RADIOACTIVE ISOTOPES AS INDICATORS IN BIOLOGY

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I. INTRODUCTION

Artificial radioactivity has come to be such a part of daily thought that it is difficult to introduce a review of the applications of radioactive isotopes as tracers in biology in terms which are adequate to the situation and not already common knowledge. It is necessary, however, to temper such general information with a critical analysis of the scope and limitations of the tracer technique.

The essential property which is the basis of all tracer applications of radioactive elements is that the tracer isotope possesses the same chemical properties as the naturally occurring stable isotopes of the particular element, while having a distinctive physical property, subject to measurement under properly selected conditions, which is independent of the chemical transformations it may undergo.

The radioactive isotopes are more generally useful as tracers than the separated heavy stable isotopes. The latter are available in only a few cases, and need to be converted to gases for determination of their relative abundance. There are available radioactive isotopes of practically all the elements of biological interest, and in most cases the measurement of very small amounts of such materials, preferably in the solid state, is relatively simple.

Although it is possible in some instances to obtain a practically pure radioactive isotope, the material used as a tracer in the usual case contains only a small amount of radioactive isotope in a large amount of ordinary stable isotope as carrier. The specific activity, the ratio of the number of radioactive atoms to the total number of atoms of that element present, is therefore rather low. For most purposes this is unimportant, since the comparison made is that of the relative specific activities of the substances isolated from the tissues with that of the material administered to the experimental animal. The relative specific activities are usually stated in arbitrary units, rather than in terms of the curie, the absolute unit of radioactivity. The curie is defined as the number of disintegrations per second from 1 g. of radium; the figure generally given for this is 3.7×10^{10}. The amounts used in tracer experiments are of the order of millicuries or, more commonly, microcuries. The smallest amount of radioactive material which can be measured with reasonable accuracy is of the order of 10^{-5} microcurie. This may represent as little as 10^{-8} microgram of the radioactive isotope.

The property by which radioactivity is detected and measured is that of producing ionization in gases. For a full discussion of the various types of measuring instruments and the properties of the different types of radiation, the

review of artificial radioactivity by Seaborg (252) should be consulted. The principal types of measuring instrument are the electroscope, the integrating electrometer attached to an ionization chamber, and the Geiger–Müller counter. In the first two types named, the measurement is made in terms of the rate of movement of a quartz fiber across a scale. In the Geiger–Müller counter, which is the most sensitive type of instrument, each single disintegration is converted to an electrical discharge. This is amplified by a vacuum-tube circuit to actuate a mechanical recorder of some type. The counter tube consists essentially of a chamber containing a hollow metal cylinder cathode with a very thin wire anode coaxial with the cylinder. The chamber contains a gas or mixture of gases under somewhat reduced pressure. Those designed for counting beta particles usually have a thin window of glass, mica, or aluminum foil opposite the open end of the cylinder cathode. The samples to be measured are placed as close to this window as is feasible, and always in the same geometric relation to it.

Beta particles have a much greater tendency to produce ions and thereby lose energy than do the electrically neutral gamma rays. Their path is therefore much shorter, and the losses through scattering and absorption in the air space between the sample and the window, and absorption by the window itself, may become quite appreciable. This is particularly the case with the low-energy radiations which characterize radioactive isotopes of long half-life, such as C^{14} and S^{35}. In such cases there is also considerable self-absorption in the material containing the radioactive isotope, especially if this contains atoms of higher atomic number than those undergoing the disintegration. For the measurement of these isotopes of low energy of disintegration, special techniques are necessary. The principles involved in such measurements have been discussed by Libby (174).

Any measurements of radioactivity must be corrected for "background" ionization or counting rate arising from the effects of cosmic rays. This background ionization is a function of the volume of the ionization chamber of the electroscope or electrometer, and of the surface of the cylinder cathode of the Geiger–Müller tube. The ionization or counting rate from the sample being measured is a function of the solid angle from it subtended by the ionization chamber. On this account, the background correction may be quite high relative to the activity of weak samples. The accuracy of the measurement is proportional to the square root of the number of disintegrations recorded. The statistical error of the measurement is usually from 3 to 5 per cent, and higher with weak samples.

The radioautograph technique has certain applications in tracer studies. For this, the material is placed in contact with a photographic or x-ray film, and after a suitable exposure time, which may be several days, the film is developed. The silver deposits correspond to the location of the radioelement in the material. The method has been used rather extensively, especially in conjunction with histological studies, in determining the localization of radioiodine within the thyroid gland.

The possible duration of a tracer experiment, from the administration of the tracer substance to the completion of the measurements on material isolated from the experimental animal, is a function of the half-life of the radioelement. Experiments may extend over a period equal to four or five times the half-life without offering any great technical difficulties, but when the half-life is short, e.g., the 21-min. half-life of C^{11}, the scope and duration of tracer experiments are quite limited.

The total amount of radioactive material used in a biological experiment must not be so great that the general radiation effect is sufficient to produce alterations in functioning of the tissues of the experimental animal. The point was raised very early (20) that such tracer experiments would be invalid because of this general radiation effect and the consequent alterations in cellular function. It was soon shown (212) in experiments on *Nitella* that the permeability of the cell membrane to ions is not affected by concentrations of radioactivity below 1 millicurie per liter. This concentration is very much greater than that needed for most biological tracer experiments. However, it is well to bear in mind the general radiation effects, particularly in experiments of long duration.

One limitation on tracer experiments with organic compounds arises from the enormous energy release that accompanies the formation of a radioactive atom. This usually results in the rupture of the bonds that hold the atom in combination, especially if the linkages are non-ionic. It is therefore necessary to obtain the radioactive isotope by bombardment of an inorganic substance, and then use this material for the synthesis of the desired organic compound. With the increasing use of C^{14} in tracer experiments, the development of syntheses which permit the placing of this tracer in the desired position in the molecule will become a challenge to the ingenuity of the organic chemist.

There is still another method of making organic derivatives containing a traceable atom: the introduction of a radioactive halogen substitutent into an aromatic ring. In any such case, the question must be asked whether the presence of the substituent will alter the metabolic fate of the parent compound. More properly, the question becomes the point at which the halogen substituent may be removed from its position and thus cease to act as tracer for the original substance.

In cases where the nature of the compound is such that laboratory synthesis is impossible, for example, the plasma proteins, attempts have been made to obtain tracers by feeding compounds containing the desired radioelement. The enormous dilutions undergone in the metabolic processes that take place have been found to render the method unworkable in general.

An appreciable number of the early experiments with radioactive tracers were purely descriptive, and had little reference to whether the data obtained could contribute materially to knowledge of the dynamics of living matter. Still others were made without regard to whether the tracer approach was capable of yielding any information of critical value that was not as readily obtainable by ordinary experimental approaches. Such a situation arises whenever a new experimental tool is developed. It is only natural that the new technique be

applied without taking cognizance of its inherent limitations. In fact, it sometimes happens that such experiments are the best means of calling attention effectively to the limitations to which the technique is subject.

The nature of many of the problems to which the radioactive isotope technique has been applied is such that the emphasis must fall on the biological aspects, rather than on the strictly chemical ones. The attempt has been made to select, from the large mass of literature available, those experiments which illustrate the particular field of usefulness of isotopic tracers in the study of dynamic processes within the living organism.

II. CARBON

The radiocarbon first discovered, C^{11}, has a half-life of only 21 min.; consequently a tracer experiment with it must be completed within 5 hr. after the cessation of bombardment of the target substance by the cyclotron. This includes the conversion of the carbon dioxide formed into an organic compound, administration of this to the experimental animal, isolation of the metabolic products formed, and determination of their radioactivity. In the counting, correction must be made not only for the self-absorption, but also for the falling off in the counting rate given by a sample during the few minutes necessary for making the measurements. In view of these limitations, the noteworthy results that have been obtained by the use of this isotope are all the more remarkable.

The other radiocarbon, C^{14}, represents the other extreme in half-life. The most probable value seems to be in the neighborhood of 5000 years. Thus, decay during the lifetime of an investigator is negligible, and material containing this isotope could conceivably be recovered and used again, subject to the limitation of dilution with ordinary carbon in the experimental procedures. The isotope is readily available from the operation of the uranium pile, both in the form of barium carbonate and as an increasing number of organic compounds. However, there are technical difficulties in its use which are by no means inconsiderable. Owing to the low energy of disintegration, there is a high self-absorption correction, and also a low efficiency of counting by the Geiger–Müller tube. This results from the scattering and absorption through even a few millimeters of air between the sample and the window, and great absorption by even the thinnest of windows (173).

Two methods have been proposed for overcoming this difficulty. Both depend on measuring the radiation from material in the form of $C^{14}O_2$. Henriques and Margnetti (125) introduce the gas into a quartz ionization chamber attached to a Lauritzen electroscope, and Miller (199) introduces it into the gas volume of a specially constructed Geiger–Müller tube. Carbon dioxide is notoriously unsuited for the gas in such counter tubes because of the lack of voltage plateau over which the counting rate is practically constant. Miller overcomes this by introducing a measured amount of carbon disulfide into the tube after the introduction of the carbon dioxide. It is stated that this gives a

good plateau suitable for accurate counting, and that the counting rates obtained are perhaps one hundred times as much above background as would be the case when the same absolute amount of C^{14} as barium carbonate is measured by the ordinary Geiger–Müller counter technique. Both of these methods require extreme care in transferring the gas sample to the chamber and in removing it prior to the introduction of the next sample.

Despite these technical difficulties, C^{14} will probably prove to be one of the most useful tracer isotopes. The possibilities for its use in the study of intermediary metabolism are almost unlimited. Together with the separated stable heavy isotope, C^{13}, it can be used to establish the mechanism of many reactions where ordinary experimental approaches cannot do more than give indications of possibilities. The design of such experiments must be carefully thought out, and careful evaluation of both the experimental procedure and the data obtained will be necessary to establish whether the experiment is capable of demonstrating the point.

An example of the type of experiment in which C^{14} will probably be of greatest value is the recent demonstration by Gurin and Delluva (102) that phenylalanine is the precursor of epinephrine. They prepared the former compound with C^{14} in the carboxyl and alpha positions, injected this in rats, and after 3 days isolated the epinephrine from the adrenals. To obtain a crystalline product, it was necessary to add a relatively large amount of ordinary epinephrine in the isolation procedure, to act as carrier. The product isolated contained C^{14}, and it was shown by oxidation with periodic acid that this was all present in the terminal carbon of the aliphatic side chain of the epinephrine.

The major portion of the work that was done with C^{11} has been concerned with the reactions by which carbon dioxide is incorporated into organic compounds by living cells. The great accomplishment has been the demonstration that this type of reaction is by no means limited to the photosynthetic reaction in green plants, but also takes place in mammalian tissues.

The field of photosynthesis has been thoroughly explored in the book of Rabinowitch (226), and Wood (283) has reviewed non-photosynthetic carbon dioxide incorporation reactions. The use of isotopic carbon in the study of intermediary metabolism has also been reviewed fairly recently, by Buchanan and Hastings (31). Developments since these were published, and differences in interpretation of the data obtained, make it desirable to present certain aspects of the material in considerable detail.

The tracer experiments on photosynthesis were carried out with C^{11}, and the data obtained and conclusions drawn from them must be regarded as only preliminary. Experiments on barley seedlings (236) showed incorporation of carbon dioxide into the carbohydrates and to a very limited extent into the chlorophyll fractions, but no short-chain carbon compounds could be isolated which might serve as possible intermediates. Repetition of the experiments with the green alga *Chlorella*, which has a much higher rate of photosynthesis, led to indications that the initial stage, which is reversible and independent of

light, is the incorporation of carbon dioxide into a compound of molecular weight between 1000 and 1500 which has the properties of a carboxylic acid. The reaction which is catalyzed by light appears to be the reduction of this acid to a primary alcohol (237, 238, 239). There were no indications obtained of any simple compounds which might be intermediates in the photosynthetic reaction.

