Croonian Lecture

Thyroxine: its biosynthesis and its immunochemistry

By C. R. Harington, F.R.S.

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The suggestion that thyroxine might be formed in nature from tyrosine through the stage of diiodotyrosine was made at an early stage of the elucidation of the chemistry of thyroxine, and was made more probable when the constitution of the latter was finally determined. Over a number of years several pieces of evidence, all indirect in character, were brought forward in support of this biogenetic hypothesis which thus came to be generally accepted. Recently two lines of direct evidence have become available which seem to place the matter beyond doubt. In the first place the transformation of diiodotyrosine into thyroxine has been effected by purely chemical methods of a character which make it possible to formulate a theory of the chemistry of the process involved. Secondly, by the application of modern biochemical technique, the actual synthesis of thyroxine from diiodotyrosine has been demonstrated in surviving thyroid tissue in vitro. The latter type of experiment incidentally offers an opportunity for the analytical study of the action of substances such as thiourea which inhibit thyroid activity supposedly by interfering with the biosynthesis of the hormone.

Accepting the mechanism of biosynthesis of thyroxine as being satisfactorily established we are left with two outstanding problems. Is thyroxine itself the actual circulating thyroid hormone, and if so, by what mechanism does it exercise its effect in the periphery? To the second of these questions no answer can yet be given. Evidence regarding the first is conflicting and in the attempt to obtain a definitive answer an approach has been made along a new line which raises matters of some general interest. The method is based on the theory, deduced from the known facts of immunological chemistry, that an antigen of which the determinant group is a physiologically active substance should give rise to an antiserum capable of inhibiting the characteristic activity of this substance. Application of this idea to the problem of thyroxine involved the development of a new technique for building up artificial antigenic complexes. Such a complex containing thyroxine as the determinant group has proved to be able to give rise to an antiserum which can inhibit the physiological action both of a protein containing thyroxine, such as thyroglobulin, and of thyroxine itself. The latter observation, together with extension of the experimental method to an entirely different compound, favours the hypothesis that thyroxine itself is in fact the actual circulating thyroid hormone.

In a recent review of the physiology of the thyroid gland an American authority on the subject (J. H. Means 1943) has discussed the question of the evolutionary development of the gland and of its specific hormone; he sums up his views in these words: 'As to which came first, the gland or the hormone, I think we can safely say the hormone, but in a form of low potency. The evolution of the gland, like the evolution of the cerebral cortex with respect to sensory and motor activity, has raised the whole process to one of high-powered efficiency.' It is not clear to me exactly what Means wishes to imply by the phrase 'in a form of low potency', but it is my main concern in this lecture to present the chemical and biochemical evidence bearing on the mechanism by which the thyroid hormone is formed in the body from simpler substances; in the course of the discussion I shall have
occasion to mention certain facts which will indicate the reasons that led Means
to make the statement which I have quoted.

Although there is still room for discussion concerning the nature of the actual
circulating thyroid hormone, the specifically active portion of this hormone is
unquestionably the iodine-containing compound thyroxine, which is the only
physiologically active substance which can be isolated in the pure state from the
gland; the problem which I wish first to consider is therefore the mechanism by
which thyroxine is synthesized in the living organism, assuming for the present
that thyroxine is itself the complete hormone.

It is convenient first to consider the biosynthesis of thyroxine as a problem in
organic chemistry. This is an aspect of the matter which I have recently had
occasion to discuss in detail elsewhere, so that I shall here confine myself to a
summarized statement of the relevant facts and a brief reference to the bearing
of modern chemical theory on their interpretation.

The broad outline of a probable process of natural formation of thyroxine
immediately became apparent when the constitution of desiodothyroxine or
thyronine was elucidated (Harington 1926). It will be recalled that this compound,
derived from thyroxine by substitution of hydrogen atoms for the four iodine
atoms of the latter, was shown by degradation and synthesis to be the \( p \)-hydroxy-
phenyl ether of tyrosine:

\[
\begin{align*}
\text{HO} & \quad \text{O} \quad \text{CH}_2\text{.CH(NH}_2\text{).COOH.}
\end{align*}
\]

Consideration of this formula, in conjunction with the fact that the only other
iodine-containing amino-acid known to exist in nature was 3:5-diiodotyrosine,
led to the deduction that thyroxine was probably formed by coupling of two
molecules of diiodotyrosine with loss of one side-chain:

\[
\begin{align*}
\text{HO} & \quad \text{I} \quad \text{CH}_2\text{.CH(NH}_2\text{).COOH} + \text{H} \quad \text{O} \quad \text{CH}_2\text{.CH(NH}_2\text{).COOH}
\end{align*}
\]

Although the process by which thyroxine was actually synthesized in the laboratory
(Harington & Barger 1927) did not employ tyrosine or diiodotyrosine as inter-
mediates, the biogenetic hypothesis was considered sufficiently plausible to deter-
mine the plan of the synthesis with respect to the orientation of the iodine atoms
in the final product, in the absence of any direct chemical evidence bearing on this
orientation; the successful outcome of the synthesis justified the reliance which
had been placed on the underlying idea. As a first piece of chemical evidence in favour of the hypothesis of formation of thyroxine from diiodotyrosine we therefore have the actual constitution of the former compound.

