first turnover studies of body constituents with the help of a radioactive tracer, which were carried out simultaneously with the above-mentioned first investigations by Schoenheimer and Rittenberg, who used deuterium as an indicator.

Blood-plasma phosphate was found to interchange very rapidly with the uppermost phosphate layer of the apatite-like cystallites. This rapid interchange is followed by a much slower one due to a slow recrystallization of the apatite crystals. Some molecular layers go into solution while others are formed with the participation of labelled phosphate. Such layers will be comparatively strongly active in the early phase of the experiment. Owing to the decrease in the ^{32}P content of the plasma and lymph with time, they will be much less active in later phases.

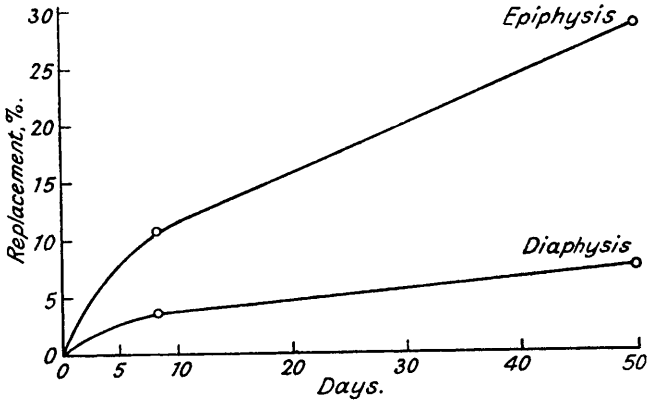
On top of a strongly active layer, slightly active layers may be deposited, protecting the first layer from dissolution. The great complexity of ^{32}P distribution in the skeleton apatite

[44] Chievitz and Hevesy, *Nature*, 1935, **136**, 754.

frustrates, or at least makes extremely difficult, a quantitative determination of the extent of renewal of the mineral constituents of the bone when labelled phosphate is administered only at the start of the experiment. We arrive at such a result when administering daily repetitions of labelled phosphate, thus keeping the level of plasma activity constant. By applying this technique, which is much less convenient, and by comparing the specific activities of the bone inorganic P and plasma inorganic P at the end of the experiment, we obtain a figure indicating the percentage of the renewed skeleton. Results obtained with rabbits, seen in Fig. 4, show that after the lapse of 50 days 74% of the soft bones (epiphysis) and as much as 93% of the hard bones (diaphysis) remained unchanged.[45] No similar experiments were carried out with human subjects but, as the extent of renewal increases only slightly with increasing time, we seem to be justified in concluding that a large part of the mineral constituents of the adult skeleton remains unchanged during life.

FIG. 4.

Extent of replacement of rabbit's bone phosphorus by labelled phosphorus (reproduced, with permission, from Biochem. J., 1940, **34**, 532).



The above-mentioned protection of highly active phosphate apatite layers by slightly active ones much resembles the protection of lead incorporated with the skeleton. From a blood plasma with comparatively high lead content, lead-replacing calcium ions are incorporated with an apatite layer. Since the lead content of the plasma decreases with time, the above-mentioned layer may be covered by others containing only negligible amounts of lead. The probability of an escape of lead from the skeleton is thus strongly reduced. It takes place, however, to some extent over long time intervals, constantly supplying the plasma with toxic lead. Not only lead but also numerous other elements, *e.g.*, uranium,[46] find more or less permanent abode in the skeleton.

Determination of the Life-cycle of Blood Corpuscles.

In the determination of the lifetime of blood corpuscles, the marked decline of the specific activity of the precursor with time proves to be most advantageous, as shown, for example, in Ottesen's work [46a] on the life-cycle of nucleated red corpuscles. After administration of labelled phosphate, the specific activity of the inorganic phosphate of the blood plasma declines, as seen in Fig. 5. It takes some time for the inorganic phosphate of the marrow to come into exchange equilibrium with the inorganic phosphate of the plasma, but after that, the specific activity of the marrow inorganic phosphate will markedly decrease with time as well. Thus, the deoxyribonucleic acid molecules formed with participation of labelled inorganic phosphate in the course of the first day are much more active than those formed later. The deoxyribonucleic acid-containing red corpuscles have a definite lifetime. As soon as this is reached they are destroyed and their nucleic acid phosphorus, which is now exposed to the effect of phosphatase, is split off and soon lost in the larger pool of inorganic phosphate present in the body. The end of the life-cycle of the red corpuscles will thus be indicated by a sudden decrease in the activity of the deoxyribonucleic acid extracted from the erythrocytes.

[45] Hevesy, Levi, and Rebbe, *Biochem. J.*, 1940, **34**, 532.
[46] Neuman and Neuman, *J. Biol. Chem.*, 1948, **174**, 711.
[46a] *Nature*, 1948, **162**, 730; Diss., Copenhagen, 1951.

