

Newer Knowledge of the Biochemistry of the Thyroid Gland.

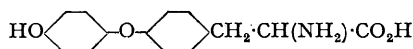
THE PEDLER LECTURE, DELIVERED BEFORE THE CHEMICAL SOCIETY AT BURLINGTON HOUSE ON MARCH 16TH, 1944, AND AT THE UNIVERSITY, MANCHESTER, ON MARCH 24TH, 1944.

By C. R. HARRINGTON, F.R.S.

I PROPOSE to take as the starting point of this lecture the completion of the work which led to the synthetic proof of the constitution of thyroxine. It might have been supposed that after the problems of the isolation of an active principle from the thyroid and of the chemical identification of this principle had been solved, the gland would not have much of new interest to offer to the chemist. It is part of my purpose to show how far such a supposition would have been from the truth and how further work, although perhaps inspired primarily by interest in biological processes, has brought to light matters of interest to the chemist.

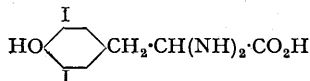
When the constitution of a naturally occurring active principle is explained, the biochemist immediately asks himself from what precursor it is likely to have arisen and what are the processes by which its formation has come about. In the case of thyroxine, indeed, speculation as to the answer to the first of these questions actually preceded the final proof of the constitution and helped to direct synthetic efforts along what proved to be the correct lines.

As soon as the constitution of thyronine or deiodothyroxine had been proved (Harrington, 1926) it became

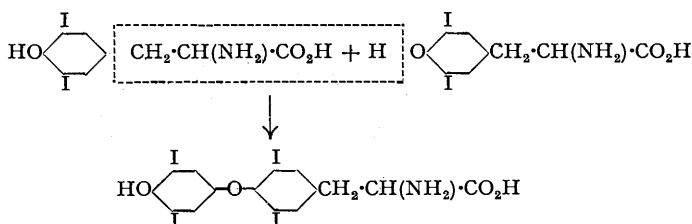


evident that tyrosine was almost certainly a biological precursor of thyroxine. The location of the four iodine atoms of thyroxine in the thyronine molecule could not be achieved with certainty by chemical means and the decision as to which tetraiodothyronine should be selected as a synthetic objective was made principally upon a biological hypothesis.

Until thyroxine was discovered and shown to be an iodine-containing amino-acid, only one other natural compound of this type was known, namely, 3 : 5-di-iodotyrosine :



Now just as thyronine could be regarded as a condensation product of two molecules of tyrosine with loss of one side chain, so thyroxine might reasonably be derived by a similar reaction from 3 : 5-di-iodotyrosine; if this were to happen, the compound produced would be 3 : 5 : 3' : 5'-tetraiodothyronine :



The synthesis of the latter substance and the proof of its identity with thyroxine (Harrington and Barger, 1927) provided circumstantial evidence of the validity of the biological hypothesis, and made it all the more desirable that direct experimental evidence should be sought.

Clearly a fundamental requirement of the theory that the biosynthesis of thyroxine proceeds through 3 : 5-di-iodotyrosine is the demonstration of the presence of the latter compound in the animal body; there is no place in the body where iodine occurs in any significant concentration except in the thyroid itself, and it was therefore in this organ that di-iodotyrosine must be sought.

Earlier work had shown that the whole of the iodine in the thyroid was in firm organic combination and that it was confined to the protein thyroglobulin which constituted the characteristic colloid material of the gland. On the other hand, even with improved methods of isolation it was not possible to account for a very large proportion of the total iodine of the thyroid in terms of thyroxine. Furthermore, in the course of the study of the isolation of thyroxine it became apparent that the iodine of the thyroid was easily separable into two distinct fractions characterised by their widely different solubilities at about pH 5. Such a separation could be effected by mild alkaline hydrolysis, as in the ordinary process of isolation of thyroxine, or by the early stages of enzymic breakdown of thyroglobulin. Of these two fractions, only that which was insoluble at pH 5 exhibited physiological activity and served as a source of thyroxine, in spite of the fact that most of the iodine in the soluble fraction remained in organic combination.

These facts then were known at the time when thyroxine was synthesised, and they carried the strong suggestion that it was among the acid-soluble hydrolytic products of thyroglobulin that di-iodotyrosine would

most probably be found. This acid-soluble fraction of the hydrolytic products was therefore subjected to complete hydrolysis with alkali, and a systematic search was made for di-iodotyrosine which was guided by the known properties of this amino-acid. Detailed description of the methods employed is unnecessary; it will suffice to say that the search was successful, di-iodotyrosine being identified among the products of hydrolysis without much difficulty (Harington and Randall, 1929).

