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Editor for the Ciba Foundation

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FOREWORD

by

SIR CHARLES HARINGTON, F.R.S., M.A., Ph.D.

The advent of an entirely new tool of research is always something of an event in science. There can be few more striking illustrations of this than the story of the advances in knowledge that have been made with the aid of isotopes. The speed of development of this branch of research is due to the realization of some of the potentialities of the method even before separated isotopes were available, and to the eagerness with which such pioneers as Hevesy exploited the possibilities as soon as the discovery of induced radioactivity made the preparation of minimal amounts of radioactive isotopes possible; it was still further increased by the dramatic circumstances of the war-time work on nuclear fission, which brought the technique within the reach of laboratories in all branches of science.

So far as biological science is concerned it was obvious at the outset that the tracer isotope technique would find its widest application in biochemistry; it offered indeed a method of attack on problems of intermediary metabolism and biosynthesis which had hitherto been unapproachable. It was unfortunate, though perhaps only natural, that the sudden general availability of isotopes for biochemical research should have led to some rather hasty experimentation in which the more obvious problems were studied without deep thought. Such work, although it may provide quick answers to some questions of interest, is far from representing the best use of the new method.

As time has passed, there has been more opportunity for reflection, and the result of this is apparent in the nature of the biochemical problems that are now being tackled with the
aid of isotopes as tracers. All of these problems require powers of biochemical interpretation; many of them demand also new work in organic chemistry, both for the synthesis of the specifically labelled precursors whose metabolic fate it is desired to study, and for the stepwise degradation of the biosynthetic product, so that the process of its formation can be analysed. Again new methods of isolation of intermediary metabolites may have to be devised. Thus the full development of the isotope technique in this field can only be achieved with the aid of concurrent advances in more conventional chemical and biochemical methods.

The collection of papers in the present volume, contributed at an informal meeting by a number of leading workers, affords as good an illustration as could be wished of the stage of isotope research in biochemistry that has now been reached by the process of development that has been outlined. The Ciba Foundation are to be congratulated on performing a useful service in assembling a number of contributions which are not only of the greatest intrinsic interest but, in so far as they indicate the potentialities of isotope research in biochemistry, will serve as an inspiration for the further cultivation of this productive field of work.
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CHAIRMAN'S OPENING REMARKS

A. S. McFARLANE

With some notable exceptions, the biological use of isotopes has lagged in Europe by comparison with America, and this is a matter which has to be put right if the high standard of European biological science is to be maintained. A step towards doing this can be made by means of conferences with our American colleagues and to-day we make an important start in this direction. We are helped by having the example of several similar post-war U.S. conferences to follow—notably the meetings held at Wisconsin in 1947, and at Cold Spring Harbor in 1948, and we are additionally fortunate in being the guests of the Ciba Foundation. This will be the tenth conference held by the Foundation and already a high reputation has been established for efficiency. You will note a main idea behind their organization, namely, strict limitation of membership to people who are actively engaged in research in the subject under discussion. Proceedings will be recorded, but nothing will be published without the approval of the speaker concerned. In the privacy of this room, and in the company of kindred workers, it is hoped that discussion will be full and only restrained by considerations of time.

Everyone will agree, I am sure, that such a conference is held most opportunely. Almost without exception, the Ciba Foundation's invitations have been accepted and difficulties of travelling largely ignored. One is tempted to enquire why leading isotope users—all of them busy people—are prepared to travel vast distances to be here. What is this conference likely to offer that cannot be obtained at home by reading the literature? In the few moments at my disposal I would like to try to answer this.

Biological application of isotopes has brought to light many surprises, with which we are all familiar, but I think most
people will agree that the result to date is by no means a simplification of our former conceptions of how the animal body works. It appears rather that the use of isotopes has uncovered problems of greater complexity than the ones they were intended to solve. Perhaps this is not quite a fair generalization since at least in the case of two elements, namely iron and iodine, relatively clear-cut metabolic pictures have emerged—and for the same reasons, namely that the body reserves of both elements are relatively small, and in each case one metabolic pathway predominates over all others.