The next piece of evidence came from further study of the chemical composition of the thyroid gland. Throughout the earlier work which had ultimately led to the isolation of thyroxine attention was naturally concentrated on those iodine-containing fractions which showed physiological activity; it was realized, however, that these fractions represented no more than a part of the total organically combined iodine of the thyroid, and after the chemistry of thyroxine had been cleared up a return was made to the study of the non-thyroxine fraction of the gland. It may be recalled that the isolation of thyroxine from the thyroid was achieved by a process of graduated alkaline hydrolysis; at an early stage in this process the iodine became separable into two fractions, one of which was acid-insoluble, physiologically active and led eventually to pure thyroxine, whilst the other was acid-soluble and showed no physiological activity. In view of the idea which had already been formed regarding the origin of thyroxine there was naturally a strong suspicion that the acid-soluble fraction derived from the thyroid might contain diiodotyrosine, and such indeed proved to be the case (Harington & Randall 1929). Moreover, careful quantitative study of the hydrolytic products of the thyroid led to the conclusion that the total iodine content of the gland was entirely accounted for by thyroxine and diiodotyrosine, the latter actually being present in preponderating amount. This work therefore provided the proof, essential to the theory which is being developed, that the hypothetical precursor of thyroxine was a natural constituent of the body and that it occurred in high concentration in the very situation where it would be required for the synthesis of thyroxine.

An entirely different mode of attack on the problem became possible when thyroxine was isolated in the optically active condition, for it is clear that if in fact thyroxine is derived in nature from tyrosine through the stage of diiodotyrosine the three compounds must be configuratively related. The original method of isolation of thyroxine by alkaline hydrolysis led to a racemized product, but it was later found possible to isolate small amounts by a long and complicated process of digestion with proteolytic enzymes; in the same way diiodotyrosine could be isolated and both these amino-acids, which were thus incidentally proved to be constituents of thyroglobulin, the characteristic protein of the thyroid, were obtained in the optically active condition (Harington & Salter 1930; Harington & Randall 1931).

At the time of which I am speaking no direct method of converting diiodotyrosine into thyroxine was known; a process was, however, worked out by which thyronine could be synthesized from tyrosine, and when this synthesis was carried out with natural \( l \)-tyrosine as the starting material an optically active thyronine was obtained which, owing to the nature of the reactions employed, could be certainly related configuratively with the original tyrosine. On the other hand, thyronine is obtained from thyroxine by a reaction (catalytic deiodination) which
does not involve the asymmetric centre, and therefore another sample of optically active thyronine could be prepared by deiodination of optically active natural thyroxine. When these two optically active preparations of thyronine were compared they were found to be identical; the proof, though indirect, was therefore complete of the configurative relationship of tyrosine and thyroxine (Canzanelli, Harington & Randall 1934).

We thus reach a point at which we have as facts supporting the general hypothesis (1) the structure of thyroxine itself, (2) the demonstration that the supposed intermediate in its biosynthesis, namely, diiodotyrosine, occurs as a normal constituent of the thyroid gland, and (3) the proof that the stereochemical configurations of natural tyrosine and natural thyroxine are such as would be required if thyroxine were derived from tyrosine; there is still lacking however a proof of the chemical feasibility of the direct transformation of diiodotyrosine into thyroxine, and it is only as the result of the work of the last few years that it is possible to offer such a proof.

The attempt to obtain physiologically active products by the action of iodine on proteins is no new thing; such attempts, indeed, date from the original demonstration of the presence of iodine in the thyroid and its association with the characteristic physiological activity of the gland, a demonstration which was first made nearly fifty years ago. From the many studies of iodination of proteins which were made up to five years ago certain facts did indeed emerge, of which the most significant, both in relation to the problem under discussion and also in respect of its application in other fields of biochemistry, was the discovery that in most cases the predominant effect of the action of iodine on a protein was to cause substitution in the tyrosine groups with production of diiodotyrosine. In so far as the search for thyroid-like activity among the iodinated products was concerned the record was, however, one of almost unrelieved failure until a few years before the present war, when Abelin (1934–8) published a series of papers in which the consistent production of physiologically active iodinated proteins was claimed. Owing to the absence of any proper chemical characterization of the products obtained by Abelin there was hesitation in accepting his claims, but in 1939 a paper appeared from Ludwig & von Mutzenbecher in which a method was described for the iodination of casein to give products which were physiologically active and of which the activity could be explained by the isolation from them of pure thyroxine. This discovery, which was rapidly confirmed in my own and other laboratories (Harington & Pitt Rivers 1939), and which, by offering a means of producing an effective thyroid substitute cheaply and easily, not only from casein but from other proteins rich in tyrosine, has led to results of some practical importance, opened up again the possibility of the direct chemical conversion of tyrosine into thyroxine.