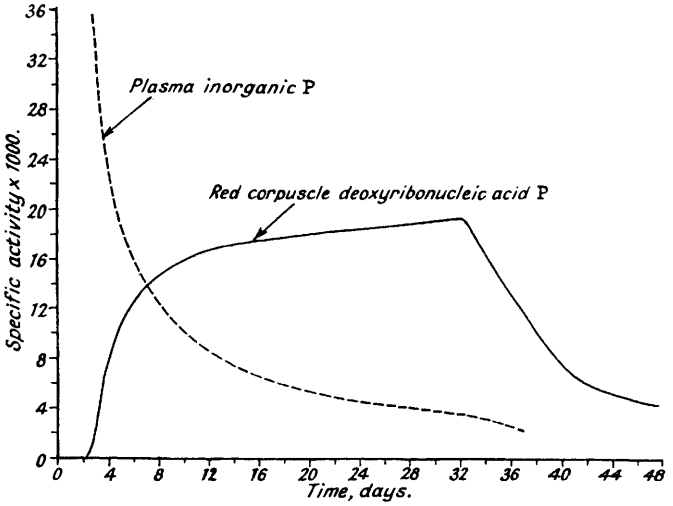
(a) *Life-time of the Avian Red Corpuscles.*—In the determination of the life-cycle of avian red corpuscles, erythrocyte samples are secured daily after injection of labelled sodium phosphate (about 15 μc.) to the hen, and the radioaticvity of their deoxyribonucleic acid P is determined. The specific activity of the plasma inorganic P is determined as well. In Fig. 5 the broken line indicates the change of the specific activity of plasma inorganic P with time, and the full line shows the corresponding values of deoxyribonucleic acid P.

The radioactivity of the deoxyribonucleic acid P of the red corpuscles formed during the first day constitutes a very large part of the radioactivity of the deoxyribonucleic acid of the total red corpuscle content of the circulation. Correspondingly, as soon as the life-cycle of the red corpuscles formed during the first day after injection of labelled sodium phosphate is accomplished, the activity of the deoxyribonucleic acid P of the total corpuscles rapidly declines, as seen in Fig. 5. The date of this rapid decline indicates almost precisely the life-cycle of the avian red corpuscles.

To arrive at a correct value of the life-cycle of the deoxyribonucleic acid, the contribution of red corpuscles formed in a later part of the experiment to the activity of the total erythrocyte deoxyribonucleic acid must be taken into account as well. This can be done by making use

FIG. 5.

Change of the specific activity of phosphorus fractions of the hen's blood with time (Ottesen).



of the following consideration. The formation of labelled deoxyribonucleic acid during the first day takes place with participation of inorganic P the specific activity of which corresponds to the mean value of the inorganic P during that time interval. This mean value, which can be experimentally determined, is denoted by $f(\frac{1}{2})$. The contribution of active deoxyribonucleic acid of red corpuscles formed during the first day of the experiment to the total activity of erythrocyte deoxyribonucleic acid measured after the lapse of 1 day is $f(\frac{1}{2})$ $(\phi\frac{1}{2})$, where ϕ is a function of the time taken by the transition of the red corpuscles from the marrow into the circulation, and also of the duration of the life-cycle of the erythrocytes. Actually, we compare, not the deoxyribonucleic acid activities of the total erythrocyte content of the circulation, but the specific activities of deoxyribonucleic acid P fractions.

The second day, the corpuscle deoxyribonucleic acid is formed from inorganic P having a much lower specific activity than the first day, the mean value of which is now $f(1\frac{1}{2})$. The contribution of active deoxyribonucleic acid formed during the second day to the total doxyribonucleic acid activity measured on the second day is now $f(1\frac{1}{2})(\phi\frac{1}{2})$. The share of the active deoxyribonucleic acid formed in the course of the first day in the total activity of deoxyribonucleic acid of the red corpuscles is somewhat different from the corresponding value during the first day and amounts to $f(\frac{1}{2})(\phi 1\frac{1}{2})$.

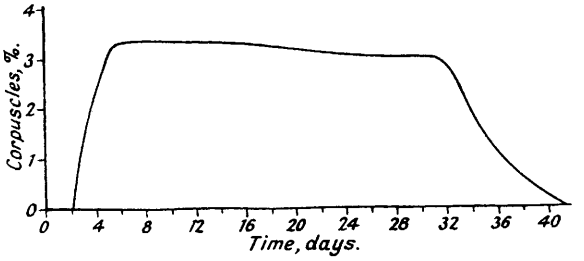
In a similar way the contribution of the active deoxyribonucleic acid formed during the third day to the total deoxyribonucleic acid activity can be calculated and also the share of the deoxyribonucleic acid formed in the course of the first and second day in the total activity

measured on the third day of the experiment can be determined. Each consecutive day we find a new (ϕ) value not represented previously.