In the case of almost all other elements and especially, of course, with compounds of carbon complex interconnected chains of biochemical events have come to light. Where these chains cross and so-called “metabolic pools” exist, the investigator’s field of interest is necessarily broadened beyond that of his immediate problem; for example, the expert in porphyrin synthesis is compelled to take an interest in the broader field of amino-acid metabolism. It seems that more than ever before the specialist has to pay attention to his general biochemistry, and herein presumably lies one reason why he welcomes such an opportunity as this to meet experts in other branches of biochemistry.

The second reason arises from the fact that the literature is remarkably disjointed and incomplete, and it is extremely difficult for the reader to correlate the findings of one tracer user with those of another. For instance, in spite of du Vigneaud’s demonstration that doubling the dietary level of methionine in the rat increases the proportion of amino-acid oxidized nine-fold, some workers still employ doses of labelled compounds which are comparable to the dietary intake. Others use the dubious procedure of starving or otherwise depleting their animals beforehand in order to enhance the isotope uptake. Far too often, also, samples are taken—especially of blood—which are large enough to disturb significantly the physiological balance.

These difficulties are all avoidable by the preparation of labelled material of high specific activity, by refinements in
counting technique or by the use of larger experimental animals. There will be advantages also in using one, or at most a few, kinds of animal, since species differences may be surprisingly great. Whereas in normal man 90 per cent of a dose of radioactive-iron is utilized for haemoglobin production, the same figure in the rat or dog never rises above 60 per cent.

There is also little uniformity in the manner of administering labelled substances. Whereas Whipple and his collaborators feed amino-acids to their dogs and find peak values for free labelled amino-acids in the plasma at 4–6 hours, Borsook uses intravenous injection and finds that the free amino-acid has largely disappeared from the plasma in 10 minutes. Even the method of expressing results is sometimes confusing. Whereas some plasma protein investigators give their results in the form of the proportion of amino-acid in the protein which was derived from the labelled amino-acid in the diet, others give the proportion of total administered isotopic amino-acid which appears in a given weight of plasma protein—and the figures cannot easily be related. These inconsistencies which may appear to be of a minor technical nature stand, nevertheless, in the way of an altogether wider view of metabolic events. If our discussions, ranging as they will over several important fields and contributed to by leading protagonists in each, can serve to smooth out the technical difficulties while at the same time keeping in view the broader principles, the effort made by many to attend the Conference and by the Ciba Foundation to arrange it will be amply repaid.
PART I—STEROIDS

METABOLISM OF $^{14}$C-LABELLED STEROIDS*

C. P. LEBLOND

The basic postulate of tracer work is that the changes exhibited by the radioactive material also take place normally under physiological conditions. This is true only if the amount of radioactivity is kept low enough to avoid radio-chemical effects, and if the amount of the labelled substance is such that it can mix with the same substance in body fluids and tissues without significantly altering the physiological level of the material under investigation. In the case of $^{14}$C labelled steroids, the first requirement is easily satisfied but not the second.‡ It is indeed quite difficult to trace physiological amounts of $^{14}$C-labelled substances which exhibit their biological activity in minute doses, as is the case with oestrogens. The problem, on the other hand, is somewhat easier in the case of substances active in larger doses, for example progesterone, with which we may hope to obtain in the future truly physiological results. At any rate, a greater amount of radioactivity per mg. of substance, i.e. a higher specific activity, may be expected from (1) the use of


‡Substances labelled with radiocarbon must of necessity contain a large amount of carrier since the half life of $^{14}$C is about 5,500 years, and therefore, one millicurie must contain at least enough atoms to have $3.6 \times 10^{7}$ atoms exploding every second over thousands of years. Thus, 1 mc. of radiocarbon as barium carbonate must weigh at least 3 mg. In practice, the available radiocarbon weighs several times that amount, due to contamination with non-radioactive carbon in the course of preparation. As a result, the dose of a $^{14}$C-labelled substance which is administered for tracing purposes will often be greater than the amount of the natural substance present in body fluids.
radiocarbon uncontaminated with non-radioactive carrier, and (2) the labelling of as many carbon atoms as possible.

