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THE USE OF ISOTOPES AS INDICATORS IN BIOLOGICAL RESEARCH[1]

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WHILE it is undoubtedly true that the chief tool and weapon in research is thought and ideas and that a large amount of experimental work in biology is more or less wasted for lack of thought, it is not less true that progress depends to a very large extent upon methods and that new methods may open up new and fruitful fields.

It is my task to-day to present some thoughts about a new and, as I believe, extremely powerful tool for biological and biochemical research: A small number of isotopes which can be readily distinguished and quantitatively determined by relatively simple physical means.

Isotopes are atomic species which differ in weight, but have the same nuclear charge and as a consequence of this last-named property they are practically identical chemically and will behave in the same way in organisms. Isotopes of lead are available in nature which can be recognized and quantitatively determined physically by their radioactivity, and recent progress is making available radioactive isotopes of a number of elements, including some of those which are of special importance in living organisms.

The methods for recognizing and estimating radioactive substances are highly developed and easy of application.

Hevesy, himself a pioneer in the chemical and physical study of isotopes, was the first to see the possibilities offered in biology by recognizable isotopes and made the classical and fundamental experiments with radioactive lead in 1921.

Attempts over several years to separate radium D from lead by chemical means had thoroughly convinced him of their identity, but the atoms of the one carried a label, so to speak, in their radioactivity. When a plant is grown in a solution containing lead, this ele-

[1] Address given at the Harvard Tercentenary Conference of Arts and Sciences, September 10.

ment will enter the roots and become distributed all through the plant, where it can be detected chemically and determined quantitatively in the ash of stems, leaves, fruits and so on. If after a certain time the plant is transferred to an ordinary nutritive solution, containing no lead, the quantity once taken up is retained and one would assume it to be firmly combined. If a radioactive variety of lead is used the detection and estimation becomes greatly simplified, but the really important point is brought out when a plant is grown first in a solution containing radioactive lead, until equilibrium is reached, and then transferred to a solution with the same concentration of ordinary lead and it is found that the radioactive atoms gradually leave the plant and are regularly exchanged with the ordinary variety, that in other words lead atoms are in reality never fixed anywhere, but are always on the move up and down the plant to and from the single cells, to and from the organic lead compounds which are continually formed and reformed. This concept of the lability of compounds and mobility of atoms within the living organism is fundamental, and it has been broadened and deepened by all subsequent research. It turns out in this particular case that lead atoms in organic combination within a plant can also be exchanged to some extent with other heavy metals, especially copper.

Lead is a decidedly foreign substance within an organism, and it might be thought that elements which were normal constituents of the tissues would settle down more permanently. Preparing a radioactive phosphorus isotope from ordinary sulfur Hevesy and his associates have utilized this substance for a number of experiments both on plants and animals which are still in progress. Professor Hevesy has kindly allowed me to mention some of his results. On plants the results correspond closely to those obtained with lead; phosphorus atoms will travel constantly throughout the plant and be transferred during growth from old leaves to new and *vice versa,* showing much more extensive transports of substances from leaves to roots as well as from roots to leaves than hitherto assumed.

On animals it is found, and was perhaps to be expected, that phosphorus introduced by injection is almost at once distributed with the blood all over the body and becomes excreted through the kidneys and also to some extent through the gut. In one experiment on a human subject 20.3 per cent. of the radioactive phosphorus introduced was eliminated in 4 days through the kidneys and 2.5 per cent. through the gut. When in another experiment the phosphorus was taken by mouth 15.5 per cent. was eliminated through the gut and 17.9 per cent. through the kidneys, showing that quite an appreciable amount had not become absorbed.

Most of the radioactive phosphorus introduced into a mammal leaves the blood within a short time, exchanging with the phosphorus of the tissues. The exchange with the inorganic phosphorus is almost instantaneous, but also the organic phosphorus present in muscles and other organs comes gradually into an exchange equilibrium with the radioactive element.

To my mind the most interesting result is the extensive exchange taking place in bones and teeth. It is, of course, well known that the organism is able to draw upon the skeletal system as a reserve of inorganic salts, but even remembering this I have never before been able to look upon the atoms deposited in practically insoluble salts and at a considerable distance from any blood vessels, in the dentine for instance, as being in constant interchange with the atoms of the salts in solution in tissue fluids and blood. This is, however, what the experiments clearly indicate. When a single dose of radioactive phosphorus is given to an adult rat and the animal is killed after one week 29 per cent. of the dose is found in the bones, 0.2 per cent. in the molars, 3.3 per cent. in the incisors (which are growing all the time) and 3.2 per cent. in the liver. When the rat is allowed to live on for one or two weeks more the content of radioactive phosphorus (corrected of course for the regular decrease in activity) gradually decreases, because replaced by the ordinary phosphorus of the diet. This decrease is conspicuous in the bones and in the liver, but has not so far been observed in the teeth, where the exchange is much slower. When the long incisors are cut in pieces and the growing roots, a middle portion and the tips, which one would expect to be quite outside any circulation, are examined separately it is found that even here the radioactive phosphorus penetrates and an exchange takes place. So far it has not been possible to examine the enamel separately and it is a matter for conjecture whether or not this, the hardest of all tissues, is taking part in the processes of exchange.