Clearly there were two alternative explanations of the formation of thyroxine under the conditions employed by von Mutzenbecher; either the protein contained thyronine, as a hitherto unrecognized constituent, which was directly iodinated
to form thyroxine, or the first effect of the iodine was to produce diiodotyrosine which was secondarily converted into thyroxine. All the evidence which I have described hitherto, together with other considerations which I cannot discuss in detail here, pointed to the second explanation as the correct one, and this conclusion was reinforced by the later observation of von Mutzenbecher (1939) that a very small amount of thyroxine was formed from diiodotyrosine itself on prolonged incubation of the latter in alkaline solution.

Both in the last-mentioned experiment and in the iodination of casein it was reasonable to suppose that the actual conversion of diiodotyrosine into thyroxine was an oxidative process; since the conditions both in the protein iodination experiment where iodine was added to an alkaline solution and in the incubation of diiodotyrosine where small amounts of iodine would be slowly split off in the alkaline medium, favour formation of hypoiodite, it could be further concluded that this was the effective oxidizing agent. On the basis of such a supposition T. B. Johnson (Johnson & Tewkesbury 1942) proposed an outline theory of the process, but without offering satisfactory experimental evidence in support. At this stage we took up the experimental study of the question in my own laboratory, and after numerous unsuccessful attempts we have found that it is possible under suitable conditions to oxidize diiodotyrosine directly to thyroxine in yields which though small are significant and greatly exceed those obtained by von Mutzenbecher; moreover, in these successful oxidations we have employed a reagent, namely, hydrogen peroxide in alkaline solution, which is well known frequently to resemble biological oxidations in the transformations which it brings about. Furthermore, by consideration of analogous oxidations of phenols in alkaline solution and by application of modern conceptions of the resonance structure of phenoxide ions of the type of diiodotyrosine as it exists at the pH of the body tissues, we have been able to develop a theory of the reaction which not only gives a reasonable explanation of the mechanisms involved but leads to the conclusion that among the various possible courses which the reaction may follow, that leading to the formation of a diphenyl ether with loss of the side-chain of one molecule (i.e. to thyroxine) is actually favoured by the presence of the iodine atoms in positions ortho to the phenolic group as they occur in diiodotyrosine (Harington 1944).

From the brief summary which I have given I think it is reasonable to claim that purely chemical methods of attack, both theoretical and practical, have done about as much as could be expected to establish the possibility, and indeed the likelihood, that the biosynthesis of thyroxine follows the course which has been suggested; attention may now be given to a consideration of the contribution which has been made by a more biochemical approach.

It is obvious that a direct in vitro demonstration of the power of thyroid tissue to convert tyrosine into diiodotyrosine and the latter into thyroxine would provide the final piece of evidence which is necessary to establish the validity of the hypothetical biosynthesis of thyroxine under discussion. Experimental results which
go a long way in this direction have recently been forthcoming from some American investigations to which I have now to refer.

Technical difficulties, chiefly of an analytical nature, make the study of the biochemical process in vitro by ordinary methods a formidable task. The bulk of the evidence at present available comes from experiments employing radioactive iodine as an indicator which have been carried out by Chaikoff and others in California. This particular problem is, indeed, one that lends itself readily to investigation by the radioactive tracer technique which has proved such a powerful weapon in the hands of American biochemists.

The experiments fall into two main groups according to whether they are carried out on the intact animal or on surviving thyroid tissue in vitro. In the first group the animal is injected with a minute amount of radioactive iodide, and the distribution of radioactivity in the body is subsequently determined; in the second group slices of thyroid tissue are allowed to respire in a medium to which has been added a tracer quantity of radioactive iodide and the fate of the latter is followed.

It has long been known that the thyroid is unique among the organs of the body in containing iodine in significant concentration, and that this fact is associated with the great power of the gland to concentrate iodine which is introduced into the body. It is therefore no matter for surprise that as soon as 2 hr. after the injection of radioactive iodine 10–15% of the total radioactive material is found in the thyroid although the latter represents less than 0·01% of the body weight; after 48 hr. the proportion of the total radioactivity in the thyroid may be as high as 50%. If the matter is pursued further by chemical fractionation of the thyroid gland it is found that the radioactive iodine is distributed over the three fractions representing inorganic iodide, diiodotyrosine and thyroxine respectively. Quantitatively, the greatest concentration is found in the diiodotyrosine fraction at all time intervals over 2 hr. after the injection; in the early stages the amount of radioactivity associated with the thyroxine fraction is small, but this increases as time goes on; thus in sheep it has been found that after 2 hr. 2–10% of the total dose of radioactive material injected is associated with the diiodotyrosine fraction and 0·1–1·0% with the thyroxine fraction; after 48 hr. the corresponding figures are 20–30 and 2·3·5% respectively, the trend being what one would expect on the assumption of a rapid formation of diiodotyrosine followed by a slower conversion of the latter into thyroxine (Perlman, Morton & Chaikoff 1941).