Two types of methods for tracing radioactive material in the body exist. The first is by the Geiger Counter tube, by which quantitative estimates are obtained with extracts of tissues and excreta. The other technique consists of the recognition of radioactivity by means of its effect on photographic emulsion. In this case, the detector is the photographic grain, the size of which makes it suitable for the microscopic localization of radioactivity. The autoradiograph technique consists of placing emulsion in intimate contact with a thin section of tissues of animals sacrificed at various intervals after administration of radioactive material. Recently, a similar method has been applied to the localization of radioactive spots in paper chromatograms. This method, which makes it possible to identify substances with a precision comparable to that of melting point determinations, can be used to locate infinitesimal amounts of a substance provided it contains radioactivity and a sufficiently long exposure is used. However, the use of both the Geiger counter and radio-autographic techniques is complicated by the low specific activity of the carbon present in radioactive steroids. Thus, in using the Geiger counter, the easiest method with the soft $\beta$-rays of $^{14}$C is to employ preparations of infinite thickness, for which corrections for absorption are unnecessary. But this method has a low sensitivity, and to detect the small amounts present in tissues, thin samples may have to be used, and complex corrections for absorption may have to be made. In autoradiography the main difficulty is that the preparations require very long exposures, a year in the case of the slide illustrated in Figs. 1 and 2.

A number of radioactive steroids have been synthesized: progesterone labelled with $^{14}$C in positions 21 or 3; cestrone labelled in position 16; and deoxycorticosterone acetate (DCA), labelled in positions 21 and 3. Thus, the chemical part of the research is well advanced, more so than the biological. There have been a few reports on the use of
radioactive steroids: (1) We examined the distribution of radio-iodine-labelled oestradiol and the outstanding findings (presented in May, 1947, before the American Association for Cancer Research) were the major role played by the gastrointestinal system in promptly disposing of the material, and the lack of any remarkable concentration in the secondary sex organs with the exception of the mammary glands. Unfortunately, when the di-iodo- and mono-iodo-a-oestradiol used were assayed in spayed rats by the vaginal smear test, which is sensitive to 0.1 μg. of oestradiol, neither of these substances displayed oestrogenic activity in doses up to 100 μg. Thus, some doubt has been cast on the significance of the results obtained with these substances (Albert, Heard, Leblond and Saffran, 1949). (2) Twombly, McClintock and Engelman (1948) obtained similar results with equilin brominated with radio-bromine. (3) A recent paper by Riegel, Hartop and Kittinger (1950) deals with the metabolism of [21-14C]-progesterone in mice and rats. Their main findings are that a considerable proportion of the material is recoverable as labelled CO₂ in the expired air. Some is excreted in the faeces but none in the urine. Finally, a significant concentration of material is present in the pituitary, adrenals and ovaries.

Like the above, the results to be reported here are of a preliminary nature and refer to pilot experiments only. Three steroids have been investigated so far: [16-14C]-œstrone, [21-14C]-progesterone, and [21-14C]-deoxycorticosterone acetate (DCA).

**Methods and Techniques**

(1) **Preparation of Radioactive Steroids.** Radioactive œstrone was prepared by Heard, Saffran and Thompson by opening ring D, addition of 14C-diazomethane to the resulting aliphatic side chain through the Arndt and Eisert reaction and pyrolysis of the corresponding homo-di-acid back to the original ketone. Radio-progesterone and radio-deoxycorticosterone were obtained by Heard and Yates. [21-14C]-progesterone
was prepared from \([21-^{14}C]-21\)-diazoprogesterone by the action of hydriodic acid, and \([21-^{14}C]^{-}\text{DCA}\) from the same labelled intermediate by treatment with acetic acid. \(21\)-Diazoprogesterone was prepared from \(3\)-keto-\(\Delta^4\)-etiocholenone chloride by an improved Arndt-Eisert synthesis.

(2) Mode of Administration. After trying a variety of solvents and doses, the following procedure was adopted. A dose of 1 mg. of radioactive steroid is dissolved in 0.25 ml. ethyl laurate and injected at 9 p.m. intramuscularly in the neighbourhood of the head of the femur. Immediately after injection the animals are placed in a metabolism chamber without food or water. The air circulating through the chamber is first freed of \(\text{CO}_2\) by being passed through barium hydroxide solutions. The air coming from the chamber is circulated through two sodium hydroxide bottles for trapping the \(\text{CO}_2\) exhaled by the animals. Contact of the feet of the animals with the floor of the chamber is prevented by a wire sheet, in such a way that the urine falls to the bottom but the faeces are retained on the wire.