The observation just described thus afforded a significant piece of qualitative evidence in favour of the biogenetic hypothesis under consideration. It seemed worth while, however, to press the matter a little further, and to attempt to make so far as possible a quantitative allocation of the iodine of the thyroid to thyroxine and di-iodotyrosine respectively; not only would this indicate the approximate proportions in which the two compounds occurred in the thyroid, but it might also be expected to reveal the existence of other organic iodine compounds if such occurred in the thyroid. With this end in view a quantity of desiccated thyroid was submitted to the step-wise hydrolytic process which had been employed for the isolation both of thyroxine and of di-iodotyrosine. At the early stage of mild hydrolysis the two main fractions, acid-insoluble and acid-soluble, were separated as sharply as possible, and from these fractions were isolated thyroxine and di-iodotyrosine respectively. Every step of the process was accompanied by an iodine analysis in order that any significant loss of iodine should be observed. The result of this experiment was to show that although the over-all losses of iodine were considerable, only one step was accompanied by a loss greater than could reasonably be regarded as due to the nature of the manipulation. This exceptional loss occurred at the stage when the hydrolytic products were subjected to intensive alkaline hydrolysis; after this operation approximately one third of the iodine appeared in the form of iodide in the acid-soluble fraction and a considerably smaller proportion appeared similarly in the acid-insoluble fraction. It was, however, demonstrable by experiments on the pure compounds that intensive alkaline hydrolysis caused some decomposition with liberation of iodine in both cases, the destruction being relatively greater with di-iodotyrosine than with thyroxine. This being so, it was unnecessary to assume that the iodine appearing as iodide at the stage of intensive hydrolysis originated from compounds other than thyroxine and di-iodotyrosine, and indeed the upshot of the whole experiment was to indicate clearly that these two compounds accounted between them for the whole of the iodine of the thyroid; moreover it appeared that they were present in somewhat similar amounts, di-iodotyrosine slightly preponderating. Thus not only have we the proof that di-iodotyrosine is a constituent of the thyroid, but we see that it is quantitatively the most important iodine-containing compound which occurs therein.

The fact that neither thyroxine nor di-iodotyrosine could be isolated from thyroglobulin except after a process of drastic hydrolysis, coupled with the observation that both compounds thus isolated were obtained in the racemic condition, gave a strong indication that they must originally have been present as constituent amino-acids of the thyroglobulin. Direct proof of this supposition was afforded by the results of enzymic degradation of thyroglobulin (Harington and Salter, 1930). By a laborious process involving successive digestion with pepsin, trypsin and finally a peptidase-containing extract of intestinal mucosa it was possible to isolate a small yield of thyroxine. Analysis of the intermediate fractions obtained for iodine and for total and amino-nitrogen made it quite clear that the process of isolation consisted of the enzymic breakdown of peptide chains containing thyroxine as a constituent amino-acid; moreover the thyroxine finally isolated proved to be optically active; similar evidence was later obtained in respect of di-iodotyrosine.

I have mentioned this proof that thyroxine and di-iodotyrosine occur naturally as constituent amino-acids of thyroglobulin, not because it has a direct bearing on the problem of the biogenesis of thyroxine, but because the availability of natural optically active thyroxine which resulted from it made possible a further chemical attack on this problem.

The optically active isomerides of thyroxine had indeed been prepared by resolution of the *dl*-compound (Harington, 1928) (or more precisely of *dl*-3 : 5-di-iodothyronine, followed by iodination of the enantiomorphs) before natural thyroxine had been isolated as described. Although, however, the *l*-vortatory synthetic isomeride exhibited a higher degree of physiological activity than the *d*-vortatory compound, the difference was not very great, and complete certainty as to which was the natural compound had to await the isolation of the latter by methods which did not involve racemisation; when this was accomplished the natural compound turned out in fact to be *l*-vortatory as had been anticipated from the biological activity.

The optical rotation of natural thyroxine having thus been established, it was clear that if thyroxine could be synthesised from natural *l*-tyrosine by a series of reactions which did not involve the possibility of an optical inversion the product obtained should be identical with natural thyroxine if the biogenetic hypothesis were correct. At the time of which I am speaking the possibility of such a direct synthesis of thyroxine from tyrosine was not in sight, but there remained an indirect method of attack of the problem; it appeared that it might be possible to synthesise thyronine from tyrosine; if this could be accomplished, the optical rotation of the product could be compared with that of a sample of thyronine prepared by the catalytic deiodination of natural thyroxine.