There have been a considerable number of tracer experiments on bacterial metabolism, many of which deal with the carbon dioxide incorporation reaction first described by Wood and Werkman (285, 286). These investigators were able to demonstrate conclusively, and without the help of tracer isotopes, the incorporation of carbon dioxide into a three-carbon-atom compound, with the formation of a four-carbon-atom chain. In the anaerobic fermentation of glycerol to propionic acid by propionic acid bacteria, succinic acid is formed. By the use of $C^{13}O_2$ in the system, they were able to show that the carbon dioxide absorbed is present only in carboxyl groups of the succinic acid (287, 288). The mechanism postulated by Wood and Werkman for the reaction is the following:

$$CH_3COCOOH + C^*O_2 \rightleftarrows HOOC^*CH_2COCOOH \quad (1)$$

$$HOOC^*CH_2COCOOH + 2H \rightleftarrows HOOC^*CH_2CHOHCOOH \quad (2)$$

$$HOOC^*CH_2CHOHCOOH \rightleftarrows HOOC^*CH{=}CHCOOH + H_2O \quad (3)$$

$$HOOC^*CH{=}CHCOOH + 2H \rightleftarrows HOOC^*CH_2CH_2COOH \quad (4)$$

By the use of C^{11} it has been shown (32) that the Wood and Werkman reaction is reversible. It has been demonstrated with C^{11} or C^{14} in three species of molds (80) and a protozoan organism (273). It has also been demonstrated in cell-free extracts of liver (67), and the data of Hastings and his collaborators (44, 260, 274) on the formation of liver glycogen in rats fed lactic acid are best interpreted on the basis that this reaction also takes place under these circumstances.

The mechanism of the Wood and Werkman reaction is apparently not as simple as that indicated in reaction 1, as Utter and Wood (271) found in extracts of liver tissue that the reaction takes place only in the presence of adenosine triphosphate (ATP). They suggest that this compound phosphorylates pyruvic acid, and that it is the phosphopyruvic acid which incorporates the carbon dioxide. Buchanan and Hastings, on the other hand, postulate direct incorporation into pyruvic acid, with phosphorylation taking place subsequently (31).

Other types of carbon dioxide incorporation reactions occurring in bacterial metabolism have been studied with radioactive carbon as tracer by Barker and his coworkers (14, 16, 17, 19) and by Foster *et al.* (80). In addition, Barker (18) showed by the use of butyric acid containing C^{14} in the carboxyl group that the conversion of this to caproic acid by *Cl. kluyveri* in the anaerobic fermentation of ethanol was brought about by condensation of the butyryl radical with the methyl carbon of acetic acid, rather than the reverse condensation. This was shown by the absence of C^{14} from the carboxyl group of the caproic acid formed, and by its presence elsewhere in the carbon chain.

Another type of carbon dioxide incorporation reaction in which the tracer

technique was needed both for the demonstration of its occurrence and for the elucidation of the mechanism, is the formation of α-ketoglutaric acid from pyruvate by pigeon liver mince under aerobic conditions. On balance, carbon dioxide is evolved by this system. Evans and Slotin (67) used C^{11}, and Wood *et al.* (289) used the heavy stable isotope, C^{13}, to demonstrate that the carbon dioxide incorporated was present in the carboxyl group adjacent to the carbonyl. Wood *et al.* (288) have postulated the following modification of the Krebs tricarboxylic acid cycle to account for the experimental findings:

$$CH_3COCOOH + C^*O_2 \rightleftarrows HOOC^*CH_2COCOOH \rightleftarrows HOOC^*CH{=}COHCOOH \quad (1)$$

$$HOOC^*CH{=}COHCOOH + CH_3COCOOH \rightleftarrows HOOC^*CH{=}\underset{\displaystyle CH_2COCOOH}{\underset{|}{C}}COOH + H_2O \quad (5)$$

$$HOOC^*CH{=}\underset{\displaystyle CH_2COCOOH}{\underset{|}{C}}COOH + \tfrac{1}{2}O_2 \rightleftarrows HOOC^*CH{=}\underset{\displaystyle CH_2COOH}{\underset{|}{C}}COOH + CO_2 \quad (6)$$

$$HOOC^*CH{=}\underset{\displaystyle CH_2COOH}{\underset{|}{C}}COOH + H_2O \rightleftarrows HOOC^*CHOH\underset{\displaystyle CH_2COOH}{\underset{|}{C}}HCOOH \quad (7)$$

$$HOOC^*CHOH\underset{\displaystyle CH_2COOH}{\underset{|}{C}}HCOOH \rightleftarrows HOOC^*CHOHCH_2CH_2COOH + CO_2 \quad (8)$$

$$HOOC^*CHOHCH_2CH_2COOH + \tfrac{1}{2}O_2 \rightleftarrows HOOC^*COCH_2CH_2COOH + H_2O \quad (9)$$

Although none of these intermediates appears to be symmetrical, there are reasons why the scheme may not be an adequate explanation for the appearance of the labeled carbon in only one carboxyl group. On purely chemical grounds, the aconitic acid formed in reaction 6 might be expected to show a tautomeric shift of the double bond, and the orienting forces should produce hydration of this compound to give symmetrical citric acid rather than unsymmetrical isocitric. Since the tissues contain enzymes which maintain an equilibrium between citric, isocitric, and aconitic acids, it might be anticipated that the incorporation of the labeled carbon as carbon dioxide by this set of reactions would result in the labeling being present in both carboxyl groups of the α-ketoglutaric acid. Further study of this system seems to be indicated.

Evans and Slotin noted in their experiments that considerable C^{11} was present in a fraction which gave reactions characteristic of α-amino acids. They were unable to isolate any compounds from this fraction, on account of the short half-life of the isotope. Recently Anfinsen *et al.* (3a) have published a preliminary report on similar experiments on liver slices incubated with $C^{14}O_2$, in which they found that practically all of the C^{14} incorporated into the proteins was present in the dicarboxylic amino acids. It may be postulated that these were

derived from secondary reactions of the α-ketoglutaric and oxaloacetic acids formed in the primary incorporation reactions, as liver is known to contain an active transaminase system for the interconversion of these compounds and the corresponding glutamic and aspartic acids (40).

One of the most interesting carbon dioxide incorporation reactions that has been studied by the isotope technique is that involved in the formation of liver glycogen when lactic acid is fed to the fasted rat. Hastings and his coworkers (44, 260, 274) have shown by the use of C^{11} that the glycogen deposited under these conditions contains small amounts of the tracer, whether this was present in the carboxyl or α,β-carbon of the lactic acid fed, or was administered as $NaHC^{11}O_3$ during the absorption of ordinary lactic acid. The glycogen formed in the liver was equivalent to about one-third the lactic acid fed, and the radioactivity present was from 0.6 to 3.2 per cent of that administered. The smallest amount was found in the $NaHC^{11}O_3$ experiments, and the highest when the tracer was present in the α,β-positions.

The experimental findings were accounted for on the basis of the occurrence of the Wood and Werkman reaction, reduction of the oxaloacetic acid formed to fumaric acid, phosphorylation of this, and decarboxylation to phosphopyruvic acid. This circuitous method of formation of phosphopyruvic acid was postulated by Kalckar (150) and Lipmann (177). The phosphopyruvic acid was considered by Hastings to undergo the reversal of the phosphorylating glycolysis, ultimately yielding glycogen. The series of reactions shown below is based on the postulates referred to, and is given first for lactic acid with the labeling in the carboxyl carbon, and then with α,β-labeled lactic acid. The phosphorylation of a dicarboxylic acid rather than that of pyruvic acid directly seemed called for by the finding of Meyerhof *et al.* (198) with P^{32} in muscle enzyme systems that the dephosphorylation of phosphopyruvic acid is irreversible. Lardy and Ziegler (161) have reported tracer experiments in a rather complicated system, in which they found some P^{32} uptake in the formation of phosphopyruvic acid. However, even if this indicated direct reversal of the reaction should be established, and the series of reactions given below should therefore require considerable modification, it would still be necessary to postulate the formation and decarboxylation of dicarboxylic acids in order to account for the experimental results obtained by Hastings and his coworkers.

$$CH_3CHOHC^*OOH + \tfrac{1}{2}O_2 \rightleftarrows CH_3COC^*OOH + H_2O \quad (10)$$

$$CH_3COC^*OOH + CO_2 \rightleftarrows HOOCCH_2COC^*OOH \quad (1a)$$

$$HOOCCH_2COC^*OOH + 2H \rightleftarrows HOOCCH_2CHOHC^*OOH \quad (2a)$$

$$HOOCCH_2CHOHC^*OOH \rightleftarrows HOOCCH{=}CHC^*OOH + H_2O \quad (3a)$$

$$HOOCCH{=}CHC^*OOH + H_3PO_4 \rightleftarrows \begin{cases} HOOCCH_2CHC^*OOH \\ \qquad\qquad | \\ \qquad\quad OPO_3H_2 \\ HOOCCHCH_2C^*OOH \\ \quad | \\ \quad OPO_3H_2 \end{cases} \quad (11)$$

$$\left.\begin{array}{l} \mathrm{HOOCCH_2CHC^*OOH} + \tfrac{1}{2}\mathrm{O_2} \rightleftarrows \mathrm{CH_2{=}CC^*OOH} + \mathrm{CO_2} + \mathrm{H_2O} \\ \qquad\quad \mathrm{OPO_3H_2} \qquad\qquad\qquad\quad \mathrm{OPO_3H_2} \\ \mathrm{HOOCCHCH_2C^*OOH} + \tfrac{1}{2}\mathrm{O_2} \rightleftarrows \mathrm{CH_2{=}CCOOH} + \mathrm{C^*O_2} + \mathrm{H_2O} \\ \quad\ \mathrm{OPO_3H_2} \qquad\qquad\qquad\quad \mathrm{OPO_3H_2} \end{array}\right\} \quad (12)$$

The use of ordinary lactic acid and labeled carbon dioxide would result in fumaric acid with the same labeling as shown above, and in the decarboxylation reaction half of the labeling would be lost, as is the case above, with the labeling originally present in the carboxyl group. In the condensation of the triosephosphate units resulting from the hydration and reduction of the phosphopyruvic acid, the statistical picture would be that half the triose molecules, and therefore each of the hexose units, would contain one C* atom. The reactions involved are shown below:

$$\left.\begin{array}{l} \mathrm{CH_2{=}CC^*OOH} + \mathrm{H_2O} \rightleftarrows \mathrm{CH_2CHOHC^*OOH}; \\ \quad\ \mathrm{OPO_3H_2} \qquad\qquad\quad \mathrm{OPO_3H_2} \\ \mathrm{CH_2{=}CCOOH} + \mathrm{H_2O} \rightleftarrows \mathrm{CH_2CHOHCOOH} \\ \mathrm{OPO_3H_2} \qquad\qquad\quad \mathrm{OPO_3H_2} \end{array}\right\} \quad (13)$$

$$\left.\begin{array}{l} \mathrm{CH_2CHOHC^*OOH} + 2\mathrm{H} \rightleftarrows \mathrm{CH_2CHOHC^*HO}; \\ \mathrm{OPO_3H_2} \qquad\qquad\quad \mathrm{OPO_3H_2} \\ \mathrm{CH_2CHOHCOOH} + 2\mathrm{H} \rightleftarrows \mathrm{CH_2CHOHCHO} \\ \mathrm{OPO_3H_2} \qquad\qquad\quad \mathrm{OPO_3H_2} \end{array}\right\} \quad (14)$$

$$\left.\begin{array}{l} \mathrm{CH_2CHOHC^*HO} \rightleftarrows \mathrm{CH_2COC^*H_2OH}; \\ \mathrm{OPO_3H_2} \qquad\qquad \mathrm{OPO_3H_2} \\ \mathrm{CH_2CHOHCHO} \rightleftarrows \mathrm{CH_2COCH_2OH} \\ \mathrm{OPO_3H_2} \qquad\qquad \mathrm{OPO_3H_2} \end{array}\right\} \quad (15)$$

$$\left.\begin{array}{l} \mathrm{CH_2CHOHC^*HO} + \mathrm{CH_2COCH_2OH} \rightleftarrows \\ \mathrm{OPO_3H_2} \qquad\quad \mathrm{OPO_3H_2} \\ \qquad\qquad\qquad\quad \mathrm{CH_2CHOHC^*HOHCHOHCOCH_2} \\ \qquad\qquad\qquad\quad \mathrm{OPO_3H_2} \qquad\qquad\qquad\quad \mathrm{OPO_3H_2} \\ \mathrm{CH_2CHOHCHO} + \mathrm{CH_2COC^*H_2OH} \rightleftarrows \\ \mathrm{OPO_3H_2} \qquad\quad \mathrm{OPO_3H_2} \\ \qquad\qquad\qquad\quad \mathrm{CH_2CHOHCHOHC^*HOHCOCH_2} \\ \qquad\qquad\qquad\quad \mathrm{OPO_3H_2} \qquad\qquad\qquad\quad \mathrm{OPO_3H_2} \end{array}\right\} \quad (16)$$

The fructosediphosphate will thus contain equal amounts of C* in positions 3 and 4, and the statistical average content will be equivalent to one labeled carbon per hexose unit. The postulate that phosphopyruvic acid is formed directly, instead of by the Wood and Werkman reaction, would require that the glyceric aldehyde phosphate and dihydroxyacetone phosphate be labeled to the same extent as the lactic acid administered, and the fructosediphosphate thus would contain a statistical average of two labeled carbon atoms per hexose unit.