As the deoxyribonucleic acid molecules present in the nucleated red corpuscles were found to be entirely stable, not showing any perceptible turnover, the above considerations permit one to determine what percentage, for example, of the labelled red corpuscles formed on the first day is still present in the circulation at any later date. The percentage share of the erthrocytes formed during the first day of the experiment in the total red corpuscle content of the circulation at different dates is shown in Fig. 6.

FIG. 6.

Percentage of hen red corpuscles, formed during the first day of the experiment, present in blood stream at the time indicated on the x*-axis (Ottesen).*

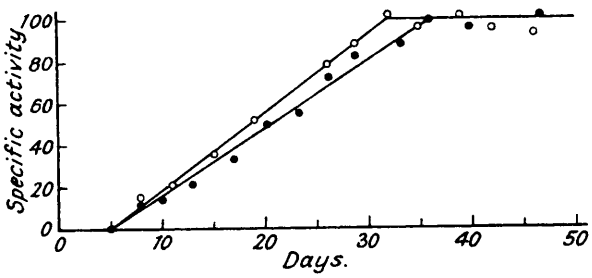


One may be tempted to explain the results obtained by assuming a successive loss of the deoxyribonucleic acid content of red corpuscles during their life-cycle. However, an investigation of the deoxyribonucleic acid content of individual erythrocytes by Ris and Mirsky [47] has shown that this value for each red corpuscle is constant within 10%.

As to a possible objection that not the inorganic but an organic P fraction of the bone marrow is the pertinent precursor of deoxyribonucleic acid, Ottesen has shown that the existence of a deoxyribonucleic acid precursor of considerably lower turnover rate than that of the inorganic P of the marrow is incompatible with the results demonstrated in Fig. 6. A precursor of more rapid turnover rate than that of the marrow inorganic P would clearly not influence the results obtained.

FIG. 7.

Life-cycle of the red corpuscles of two hens. Abscissæ : days after start of experiment; ordinates : specific activity of deoxyribonucleic acid phosphorus extracted from the corpuscles secured at different dates (reproduced, with permission, from Nature, 1945, **156**, 534).



A method very similar to that described above had been used by Shemin and Rittenberg [48] in the determination of the life-cycle of human red corpuscles. They administered glycine containing ^{15}N to human subjects and followed the change with time of the ^{15}N content of hæmin isolated from the erythrocytes. They found this content to decline rapidly after the lapse of 109—127 days. Ottesen based his considerations on the ratio of the ^{32}P content of deoxyribonucleic acid P and its precursor (inorganic P), but Shemin and Rittenberg could not follow such a procedure since the ^{15}N content of the precursor of the hæm nitrogen at different times of the experiment is not known.

Shemin *et al.*[49] applied their ^{15}N technique also to the study of the life-span of the nucleated

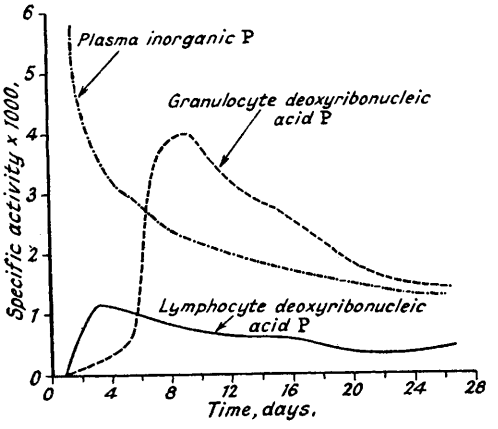
[47] *J. Gen. Physiol.*, 1949, **33**, 125.
[48] *J. Biol. Chem.*, 1946, **166**, 627.
[49] *Cold Spring Harbor Symp. Quant. Biol.*, 1948, **13**, 185.

red blood corpuscles of the chicken. The life-cycle of such corpuscles was found to be about 28 days, in good agreement with the results obtained when using deoxyribonucleic acid ^{32}P as an indicator.

Radio-phosphorus was also applied in life-span determinations in a different way, as described above.[50] By injecting, twice daily, labelled sodium phosphate into the hen, the

FIG. 8.

Change of the specific activity of human-plasma inorganic phosphorus and white-corpuscle nucleic phosphorus with time (Ottesen).



specific activity of the plasma inorganic P was kept at a fairly constant level. As demonstrated by Fig. 7, after the lapse of 33 days the specific activity of deoxyribonucleic acid no longer increased, indicating that only red corpuscles formed during the experiment were now present in the circulation. As significant amounts of labelled red corpuscles were observed only 5 days after the start of these experiment, the life-span of the erythrocytes works out again to be 28 days.