The method by which this object was achieved is as follows (Canzanelli, Harington, and Randall, 1934). The ethyl ester of *N*-benzoyl-*l*-tyrosine was condensed with 3 : 4 : 5-tri-iodonitrobenzene by boiling in methyl ethyl ketone solution with potassium carbonate to give ethyl α -benzamido- β -[4-(3' : 5'-di-iodo-4'-nitrophenoxy)-phenyl]propionate; the object of employing 3 : 4 : 5-tri-iodonitrobenzene instead of the more obvious *p*-iodonitrobenzene is simply to improve the yield in the condensation; the subsequent removal of the 3' : 5'-iodine

atoms can be effected in quantitative yield and without difficulty. The ester thus obtained was hydrolysed to the free acid, which in turn was reduced with ferrous sulphate and baryta to the corresponding amino-compound; the latter was deiodinated by catalytic hydrogenation in the presence of palladised calcium carbonate and the resulting iodine-free acid was converted into *N*-benzoylthyronine by diazotisation, followed by boiling of the aqueous solution of the diazonium salt; thyronine obtained by hydrolysis of the *N*-benzoyl derivative had $[\alpha]_{5461} + 13.3^\circ$. A sample of thyronine, which was obtained, on the other hand, by catalytic deiodination of natural *l*-thyroxine had $[\alpha]_{5461} + 12.2^\circ$. It is clear, therefore, that the preparations of thyronine obtained from natural tyrosine and from natural thyroxine are configuratively identical, from which it follows that natural tyrosine and natural thyroxine are configuratively related.

It will be seen from what I have to say later that a more direct chemical proof of the configurative relationship of tyrosine and thyroxine has recently become available. The establishment of the configurative relationship, however, by the indirect method which I have described sufficed to bring strong chemical support to the idea that tyrosine was in fact the biological precursor of thyroxine.

In thinking of the biosynthesis of thyroxine from tyrosine it is necessary to consider the reaction in two phases, the first consisting of the iodination of tyrosine and the second of the coupling of two molecules of the latter to give thyroxine. As to the first phase there is little to be said, except that it must be presumed to occur in the thyroid gland and that it must involve an oxidative system which will liberate iodine from iodide, which is the form in which iodine taken into the body reaches the thyroid. It has already been stated that the thyroid gland is the only situation in the body where iodine occurs in significant concentration. The avidity of the thyroid for iodine is indeed very striking; for instance, if a dose of 50 mg. of potassium iodide is administered to a dog, 18.5% of the iodine can subsequently be recovered from the thyroid although the latter represents less than 0.15% of the total body weight; moreover, if the animal receiving the iodine has previously been in a state of iodine deficiency, the administration of iodide results in rapid storage of colloid (thyroglobulin) and in enhancement of the physiological activity of the gland. It is clear, therefore, that iodine introduced into the body as iodide is specifically taken up by the thyroid and there enters into organic combination which may lead to the production of the active principle; whatever the later stages of the synthesis of thyroxine may be, the first event must be substitution of the iodine in the molecule of an organic compound, and such substitution can only occur after oxidation of the iodide. Direct evidence that the first reaction which occurs is in fact the formation of di-iodotyrosine has recently been provided by the work of two groups of American investigators (Perlman *et al.*, 1941, a & b; Morton and Chaikoff, 1943; Mann, Leblond, and Warren, 1942), who have employed radioactive iodine for the study of the process. Immediately after the administration of iodide containing a proportion of radioactive iodine, part of the radioactivity is found in iodide in the thyroid; a few hours later radioactivity appears in the fraction of the hydrolytic products of the thyroid which contains di-iodotyrosine, and still later in the fraction containing thyroxine, whilst at the same time the proportion of iodine present as iodide diminishes. In addition to the experiments on the intact animal the metabolism of iodine has been studied in thyroid slices. By the use of radio-iodine the same result has been demonstrated, namely, rapid conversion of iodide into di-iodotyrosine, followed by later formation of thyroxine.