When the labeling agent is present in the α,β-positions instead of the carboxylic acid group, the degree of labeling of the hexose unit is independent of the occurrence of the carbon dioxide incorporation reaction. This can be seen from the following set of reactions:

$$C^*H_3C^*OCOOH + CO_2 \rightleftarrows HOOCC^*H_2C^*OCOOH \quad (1b)$$

$$HOOCC^*H_2C^*OCOOH + 2H \rightleftarrows HOOCC^*H_2C^*HOHCOOH \quad (2b)$$

$$HOOCC^*H_2C^*HOHCOOH \rightleftarrows HOOCC^*H{=}C^*HCOOH + H_2O \quad (3b)$$

In the subsequent decarboxylation of this symmetrically labeled fumaric acid, no labeled carbon is lost, and the final condensation of the two triosephosphates becomes:

$$\begin{array}{l} C^*H_2C^*HOHCHO \\ \quad | \\ OPO_3H_2 \end{array} + \begin{array}{l} C^*H_2C^*OCH_2OH \\ \quad | \\ OPO_3H_2 \end{array} \rightleftarrows$$

$$\begin{array}{l} C^*H_2C^*HOHCHOHCHOHC^*OC^*H_2 \\ \quad | \qquad\qquad\qquad\qquad\qquad\quad | \\ OPO_3H_2 \qquad\qquad\qquad\qquad\quad OPO_3H_2 \end{array} \quad (16a)$$

The fructosediphosphate has four C* atoms, in positions 1, 2, 5, and 6. Vennesland *et al.* (274) performed the experiment with lactic acid prepared by the method of Cramer and Kistiakowsky (54). This involves reduction of $BaC^{11}O_3$ to BaC_2^{11}, conversion to acetylene, acetaldehyde, its cyanohydrin, and finally lactic acid. The C^{11} must be regarded as being equally distributed between the two carbons of the acetylene, and therefore equally between the α- *and* β-carbons of the lactic acid, rather than being present in the α- *or* β-position, as was assumed by Vennesland *et al.*

Since the experimental conditions were the same whether carboxyl-labeled or α,β-labeled lactic acid was fed, it is reasonable to assume that the lactic acid fed constituted the same fraction of the metabolic pool which gave rise to the liver glycogen. The data obtained with α,β-labeled lactic acid are independent of the occurrence of a carbon dioxide incorporation reaction, and can thus be used for the calculation of the contribution of the fed lactic acid to this metabolic pool. This is done by comparing the relative specific activities of the labeled carbon fed and found. The data of Vennesland *et al.* have been recalculated on the basis indicated above, and the average contribution of the lactic acid fed to the metabolic pool is found to be 14.3 per cent.

Similar calculations from the data on carboxyl-labeled lactic acid (44) indicate

an average contribution of 11.6 per cent by the fed lactic acid to the metabolic pool on the basis of the Wood and Werkman reaction, and only 5.8 per cent if no such incorporation reaction is postulated. These calculations show that a carbon dioxide incorporation reaction is involved in the formation of liver glycogen from fed lactic acid in the fasted rat. They do not, of course, indicate whether the phosphorylation involved is that of some dicarboxylic acid intermediate, as assumed by Hastings and his coworkers, or of pyruvic acid directly, as postulated by Lardy and Ziegler (161).

The calculations just given present a suggestion of the possible quantitative significance of the carbon dioxide incorporation reaction in mammalian metabolism. It is obvious that a vast amount of work must be done before an assessment of the significance of such reactions under a wide range of conditions can be had. Such experiments can be carried out only with the use of tracer isotopes.

There is evidence from some recent experiments by Hastings *et al.* (120) that indicates direct combination of three-carbon-atom fragments as an alternative mechanism for the formation of the units which form liver glycogen. When rabbit liver slices were incubated with pyruvate and $C^{14}O_2$, the tracer content of the glycogen formed indicated that 10 per cent of its carbon was derived from carbon dioxide. These data can be taken as indicating that 60 per cent of the glycogen formation proceeded through the incorporation reactions, and 40 per cent by direct combination of three-carbon-atom units.

With glucose as substrate, 2 per cent of the glycogen carbon was found to originate in carbon dioxide, corresponding to 12 per cent of the glycogen arising through incorporation reactions under these conditions. These probably took place with pyruvate arising during the course of oxidation of carbohydrate. In the same way, the finding of Vennesland *et al.* (275) that some C^{11} was present in the liver glycogen deposited when fasted rats were fed glucose and injected with $NaHC^{11}O_3$ can be explained on the basis that the incorporation took place with pyruvate arising from lactic acid reaching the liver from the breakdown of muscle glycogen. The assumption of Buchanan and Hastings (31) that the data of Vennesland *et al.* indicate an obligate conversion of glucose to three-carbon-atom fragments which then undergo the Wood and Werkman reaction scarcely seems necessary.

The method indicated above for calculating the contribution of the metabolite fed to the metabolic pool which forms liver glycogen is not applicable to the experiments of Solomon *et al.* (260) in which ordinary lactic acid was fed to fasted rats and $NaHC^{11}O_3$ injected at intervals during the absorption period, nor to the similar experiments of Vennesland *et al.* (275) with glucose and $NaHC^{11}O_3$.

There still remains to be determined the total composition of the metabolic pool which goes to form liver glycogen when glucose or lactate is administered to the fasted rat. The major possibility is, of course, the fatty acids and their derivative ketone bodies. Buchanan *et al.* (30) have attempted to evaluate the contribution of the lower fatty acids to this metabolic pool, by administering acetic, propionic, and butyric acids containing C^{11} in the carboxyl group, together with glucose, to the fasted rat. The experimental procedure reduced the possible

contribution of the fatty acids to a small fraction of the metabolic pool, as they were fed with more than their own weight of glucose, and the glycogen formed was equivalent to less than 20 per cent of the glucose fed. The method of calculation used did indicate to them that propionic acid did contribute directly, and acetic and butyric acids probably did contribute, to the metabolic pool, beyond the amount that could be expected on the basis of carbon dioxide from the oxidation of these acids during the absorption period. A recalculation of their data on the basis of relative specific activities of carbon fed and found fails to show definitely that these fatty acids were direct precursors of liver glycogen.

Wood and his collaborators have carried out experiments with fatty acids labeled with heavy carbon, C^{13}, which demonstrate that these do contribute to the formation of liver glycogen, and have also clarified the mechanism of the process to a very considerable extent. Their experimental conditions were such that the quantitative significance of this process under steady-state conditions cannot be evaluated. The first experiment consisted of feeding ordinary glucose to fasted rats and injecting $NaHC^{13}O_3$ (284). The glycogen isolated from the liver was subjected to hydrolysis, and part of the glucose was converted to methyl glucoside. By bacterial degradation of the glucose, and stepwise oxidation of the methyl glucoside with periodic acid, they were able to show that all the C^{13} excess was present in positions 3 and 4 of the glucose. Reference to reactions 1–3 and 13–16 will show that this is exactly the result to be anticipated on the basis of the Wood and Werkman reaction followed by a reversal of the pathway of the phosphorylating glycolysis.

The next step was the feeding of $C^{13}H_3C^{13}OOH$ (179). The liver glycogen isolated in this case had C^{13} excess present in all six positions. Since that in positions 3 and 4 came from the Wood and Werkman reaction, that in the remaining four positions could have come either from the acetic acid directly or from the methyl group alone. Positions 1, 2, 5, and 6 would be labeled if the triose-phosphate molecules which condense to form the fructosediphosphate contain the labeling in the 2,3-positions, as shown in reaction 16a.

More recently (175) experiments with C^{13} present in the α,β- or carboxyl position of butyric acid have been reported. The glycogen obtained from either carboxyl- or β-labeled butyric acid contained C^{13} excess in only the 3,4-positions, while that from the feeding of the α-labeled acid contained C^{13} excess only in positions 1, 2, 5, and 6.

These experimental findings are all consistent with the hypothesis of Wood *et al.* (289) that the pathway for the conversion of the ketone bodies, and thus also the fatty acids, to glycogen is through a modification of the tricarboxylic acid cycle. The initial step is the condensation of acetate (or possibly acetyl phosphate) with oxaloacetic acid, yielding aconitic acid. The further series of reactions (6–9) would yield α-ketoglutaric acid, from which fumaric acid would be formed by oxidative decarboxylation and dehydrogenation. Since this is a symmetrical compound, the labeling agent originally present in the α-carbon of the acetic and butyric acids would be equally distributed between the two central carbons of the fumaric acid, and hence in the α,β-positions of the decarboxylation

product. In the final condensation of the two triosephosphates, the labeling agent would be equally distributed between carbons 1, 2, 5, and 6.

These experiments indicate that when lactate is fed to the fasted rat, the principal source of the metabolic pool which goes to form liver glycogen is furnished by the ketone bodies which are being produced by the liver under these conditions. In this sense, and under these conditions, the conversion of fat to carbohydrate in the animal body has been demonstrated. It still remains to be ascertained whether ketone bodies are such quantitatively important precursors of liver glycogen in other species, and in the postabsorptive state. Properly designed experiments with either heavy carbon or radiocarbon are the only means which offer definite prospect for establishing the facts.

III. SODIUM, POTASSIUM, AND CHLORINE

The radioactive isotopes of these elements have given information concerning the passage of ions across capillary walls and cell membranes which could not be obtained without the use of such tracers. The experimental data show that such movements of ions are best explained in terms of active transfer rather than as simple physical diffusion processes, in the case of cell membranes and some capillaries.

The rate at which isotope equilibrium is established between plasma and the extracellular phase of the tissues is greatest in small animals. Such equilibrium is reached within 5 min. after the intravenous injection of $Na^{24}Cl$ in the rat (197), but requires about an hour in the dog (152). In the normal human subject the equilibration time is about 3 hr. (152). The capillaries of the choroid plexus have been found to be as freely permeable to cations as are those of the tissues generally. This has been shown in an extensive series of experiments by Greenberg *et al.* (98), in which the time course of the isotope concentration in freshly secreted cerebrospinal fluid was compared with that of plasma. The cations studied were sodium, potassium, strontium, and rubidium. The situation with respect to anions is much more complex. Bromide ion behaved like sodium ion, but iodide and phosphate ions were present in much lower concentration in the CSF than in plasma, at apparent equilibrium.

The capillaries within the brain substance show a very limited permeability to ions. This had been demonstrated by a variety of other techniques, with respect to cations. These are summarized in the review by Friedemann (85). It remained for the radioactive isotope technique to demonstrate that the anion-transfer rates are very low.

Determinations of the total extracellular phase of the body by the isotope dilution method with $Na^{24}Cl$ have generally given results in the human and in the laboratory animal which are comparable to those obtained by the thiocyanate method (99, 104, 152, 184, 204). The results of such determinations with $LiCl^{38}$ (185) and $NaBr^{80}$ (104) on laboratory animals have given comparable results also, but Moore (204) has reported that in the same human subject Cl^{38} was distributed through only about 70 per cent as much volume as Na^{24}.

There has been considerable discussion in the literature on the question of

whether sodium ion is present within the cells of certain tissues. Experiments with $Na^{24}Cl$ have thrown some light on the matter. In the case of the testis, for instance, it has been found (184) that the time course of the uptake of sodium ion shows a rapid phase which corresponds to the equilibration between plasma and extracellular fluid, followed by a slow phase which indicates transfer across the cell membrane by osmotic work. A similar phenomenon has been shown in the muscles of rats which had been maintained on a diet low in either sodium (99) or potassium (126).

Studies with radioactive tracers on the passage of ions across the cell membrane of the erythrocyte have shown that chloride ion passes across this membrane quite rapidly (258), but the rates of transfer of sodium ion and potassium ion are quite low (41, 61, 103, 211). The experiments with K^{42} illustrate one pitfall encountered in the use of radioactive isotopes. Hahn, Hevesy, and Rebbe (103) first reported the extremely low exchange rate of 3 per cent in 24 hr. in the rabbit. Fenn and his coworkers (57, 214) found about 50 per cent exchange in 24 hr. in this species, and also reported that there were marked species differences in the transfer rate. Fenn has pointed out that the discrepancy could be accounted for by the presence of as little as 0.02 per cent sodium chloride in the potassium chloride subjected to bombardment by Hahn *et al.*, because the yield of Na^{24} per microampere hour of bombardment by deuterons is about 200 times that of K^{42}. Fenn and his coworkers freed their $K^{42}Cl$ from contaminating sodium by precipitation as perchlorate and ignition of this to the chloride.