FIG. 9.

Percentage of human lymphocytes, formed during the first day of the experiment, present in the blood stream at the time indicated on the x-axis (Ottesen).

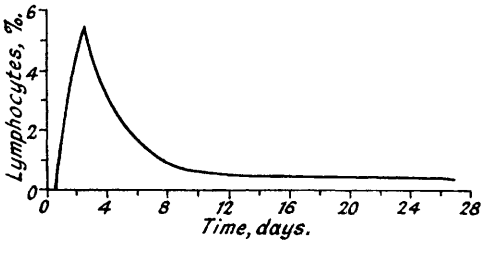
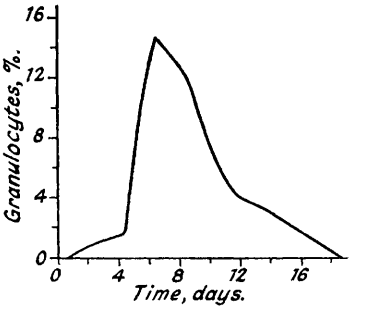


FIG. 10.

Percentage of human granulocytes, formed during the first day of the experiment, present in the blood stream at the time indicated on the x-axis (Ottesen).



(b) *Life-time of Human White Corpuscles.*—The same considerations as described above were applied by Ottesen in the determination of the lifetime of human lymphocytes and granulocytes. His results are illustrated by Figs. 8, 9, and 10. In these experiments, not the specific activity of inorganic P of the blood plasma but the corresponding value of the urine inorganic P was determined, as well as the specific activity of deoxyribonucleic acid P of lymphocytes and granulocytes. As with nucleated erythrocytes, the deoxyribonucleic acid molecules present in the white corpuscles were found to remain unchanged during the lifetime of the corpuscles, all turnover being absent. Correspondingly, the ratio of the labelled deoxyribonucleic acid

[50] Hevesy and Ottesen, *Nature*, 1945, **156**, 534.

content of the white corpuscles formed during the first day to that of the totality of circulating white corpuscles is identical with the ratio of the number of white corpuscles formed within the first day to the total number of white corpuscles present in the circulation. It requires a few days before the white corpuscles are formed and released into the circulation. Owing to this, the labelled granulocyte content of the circulation first increases and reaches a maximum after the lapse of 6 days. About 14% of the granulocytes then present are such as were formed during the first day. Their number decreases, however, fairly rapidly with time. After a further 4 days their proportion is reduced from 14 to 7%.

A closer investigation of the number of lymphocytes formed during the first day and still present in the circulation revealed that an appreciable part of lymphocytes present is formed many months before the start of the experiment; thus lymphocytes contain an appreciable proportion of cells of long life. This conclusion is based on the figures obtained for the ratio of the specific activities of plasma (urine) inorganic P and the corresponding value of deoxyribonucleic acid P extracted from lymphocytes. This ratio should be equal to or less than unity for all lymphocytes formed during the experiment. A ratio appreciably higher than unity was, however, found even after the lapse of more than a month.

Determination of the Amount of Circulating Red Corpuscles.

Although the determination of the life-cycle of the red corpuscles necessitates a type of labelling which remains in the corpuscles throughout their life, the measurement of the amount of red corpuscles circulating in the body can be carried out in a few minutes. Hence it suffices to fix the radioactive label to the corpuscles for a comparatively short time. This procedure can be carried out *in vitro*.[51] We secure a blood sample of a human subject, add a few microcuries of labelled sodium phosphate of negligible weight, and shake the material for 1 hour at body temperature. Let us denote the number of red corpuscles injected into the circulation by A, and the ratio of red corpuscles in 1 g. of the injected blood to those in 1 g. of blood secured from the circulation after the injection by B; then the total amount of red corpuscles present in the circulation (x) is given by $x = AB$.

If to 100 ml. of a blood sample kept at body temperature we add labelled sodium phosphate of negligible weight, about one-third of the ^{32}P atoms added are found in the red corpuscles after 1 hour. It follows that in 1 hour—it being assumed that the inorganic P content of the plasma is 4 mg. % and the weight of the plasma constitutes 55% of that of the blood—about 0·7 mg. of inorganic P moves from the plasma into the corpuscles, and *vice versa*. In the course of this interchange some of the ^{32}P added to the plasma penetrates into the red corpuscles and is replaced by ^{31}P atoms moving in the opposite direction.