All the animals used for complete distribution studies were sacrificed twelve hours after injection by exsanguination from the inferior vena cava under ether anaesthesia. In addition, 5 mice were given 1 mg. of cestrone, and the faeces and urine were collected for 144 hours, after which the animals were sacrificed. \(\text{CO}_2\) was collected for the first 12 hours and again for 12 hours starting at 97 hours after injection. Five mice were similarly treated with progesterone and 5 other mice with DCA.

(3) Preparation of Samples of Tissues and Excreta for Counting. In the animals sacrificed 12 hours after injection, about 40 tissues and excreta were examined with the Geiger counter. In the animals sacrificed at 144 hours, only plasma, kidney, adrenal, thyroid, hypophysis, liver and gall bladder were investigated. The organs were removed from the animals and the excreta digested in sodium hydroxide, plated on a brass planchette and the plates carbonated. The more reliable results were obtained with infinitely thick preparations.
The technical details will be published elsewhere. The results were expressed as the percentage of the recovered dose and therefore did not include the amount retained at the site of injection. The concentrations were also recorded as the number of counts per mg. of organ over the number of counts recovered in tissues and excreta per mg. of body weight.

(4) Preparation of Autoradiographs. The tissues removed from the animals for autoradiography were fixed in 10 per cent formalin at pH 5–6. The tissues were cut on the freezing microtome and coated with photographic emulsion according to a previously described method. The slides were then placed in a light-tight box containing a dehydrating agent and were stored in a low temperature cabinet for several months. The preparations were developed and fixed according to routine photographic procedures. They were then mounted under balsam and protected with a coverslip (Leblond, Percival and Gross, 1948).

Results

(1) Distribution of Òestrone. The distribution of radioactive Òestrone in about 40 organs and tissues was examined in two tumour-bearing C₃H mice. Animal number 1 had several very large tumours, and animal number 2, a rather small one. The plasma contained a fair amount of radioactivity, approximately equal to that found in the red blood cells. An outstanding feature of the metabolism was the major role played by the gastro-intestinal tract. There was little radioactivity in the stomach but a considerable amount in the intestine and fæces. In animal number 2, the bile was highly radioactive, thus indicating a major role of biliary excretion. The secondary sex organs, uterus, vagina and mammary gland contained a small amount of radioactivity. Urinary excretion of Òoestrogens has received much emphasis in the past. Indeed, 27 per cent of the dose was present in the urine of the first animal and 10 per cent in the second. A fair concentration was found in the kidney, especially in animal number 1.
The nature of the radioactive material present in urine and faeces was especially investigated in the animals sacrificed at 144 hours. Thus, in the urine only 15 per cent of the activity was removable by ether and, after acid hydrolysis in the autoclave, only another 10 per cent became ether-soluble, the rest, or roughly 75 per cent, being water-soluble. The situation in the faeces was very similar, with only a small portion soluble in ether.

In the long duration experiment with 5 mice the loss of CO$_2$ was 2.0 per cent in the first 12 hours following injection. Another estimation at 97 hours after administration gave an elimination of 1.1 per cent for a 12-hour period.

(2) *Distribution of Progesterone.* A complete distribution of radioactivity after administration of radioactive progesterone was determined in one tumour-bearing C$_3$H mouse. The following points were observed: (a) the radioactivity in the plasma was not high and was within the range of that of the red blood cells. The blood level may have been maintained for a very long period since it remained appreciable at 144 hours. (b) A major role of biliary excretion was indicated by a high concentration in the bile and large amounts in the intestine at 12 hours after injection. (c) However, urinary excretion tended to be more pronounced than faecal excretion, at least during the first 12 hours following injection. (d) As in the case of oestrone, partition of urine and faeces revealed that the greatest proportion of the excreted material was water-soluble and remained so after hydrolysis. (e) Neither secondary sex organs, nor endocrines showed a high concentration of radioactivity. (f) The loss of radioactivity as CO$_2$ was 2.5 per cent within 12 hours after injection in the long duration experiment with 5 mice. Another estimation at 97 hours after administration gave an elimination of 0.9 per cent for a 12-hour period.

In another experiment, two mice were castrated and three weeks later one of them received 2 $\mu$g of oestradiol a day for 3 days, after which they both received the standard dose of radioactive progesterone. The oestradiol-treated animal was
the only one to have a concentration above background in the uterus.