These observations and a number of others with Na^{24} and Cl^{38} indicate that osmotic work is necessary for the transfer of ions across membranes, and that simple physical diffusion is inadequate to explain the observed rates of movement. For example, Visscher *et al.* (278) found transfer rates between the lumen of the ileum and the plasma to be far higher than those expected from simple diffusion, and not necessarily dependent on concentration gradients. Cope *et al.* (47) found that $Na^{24}Cl$ was absorbed at different rates from pouches of the body and the pyloric antrum of the stomach: per unit area of mucosa the pyloric region absorbed about one hundred times as much as did the body, and this high absorption rate continued during the active secretion of hydrochloric acid.

Other studies which indicate that the passage of ions across cell membranes involves osmotic work are those of Kinsey *et al.* (158) on the aqueous humor, and of Wills (282) on saliva. In these experiments Na^{24}, K^{42}, F^{19}, Cl^{38}, and P^{32} as phosphate were used. The data of Ussing (270) with Na^{24} on axolotls show that the rate of transport of this ion into the tissues can be increased by much as 200 per cent by the injection of the antidiuretic hormone of the posterior pituitary.

Flexner and his collaborators (76) have made an extensive study of the rate of transfer of $Na^{24}Cl$ across the placenta in a number of species. The transfer was found to be relatively slow in comparison with the rate of passage across the capillary wall. For example, only 4 min. are required to reach 90 per cent equilibrium between plasma and extracellular phase in the cat, but a similar degree of equilibrium between maternal and fetal plasma is reached only in 12–18 hr. The absolute transfer rate was found to be related to the number of

cell layers in the placenta, and in most species the total sodium ion transfer during pregnancy was computed to be about twenty-five times the sodium content of the fetus at term. In contrast, the total transfer of phosphate across the placenta was found (281) to be only slightly in excess of that retained by the fetus.

Studies with $K^{42}Cl$ (70, 149, 215) have shown that potassium exchange between tissues and extracellular phase takes place at fairly rapid rates despite high concentration gradients. This applies to the heart, liver, and kidney. In striated muscle the exchange rate is slower, possibly because of the relatively low blood flow.

When $K^{42}Cl$ is administered by mouth or intraperitoneally, there is a brief period during which the specific activity of the potassium ion of liver exceeds that of plasma (70), after which the specific activity falls rapidly to that of the plasma potassium. The initial accumulation of potassium in the liver that these findings indicate had been shown earlier by Fenn (68) without benefit of radioactive tracers, but the time course of the relative specific activities of liver and plasma potassium can only signify that this accumulation takes place on the cell membrane, rather than within the cells. Teleologically, such a storage may be postulated to have the function of protecting the heart against the depressant action of a sudden influx of potassium ions into the plasma.

Another illustration of the value of the tracer technique in demonstrating ion-transfer effects that could not otherwise be shown is in connection with the finding by Fenn and Cobb (69), by ordinary experimental procedures, that prolonged stimulation of muscles of rats *in situ* results in net loss of potassium to the plasma. Repetition of the experiments with $K^{42}Cl$ by L. Hahn and Hevesy (105) and by Noonan *et al.* (216) showed that this loss of potassium took place in spite of a markedly accelerated rate of entry of this ion into the muscle cells. Noonan *et al.* ascribe this accelerated exchange to increase in blood flow rather than to change in permeability of the muscle cell membrane, as it was not seen in isolated frog muscle stimulated in Ringer solution containing $K^{42}Cl$. On the other hand, both these workers and Lyman (183) found an accelerated rate of exchange of potassium ion between plasma and denervated muscle which can best be explained on the basis of altered permeability.

Another example of a problem in this field which can be handled adequately only by the isotopic tracer technique is that of the state of sodium in bone. Kaltreider *et al.* (152) showed by the use of $Na^{24}Cl$ *in vivo* that bone contains a reservoir of sodium that exchanges only slowly with plasma sodium. This finding, and the additional observation that the sodium content of bone is rather high, are adequately accounted for by the showing by Hodge *et al.* (141) that ground bone *in vitro* adsorbs considerable sodium. The adsorption follows the Freundlich isotherm, and at body temperature and the concentration of sodium present in plasma, it is of sufficient magnitude to explain the sodium content of bone.

In plant materials it has been shown by the use of Na^{24}, K^{42}, and $Rb^{86,88}$ that both simple cation exchange and metabolic uptake of these ions takes place

(27, 28). The exchange is independent of temperature and oxygen tension, but the metabolic uptake takes place only in the presence of oxygen, is increased by exposure to light, and has a high temperature coefficient. There is also a related observation by Hevesy and Nielsen (138) that yeast cells show a more rapid exchange of K^{42} with the suspending medium during active fermentation than during the resting state.

IV. PHOSPHORUS

Compounds of phosphorus are of such great biological importance that it is fortunate that P^{32} combines the desirable features of relatively long half-life and high energy of disintegration. Almost half of the total number of papers on biological applications of radioelements as tracers describe experiments with P^{32}. The very first such paper, that of Chievitz and Hevesy (39) in 1935, was concerned with the distribution of labeled phosphate among the tissues of the rat.

The problems that have been studied with this tracer range from the demonstration of the mechanism of an enzyme reaction to the study of the effects of x-ray radiation on the rate of formation of nucleoproteins in the nuclei of tumor cells. The wealth of material available makes it necessary to limit the discussion to those matters in which the tracer technique has given definitive information not attainable by other experimental methods.

One limitation on the value of P^{32} as a tracer is that organic phosphate compounds injected into the animal undergo hydrolysis to inorganic phosphate in the process of absorption, so that their fate and distribution are not directly ascertainable (2, 37, 132, 235).

A. Enzyme reactions

The simplest system in which P^{32} has given definitive results not otherwise demonstrable is in the conversion of α-glycerophosphoric acid to the β-derivative. Chargaff (36) incubated the α-compound with alkaline phosphatase in the presence of P^{32} as inorganic phosphate. The β-compound formed contained no P^{32}; the reaction must therefore have been an intramolecular one. Meyerhof *et al.* (198) used a similar technique to show that the conversion of 3-phosphoglyceric acid to the 2-derivative also takes place intramolecularly. They also obtained evidence by this means that the enzymatic dephosphorylation of phosphopyruvic acid is irreversible. However, Lardy and Ziegler (161) have demonstrated with P^{32} that some of the tracer was taken up by the phosphopyruvic acid formed in muscle extract under certain conditions. While the conclusion they drew, that this reaction is reversible, may be correct, the enzyme system used was so complex and the P^{32} uptake by the phosphopyruvate was so low, that the point cannot be considered to be established.

B. Bone and teeth

There have been several investigations of the metabolism of phosphate in these structures by the tracer technique. One quite thorough study of the time course

of the relative P^{32} contents of these in comparison with plasma inorganic phosphate was made by Manly *et al.* (186, 187). The conclusion drawn was that bone and teeth consist of a stable portion in which phosphate once deposited tends to remain indefinitely, and a labile portion which is in dynamic equilibrium with plasma inorganic phosphate. The subject has recently been reinvestigated by Neuman and Riley (214a) in the same laboratory. They find that exchange adsorption takes place on the surface of bone salt with great rapidity. Most of this process is complete within 2 hr. after the intravenous injection of tracer, and it involves about 20 per cent of bone. On this account, it is concluded that P^{32} is not particularly useful in studies on the deposition of bone in growth.

Hodge and his coworkers have made extensive studies of the adsorption of various ions on bone and teeth *in vitro* with radioelements as the adsorbed ion. Studies on phosphate (142, 145, 188), sodium (141), strontium (140), and fluoride (279) have shown that the process follows the Freundlich isotherm. In the cases of sodium and strontium, it is possible to account for the amounts normally present in bone on the basis of such adsorption.

There have been a number of studies on rickets in which P^{32} has been used, but in general the information obtained could have been inferred, if not necessarily clearly demonstrated, by ordinary experimental methods. As examples of this, the following may be cited: Cohn and Greenberg (42) found that the administration of vitamin D to rachitic rats resulted in a shift in distribution of the phosphate absorbed from the intestine, so that less exchanged with soft tissue phosphate and more was deposited in bone, and also there resulted decreased reëxcretion of phosphate into the intestine. Morgareidge and Le Fevre (202) found that the maximum increase in the P^{32} content of the metaphysis of the bones in rachitic rats which followed the administration of a single dose of vitamin D took place in from 54 to 72 hr. Histological examinations of tissues taken at this time showed healing of the lesions. Manly and Levy (189) gave a single dose of P^{32} phosphate to pregnant rats shortly before term, and found that the relative specific activity of the phosphate of the young at birth was comparable to that of the maternal soft tissues, rather than to that of the bones. This held whether the rats were on a normal or rachitogenic diet.

C. Mechanism of entry of phosphate into tissue cells

The entrance of phosphate into the cell interior from the extracellular phase is a considerably more complicated matter than the entry of cations. Most tissues contain higher concentrations of inorganic phosphate than is present in plasma, and still larger amounts are present in organic combination. When a dose of tracer phosphate is injected into an experimental animal, the distribution of the P^{32} found in the tissues is the resultant of the transfer across the cell membrane and the metabolic interchanges within the cell. On this account it is necessary to use caution in the interpretation of such experiments. Critical analysis of the data obtained on various tissues by this means indicates that simple diffusion processes are less adequate to account for the phosphate ex-

change between the cell and its environment than is the case with cations. It is necessary to postulate some enzymatic mechanism for bringing about the entry of phosphate. In the erythrocyte, for example, Eisenman *et al.* (61) found that the amount of phosphate transferred to the cell interior was entirely too great at 37°C. to be explained on the basis of diffusion, and also that the process had the high temperature coefficient characteristic of chemical reactions. In yeast cells, both Hevesy *et al.* (137) and Mullins (213) report that phosphate enters the cell only during active metabolism of carbohydrate, and that the rate of entry shows a high temperature coefficient.

The rate of exchange of phosphate between the brain and blood plasma is extremely slow in comparison with that of other tissues (*10*, *21*, *184*), and the uptake of P^{32} by the phospholipids of brain is very slow in the adult animal (34). In the new-born rat, however, the uptake by both total brain phosphate (88) and phospholipids (89) is many times the rate found in the adult animal. Slices of brain incubated with P^{32} phosphate show a fairly rapid incorporation of the tracer into the phospholipids (90), and the rate is increased considerably in the presence of glucose or other readily oxidizable sugar (249).

These results show that it is necessary to separate the metabolic interchanges between the various phosphorus compounds present within the cell, from the transfer of phosphate across the membrane. In brain, the metabolic interchanges apparently have a high rate, whereas the rate of transfer across the cell membrane is limited by the very low permeability of the brain capillaries.

The necessity for making this distinction is emphasized by the conflicting interpretations of data on these processes in heart and striated muscle. The experiments in point are those of Furchgott and Shorr (91) on slices of dog heart incubated with tracer phosphate, of Kalckar *et al.* (151) on intravenous injection of the tracer in rabbits, of Sacks and Altshuler (246) on the time course of the relative specific activities of the acid-soluble phosphorus compounds of heart and striated muscle in cats, and of Bollman and Flock (23) on the effect of prolonged circulatory occlusion on the subsequent uptake of P^{32} by muscles.

Furchgott and Shorr found that in incubation of heart slices at 37.5°C. the specific activities of the phosphocreatine (PC) and terminal phosphate group of the adenosine triphosphate (ATP) at equilibrium were about one-fifth that of the inorganic phosphate of the medium. They interpreted the data in terms of diffusion of inorganic phosphate into the cell interior, followed by rapid conversion to the organic compounds by the reactions of the phosphorylation cycle. It should be pointed out that this interpretation carries with it the implicit corollary that only one-fifth of the intracellular inorganic phosphate is available for such free diffusion across the cell membrane, while the entire amount is available for the interchange reactions with PC and ATP.

Kalckar *et al.* injected the tracer phosphate, after a short time chilled the hind legs of the animal, and then perfused one leg with ice-cold phosphate-free Ringer solution, in an attempt to wash all the P^{32} out of the extracellular phase, so that comparison could be made of the relative specific activities of intracellular inorganic phosphate and ATP. Most of the PC in the muscles underwent hydrol-

ysis during the perfusion period. The experiments are subject to the criticisms that the anoxia and low temperature during the perfusion can hardly be expected to leave the permeability of the cell membrane unimpaired, that the hydrolysis of PC added to the inorganic phosphate a relatively large amount of material of low specific activity, and that the data show that the washing out of the extracellular inorganic phosphate was by no means complete. The experiments are therefore of no critical value for the determination of how phosphate enters the muscle cell.