When one comes to the experiments with surviving thyroid tissue one finds that a similar series of changes can be demonstrated in vitro. Experiments of this type are necessarily of short duration, the effective limit being 3 hr.; nevertheless, it has been shown by Morton & Chaikoff (1941–3) that during such a brief period a very large proportion of radioactive iodine added to the medium in which thyroid slices are respiring is incorporated into organic combination in the tissue; fractional analysis of the tissue shows that, as in the experiments with the intact animal to which reference has been made, the greater part of the radioactivity is associated with diiodotyrosine; the change with time of the relative amounts of radioactive
iodine found in diiodotyrosine and in thyroxine again accords with the hypothesis of conversion of the former into the latter.

A significant fact emerging from these experiments is that the reaction by which the incorporation of radioactive iodine into organic combination is brought about is associated with the state of organization of the tissue; whilst the reaction occurs, as we have seen, readily with slices of tissue it is much slower when the tissue is minced and is no longer demonstrable at all when it is homogenized. This observation not only disposes of the criticism that the changes in distribution of radioactive iodine represent no more than chemical interchange reactions, but it indicates the participation of an intracellular enzyme system.

The suggestion that an intracellular oxidizing system is involved is further reinforced by the finding that the reaction does not occur under conditions of complete anaerobiosis, and that in presence of oxygen it is inhibited if the medium contains such substances as cyanide, azide or hydrogen sulphide, which are typical inhibitors of cytochrome oxidase (Schachner, Franklin & Chaikoff 1943).

Apart from the light which they have thrown on the processes occurring in the thyroid gland, experiments with radioactive iodine have revealed an interesting and unexpected phenomenon, namely, the apparent possibility of biosynthesis of thyroxine outside the thyroid gland. In these investigations which are again due to Chaikoff and his collaborators (Morton, Chaikoff, Reinhardt & Anderson 1943) young rats were thyroidectomized at 4-6 weeks of age; after periods of 2-8 months the animals, whose basal metabolic rate was 40-50 % below normal, were injected with small amounts of radioactive iodide and killed at times from 2 to 96 hr. after the injection. Various tissues were then fractionated by the method employed to determine the distribution of iodine in the thyroid. It was found that in tissues such as liver, muscle and intestine significant quantities of radioactive material were associated with the fractions corresponding to diiodotyrosine and thyroxine.

Clearly these experiments are open to two criticisms: (a) the observed distribution of radioactivity may be fortuitous; (b) the thyroidectomy may not have been complete. Further evidence is offered, however, which goes far to meet these objections. As to (a) it can be shown that if one of the pure non-radioactive iodine compounds is crystallized in presence of the hydrolytic fraction of tissue which corresponds to it in properties the radioactivity becomes associated with the crystalline compound and remains associated with it in constant proportion through a series of recrystallizations. As to (b) the completeness of thyroidectomy was checked with the greatest care by histological examination of serial sections of the whole neck and chest regions of the animals, and in some cases by the radiographic method in which the tissues are rolled out and exposed to an X-ray film; under such circumstances a high concentration of radioactive iodine such as occurs in residual fragments of thyroid tissue is automatically revealed. Finally, the results of the experiments were not in any way influenced by previous hypophysectomy as would have been expected if they had been due to undetected thyroid tissue. It is difficult, therefore, not to accept the genuineness of the
phenomenon of extra-thyroidal formation of thyroxine, and it is these observations probably which mainly influenced Means in making the generalization which I have quoted at the beginning of this lecture, namely, that in the course of evolution the biosynthesis of thyroxine may have been developed as a general property of living tissues, the process later becoming concentrated in the thyroid gland.

Before leaving the question of the biosynthesis of thyroxine it is necessary to refer to some remarkable recent work which bears on the problem and which promises to be of great therapeutic importance. It has been known for some time that the administration of various chemical substances, among which may be mentioned nitriles and thiocyanates, produces enlargement of the thyroid gland; such enlargement in these cases can be prevented, however, by the simultaneous administration of iodine, and it has therefore been assumed that the thyroid enlargement was identical in character with the compensatory hypertrophy which occurs on simple withdrawal of iodine from the diet, and that it was brought about by interference of the compound administered with absorption of dietary iodine or with access of iodine to the thyroid. Recently, there has been discovered an entirely different group of goitrogenic agents, the action of which is independent of the exogenous supply of iodine.