The red corpuscles contain appreciable amounts of labile organic phosphorus compounds. In glycolytic and other enzymic processes taking place in the erythrocytes these compounds are degraded and resynthesized at a remarkable rate. Shortly after their intrusion as inorganic phosphate most of the ^{32}P atoms participate in the resynthesis of labile organic phosphorus compounds and are incorporated with them.[52] The presence of a comparatively large amount of labile organic phosphorus molecules makes it possible to fix ^{32}P in red corpuscles during an interval which amply suffices to carry out a determination of the circulating erythrocyte volume.

If we suspend the labelled corpuscles obtained in inactive plasma, or inject them into an inactive circulation, the interchange of inorganic P between the labelled red corpuscles and the inactive plasma continues, involving respectively 0·7 mg. of inorganic plasma and corpuscle P in 100 ml. of blood per hour. While, however, during the activation process, together with 0·7 mg. of inorganic phosphorus, ^{32}P of negligible weight and having an activity of 1 μc. moves into the corpuscles, after injection of the labelled corpuscles into an inactive circulation the migration of 0·7 mg. of inorganic P from the red corpuscles into the plasma will be followed by that of about 0·1 μc. only (cf. Figs. 11 and 12). This is because most of the ^{32}P (and ^{31}P) after having penetrated into the red corpuscles finds a temporary abode in the organic phosphorus compounds present in the erythrocytes. About 1/10 represents the ratio of the inorganic P content of the plasma and labile P content of the red corpuscles. This ratio, and correspondingly the time during which ^{32}P is kept in the erythrocytes, may vary for the blood of different subjects. The permeability of the red corpuscle membrane, which is also of importance for the temporary

[51] Hevesy and Zerahn, *Acta Physiol. Scand.*, 1942, **4**, 376; Bohr, *Kgl. Danske Vidensk. Selskab Biol. Medd.*, 1950, **18**, Nr. 1.

[52] Aten and Hevesy, *Nature*, 1938, **142**, 871.

conservation of ^{32}P in the corpuscles, may vary as well. The loss of the ^{32}P content of the corpuscles in the course of 20 minutes is, however, in no case larger than 3%, and in most cases even appreciably less. When not labelled corpuscles but labelled blood is injected into the circulation, loss of ^{32}P by the corpuscles during the experiment can furthermore be compensated to an appreciable extent by uptake of ^{32}P from labelled plasma present in the circulation.

FIG. 11.

Distribution of inorganic phosphate intruded into the red corpuscles between organic and inorganic fractions.

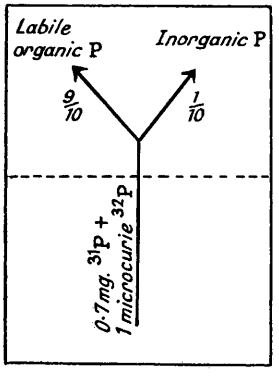
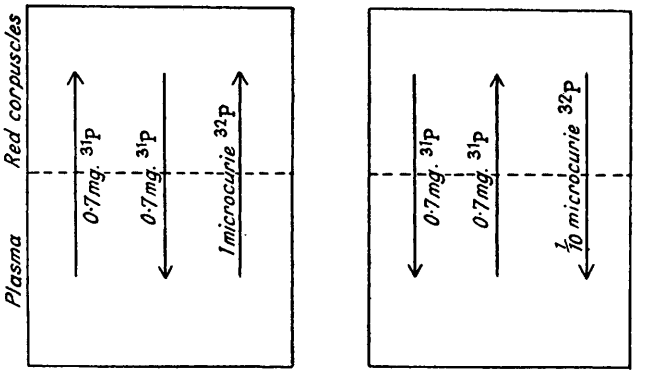


FIG. 12.

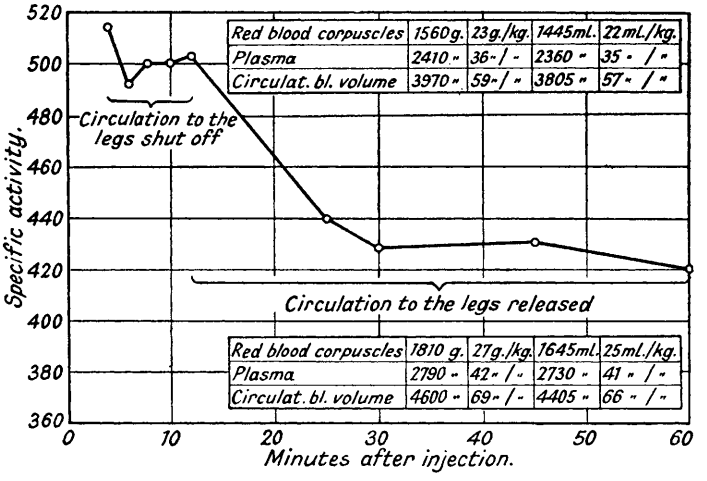
Interchange of ^{31}P and ^{32}P between plasma and red corpuscles.