The data of Sacks and Altshuler show that the highest specific activity found in the PC and ATP of the heart was less than one-fourth that of the plasma inorganic phosphate sampled at the same time, and that the specific activities of these compounds in muscle were only about one-tenth as high as those of heart. At no time was there any evidence that isotope equilibrium had been established between plasma phosphate and PC or ATP in either muscle or heart; 24 hr. after the injection of the tracer, the relative specific activities of PC and ATP in the heart were significantly higher than that of plasma phosphate. Such a situation could not obtain if phosphate entered the cell by simple diffusion; once isotope equilibrium had been established, there could not subsequently be any change of specific activities of plasma and intracellular phosphates with respect to each other. The conclusion drawn from these experiments was that phosphate entered the cell by formation on the membrane of organic phosphate compounds, so oriented spatially that the compound could enter the cell interior and then take part in metabolic interchanges with other phosphate compounds.

On this hypothesis, the equilibrium ratios of relative specific activities of plasma and intracellular phosphates, under conditions of constant P^{32} content of the plasma, would give a measure of the relative frequency of intracellular metabolic interchanges and those across the cell membrane. The slice experiments of Furchgott and Shorr approximate this condition and indicate for heart muscle a ratio of five intracellular exchanges to one across the membrane. The data of Sacks and Altshuler indicate a similar ratio in the intact heart, and by extension to the muscle, a ratio of some 50 to 100 intracellular exchanges for each one across the membrane. The situation is analogous to that found in brain by Fries and Chaikoff (88, 90): a high rate of metabolic interchange within the cell coupled with a low rate of transfer across the membrane. In the case of brain it is the capillary wall rather than the cell membrane which is the barrier to free exchange with plasma phosphate.

To explain the data on intact muscle by the hypothesis of free diffusion of phosphate ion across the cell membrane would require the postulate that only 1 or 2 per cent of the entire intracellular inorganic phosphate is available for the metabolic interchanges of the phosphorylation cycle.

The only experiments with tracer phosphate on muscle permeability which show unequivocally that this membrane can become permeable to phosphate ion are those of Bollman and Flock (23). They occluded the circulation to one extremity for periods up to 4 hr., released the occlusion, and injected tracer

phosphate. Under these conditions it could be shown that the muscles which had undergone such drastic treatment did become permeable to phosphate ion.

Kaplan and Greenberg (154, 155) have made extensive studies of the distribution of P^{32} phosphate in liver under a wide variety of conditions. No data are given with respect to the P or P^{32} contents of plasma, and it is therefore difficult to evaluate the data in terms of metabolic interchanges. One fact definitely shown is that the administration of glucose to the fasted rat results in an increase in the ATP content of the liver, and that the phosphate for this is derived from plasma inorganic phosphate rather than from the intracellular inorganic phosphate of the organ.

Lundsgaard (182) has obtained data from perfusion experiments on the livers of cats indicating that the addition of fructose to the perfusing fluid leads to a similar effect. In this species fructose is said to be a glycogen former.

The administration of phlorhizin results in a diminished rate of uptake of P^{32} from plasma phosphate by the liver (155) and kidney (227). These findings may be significant in relation to the hypothesis of Verzar (276) that absorption processes in the intestine, and presumably elsewhere in the body, take place by way of intermediate phosphorylations which are inhibited by phlorhizin.

D. Phosphate compounds in muscle metabolism

Implicit in the formulations given above for the mechanism by which phosphate enters the muscle cell is the assumption that PC and ATP enter into interchanges with other compounds which are essential parts of the metabolic cycle of resting muscle. The exact function of these compounds is still a matter of dispute. Studies of enzyme systems isolated from muscle have led to one formulation, and investigation of the time course of the chemical changes taking place during muscular activity has led to an entirely different one. The use of P^{32} as a tracer in the intact animal has pointed the way to an integration of these apparently opposite formulations. Much remains to be done to complete this synthesis.

The enzyme studies, which have recently been summarized by Potter (225), have led to a concept of interrelated phosphorylation and oxidation reactions, based on ATP as the central point. It is assumed that the same metabolic cycle is operating during contraction as in the resting state. The study of the changes in the phosphate compounds in mammalian muscle contracting *in situ* with normal blood supply has led to the formulation that the energy for contraction is furnished by reactions which do not involve phosphate transfers from and to ATP. The evidence for this view has been summarized by Sacks (241).

Conditions were found under which the injection of tracer phosphate into the anesthetized animal resulted in a differential distribution of the P^{32} among the PC, ATP, and hexosemonophosphate (HMP) (240). Under these conditions, neither a fairly prolonged tetanus under essentially anaerobic conditions (240) nor a long series of twitches under essentially oxidative conditions (242) led to any equalization of the distribution of the P^{32} between these compounds. It follows from this that the reactions which take place during contraction do not involve

the phosphate transfers that obviously do take place in resting metabolism, and there is a qualitative separation of the pathways of carbohydrate metabolism in resting and contracting muscle. It may be speculated that the enzyme systems of the activity metabolism are more closely linked to cellular organization than are those of the phosphorylating resting metabolism.

Further evidence for the existence of separate metabolic pathways in resting and activity metabolism is furnished by the observations of Bollman and Flock (22, 77) and Sacks (244) that no increased uptake of phosphate from the plasma takes place during activity, but does occur in a subsequent recovery period.

The tracer experiments on resting metabolism have shown some divergences from the present formulation of the phosphorylation cycle. For example: in cat muscle the tracer experiments show free interchange between fructose-6-phosphate and the PC and ATP, but no interchange between these latter compounds and glucose-6-phophate (244), whereas in frog muscle no interchange was found between the phosphorylated sugars and the PC and ATP (246).

There have been a number of observations with P^{32} showing that the rate of new formation of ATP can be modified in muscle (243, 244, 245) and in liver (154, 155) by the nutritional state of the animal, by the administration of glucose or insulin, or by various enzyme poisons. Some of the data are at variance with current concepts of the phosphorylation cycle. More thorough study on several species may be necessary before the findings on isolated enzyme systems and in the intact animal can be integrated.

E. Mechanism of absorption of glucose

Until the advent of the tracer technique, no clear-cut demonstration had been obtained that the absorption of glucose by the cell involves intermediate phosphorylation reactions. It has been shown (246) that when glucose and tracer phosphate are administered to the fasted cat, there is a temporary accumulation of glucose-6-phosphate on the muscle cell membrane. This effect is absent in the animal in the postabsorptive state (244). The proof that the accumulation of the phosphorylated sugar is on the membrane and not within the cell is that the specific activity of this fraction subsequently falls to a value below that of the PC or ATP. The most satisfactory interpretation of the data is that glucose enters the muscle cell by the formation of the 6-phosphate derivative on the membrane with such a spatial orientation that the glucose portion is directed toward the cell interior and the phosphate portion directed away from the cell. The hydrolysis of the compound then results in the entry of the glucose molecule, while the phosphate remains in the extracellular phase. Application of the same experimental technique to the small intestine and the kidney might determine whether phosphorylation does take place in the absorption of glucose by these organs.

F. Mechanism of insulin action

The use of P^{32} in the intact animal has shown that insulin accelerates the transfer of phosphate across the cell membrane of muscle in the forms of PC and ATP.

This was shown in the fasted cat given glucose by Sacks (245) and in the rat by Goranson (93). The effect is less marked in the cat in the postabsorptive state, because the administration of glucose to such animals depresses the rate of exchange across the membrane (244). Insulin also seems to depress the increased exchange that normally takes place during recovery from muscular activity (245). In traumatic shock, the stimulating effect of insulin on phosphate exchange is not seen (93).

In rabbits given insulin, Kaplan and Greenberg (153) noted an increase in the amount and relative specific activity of the "barium-soluble" fraction of the phosphate compounds of muscle. The data indicate that the largest part of the increase is derived from the PC, but there must also have been some derived directly from plasma phosphate. This fraction consists largely of HMP. The published data are inadequate for an analysis of the process taking place under the influence of insulin, but do indicate species differences. Further application of tracer phosphate to this problem should throw considerable light on both the details of the phosphorylation cycle in the intact muscle cell, and the effects of insulin thereon.

G. Thyroid hormone effects

Greenberg *et al.* (101) have found an increase in the rate of transfer of phosphate across the cell membrane of muscle in hyperthyroid rats, without any effect on the transfer rates in liver and kidney. Conversely, the transfer rate across the muscle cell membrane is decreased in hypothyroidism.

H. Phospholipid metabolism

Although other types of tracer substance have been used in the study of phospholipid metabolism, the ease of use and wide range of applicability of P^{32} have resulted in the abandonment of the other tracers and extensive use of the isotope technique. The simplest application has been to measure the rate of incorporation of P^{32} into the tissue phospholipids following the administration of the tracer as inorganic phosphate (9, 34, 38). A refinement of this consists in obtaining blood plasma from dogs after such treatment, injecting it intravenously into recipient dogs, and following the fate of the labeled phospholipids there present (291). This procedure has given results which are of physiological significance. On the other hand, attempts to isolate such labeled phospholipids from the tissues of animals given P^{32} phosphate and inject them into others have been less successful. The rapid disappearance of such material from the plasma (135) and the distribution of the labeled material among the tissues indicate that it has been treated as foreign particulate matter (122). Very high concentrations were found in the spleen and lung, organs which show low rates of incorporation when P^{32} phosphate is given. Two attempts to trace labeled phosphoric esters, those of ethanolamine (37) and choline (235), failed because the injected substances underwent hydrolysis in the body at fairly rapid rates.

Flock and Bollman (78) found that the glycerophosphate of liver acquired P^{32} from administered inorganic phosphate at a greater rate than did the phos-

pholipids of this organ. This indicates that the esterification of glycerol with phosphate precedes the esterification with fatty acids in the formation of phospholipids. Experiments on slices of kidney and liver (90, 209) have shown that phospholipid formation takes place only under oxidative conditions. It has also been shown (75) that cellular organization is necessary for this synthesis, as it does not take place in homogenates.

A series of experiments by Chaikoff and his collaborators has shown that the phospholipids of plasma are both supplied and removed almost entirely by the liver, although the kidney and small intestine form these compounds at rates only slightly lower than does the liver itself. They found (74) that practically no labeled phospholipid appeared in the plasma of the hepatectomized dog maintained with glucose and given tracer phosphate; the kidney and intestine showed normal rates of phospholipid formation under these conditions. Conversely, the rate of disappearance of injected labeled phospholipid from the plasma, which is normally about 10 per cent per hour (291), is very much less when the liver is excluded from the circulation (64).

The data of Bollman and Flock (24) indicate that in the rat about one-fourth of the total amount of phospholipid formed in the liver is transferred to the plasma. These data, together with the observation that the turnover rate of intestinal phospholipid is higher in animals on a high fat diet than on a high carbohydrate diet (10), and the further finding (222) that administration of fat increases the rate of formation of phospholipid by the intestine, are consistent with the hypothesis that phosphorylating reactions are involved in fat transport and metabolism. However, Bollman and Flock calculated from their data that the total amount of phospholipid formed in the liver was adequate to account for only about 3 per cent of the fat undergoing transport in the course of metabolism. This would seem to exclude the possibility that phosphorylation is an obligate stage in fat transport.

Studies by means of injection of plasma containing labeled phospholipids (234) indicate that these substances pass directly from plasma to intestinal lymphatics. The major portion of the phospholipids present in thoracic duct lymph, however, are synthesized by the intestine, with a smaller portion arising in the liver (79). The greatest part of the increased phospholipid content of thoracic duct lymph which appears after feeding fat was found to arise in the intestine also (79). The inflow from the liver was increased somewhat by feeding fat.

In the adrenalectomized rat, Stillman *et al.* (262) found normal rates of formation of phospholipid in the intestine, irrespective of whether the animals were suffering from untreated cortical insufficiency or were maintained by the administration of sodium chloride. These observations, together with those of Bollman and Flock (24) on the quantitative unimportance of phosphorylation in fat transport, would seem to dispose of the hypothesis of Verzar that the decreased intestinal absorption of fat in adrenal insufficiency is due to impairment of the phosphorylating mechanism.

The time course of the uptake of P^{32} by the phospholipids of muscle, when the

tracer is administered as inorganic phosphate, does not permit an answer to the question of whether the phospholipids are formed there or merely transported to muscle by the plasma. This follows because the rate of appearance of the P^{32} in muscle phospholipids is low in comparison to the rates for liver and plasma (9, 38). Indirect evidence, obtained from the intravenous injection of plasma containing labeled phospholipid (291), shows that the musculature accounts for an appreciable fraction of the phospholipid removed from the plasma, and denervated muscle shows a higher uptake than normal muscle (6, 87). These findings would seem to indicate that the phospholipids present in muscle are acquired by transport rather than by synthesis *in situ*.

Treatment of the rooster with the synthetic estrogen diethylstilbestrol results in a marked increase in the rate of formation of phopsholipids by the liver and in the amount of phospholipid transferred to plasma by the liver (78). The removal of phospholipid from plasma by the liver is increased slightly by this treatment of the animal. The increased rate of formation of phospholipids has also been shown (268) in slice experiments using livers of estrogen-treated roosters.