The first observation of this kind was made by Mackenzie, Mackenzie & McCollum (1941) in Baltimore, who observed that prolonged administration of sulphaguanidine to rats caused a marked enlargement of the thyroid which was not affected by administration of iodine. This action was later found by the Mackenzies (1943) and by Astwood and his co-workers (1943) to be common to a number of sulphanilamide derivatives, and to be much more fully developed in thiourea and its substitution products. Histological examination of the enlarged thyroids produced by one or other of these compounds revealed a condition of extreme glandular hyperplasia with complete loss of colloid, that is to say, a histological picture such as one might expect to find in a severe untreated case of Graves's disease; paradoxically, however, this state of apparent hyperactivity of the gland was associated with hypofunction, the basal metabolic rate being considerably diminished. Other significant observations with regard to the action of this group of goitrogenic agents are that the thyroid enlargement does not take place in hypophysectomized animals, and that, although it is unaffected by iodine, it is inhibited by administration of thyroid or thyroxine; moreover, the normal physiological response to thyroxine is unaffected by the simultaneous administration of thiourea or other goitrogenic agents of this group. From these facts it may be deduced that the action of such agents is not to interfere with the peripheral action of thyroxine but rather to inhibit the synthesis of the hormone in the thyroid gland. According to the explanation which is currently accepted such an inhibition will result, after exhaustion of the store of colloid in the gland, in diminished circulating thyroxine and a fall in metabolic rate; the lower concentration of circulating thyroxine in turn causes an increased output of thyrotropic hormone from the anterior pituitary which brings about the hyperplasia of the thyroid.
I do not myself feel that this explanation is completely satisfactory. In the first place it would seem on general grounds that the inevitable end-result of a process such as is supposed to be taking place must be atrophy of the thyroid, no signs of which have been observed even in the most drastic and prolonged animal experiments. Furthermore, it might be supposed that depression of thyroid activity as the result of administration of the thiourea type of goitrogenic agent would not supervene until complete exhaustion of the gland had taken place; such a conception does not accord with the observed results of therapeutic use of these compounds in Graves’s disease, for the control of thyroid hyperactivity is achieved much earlier and is of a more delicate character than would be expected if complete exhaustion of the gland were necessary; moreover, the establishment of this control is not necessarily accompanied by conspicuous enlargement of the gland.

On the other hand, it is certainly reasonable to regard the over-all effect of thiourea and analogous drugs as ‘chemical thyroidectomy’. If the results of treatment of Graves’s disease with thiouracil are compared with those following thyroidectomy a very close similarity is observed, including the persistence of exophthalmos during the disappearance of other signs of thyrotoxicosis. The latter point is of special interest, since the symptom of exophthalmos is generally believed to be due to excess of thyrotropic hormone and not to excess of thyroxine; persistence or even exacerbation of exophthalmos is therefore precisely what would be expected to result from treatment with thiouracil if the theory of the mode of action of this drug which I have indicated is correct. Indeed, exophthalmos from anterior pituitary stimulation might be expected to result from administration of the drug to a normal animal, although this has not yet been observed.

It should be possible to obtain direct experimental evidence of the nature of the action of goitrogenic agents by studying their effects on surviving thyroid tissue; attempts have indeed been made in this direction, but hitherto the results have been inconclusive. Thus Chaikoff and his collaborators (Franklin, Chaikoff & Lerner 1944) have shown by the radioiodine technique that incorporation of iodine into diiodotyrosine and thyroxine by thyroid slices is inhibited both by thiocyanate on the one hand and by the thiourea group of drugs on the other; in the former case uptake of iodine by the tissue is prevented, whilst in the latter iodide is taken up but remains in the inorganic form. This observation would afford complete confirmation of the theory that the thiourea group acts by interfering with the synthesis of thyroxine were it not for the fact that there are indications in the intact animal of interference of access of iodide to the thyroid after treatment with thiouracil (Franklin, Lerner & Chaikoff 1944). The discrepancy in these observations may be more apparent than real, since in the experiments on the intact animal the effect of administration of iodine was observed after a long period of treatment with thiouracil, i.e. at a time when the normal power of the thyroid to store iodine may well have been destroyed.