In young rats the exchange takes place more quickly, as one would expect, and relatively more is taken up by the growing bones and teeth, but on the whole the same relations are observed.

The heavy hydrogen isotope, called deuterium to distinguish it conveniently from the ordinary hydrogen or protium, is as an isotope in a class by itself. On account of the 100 per cent. difference in atomic weight and the fact that D and H may be present and act as naked nuclei, its chemical behavior is not exactly the same as that of protium. Several reactions carried out with deuterium are definitely slower than the corresponding ones with protium. The rate of hydrogenation of fats with deuterium gas, for instance, is only about half the rate for protium. The equilibrium

constant of numerous reactions in which deuterium participates is markedly different from that obtained with protium.

Several vital processes are slowed down by heavy water, deuterium oxide, and in high concentration it is harmful or even lethal to many organisms. With this aspect of the problem I do not propose to deal. The observations go to show that in concentrations below 10 per cent., where the deuterium is mainly present as DHO, heavy water behaves in the organism just as ordinary water and can safely be used as an indicator. Deuterium can be estimated with great accuracy as "heavy water" in mixtures with ordinary water by specific gravity determinations, and the chief difficulty is to purify the sample so that it contains only water. For the specific gravity determination itself fairly simple methods are available which are accurate to the 6′ decimal place, corresponding to 0.001 per cent. heavy water in samples containing from 0 to 5 per cent. over and above the 0.05 per cent. present in all natural waters. The falling drop method of Barbour can attain this accuracy on small fractions of 1 ml.

Heavy water can be utilized in the study of a variety of biological problems. It was shown by Hevesy and Hofer that D_2O either taken by mouth or, in aquatic animals, diffusing in through the integuments and gills, is rapidly and evenly distributed all over the body so that the concentration in the urine measures exactly the concentration in all the water in the body. We have attempted in collaboration with Hevesy to use D_2O to measure permeability, and important information can be gained, but there is a definite limitation to its use. It can show whether a membrane is permeable to water or not. In the eggs of certain fishes, notably the trout, there is a stage in which the vitelline membrane is stated to be impermeable to water. We have confirmed and extended this statement by means of D_2O. Immediately after laying, trout eggs swell by taking up water. Heavy water will penetrate at a rapidly decreasing rate, and after a couple of hours the penetration ceases. When at this state trout eggs are transferred to a heavy water solution not a trace of D_2O is found, even after a day or more, in the water to be distilled off from the eggs after they have been washed superficially with ordinary water. At a much later time, when the development of the embryo has reached the stage with just visible eyes, the membrane again becomes permeable so that water can pass in osmotically and, as no swelling is observed, excretion of water must be taking place. In all cases where it is desirable to find out qualitatively whether or not water can penetrate a membrane the heavy water can be used as an indicator of very high sensitivity.

In experiments on frogs we have been able to solve the much debated problem whether or not there is a selective permeability for water in one direction. When the legs of a frog are immersed in a known volume of, say 3 per cent. D_2O, the rate at which D_2O diffuses in can be ascertained and compared with the rate at which it diffuses out, observed when a frog is saturated with 3 per cent. D_2O and the legs immersed in H_2O. All the experiments made go to show that there is no significant difference in the diffusion rates outside $\rightarrow$ in or inside $\rightarrow$ out.

The diffusion rates measured with heavy water on living membranes are of a very low order compared, for instance, with collodion membranes, but unfortunately the heavy water can not be used to measure the rate at which water passes through a membrane by osmotic pressure differences. It is tempting to assume that a certain concentration gradient, say of one mole per liter of D_2O, can cause the same movement of D_2O molecules across a given membrane as the water movement brought about by a pressure difference of one mole of a substance which can not pass the membrane, but Jacobs has pointed out that the conditions are not comparable and it is certain that in the cases examined by us, mainly on frog's skin and on artificial membranes, the rates are very different and the osmotic water transport for a given pressure difference generally larger, while the proportion varies from one membrane to another.