Experiments with P^{32} phosphate by Hevesy and L. Hahn (133), by Chargaff (35), and especially by Lorenz *et al.* (180) have shown that phospholipids once incorporated into the growing egg yolk in the hen do not undergo any subsequent breakdown reaction which releases phosphate or phospholipid to the plasma. Lorenz *et al.* also showed that the phosphoproteins of the white are formed in the early stages of growth of the yolk, ready for deposition around the yolk at the time the yolk is fully grown. Hevesy *et al.* (136) injected P^{32} phosphate into the egg, and after incubation found that the tracer was present in the phospholipids of the embryo but not in those of the yolk. This demonstrates that the phospholipids of the yolk were not transported as such to the embryo.

Radiophosphorus has been used extensively in the study of lipotropic effects of choline and other compounds, but in many of the earlier papers describing such studies, the experimental data were incomplete, and therefore do not establish any necessary connection between lipotropic activity and phospholipid turnover. The theoretical basis for such connection implies phospholipid formation as an obligate stage in fat transport. The early reports (220) that choline increased the P^{32} content of the liver phospholipids in rats with fatty livers included no determinations of lipid or phospholipid content of the liver, and hence are not of critical value. More recent experiments by Horning and Eckstein (143) indicate that the increased P^{32} content of the liver which results from the administration of choline or methionine in such animals can be accounted for by an increase in the phospholipid content of the organ, which is not always accompanied by a fall in total lipid content. These authors state that there may be no relation between this phospholipid effect and lipotropic action. Artom and Cornatzer (7,8) conclude, from their experiments on animals on a low-fat, low-protein diet, that the effect of choline is to stimulate phospholipid formation, rather than to accelerate the turnover. Both methionine and cystine have been found (222) to increase the uptake of P^{32} by the phospholipids in the liver of animals on a diet producing fatty livers. Methionine has a lipotropic action, but cystine has the

opposite effect. It is therefore difficult to relate the observed effect to lipotropic activity.

Bollman and Flock (24) have made a very thorough study of phospholipid turnover in the liver. By proper design of experiment, following the relative specific activity of plasma and liver inorganic phosphate as well as that of liver phospholipids, they have established that the rate of formation of liver phospholipids in the rat is remarkably constant. Their data have been calculated in terms of phospholipid phosphorus formed per hour per 100 g. of body weight. Changes in composition of diet which influence the size and phospholipid content of the liver were found to be without effect on the rate of formation of phospholipid in this organ, in the terms given above. Partial hepatectomy was found to increase the rate of formation per gram of liver and carbon tetrachloride injury, which gives livers of increased size, resulted in decreased formation rate per gram of liver tissue, but in both cases there was no departure from the normal rate of formation of liver phospholipid per 100 g. of body weight of animal. An effect of choline on turnover rate could be demonstrated in the fatty livers resulting from a choline-free diet, but not in animals whose diet contained adequate choline to prevent the formation of fatty livers.

The turnover rate of lecithin was found to be higher than that of cephalin both in normal tissues (38, 134) and in tumors (121). It has also been reported that choline administration increases the turnover rate of the choline-containing phospholipids of liver (63) and of plasma (187), without affecting the turnover rate of the non-choline-containing phospholipids. The dose of choline chloride used, 300 mg. per kilogram, is so high that the observed effect may be a mass-action one rather than one of physiological significance.

I. Nucleic acid metabolism

The data obtained on the time course of the uptake of P^{32} by these compounds indicates that the ribosenucleic acids of cytoplasm undergo metabolic turnover, while the new formation of desoxyribosenucleic acids in cell nuclei represents primarily growth rather than metabolic turnover. This is indicated by the relatively rapid uptake of P^{32} by the desoxyribosenucleic acids of liver and spleen of rats 3 to 4 days old, as compared to the slow uptake in adult animals (1). Also, Brues *et al.* (29) found rather close parallels between the uptake of P^{32} by the desoxyribosenucleic acids and the mitotic activity, both in liver regenerating after partial hepatectomy and in the tumor Hepatoma 31. In adult animals, Ottesen and his collaborators (3, 139) found the highest uptake rates in those tissues in which new cells are formed throughout the life of the animal: bone marrow, mucosa of the small intestine, etc. Radiation with x-rays produces an immediate decrease in the rate of incorporation of P^{32} into desoxyribosenucleic acids, but the rate returns rapidly to normal. The effect was seen both in normal tissues (1) and in tumors (65, 66). On the other hand, Marshak (191) reported that x-radiation led to decreased uptake of tracer phosphate by the cytoplasm and increased uptake by cell nuclei.

Marshak and Walker (193) have used P^{32} in a very ingenious experiment on

chromatin. They injected tracer phosphate in rats, and after some time isolated from the livers, chromatin strands which contained the tracer. When suspensions of these chromatin strands were injected intravenously into rats which had previously undergone partial hepatectomy, the P^{32} uptake by the liver was several times as high as when the tracer was injected as inorganic phosphate or ATP. The result found may represent either the incorporation of intact chromatin into regenerating liver cells, or the engulfment of the chromatin strands as foreign particles by the reticuloendothelial cells of the liver. If the former be the case, it represents the only reported instance in which a labeled organic phosphate compound not normally found in plasma escaped hydrolysis after injection. Labeled nucleic acids from liver, obtained by a similar technique, were found to undergo rapid hydrolysis after intravenous injection or on incubation with liver slices (2).

J. Other applications of P^{32}

Lindsay and Craig (176) used the radioautograph technique to study the distribution of phosphate in various stages of development of a number of insect species. Born *et al.* (25) and Stanley (261) attempted without success to obtain differential distribution of this tracer between tobacco mosaic virus and the host plant, by adding tracer phosphate to the nutrient solution in which the young plants were growing. In plant materials a non-metabolic uptake of P^{32} phosphate from nutrient solution has been demonstrated (27, 130, 217). It has also been shown (27) that light and favorable environmental temperatures increase the metabolic uptake of phosphate by plants. Biddulph (21) and Arnon *et al.* (5) were able to show shifts of phosphate taking place between plant roots and leaves and from leaves to fruit. Stout and Hoagland (263) showed by the following experiment that migration of phosphate in woody plants takes place only through the wood and not the bark. They separated bark and wood for a short space by inserting waxed paper, then added tracer phosphate to the nutrient solution. After some time, relatively high concentrations of P^{32} were found in the bark above and below the separated portion, but practically none in the separated portion itself.

V. SULFUR

Most of the tracer experiments with this element involve the synthesis of labeled cystine or methionine, by methods capable of giving the greatest possible conversion of sulfur to the desired compound. The relatively long half-life, 87 days, allows adequate time for the carrying out of the synthetic reactions and even rather prolonged animal experiments. On the other hand, the low energy of disintegration of S^{35} requires special methods for measurement of the radiation.

Two experiments with this tracer point out the necessity for caution in translating the results of *in vitro* experiments into terms of metabolic reactions taking place in the intact animal. One of these concerns the formation of cystine from pyruvate, ammonia, and sulfide sulfur by an enzyme present in liver. Smythe and Holliday (259) used sulfide containing S^{35} to demonstrate that the reaction

does involve the incorporation of sulfur present in this form into the cystine formed. However, Dziewiatkowski (60) was able to show that in the intact rat only a very small fraction of the cystine present in hair, skin, muscle, or the liver itself could have come from this reaction. Even when the formation of cystine was promoted by feeding bromobenzene, this possible mechanism of synthesis had little quantitative significance.

The other illustration comes from the experiments of Melchior and Tarver (196) on the incubation of S^{35} cystine with homogenates of liver. Under these conditions, a rather rapid incorporation of cystine into the proteins took place. It was shown to be present there through sulfhydryl linkages and not by peptide bonds. When S^{35} methionine, which has the sulfhydryl group blocked, was used, none of the amino acid was incorporated into the proteins of the homogenate.

The biological conversion of methionine to cystine has been shown in the intact animal by Tarver and Schmidt (265) and in liver slices by Melchior and Tarver (196), by the use of methionine containing S^{35}. Tarver and Schmidt (266) also showed by this means that the taurine of the bile may arise from methionine.

The mechanism postulated by Tarver and Schmidt for the conversion is the following: demethylation of the methionine to homocysteine, migration of the sulfhydryl group from the gamma to the beta carbon, and demethylation of the resulting β-methylcysteine. However, du Vigneaud *et al.* (277) were able to show, by the use of doubly labeled methionine, that this postulated mechanism is not the one used by the body. They prepared methionine with excess C^{13} in the β- and γ-positions and excess S^{34}. This was fed to rats; after some time the hair was clipped, and from it the cystine was isolated. It contained excess S^{34}, but no excess C^{13}, and therefore the carbon chain of the methionine was not used in the cystine formation. du Vigneaud *et al.* discuss a number of other possible mechanims for this conversion which have been postulated at various times.

Radiosulfur has been used to throw more light on the origin of the plasma proteins than was possible to obtain from ordinary experimental methods. There is overwhelming evidence that the liver is the site of formation of most of these, and its contribution is so great as to mask other possible sources when ordinary techniques are used. However, Tarver and Reinhardt (264) have recently shown, by the use of S^{35} methionine in the hepatectomized animal, that an appreciable fraction of the globulins and a smaller fraction of the albumin of plasma have an extrahepatic origin. Their results indicate that fibrinogen is formed exclusively by the liver. They have also been able to demonstrate by this means that other tissues than the liver, in particular the intestinal mucosa, can convert methionine to cystine.

Mention should also be made of the ingeniously planned but only partly successful attempts of Seligman and Fine (254) to obtain plasma proteins labeled with S^{35}. They synthesized cystine, homocystine, and methionine with S^{35} and fed these, together with casein, to dogs which had been rendered hypoproteinemic by plasmapheresis. After a few days there was obtained from these

animals plasma which did contain some S^{35} in protein combination. The yields were too low to be practical for use in their program of studying changes in capillary permeability in traumatic shock. They were able to carry out a few experiments in which the rate of disappearance of the labeling agent from the plasma was followed in dogs which had been injected intravenously with the labeled plasma proteins. The rate of disappearance found (72) indicates a turnover rate of about 1.5 per cent per hour for the plasma proteins.

VI. CALCIUM AND STRONTIUM

The only radioactive isotope of calcium that has a half-life long enough to permit its use in biological tracer experiments is Ca^{45}. The energy of disintegration of this radioelement is so low that measurements on it can be made only with great losses due to self-absorption, scattering in air, and absorption by the window of the Geiger counter tube. It has therefore not been used to any great extent. Instead, a number of experiments have been carried out with Sr^{89} as a substitute tracer. This isotope has both a favorable half-life and high energy of disintegration.

The findings have been that radiostrontium is only a qualitative, and not a quantitative, substitute for radiocalcium in tracer experiments, both in plant (257) and in animal (218) material. A survey of the data obtained in many of the experiments raises serious questions about the effectiveness of this substitute tracer. Most of the information obtained would appear to have been as readily obtainable by ordinary methods of investigation.

Studies on calcium metabolism and transport in pregnancy and lactation (219), in rickets (97, 280), and in the healing of fractures (49, 192) are among those which have been made with these radioelements.

VII. IRON

Studies on the metabolism of iron have been concerned principally with the factors influencing absorption from the gastrointestinal tract, storage in the tissues, and conversion to hemoglobin. Decades of research by ordinary experimental methods have yielded an extensive literature characterized by controversy and confusion. A few years of experimentation with radioiron have led to a clearly defined picture. By this means it has been established beyond a reasonable doubt that the principal factor which determines the absorption of iron from the gastrointestinal tract is the need of the body for iron for the formation of the hemoglobin of the red cell. The mechanism by which this need is made effective remains to be found.

The key to the problem was given by the experiments of P.F. Hahn, Bale, Lawrence, and Whipple (107). They fed Fe^{59} as ferric sulfate to normal dogs and to others rendered anemic by low-iron diet plus repeated bleedings. Only traces of iron appeared in the blood of the normal animals, but prompt absorption of a rather large percentage of the tracer took place in the anemic animals. It appeared in the plasma within a few hours; by 24 hr. the iron had practically disappeared from the plasma and was present in large amount in the circulating

red cells. The concentration in the red cells increased for several days following the administration of a single dose of labeled iron. Since no exchange reaction took place between plasma and corpuscle iron (108), the data must signify transport by the plasma to the bone marrow and incorporation there into newly forming red cells. Some details have been added by further study, but the essential results have been fully confirmed by subsequent experiments. For example, Austoni and Greenberg (12) found that relatively more iron is absorbed by normal rats than Hahn *et al.* noted in normal dogs. They also observed greater differences in absorption of iron between normal and anemic rats than had been found in dogs. Copp and Greenberg (50, 51) used Fe^{55} preparations of relatively high specific activity to show that anemic rats might absorb up to 90 per cent of a single 50-γ dose of iron.