The ultimate explanation of the details of the action of goitrogenic agents is not, however, an essential part of my main theme, and whatever this explanation
may turn out to be I think that it will be clear that the work of Chaikoff has provided satisfactory biochemical evidence of the over-all reaction constituting the biosynthesis of thyroxine. I emphasize the word ‘over-all’ in this connexion since the process awaits closer analysis. The experiments which I have described do nothing to indicate whether both phases of the synthesis, i.e. the iodination of tyrosine and the subsequent conversion of diiodotyrosine into thyroxine are enzymic, and if so whether they are controlled by the same or different enzyme systems or whether one phase only is enzymic, the other being a purely chemical reaction. I can offer one comment on this matter from the results of some experiments recently carried out in my own laboratory. Using ordinary analytical technique we have been able to demonstrate the formation certainly of diiodotyrosine and probably of thyroxine by thyroid slices respiring in a medium containing iodide, thus confirming the main observation of Chaikoff; on the other hand we have been quite unable to observe increase of thyroxine in thyroid slices respiring in a medium containing diiodotyrosine. Whilst these results are of a preliminary nature and must be accepted only with reserve, it is clear that they convey the suggestion that the enzymic reaction is concerned with the formation of diiodotyrosine and not with its conversion into thyroxine. I would therefore tentatively advance the idea that the essential biochemical reaction leading to the synthesis of thyroxine may be the liberation of iodine from iodide by an oxidizing enzyme system; if this were to occur conditions would be set up, namely, the presence of iodine in a faintly alkaline medium, which would not only be suitable for the iodination of tyrosine but would be analogous with those which, as has already been seen, will effect the formation of thyroxine from diiodotyrosine in vitro.

If the evidence which I have presented be accepted as conclusively establishing the fundamental facts of the biosynthesis of thyroxine, leaving only matters of detail to be elucidated, there remain two outstanding problems of the physiology of the thyroid, to one of which I wish to give some attention. The problems are the real nature of the normal circulating thyroid hormone and the exact mechanism by which it exercises its effect.

To the second of these problems no real solution can be offered, the one fact which can be accepted without question being that the hormone exercises its effects peripherally on the tissues and not through the intermediation of a central mechanism. The general nature of the effect is one of stimulation of metabolic processes, as reflected for instance in increased oxygen consumption, increased rate of beat of the heart and so on, but the intimate details of the way in which this effect is brought about are quite unknown. The fact that the effect is peripheral in nature has been demonstrated in many ways, into a discussion of which I cannot enter, but most strikingly perhaps by its persistence in isolated tissues of animals which have been treated with thyroid or thyroxine.

It is not perhaps at first obvious why there is a problem at all with regard to the nature of the circulating hormone. It is known that administration of thyroxine to an animal by injection will eventually produce all the symptoms which arise
from over-dosage with whole thyroid gland and all but one of those which appear in Graves's disease; the one exception is the symptom of exophthalmos which, as has been seen, is probably the result of excessive output of thyrotropic hormone rather than of thyroid hormone. It is also known that thyroxine can restore an animal from which the thyroid has been removed to the normal state. Why then should there be any hesitation in accepting the simple hypothesis that the circulating thyroid hormone under ordinary conditions is thyroxine itself? The answer lies partly in certain peculiar features of the physiological action of thyroxine and partly in discrepancies which have been observed between the apparent thyroxine iodine contents of samples of thyroid and their physiological activities.

The main peculiarities of the physiological action of thyroxine are the delay which elapses after its administration before its effects become manifest, the prolongation of these effects when once established, and the difficulty of demonstrating its direct action on isolated tissues. As I have already indicated there is no difficulty in demonstrating the persistent local action of thyroxine in a tissue excised from an animal which has been treated for a sufficient length of time with thyroxine; all attempts, however, to find a direct effect of thyroxine on a normal surviving tissue in the typical 'acute' experiment of which the duration does not exceed 3 hr. or so have failed; indeed, the only satisfactory claims to have observed direct action of thyroxine on normal tissues relate to special cases, such as tissue cultures and surviving frogs' hearts where contact between thyroxine and the living tissue can be maintained over many hours. As a result of these observations and of the apparently closer relationship of the physiological activity of the thyroid with the total iodine than with the thyroxine iodine as chemically determined, it has been supposed, without any satisfying direct evidence, that the normal circulating thyroid hormone might either be thyroglobulin itself, or a peptide derived therefrom, possibly containing diiodotyrosine as well as thyroxine. If this supposition were correct thyroxine should be regarded as an artefact in the sense that, while retaining characteristic physiological activity, it represents a degraded form of the true hormone.

There are two considerations which make it most unlikely that thyroglobulin should itself be the circulating hormone. Immunological observations have consistently failed to reveal the presence of circulating thyroglobulin even in blood from the thyroid vein save in the totally abnormal conditions existing immediately after partial thyroidectomy. Although it may be argued that this evidence is not very conclusive owing to the weakly developed antigenic properties of thyroglobulin, there is the further fact that thyroglobulin is active when given by mouth to the thyroidless animal; in such a case it does not seem reasonable to suppose that the characteristic thyroid protein should be broken down, as it must be before absorption can take place, and then resynthesized in the absence of the thyroid gland. For these reasons the supposition that the true hormone may be a peptide containing thyroxine has seemed to many workers to be the most acceptable hypothesis.
Although I have myself been partly responsible for the development of this idea, I am now inclined to think that it represents an unnecessary complication and one which is not justified by the facts. It is a complication because it forces us to make unsupported assumptions to explain such simple observed phenomena as the power of thyroxine to restore a thyroidectomized animal to the normal state; moreover, the facts which have been advanced in its support do not bear close scrutiny in the light of more recent knowledge.