A very large and, as I believe, very fruitful field of research is opened up by the observation that an exchange will take place between the deuterium atoms of heavy water and certain protium atoms of organic substances.

If a definite amount of an organic substance, a protein say, is dissolved in a suitable amount of water with a known content of D_2O and the water thereupon distilled off the D_2O content is found to be reduced, and when the dry residue is burned and the water formed by combustion from the hydrogen in the protein molecule is also tested the missing D_2O is found there.

At least with dilute solutions there is a definite relation between the D_2O percentage of the combustion water and the D_2O content of the water with which the protein was in equilibrium. For albumin we have found that the D_2O content of the combustion water is 40 per cent. of the distillate, and we take this to mean that 40 per cent. of the hydrogen atoms in the protein are in a labile state which allows them to continually change places with the hydrogen atoms of the surrounding water.

The experiments of Bonhoeffer and others have shown that hydrogen atoms directly attached to the carbon chain or ring are generally not liable to ex-

change, while the hydrogen of organic acids, hydroxyl, amino and aldehyde groups are readily exchangeable. In certain cases conditions are more complicated, as in the enol form of acetone or in maleic acid, where one or two hydrogen atoms are readily exchangeable to the outside, while a slow exchange can take place within the molecule between this and all other hydrogen atoms. In suitable conditions all the hydrogen atoms can therefore be exchanged with deuterium.

In a recent paper by Münzberg this slow exchange was specially studied on pyrogallic acid with the result that the exchange in 3 hydroxyl groups was practically instantaneous. One of the D atoms thus introduced could change places with one of the fixed H atoms by a keto rearrangement taking place at intervals, and this again could change places further by a spontaneous change in the place of the double bond, occurring at very long intervals. The final result was that all the 6 hydrogen atoms could be exchanged, but at ordinary temperatures this would take years.

It is possible to utilize compounds in which deuterium atoms have been built into stable positions and also the exchange processes themselves for the solution of important biochemical problems.

Schoenheimer and Rittenberg are working along the first of these lines. By the well-known process of hydrogenation they have built deuterium atoms into linoleic acid and fed the deuterium containing fat to mice. They expected to find that small amounts of fat given to animals on an insufficient diet would be readily oxidized, but they did find that even in these circumstances most of the fat was deposited before being utilized. When the fat is broken down in the body the deuterium is set free as heavy water which will become uniformly distributed in the body. In rats and mice in which the D_2O concentration was kept approximately constant over a period of a week or 10 days we (Ussing and Krogh) found small amounts of D in the body fats, indicating a new formation of fat from carbohydrate, a formation which it should be possible to measure quantitatively when the relative proportion of deuterium in carbohydrate and in fat formed from it can be determined. Schoenheimer and Rittenberg have also recently solved the long-debated problem of desaturation of fatty acids as a normal process in the living organism by showing that when saturated fatty acids containing deuterium were fed to mice, and the body fats extracted after a suitable period and fractionated so as to separate saturated and desaturated acids, an appreciable proportion of the deuterium was found in the desaturated fraction.

Schoenheimer and Rittenberg have pointed out the great possibilities for studying intermediary metabolism opened by introducing into the body substances suspected of being links in the chain of conversion and having these "labeled" with deuterium in a stable position. When this D is afterwards found in the normal end product of the series at least the possibility of the conversion in the body is proved. They have applied the procedure in the cholesterol-coprosterol series with very promising results, and I am convinced that they have hit upon a principle of very general applicability. I expect that in a not too distant future a series of organic substances containing D atoms in suitable stable positions will become available commercially.

In my laboratory Ussing and myself, in regular consultation with Hevesy, have made a number of preliminary experiments along the second line indicated. We exposed organisms to definite concentrations of D_2O to study the exchange between the water and the tissue substances. We hoped to be able to distinguish between a more or less permanent uptake of deuterium by new formation of tissue elements and a simple exchange, of the same type as that observed *in vitro*, but possibly different in amount, owing to essential differences in constitution between proteins as isolated and proteins built into living systems.

Our first experiment was done on four equal lots of peas which were soaked in water containing D_2O and then allowed to sprout in the dark for different lengths of time up to 10 days. Contrary to our expectation the maximum of deuterium in the dry substance was found just after soaking when deuterium was present in the dry substance corresponding to an exchange percentage of 26. Later on about 20 per cent. were found so that, apparently at least, no building in of deuterium into stable positions took place.

Frogs were saturated with about 1.2 per cent. D_2O, which is accomplished simply by keeping a small amount of water with an appropriate percentage circulating about them for several days. One frog was killed and analyzed while exposed in this way, and another transferred to a small volume of ordinary water, bringing the concentration down to approximately 0.4 per cent. In both cases the percentage saturation of the combustion water corresponded to 30 per cent., showing, apparently, that we have to do, at least in the main, with a simple reversible exchange.