So well established is this difference in absorption of iron between the anemic and normal, that radioiron is being used clinically as a test for iron-deficiency anemia (203). A normal individual may absorb 1 or 2 per cent of a dose of 2 mg. per kilogram, whereas the anemic subject may absorb as much as 50 per cent of such a dose. In pregnancy, the amount absorbed may be as much as ten times the normal, even in cases which show no hematologic evidence of iron deficiency (13).

Absorption of iron appears to take place from the stomach as well as from the intestine. P. F. Hahn *et al.* (110) demonstrated active absorption from pouches of the stomach, duodenum, and jejunum in dogs, and Copp and Greenberg (50) found evidence in rats that absorption of iron also takes place from the colon.

In anemic human subjects, both P. F. Hahn *et al.* (114) and Moore *et al.* (203) found greater percentage absorption of ferrous iron than of ferric, and Hahn *et al.* found the same situation in anemic dogs. Moore *et al.* found such a preferential absorption of ferrous iron in only some of their anemic dogs. Both groups are in agreement that the anemic human subject shows a similar preferential absorption of ferrous iron, and Moore *et al.* found it in their normal male subjects also.

The mechanism for the absorption of iron may be "saturated". This was shown by P. F. Hahn *et al.* (110) by administering a dose of an ordinary iron salt by mouth to anemic dogs 1 to 6 hr. before feeding a tracer dose. Under these conditions, the percentage absorption of the tracer dose was considerably less than anticipated.

Little storage of orally administered iron takes place in the liver or other organs (51). Rather high concentrations are found in the bone marrow during the first several hours after oral administration, but most of this disappears in a day or two, especially in anemic animals. Transport of iron from mother to fetus takes place principally through the plasma: Pommerenke *et al.* (224) gave radioiron salts by mouth to pregnant women shortly before anticipated delivery. In the three cases in which the birth of the child took place within 2 hr. after giving the tracer, the Fe^{59} content of umbilical vein plasma was very close to that of maternal plasma taken at the moment of delivery.

The utilization of intravenously injected iron for red cell formation takes place

fairly promptly and completely in normal dogs (58) under conditions in which orally administered iron would not be absorbed. This finding is considered as evidence that there is a preferential utilization of recently stored iron for hemoglobin formation over that which had previously been stored in the tissues.

The immediate fate of ferric iron injected intraperitoneally is quite different from that of orally administered iron. Copp and Greenberg (51) found in rats that a large portion of the injected dose appeared in the liver within a few hours, and that it was released to the marrow only over a period of several days. Some storage of the injected iron was found in the spleen. The administration of a copper salt together with the iron salt was found to reduce the storage in the liver quite markedly, even though the animals were not showing any signs of copper deficiency.

The principal storage form of iron in the liver, spleen, and bone marrow is the complex of protein and colloidal ferric hydroxide, ferritin (96). Granick and his collaborators (95, 113) have shown, by the use of Fe^{59}, that the liver rapidly converts the major portion of a dose of ferric salt, given intravenously, into this complex within a very few hours, even though large amounts are already present within the organ. The iron liberated from the destruction of red cells was also found to be stored in this form, principally in the liver and to a lesser extent in the spleen.

Copp and Greenberg (51), working with rats, and P. F. Hahn *et al.* (109), using dogs, found that practically no iron is excreted by the body. The iron liberated from the normal destruction of red cells is retained and utilized for the hemoglobin of newly forming ones. Of the iron liberated by normal red cell destruction, only about 3 per cent is excreted in the bile (124) and this is absorbed again by the intestine. For this reason, it is not possible to use this tracer to determine the average life of the red cells. Even when excessive red cell destruction is caused, for example, by the action of acetylphenylhydrazine (55), the iron is re-utilized completely.

The fate of intraperitoneally injected red cells has been studied by P. F. Hahn *et al.* (115) by injecting tracer iron in anemic dogs, and after several days injecting blood from them into recipient animals. In some instances the labeled cells were found in the circulating blood within a few hours; in others, the appearance of Fe^{59} in the circulation was delayed for several days, indicating that the injected cells had first been destroyed and their iron used to form new ones. It is quite likely that these cases of delayed appearance of the tracer in the blood of the recipient dog represent transfusion incompatibilities.

The only exception to the general rule that iron is absorbed from the gastrointestinal tract when needed for hemoglobin formation is in the case of inflammatory reactions. In chronic infections accompanied by microcytic anemia (13) and in the presence of an acute sterile abscess produced by the injection of a turpentine emulsion in anemic dogs (111), there is failure to absorb orally administered iron. In febrile conditions, even intravenously injected iron is utilized only very slowly and incompletely for hemoglobin formation, even when an iron deficiency anemia is present (58).

VIII. BROMINE

The use of radiobromine in studies on diffusion and permeability has been discussed in Section III. A somewhat related case is the demonstration by Perlman *et al.* (222) that the thyroid gland concentrates bromide in the same way that it concentrates iodide, and that in the hyperplastic state produced by thyrotropic hormone the capacity of the gland to concentrate bromide is increased in the same manner as the capacity to concentrate iodide.

Most of the tracer uses of radiobromine have been in the nature of a substitute tracer, either for chlorine or for labeling some organic compound which could not otherwise be readily prepared with a radioelement. For example, Hansen *et al.* (117) prepared monobromobenzene containing Br^{82}, and condensed this with chloral to give a dibromo analog of DDT. This compound, which is only slightly inferior to DDT as an insecticide, was used to kill susceptible insects, and the distribution in the insects determined by the radioautograph technique. The half-life of Br^{82} is 34 hr., whereas that of Cl^{38} is only 37 min.; hence the experiment with the chlorine derivative would not be feasible.

As illustrations of the use of radiobromine as a substituent to make an organic compound traceable, the preparation of brominated Trypan Blue and Evans Blue (205, 206) and of the dibromo derivative of 1-amino-8-naphthol-3,6-disulfonic acid (159) may be cited. These dyes were used to study the capillary permeability to colloids that takes place in sites of acute inflammatory processes. The γ-rays from the disintegration permit determinations of relative concentration of the dye in different areas of the body, by placing the proper type of Geiger tube over the area. It was found possible to localize inflammatory lesions in the extremities by this means, but the method failed when applied to deep lesions. It was also found by this means that tumors do not concentrate these colloidal dyes to any abnormal extent, indicating that the capillaries in the tumors do not have qualitatively abnormal permeability (206).

Fine and Seligman (72) brominated plasma proteins with radiobromine to study the question of plasma leakage in shock. They were able to obtain preparations in which up to 97 per cent of the bromine present was in protein combination. These bromoproteins disappeared more rapidly from the blood stream than did the labeled proteins obtained by feeding S^{35} amino acids to dogs (254). The rate of disappearance of the brominated proteins was roughly proportional to their bromine content, indicating that they were treated as foreign substances. Attempts to calculate the turnover rate of plasma proteins from the use of such labeled material would give erroneously high values.

The one instance in which a compound containing radiobromine has been used in relation to its action in the body is that of the synthetic estrogen α-bromotriphenylethylene. Daudel *et al.* (56) used Br^{82} in the synthesis of this compound, and studied its distribution in the tissues of the mouse. The highest concentration was found in the ovary, and in this organ was reached in about 15 hr. The concentration in the uterus was not as high as in the ovary, but was reached within 3 or 4 hr. after injection, and remained at the peak level for the

30 hr. over which observations were made. The estrus response appeared about 48 hr. after injection, as was to be expected.

IX. IODINE

An ingenious application of radioiodine as a tracer has been made by Reiner (231, 233), based on his discovery (232) that insulin can be coupled with aromatic diazo compounds under certain conditions and retain its physiological activity. *p*-Iodoaniline was made with radioiodine, diazotized, and coupled with insulin. The resulting product was injected subcutaneously, and the rate of absorption from the injection site followed by placing a γ-ray-sensitive Geiger counter tube over the injection site. The absorption of ordinary insulin was found to be proportional to the logarithm of the amount remaining, up to about 80 per cent disappearance from the injection site, indicating absorption by simple diffusion. With protamine zinc insulin and globin insulin, however, the absorption process was found to be considerably more complex.

Experiments such as this illustrate the value of the tracer technique in certain cases which could not otherwise be subjected to experimental investigation, and at the same time point out the necessity for extreme caution in the interpretation of such data. The question arises, at what point in the metabolism of the tracer compound did a separation of the aromatic group containing the tracer from the rest of the molecule take place? There is no way of ascertaining whether the diazo group was reduced in the process of absorption or only after the azo insulin had been transported to the liver. Also, the question must remain unanswered as to whether it was the azo insulin or the regenerated insulin which was physiologically active.

The great interest in tracer applications of iodine has naturally been in the study of thyroid physiology. The first such application was made by Hertz and his coworkers (128). They showed that in the normal rabbit there is an extremely marked concentration of intravenously injected iodide by the thyroid within a few minutes after injection. In animals with thyroids made hyperplastic by a cabbage diet or the injection of thyrotropic hormone, there is an even greater concentration of the iodide by the gland. The fundamental observations of Hertz *et al.* have been confirmed in a number of species (4, 163, 172, 173, 210), and have also been shown in slices of thyroid tissue by the Warburg technique (248).

The greatest part of the experiments has been in the study of the effect of alterations in the state of thyroid function on the capacity of the gland to concentrate iodide, and to convert it to diiodotyrosine and thyroxine. With the development of antithyroid substances of the type of thiouracil (11), the emphasis of the tracer studies has shifted to attempts at elucidation of the mode of action of these substances.

Conditions which increase the functional activity of the thyroid bring about an increase in the capacity of the gland to concentrate iodide, and decreased functional activity diminishes the concentrating capacity. For example, exposure of rats to a cold environment increases and exposure to a warm environment

decreases the iodine-collecting capacity of the gland (167). These changes are noted only during the period before adaptation to the new environmental temperature takes place. Treatment with thyrotropic hormone, as might be expected, increases the iodine-collecting capacity of the thyroid (127, 128, 156, 171, 210). Hypophysectomy reduces, but does not abolish completely, the iodine-collecting capacity of the thyroid (170, 172, 210). It is stated that the thyroid of the hypophysectomized animal is unable to convert diiodotyrosine to thyroxine (210).

When iodide is administered to animals which have previously been on a diet deficient in iodine, there is a marked acceleration in the rate of iodine uptake by the thyroid (168). The percentage uptake of the administered dose increases with dose up to a certain point; beyond this the absolute amount taken up continues to rise, although the per cent of total dose collected may show a decrease (172). When the tracer iodine was present as iodate or diiodotyrosine, the collection by the thyroid showed a lag, indicating that these were probably converted to iodide before fixation (172). Even when labeled thyroxine was injected into rabbits, some of the iodine was found fixed in the thyroid as inorganic iodide (147).

Experiments with thyroid slices (247, 248) have shown that fixation of iodine takes place only when the cytochrome system is operating. However, concentrations of azide which inhibit the cytochrome system were found by the same investigators not to affect significantly the uptake of iodine in the way that cyanide and sulfide did.

The initial fixation of inorganic iodide by the thyroid is followed by fairly rapid conversion to organic forms. Lein (173) found more organically bound iodine present in the gland 12 hr. after the administration of the tracer than had been initially fixed as iodide. Leblond *et al.* (169) showed by a combination of chemical and histological techniques that the principal storage form is diiodotyrosine. Chagas *et al.* (33) have followed the time course of the accumulation of tracer iodide in the colloid by a micromanipulation technique which permitted the aspiration of the contents of single follicles. Leblond (165, 166) used histological and radioautographic techniques together, to show that acidophilic colloid contains higher concentrations of tracer iodine than does basophilic colloid. Since it is generally considered that the basophilic colloid represents a more active state, Leblond interpreted his findings to mean that the lower concentration signified a more rapid secretion of thyroid hormone into the blood stream from the basophilic colloid.

In the normal dog, Mann *et al.* (190) calculated from the time course of tracer iodine content of the thyroid, that the gland forms about 1.5 per cent of its thyroxine content per hour.

Joliot *et al.* (147) report that the thyroid collects a much smaller fraction of a dose of tracer iodine when this is administered as thyroxine than when it is given in inorganic form, and also that the urinary excretion of the tracer is much greater in the former case. These workers also found (148) that several days' treatment of an animal with thyroxine resulted in a "resting state" of the thyroid

during which there was a greatly reduced uptake of a tracer dose of iodide. These observations are hardly to be expected from what is known of the duration of action of a single dose of thyroxine, and it is difficult to see how a substance which is rapidly excreted can bring about a resting state in the thyroid.