The analytical evidence to which I have referred, supposedly relating the activity of thyroid gland to its total iodine rather than to its thyroxine iodine content is scanty and, in itself, quite insufficient to form the basis of any theory. The facts which I have mentioned relating to the physiological action of thyroxine appeared more surprising when they were first observed than they would have done if they had been discovered to-day. If the delayed and prolonged action of thyroxine in the whole animal and the difficulty of demonstrating its direct action on isolated tissues are considered in the light of what is now known concerning the action of the anterior pituitary hormones and of the sex hormones, they do not appear so remarkable as they do when compared with the rapid and highly specific actions of secretin and adrenaline which were the only hormones to have been thoroughly studied at the time when thyroxine first became available. Indeed, if one thinks of thyroxine in the only way which is justified by present knowledge, as a substance exercising a general stimulating effect on oxidative mechanisms throughout the body, it no longer seems unreasonable that a significant time interval should be required for its action to become manifest. I do not therefore find, in the facts as known to-day, any convincing reason why the straightforward hypothesis that thyroxine itself is the normal circulating thyroid hormone should not be accepted.

Since, however, the question still requires clinching I should like, as an addendum to these theoretical considerations, to describe some immunological experiments which have a bearing on it, although they were planned from an entirely different point of view.

It will be recalled that the fundamental work of Landsteiner on artificial antigenic complexes proved that the property of immunological specificity was an essentially chemical phenomenon depending on the presence in the antigen molecule of definite groupings; it is supposed that these groupings determine the configuration of the antibodies produced by immunization against the individual antigens and themselves participate in the combination which occurs between antigen and antibody; for this reason they are termed determinant or hapten groups. It will also be remembered that the observations made by Landsteiner on artificial antigens were extended into the field of natural bacterial antigens when it was shown, chiefly by the work of Avery and Heidelberger, that these usually consisted of protein-carbohydrate complexes in which the carbohydrate group performed the determinant function. The validity of this conception has even been susceptible of synthetic proof by the experimental study of antigens formed by combination of a bacterial carbohydrate with a non-bacterial protein; such
antigens give rise to antisera which protect animals against infection by the bacteria from which the carbohydrate is derived, a clear demonstration that it is the hapten groups of an antigen rather than the nature of the protein carrying these groups which determine the properties of the corresponding antibody.

Reflection on these facts led me to the idea that interesting results might be obtained if an artificial antigen were constructed in which the hapten groups themselves had a definite physiological activity. Immunization of an animal with such an antigen should give rise to an antiserum in which the antibodies were specifically adapted to combine with the molecule of the physiologically active substance and might therefore be able to interfere with the action of this substance in another animal by a process analogous with passive immunization.

It happened that in the course of some work in my laboratory on the theoretical question of the nature of the chemical groups necessary for the development of antigenicity a new technique had been devised which made it possible to test the idea which has been outlined in the case of thyroxine. This technique consisted in coupling the azide of an acylated amino-acid, which might in turn be combined with the group whose hapten properties were to be studied, with a protein in alkaline solution. Under these conditions interaction occurred with the free amino groups of the protein molecule with formation of a new peptide linkage through which the hapten group became attached to the protein. Thus in the theoretical study to which I have referred glucose residues were successfully attached to proteins, in the form of the \( O-\beta \)-glucoside of an acylated tyrosine (Clutton, Hargranton & Mead 1937). The new method offers two theoretical advantages over the diazonium coupling employed throughout by Landsteiner and those who followed his technique; in the first place it makes use of no chemical linkage which is foreign to nature, and secondly it does not involve the tyrosine groups of the protein, any alteration to which will in itself affect antigenic specificity; there is therefore reason to expect that artificial antigens prepared by its means will be more highly specific with respect to the hapten groups introduced. In practice it is found that the technique is also superior to that of Landsteiner in that the masking of the original specificity of the protein from which the new antigen is constructed is more complete.

It was for long a matter of doubt whether thyroglobulin itself possessed antigenic properties. The question now seems to have been settled in the affirmative by the careful work of Heidelberger (Stokinger & Heidelberger 1937), but the very difficulty which has been encountered in obtaining an unequivocal answer is an indication that the antigenic properties are very weakly developed. There was, at the time when the work which I am describing was undertaken, no evidence to show how far they might be determined by the thyroxine which it contains. It could be deduced, however, from the earlier experiments of Landsteiner on the antigenic properties of halogenated proteins that if an artificial complex could be built up containing numerous thyroxine groups attached to a protein, these groups would act as powerful determinants in the immunological sense.
In applying to the synthesis of such complexes the new technique to which I have referred (Clutton, Harington & Yuill 1938), it was a matter of chemical convenience not to attempt to introduce thyroxine groups directly into the protein but first to attach N-carbobenzyloxy-3:5-diiodothyronine residues and then to iodinate the whole complex; in this way products were obtained in which N-acylated thyroxine residues were attached to the free amino-groups of the original protein whilst the tyrosine groups of the latter were simultaneously converted into 3:5-diiodotyrosine residues. Complexes of this kind were built up both on thyroglobulin itself and on unrelated proteins such as horse-serum albumin and globulin.