Nylin [53] made a very extended application of the method described. He determined, besides the total circulating erythrocyte volume, that of single organs such as lungs and legs. A blood sample of a human subject was secured and labelled with ^{32}P as described above. Before injection of an aliquot of the labelled red corpuscles into the subject, the vessels were clamped. The red corpuscles circulating in the legs were thus prevented from participating in

FIG. 13.

Determination of the circulating corpuscle (blood) volume of the legs (Nylin) (reproduced, with permission, from Arkiv Kemi, 1945, **20**, *A*, No. 17).



the " dilution " of the injected labelled erythrocytes, which takes place after injection of labelled red corpuscles into the non-radioactive circulation and the extent of which indicates the amount of circulating erythrocytes. As seen in Fig. 13, 10 minutes after injection of labelled corpuscles, the amount of circulating erythrocytes, excluding that of the legs, is found from the " dilution " figures to be 1560 g. After removal of the clamps, there was a marked decrease in the activity of a 1-g. sample of corpuscle (denoted in Fig. 13 as specific activity). Owing to the participation

[53] *Arkiv Kemi*, 1945, **20**, *A*, Nr. 17, cf. Reeve and Vrall, *J. Physiol.*, 1949, **108**, 12.

of the red corpuscles of the leg in the " dilution " process, the specific activity of the corpuscles decreases from 550 to 428, indicating that the red corpuscle volume of the body, which now includes that of the legs as well, amounts to 1810 g. From the above figures the weight of the erythrocytes circulating in the legs is determined to be 250 g.

The labelling of a red corpuscle sample *in vitro* is made possible by the presence in the red corpuscles of an enzymic mechanism which is instrumental in alternately degrading and building up the comparatively large amounts of labile organic phosphorus compounds in the erythrocytes, and also by the low inorganic phosphate content of the corpuscles, and finally by the fairly slow rate of penetration of phosphate from the plasma into the corpuscles, and *vice versa*.

Red corpuscles can also be labelled by introducing radio-iron, ^{55}Fe or ^{59}Fe. Iron-labelled red corpuscles keep their label throughout their life. As, however, the labelling of erythrocytes with radio-iron can only be carried out *in vivo* it necessitates having blood donors to whom iron of appreciable radioactivity must be administered. Iron-labelled red corpuscles are not applied in the determination of the erythrocyte content of the human circulation. Such corpuscles proved to be useful, *inter al.*, in the study of the post-transfusion survival of erythrocytes preserved during several weeks.

Application of ^{14}C in the Study of Metabolic Depressors and Accelerators.

Although the rapid change in the sensitivity of the indicator during the experiment may be disturbing, as in the study of the mineral constituents of the skeleton (see p. 1630), it is most advantageous when we are interested in restricting the formation of labelled components to a short time interval. This is the case, as we saw, in the study of the life-cycle of blood corpuscles. The rapid decrease with time in the specific activity of the precursor proves also to be useful in the study of the change of the metabolic rate produced by metabolic depressors or accelerators.

FIG. 14.

Effect of dinitrocyclopentylphenol on the incorporation of ^{14}C into total fats and total tissue of mice liver. Groups of animals killed at different times after injection of $CH_3 \cdot {}^{14}CO_2Na$ (reproduced, with permission, from Arch. internat. Pharm. Therap., 1951, **86**, 33).

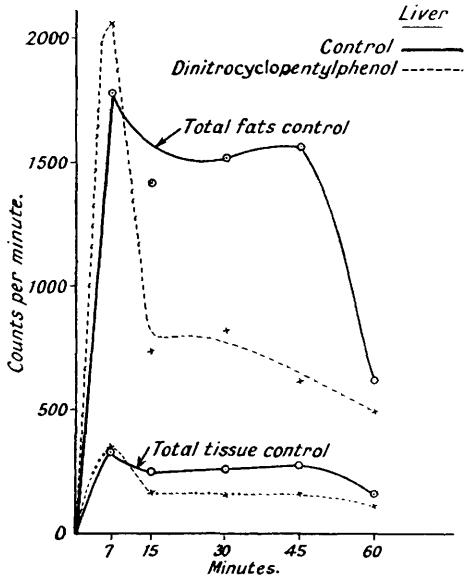
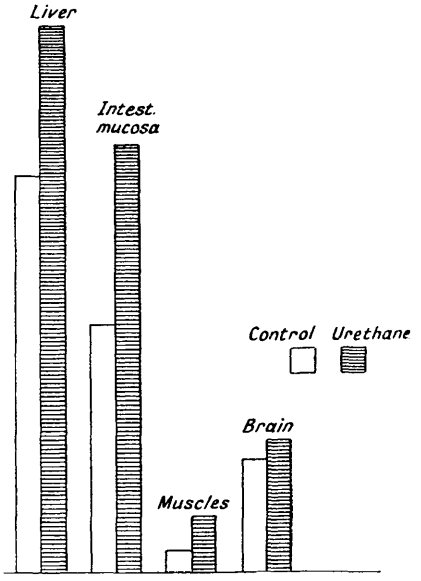


FIG. 15.