In the fetal rat, Gorbman and Evans (94) report that the capacity of the thyroid to collect iodine is first noted on the nineteenth day of the 22-day gestation period, at the same time that colloid follicles make their appearance.

Studies on thyroid slices by Chaikoff and his collaborators (208, 209) have shown that the conversion of iodide to diiodotyrosine and thyroxine is a function of cellular organization, as it does not take place in homogenates. These workers also noted that high concentrations of iodide in the medium in which the slices are suspended tend to inhibit the conversion reactions.

Morton *et al.* (211) report that in the thyroid made hyperplastic by thyrotropic hormone treatment, there is both an acclerated conversion of iodide to diiodotyrosine and thyroxine and an accelerated rate of secretion of thyroxine by the gland. Keating *et al.* (156) were able to show the latter effect in chicks after a single dose of thyrotropic hormone. It should be pointed out that this accelerated loss of iodine from the thyroid under the influence of thyrotropic hormone had been demonstrated by Schockaert and Foster (250) by ordinary analytical methods.

The thyroid hyperplasia caused by goitrogenic substances such as thiouracil has been studied extensively with radioiodine. On the basis of such studies, the goitrogens can be separated into those which decrease the iodine-collecting capacity of the thyroid and those which increase it. Most of the clinically used compounds, such as thiouracil and its homologues, and thiourea, are in the former group (52, 53, 84, 157, 162, 163, 228, 230). On the other hand, in the hyperplasia resulting from the administration of acetonitrile or thiocyanate, the iodine-collecting capacity of the thyroid is markedly increased (128, 228), but the capacity to convert the iodide into diiodotyrosine is blocked (230, 251). Still other substances, such as 1-aminothiazole, which is used clinically in France, appear to block the conversion reaction without affecting the capacity of the gland to collect iodine (147). To complicate the situation still further, some antithyroid compounds have been found (228) to result in decreased iodine-collecting capacity in rats and increased capacity in chicks.

The data referred to above have all been gained from chronic administration of the antithyroid substance to the experimental animal. Rawson *et al.* (228) have reported preliminary observations which indicate that a single dose of practically any of the goitrogens has a similar effect over a brief period during which it is present in effective concentration. Experiments on thyroid slices by Chaikoff and his collaborators have given results which are not altogether in harmony with the data obtained on the intact animal. They found (82, 83) that thiouracil, thiourea, *p*-aminobenzoic acid, and the sulfonamides do not affect the uptake of tracer iodide by the slices, but depress the conversion to diiodotyrosine. Acetonitrile was found to be without effect on either the uptake or the conversion, and thiocyanate to inhibit the uptake of the tracer. They

also state (267) that of the compounds they tested, every one which Astwood (11) had reported to be effective as a goitrogen in the intact animal was able to inhibit the conversion reaction.

No completely satisfactory theory has yet been offered for the mechanism of this goitrogenic or antithyroid action, or for the beneficial effect this clsas of substances has on clinical hyperthyroidism. A full account of this subject would be beyond the scope of this review. The facts that have been established by the use of tracer iodine in this disease do call for mention. There is general agreement (116, 118, 129) that in the clinical condition there is an increased uptake of tracer iodine by the thyroid and an increased rate of secretion of thyroid hormone into the circulation. Accompanying this, the urinary excretion of the tracer dose is diminished (229). When thiouracil or some similar compound is administered and the symptoms of hyperthyroidism are reduced, the urinary excretion of a test dose of tracer iodide is raised to the normal level (116), and presumably the fixation by the thyroid is reduced to a corresponding degree.

X. MISCELLANEOUS ELEMENTS

Included in this section are the heavy metals and the metalloids. Radioactive isotopes are particularly valuable in the study of the distribution and excretion of such trace elements, which are present in the tissues in such minute amounts that ordinary chemical methods are unsatisfactory for their quantitative estimation. Kurbatov and Pool (160) have presented a theoretical discussion of the principles involved in the use of radioactive isotopes of trace elements for metabolic studies.

Among the studies which have been made by this means are: those on manganese by Greenberg *et al.* (100) and Mohamed and Greenberg (199); on cobalt by Copp and Greenberg (48), Comar *et al.* (43), and Sheline *et al.* (256); on zinc by Montgomery *et al.* (201) and Sheline *et al.* (255); on selenium by McConnell (194, 195); and on gold by Ely (62).

Distribution and excretion studies have been made with radiocopper in connection with its function in erythropoiesis. Yoshikawa *et al.* (290) found that it appeared in the plasma within a few hours after oral administration, but was not present in the circulating red cells until after the plasma content declined below the peak value. Schultze and Simmons (251) found a greater percentage retention of an orally administered dose in animals which had been on a copper-deficient diet than in those with dietary iron deficiency. The highest concentrations were found in the liver, kidney, and bone marrow. The latter probably represents concentration in connection with the function in red cell maturation.

The distribution and excretion of arsenite has been studied by Hunter *et al.* (144) and Lowry *et al.* (181); that of arsenate has been studied by Du Pont *et al.* (59). Lawton *et al.* (164) have made some preliminary observations on an experimental approach to the mechanism of the chemotherapeutic effect of arsenicals in experimental filariasis, by demonstrating that injected arsenic compounds were concentrated to a relatively great extent by the parasites within the

infested animal. A higher concentration of the arsenic was found in the parasites than in any tissues of the host other than the liver and kidney. In this connection, it should be noted that Hawking (123) demonstrated by ordinary experimental techniques that susceptible strains of trypanosomes concentrate therapeutically active arsenicals, whereas resistant strains do not. The compounds used by Lawton *et al.* are not therapeutically active. Similar studies with active compounds containing radioarsenic may yield useful information on relative host–parasite concentrations.

Brady *et al.* (26) have used radioantimony in the same manner as was done for radioarsenic by Lawton *et al.*, to show that filaria in a host animal show a concentration of this element.

The natural radium D isotope of lead was used by Mortensen and Kellogg (207) to study the uptake of lead by the erythrocyte *in vivo* and *in vitro*. In dogs they found that doses of lead up to 1 mg. per 100 cc. of blood were completely taken up by the red cells within 10 to 15 min. after intravenous injection. *In vitro*, the process was found to have the characteristics of a first-order reaction.

In this connection it should be pointed out that the very first biological tracer application of a radioactive isotope was the study by Hevesy (131) in 1923 of the uptake of radium D by plants. Hevesy was also the first to use artificially produced radioactive isotopes as tracers in biology (39).

Even the radioactive isotopes of the rare gases have been used as tracers. Cook and Sears (45) attempted to use radiokrypton to study the mechanism of bends. They found that agents which altered blood pressure, blood flow, or capillary tone modified the time course of the concentration of the gas in the peripheral tissues in the anticipated direction. The magnitude of the changes found was not great, and the possible value of this investigation must await similar studies with other inert gases.

Other unusual instances of tracer applications include the preparation by Hamilton and Soley (117) of the hitherto unknown element 85 as a radioactive isotope, and the study of its uptake by the thyroid. The physical properties of the element are those of a heavy metal rather than of a halogen, yet it is taken up selectively by the thyroid, and treatment of the animals with thyrotropic hormone increases the relative uptake by the gland in a manner qualitatively similar to the effect on iodine uptake.

Recently Van Middlesworth *et al.* (272) reported preliminary observations on the time course of the uptake of radioactive strontium, yttrium, and plutonium in the callus of healing fractures. They injected all three isotopes simultaneously in the same animal, and were able to make determinations of all three in the same tissue samples, because the radiations are respectively pure β, γ, and α. The uptake of strontium was found to be associated with calcification, but the other two elements are concentrated in the site of fracture several days before the onset of calcification.

XI. TRACER STUDIES ON SHOCK

A number of radioelements have been used in studies to determine whether the capillary dilation that is present in shock is accompanied by a qualitative

change in the permeability of the capillaries. The data obtained from the use of Na^{24}, P^{32}, S^{35}, Fe^{59}, Br^{82}, and I^{131} are in rather general agreement and will be discussed together.

P. F. Hahn *et al.* (109, 112) found by the use of red cells labeled with Fe^{59} that the immediate rise in plasma volume which follows hemorrhage restores the total blood volume, and that as red cells are regenerated there is a corresponding decrease in plasma volume. They were also able to show that no appreciable sequestration of transfused red cells takes place in the spleen or bone marrow. However, the administration of pentobarbital was found (106) to lead to the sequestration of up to 30 per cent of the total circulating red cell mass in the spleen of the dog. Injection of epinephrine brought about contraction of the spleen and return of the red cells to the circulation.

This effect of pentobarbital, and presumably of other barbiturates, may explain why Seeley, Essex, and Mann (253) found that there was a delay in the onset of shock from manipulation of the intestines in animals under barbiturate anesthesia, in comparison with those under ether. The increase in plasma volume under barbiturates, consequent upon the removal of the red cell mass by the spleen, may have enabled a greater loss of fluid into the dilated capillaries to take place before critical degrees of hemoconcentration developed than would be the case under ether anesthesia.

Gellhorn *et al.* (92) found that the total rate of transfer of Na^{24} across the capillary membrane was reduced in shock to about half the normal value. This can readily be accounted for by the reduction in cardiac output, without reference to altered capillary permeability. Fox and Keston (81) found that, in burn or tourniquet shock in mice, about half of the extracellular sodium ion became unavailable to the circulatory system; by the use of Na^{24} they showed that the cell membranes of the tissues became permeable to this ion only in those structures which had undergone the trauma.

Bollman and Flock (23) performed similar experiments with P^{32} phosphate in rats, and arrived at a similar conclusion. They occluded the circulation to one limb for periods up to 3 hr., released the tourniquet, and then injected tracer phosphate intravenously. The distribution of the P^{32} found 1 hr. later indicated that the membranes of the traumatized muscle, and only this tissue, had become permeable to phosphate ion. At the same time the plasma inorganic phosphate showed a marked rise, indicating passage of this ion from the traumatized muscles to the extracellular phase. No evidence was found that PC or ATP diffused out of the muscles.

Seligman and Fine (254) attempted to use plasma proteins containing S^{35} to determine whether there was any loss of plasma as such to the extracellular fluid in shock. They synthesized cystine, homocystine, and methionine containing S^{35}, fed these along with casein to dogs with low plasma proteins, and some days later took plasma from these dogs for transfusion into normal recipient dogs. The over-all conversion of the fed amino acids to plasma proteins was so poor that they were unable to use this labeling agent effectively. They turned first to Br^{82} (71, 72) and then to I^{131} (71, 73) as a means of labeling the plasma proteins, by treating them with these halogens. The rate of disappearance of

these halogenated proteins from the plasma of recipient dogs was roughly proportional to their halogen content. Comparison of the rate of disappearance in normal dogs and in dogs in shock indicated that there was no leakage of proteins from the plasma into the tissues generally. Only in the traumatized tissues did the capillaries become permeable to plasma proteins.

Fine and Seligman were also able to show (71), by means of red cells labeled with Fe^{59}, that the onset of shock is accompanied by the stagnation of about one-fifth of the capillary blood volume, and concluded that the downhill course in shock results from this initial removal of blood from effective circulation, rather than to a progressive increase in the blood content of the tissues consequent on increasing capillary dilatation.

Cope and Moore (46) have confirmed the general results of Fine and Seligman by the use of brominated Evans Blue and Trypan Blue, and of iodinated plasma proteins, all containing radioactive halogen. They studied the relative specific activities of lymph and plasma following the intravenous injection of the tracer. In the normal dog, the tracer content of lymph from the extremities was much lower than that of plasma. After one leg was subjected to burn with hot water, the radioactivity of lymph from that leg rose abruptly to the level of plasma; in the resulting shock, the lymph from the uninjured extremities showed no such increase in its tracer content.

All these findings agree that there is no generalized increase in membrane permeability in shock, but that the capillaries of the traumatized area do become permeable to plasma proteins and the tissue cell membranes acquire an abnormal permeability to both cations and anions. These findings should be considered definitive in this important problem.

Another application of radioactive tracers to the study of shock is in connection with the theory that has been proposed, that the "biochemical failure" which determines the fatal outcome is a depletion of the "energy-rich" phosphate bonds, represented by ATP and PC, and the failure of the phosphorylation mechanism in which ATP occupies the central position. Studies with P^{32} phosphate by Goranson (101) have shown that in rats subjected to tourniquet shock there is no decrease in the PC or ATP content of the uninjured muscles, nor in the rate of turnover across the cell membrane. There is, however, a failure of the increase in membrane exchange produced by insulin. These observations make it necessary to exercise caution in accepting the postulate that the failure of phosphorylation mechanisms is the determining factor in the fatal outcome in shock.

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