In a series of experiments on rats and mice kept in a cage in a sealed metabolism chamber and maintained for varying lengths of time at an approximately constant concentration of D_2O in their body fluids the deuterium concentrations in single dry organs were measured and compared with the concentrations in the distillates from them, which were always identical for the whole body. In most organs a regular exchange took place, so that an approximate equilibrium in the neighborhood of 50 per cent. was reached within a few days, but the muscles behaved differently. In the

first experiments, which lasted a week or more, the deuterium concentration reached very high figures of about 70 per cent., but the increase was very slow, as shown in a one-day experiment on a rat in which the muscles had reached only 19 per cent. when the liver was 47. An experiment on three mice is especially instructive. These mice were brought by injection to about the same concentration of D_2O and kept together in the same metabolism chamber. One was killed after 1 day and showed in the proteins of muscle and bone an exchange of 11 per cent., while in the internal organs it had reached 20 per cent. The second mouse was killed after four days when the percentage saturation in the muscle and bones was 25 per cent. and in the internal organs 37 per cent. The remaining mouse was now given ordinary water to drink, which in 5 days reduced the concentration of D_2O in the body fluids from about 2 to about 1 per cent. The deuterium content in the proteins of the internal organs went down very nearly in the same proportion, showing now a 40 per cent. concentration, but in the muscle (and bone) the absolute content of D went up further, raising the proportion to 76 per cent.

It seems out of the question that a breakdown and reconstruction of muscular tissue should proceed at anything like this rate, and we are reminded of the slow exchange taking place within molecules referred to above. An exchange of this type might in the living organism be correlated with the activity, and to test this suspicion the following experiments were made on frogs with a suitable concentration of D_2O, in which one leg was denervated, while the other was stimulated to twitches at two to three seconds interval over 24 hours. We found an exchange of about 9 per cent. in the leg kept quiet and 12 per cent. in the leg which had performed about 36,000 twitches with an aggregate duration of less than 30 minutes.

It can not be sufficiently emphasized that the experiments so far made are preliminary and tentative. At the same time it seems to me that the general lability of substances and tissues in the organism already revealed is of very great significance and that we may look forward to important developments.

With regard to the utilization of heavy water as an indicator we are strongly in need of a comprehensive study of the exchange in protein substances *in vitro* both static and dynamic, studying the influence of conditions like pH, temperature, salts and so on on the final equilibrium and the rate at which it is approached.

There are, I believe, great possibilities for the further use of the hydrogen isotope in biology, but it must be admitted that the somewhat cumbersome technique of purification and determination of the deuterium oxide is in the way of rapid progress along this line.

From this great country with its enormous resources we may perhaps even look forward to the separation of other biologically important isotopes which can be determined by specific gravity methods. Still I think that the radioactive isotopes are likely to become of paramount importance because the determination is comparatively easy and the activity remains unaffected by any chemical treatment, including ashing.

The radioactive isotopes to be used in biology must possess a fairly strong activity which generally means a short radioactive life. On the other hand, the life, as characterized by the time of reduction of the activity to one half, can easily become too short for biological or even chemical purposes.

A large number of isotopes have been prepared with half times between a fraction of a second to a few hours. These will not as a rule be available for biological research.

The half time of radioactive lead (thorium B) is 11 hours and of phosphorus 16 days, which is very convenient for our purposes. A radioactive sulfur can be generated having a half time of 60 days and reports are presented of carbon with a somewhat similar length of life.

I am exceptionally fortunate in having become associated with Professor Hevesy and through him also with Bohr. The study of radioactive isotopes is to be pushed forward in Copenhagen, and a powerful plant is being erected for their generation. We are determined to do the best we can, but we cordially invite both competition, cooperation and criticism.

SCIENTIFIC EVENTS

LETTERS AND MANUSCRIPTS OF T. H. HUXLEY

IN a letter to the London *Times* dated December 31, 1936, Lord Rayleigh, chairman of the Governing Body of the Imperial College of Science and Technology, London, and Sir Frederic G. Kenyon, chairman of the Friends of the National Libraries, have made an appeal for subscriptions to a fund to make possible the preservation of a unique collection of Huxley's letters and manuscripts, now in the possession of Mrs. Leonard Huxley. The letter follows:

"In your issue of February 14, 1936, you published an article by Sir Frank Heath describing the very interesting and historically valuable collection of letters and manuscripts relating to T. H. Huxley which are now in the possession of Mrs. Leonard Huxley.