The autoradiographic detection of pure progesterone has not yet yielded results. However, impure progesterone containing methyl 3-keto-4-etocholestanate showed interesting autoradiographic reactions. The most precise localization was observed in liver and uterus (Figs. 1 and 2).

(3) Distribution of Deoxycorticosterone Acetate. The results at 12 hours after injection were very similar to those reported above. However, with this substance the urinary loss was greater than the faecal loss. The intestinal content of radioactivity at 12 hours after injection was correspondingly lower than with other steroids. The material in urine and faeces was mainly water-soluble. The smallest concentration to be found in any organ was in the adrenal. The radioactive material persisted in blood until 144 hours after injection, while it had vanished from other organs and tissues. The loss as CO₂ was 6.7 per cent within 12 hours after injection in the 5 mice in the long duration experiment. At 97 hours no activity could be detected in the CO₂ over a 12-hour period.

Discussion

The most clear cut results were those obtained in relation to excretory processes. Faecal excretion played an outstanding role, especially with oestron and progesterone; and, since the highest concentrations by far were found in the bile, there was little doubt that the bile was the source of the material excreted in the faeces through the intestine (as demonstrated by Albert et al. for the iodo-oestradiols). The fact that the faecal material, as well as the urinary material, was mainly insoluble in fat solvents even after hydrolysis indicated that the metabolism of oestron, progesterone and deoxycorticosterone in mice differed from what was known to occur in man. This was indeed found to be the case in the work reported by Gallagher in this volume. The excretion as CO₂ was appreciable, but smaller than that by faecal and urinary routes. The fact that we obtained smaller CO₂ excretion and greater
Fig. 1. Unstained autoradiographs of kidney (upper right), uterus (U, upper centre), and liver (L, lower). Low magnification.

A light reaction is present in kidney and most of the uterus. A pronounced reaction is present in liver tissues. The reaction is minimal near the hepatic vein and portal veins, and maximal half way between the two.

Fig. 2. Unstained autoradiograph of uterus. Higher magnification. An intense reaction of the endometrium is apparent.

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urinary elimination of progesterone than did Riegel and co-workers could probably be explained by the difference in injection routes, since these authors used intraperitoneal administration. The absorption through the liver which occurred in this case would lead to a greater breakdown to CO₂ and a greater excretion into the faeces through the bile. It is indeed well known that progesterone and its derivatives are detoxified in the liver (Leblond, 1942).

The radioactivity figures for organs were not very informative. They may be expected to become most useful when two further steps in the progress of this investigation are taken: the chemical analysis of the nature of the radioactive material present, a study which will require the use of such techniques as paper chromatography; and the autoradiographic localization of the radioactive material. Thus, in the case of the uterus, sections of which are shown in Figs. 1 and 2, the radioactivity content as estimated with the Geiger counter did not significantly differ from that of other organs. Nevertheless, some radioactive material was selectively concentrated in the epithelial cells.

Summary
Preliminary experiments have been carried out with cestrone, progesterone and deoxycorticosterone acetate labelled with ¹⁴C. The results obtained and some possibilities for future work are discussed.

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REFERENCES
DISCUSSION

Boscott: Have you tried tritiated oestrone or oestradiol? It presumably should be available from $\Delta^4$-oestrone, which Dr. Djerassi of Syntex Ltd., Mexico, has recently synthesized from cholesterol. Also, Dr. Miescher's work on the $\Delta^{11}$-progesterone series should give another route of tritiated progesterone, deoxycorticosterone and so forth. Would tritium have any advantage over $^{14}\text{C}$?

Leblond: These possibilities have not been examined, since there is a considerable difference in mass between hydrogen and tritium, and physiological behaviour may be different. Even with deuterium results different from those obtained with hydrogen have been recorded, for instance in the case of water metabolism (Barbour).

Boscott: Tritiated hexaestrol has been recently synthesized at Oak Ridge.* Have you heard anything about the results?

Leblond: I am not acquainted with the biological results.


* D. L. Williams, A. R. Ronzio. Unclassified publication, AECU-714 LADC-750; Micro-syntheses with tracer elements. XVI. The synthesis of hexaestrol labelled with tritium.