Effect of urethane (20 mg.) on the incorporation of ^{14}C into tissue phosphatides. Mice killed 100 mins. after injection of $CH_3 \cdot {}^{14}CO_2Na$.



Numerous carbon compounds present in the organism are metabolized at a spectacular rate. Though, as shown by Bloch and Rittenberg,[54] a 100-g. rat by catabolizing fatty acids produces 1 g. of acetate and other tissue constituents per day, the body acetate level of the organism remains very low, owing to the rapid utilization of the acetate produced. This compound is a

[54] *J. Biol. Chem.*, 1945, **159**, 45.

precursor of acetoacetic acid, cholesterol, fatty acids, glycogen, the dicarboxylic amino-acids, proto-porphyrin, uric acid, and the acetyl group formed in many acetylation reactions, with some of which the ^{14}C atoms of acetate are incorporated at a remarkable rate. If we inject labelled acetate into the mouse, the injected radioactive acetate is diluted by non-radioactive endogenous acetate. This dilution rapidly increases owing to the fact that, while the radioactive acetate injected only at the start of the experiment is rapidly metabolized, the endogenous non-radioactive acetate, though rapidly metabolized as well, is newly formed throughout the experiment. The decrease in the specific activity of the body acetate is necessarily reflected in the specific activity of those products with which acetate carbon is incorporated. As resorption, distribution, and conversion of acetate take some time, the ^{14}C content of a rapidly metabolizing fatty acid fraction found to be present in the liver of the mouse after intraperitoneal injection of labelled acetate increases during the first minutes; soon, however, a very rapid decline in the activity figure of the fatty acids is observed.[55] The strongly active fatty acid molecules formed in the initial phase of the experiment are soon metabolized and replaced by molecules formed from less active precursors; correspondingly, a rapid decrease in the fatty acid activity takes place.

FIG. 16.

Effect of urethane on the incorporation of ^{14}C into total fat. Mice killed 110 mins. after injection of $CH_3 \cdot {}^{14}CO_2Na$.

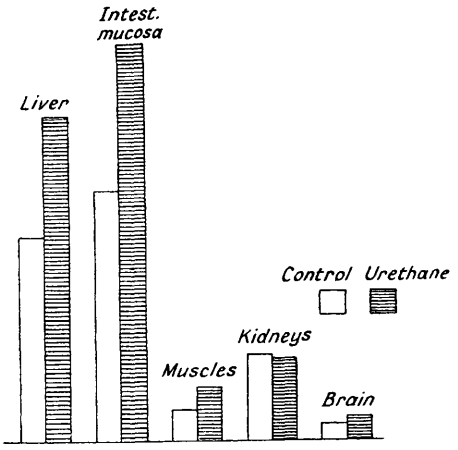
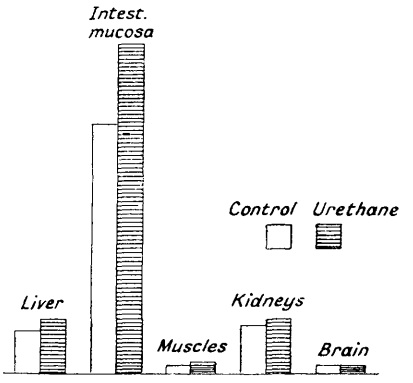


FIG. 17.

Effect of urethane on the incorporation of ^{14}C into tissue proteins. Mice killed 110 mins. after injection of $CH_3 \cdot {}^{14}CO_2Na$.



Dinitro-compounds, *e.g.*, dinitro*cyclo*pentylphenol, when given in proper doses, are metabolic accelerators. Consequently, they enhance the incorporation of acetate ^{14}C into liver fats of the mouse, as seen in Fig. 14.[56] Soon, however, the accelerated metabolism leads to an accelerated replacement of the labelled fatty acid molecules (in which most of the ^{14}C of the total fats is to be found) formed in the early phase of the experiment by molecules formed from less active precursors. The descending part of the curve correspondingly takes a steeper course in the case of the dinitro*cyclo*pentylphenyl-treated animals than in that of the controls. Urethane, which is a metabolic depressor, acts in an opposite way.[57] In experiments taking 100 minutes, thus in the descending region of the curve (Fig. 14), the phosphatide fractions or the total fat (Fig. 16) extracted from various organs of urethane-injected mice take up more ^{14}C than those of the controls. A similar, but less pronounced effect is found (Fig. 17) when comparing the incorporation of ^{14}C into the proteins of organs of urethane-injected mice with the incorporation into corresponding fractions of controls.