In so far as the serological precipitation reactions are concerned these thyroxine-protein complexes exhibit the following points of interest. As is to be expected they are all powerful antigens in which the immunological specificity of the original protein is entirely lost. Almost complete cross-reactions are exhibited between the thyroxyl derivatives of thyroglobulin and serum globulin with their respective antisera, and both these sera react with iodinated serum globulin to similar dilutions. It is further to be observed that both the antisera give definite reactions with thyroglobulin.

The serological reactions can be analysed further by the study of specific inhibition. This shows that the reaction between both the antisera against thyroxyl thyroglobulin and thyroxyl serum globulin on the one hand, and the homologous or heterologous antigens or thyroglobulin on the other, are inhibited to some extent by diiodotyrosine alone and to a greater extent by diiodotyrosine together with thyroxine in the form either of an artificial mixture or of an enzymic digest of thyroglobulin.

From these results we may conclude that the thyroxine-protein complexes are antigens of the type required in that their antigenic specificity is determined by the thyroxine and diiodotyrosine groups which they contain. From the fact that thyroglobulin itself gives precipitation with the antisera against the artificial complexes, even when these are built up on unrelated proteins, it may further be deduced that the linkages by which the iodine-containing amino-acids are attached in natural thyroglobulin and in the artificial complexes are not greatly different.

It now remains to describe briefly the physiological properties of the antisera raised against artificial thyroxine-protein complexes such as I have described. The first possibility envisaged was that the injection of such antisera into a normal animal might lower its metabolic rate by interference with the action of its endogenous thyroid hormone; such a result was not obtained, however, no detectable change in the metabolic rate being observed. In view of the great reserve power of the thyroid gland, in virtue of its store of thyroglobulin, to pour out more active secretion in response to an additional demand, the negative outcome of these experiments is perhaps not surprising. In any event, by a slightly different technique a clear-cut antagonism between the antisera and thyroid hormone could be demonstrated.
The way in which this was done was as follows. Pairs of rats as similar as possible were trained to the conditions of the apparatus for measuring basal metabolic rate until a series of reasonably constant readings was obtained. One animal then received a course of injections of the antiserum under test, whilst its fellow received a similar course of normal serum; both animals were then injected with the selected preparation of thyroid hormone, and the course of the metabolic rate was followed. The general result was that whilst the animals which had been treated with normal serum showed the marked increases in metabolic rate which are the characteristic response to a dose of thyroid hormone, such increases were almost completely absent from the animals which had received antiserum; moreover, this type of result was obtained whichever antiserum had been employed for the passive immunization and whether the thyroid hormone was given in the form of thyroglobulin or of thyroxine.

The fact that a physiological antagonism can be demonstrated between the antiseras and thyroglobulin is no more than might be expected from the serological observations which I have described, and although interesting in itself does not have any bearing on the nature of the normal circulating hormone. More significant in my view is the fact that the antiseras also neutralize the effect of thyroxine, since the simple and direct explanation is that the circulating antibodies, containing combining sites adapted to thyroxine, interfere with the access of the latter to its normal sites of action in the tissues. Such a simple interpretation can only be avoided by the assumption that injected thyroxine follows the devious route of synthesis into thyroglobulin followed by release as such (which seems on other grounds to be unlikely) or as a peptide which is the real hormone. Collateral evidence that such a complicated process is at least unnecessary to account for the immunological phenomena observed can be provided from another instance of a similar biological phenomenon in which there seems to be no question that the active hapten group can ever be incorporated in the molecule of a protein in the normal body. Thus it has been found (Butler, Harington & Yuill 1940) that an antiserum prepared against an artificial aspirin-protein complex, in which aspirin can be proved serologically to be the determinant group, has the power of passively immunizing an animal against the antipyretic effect of aspirin in precisely the same way as the anti-thyroxine-protein sera can immunize against the physiological effect of thyroxine.

The main theme of this lecture has been the biosynthesis of thyroxine. In the description and interpretation of the immunological experiments of which I have just spoken, and in the general considerations which I have brought forward, I have given my reasons for believing that the process which I have tried to explain is the biosynthesis not of a physiologically active artefact but of the true thyroid hormone as it circulates in the body.
References

Abelin, A. 1934 Arch. exp. Path. Pharmak. 175, 151.