In a similar way, acetate labelled in the carboxyl group with ^{14}C was applied in the study of the biochemical effects of ionizing radiation.[58] These investigations revealed an interference, not only, as mentioned previously with the rate of incorporation of ^{14}C into purines of nucleic

[55] Hevesy, Ruyssen, and Beeckmans, *Experientia*, 1951, in print; Beeckmans and de Elliott, *Nature*, 1951, **167**, 200.
[56] Beeckman, Casier, and Hevesy, *Arch. Int. Pharmacodyn.*, 1951, **86**, 33.
[57] Hevesy, *Nature*, 1949, **164**, 1007; Hevesy, Ruyssen, and Beeckmans, *Experientia*, 1951, in print.
[58] Hevesy, *Nature*, 1949, **163**, 869.

acids, but also of other tissue constituents. In the *non-fasting* mouse, for example, irradiation produced a similar effect on the incorporation of ^{14}C into fats and proteins of the brain to that of administration of urethane.

While the disentanglement of the numerous, often competing, metabolic steps involving ^{14}C incorporation and the determination of the extent to which this incorporation is influenced by metabolic depressors and accelerators is a very difficult task, the method outlined above may prove to be a promising approach to the study of metabolic interferences.

The application of radioactive indicators in biochemistry covers a large field and I had to restrict myself to the discussion of a few examples only.

Possible interference of ionizing radiation with the normal biochemical pattern is often considered a disadvantage of the method of radioactive indicators. Such an interference can, however, be avoided by using radioactive tracers of restricted activity. A much more dangerous source of error in the application of radioactive indicators is the non-identity of the chemical properties of isotopes. Tritium, for example, having an atomic mass three times as high as hydrogen, differs from hydrogen to a non-negligible extent in many of its chemical properties. We are witnessing the establishment of a new branch of chemistry which in the course of time may become a very important implement for classical chemistry, namely, that of the rare isotopic constituents. In the long run the claim for accuracy in the biochemical application of isotopic inidcators is bound to increase and it may become necessary to make use of the advances in this new branch of chemistry.

The application of isotopic indicators in biochemistry opened new lines of approach, not only to the solution of known problems, but also by directing our attention to trains of thoughts not considered previously. Some of these ideas I have tried to outline in this lecture. It also induced the experimental chemist to take an interest in the history of the atoms and molecules with which he is dealing.

The chemist is not a historian, he is not interested in the problem whether the carbon atoms of the benzene he is experimenting with were formerly stored in Welsh coal deposits, in carbon dioxide of a volcanic outburst, in the carbonate of crustacea shells, or in a mammalian skeleton. In contrast to the classical chemist, the indicator chemist is to some extent a historian, highly interested in the past of atoms, molecules, and molecular aggregates. He has a great concern in the distinction of how far molecules present in the tissue are " old " or " new." He wishes to know when the potassium atoms present in the tissue cells left the circulation, when the nucleic acid molecules present in the nuclei of thymus cells were formed. He is interested in questions like the former abode or abodes of the carbon atoms of glycogen, the origin of fæces constituents, whether they originate from undigested food or are due to endogenous secretion. He may desire to know which calcium, phosphorus, nitrogen, or sulphur atoms of the plant originate from the soil and which from the added fertilizer, possibly even which from the fertilizing pollen.

Many of the problems attacked by the tracer chemist are such as had been solved previously. The application of isotopic indicators often led to a remarkable simplification of earlier methods. This is the case when measuring circulation rates, water contents, blood volumes, etc. On the other hand, the application of isotopic indicators opened the only way to determine the rate, place, and sequence of formation of many molecular constituents of the living organism. The very existence of such methods was instrumental in opening new trains of thought, in demonstrating the dynamicity of metabolic processes, in confirming Hopkins's statement that " life is a dynamic equilibrium in a polyphasic system," in concentrating our interest on the problem of the velocity of the fundamental biological processes.

Lord Rutherford, who—as Niels Bohr, the preceding Faraday lecturer, so appropriately stated—contributed more than anyone else to the most remarkable development which followed Becquerel's discovery, remarked, when delivering his Faraday Lecture in 1936 : " We can now look back with some sense of perspective and recognize the extraordinary importance of the discovery of radioactivity and the profound influence on a knowledge of atoms and the relation of the elements which has followed from a detailed study of the radioactive bodies." It was this detailed study and the following most remarkable development which made possible the application of radioactive substances as indicators.