So far as the general thesis is concerned, therefore, we see that there is now an impressive body of evidence, both chemical and biochemical, that thyroxine is in fact formed in the body from tyrosine and iodide through the intermediate stage of di-iodotyrosine. The details of the biological process remain to be elucidated; observations on the effect of typical enzyme inhibitors (Schachner, Franklin, and Chaikoff, 1943) indicate that it is in part at least controlled by an oxidising enzyme system; there is, however, nothing to show whether one and the same enzyme catalyses both phases of the reaction, whether there are separate enzyme systems which catalyse the oxidation of iodide required for iodination of tyrosine and the coupling of two molecules of di-iodotyrosine to give thyroxine, or whether, by analogy with the formation of melanin from tyrosine by tyrosinase, only one phase of the over-all reaction is enzymic in character. I do not propose to pursue these questions further on this occasion; rather do I wish to direct attention to the chemistry of the second part of the reaction, namely, the conversion of di-iodotyrosine into thyroxine.

The first aspect of this matter which I wish to discuss concerns the action of iodine on proteins. When it was first discovered that the physiological activity of the thyroid was associated with iodine and that this iodine was organically bound in thyroglobulin, a discovery which preceded the isolation of thyroxine by 20 years, interest was at once aroused in the possibility of obtaining products having similar physiological properties by treatment of other proteins with iodine. Many investigations were made in which proteins were treated with iodine under various conditions of pH and temperature. In all cases products were obtained which contained more or less iodine in organic combination, but in the earlier work no indication, or only doubtful indications, of physiological activity were observed. Study of the chemistry of the iodinated proteins did, however, reveal certain points of interest. The most significant observation from the point of view of the present discussion was that of Oswald (1910), who found that iodination of albumin, gliadin and casein in alkaline solution gave products from which he was able to isolate 3 : 5-di-iodotyrosine; this revealed the predominant importance of tyrosine as an iodine-binding constituent of the protein molecule. No pure iodine-containing compound other than di-iodotyrosine could be isolated from iodinated proteins, but in some cases there was evidence that the total amount of iodine bound was more than could be accounted for by the tyrosine content of the protein. A plausible explanation of this observation was offered by the dis-

covery of Pauly (1910) that the iminazole ring is readily iodinated, and that this was the true explanation was indicated by the fact that the discrepancy between the iodine bound and the tyrosine content was greatest in proteins such as globin which contained a large proportion of histidine. With increasing knowledge of the iodination of tyrosine it has been shown in recent years that, except when an unusually large amount of histidine is present, it is possible by choosing the proper conditions almost to titrate the tyrosine in a protein with iodine and to obtain a product in which the combined iodine is stoichiometrically equivalent to the tyrosine, assuming conversion of the latter into di-iodotyrosine. This reaction has found several useful applications; it has been used by Neuberger to expose the rôle of tyrosine in the base-binding properties of zein and insulin (Neuberger, 1934; Harington and Neuberger, 1936); it has revealed the essential character of the tyrosine groups for the specific activities of insulin (Harington and Neuberger, 1936) and pepsin (Herriott, 1941—42), and it has found wide application in immunochemical studies.

It will be seen that the early disappointment regarding the production of physiologically active substances by iodination of proteins led the further study of this reaction into other channels, and, although occasional claims to have produced physiological activity were made, serious attention was not given to this part of the problem until comparatively recently when, in a series of papers, Abelin (1933, 1934, 1936, 1938) claimed to obtain physiologically active iodinated proteins from which, by alkaline hydrolysis, he could separate fractions having the general physical and chemical properties of thyroxine; in only one instance, however, was a crystalline substance having physiological activity isolated and this was not satisfactorily characterised. The matter therefore remained in doubt and it was not until 1939 that a paper was published by Ludwig and von Mutzenbecher which changed the whole situation. These workers published a detailed description of a method for the iodination of casein and other proteins to give products which were not only physiologically active themselves but from which it was actually possible to isolate thyroxine. I must confess that this result seemed to me at first to be so surprising that I was not prepared to accept it until I had repeated it in my own laboratory (Harington and Pitt-Rivers, 1939); I had, however, no difficulty at all in convincing myself that the observation was correct.

There were of course two possible explanations of the formation of thyroxine during iodination of a protein, namely, (1) that thyronine was already present in the protein molecule and was directly iodinated, and (2) that the di-iodotyrosine which must have been formed had in part undergone secondary conversion into thyroxine. Of these two explanations the second seemed for many reasons to be the more acceptable. In the first place all the evidence pointing towards the biological formation of thyroxine from tyrosine which I have recounted was available except for the experiments with radio-iodine; secondly, the direct iodination of thyronine to thyroxine had previously been attempted on many occasions and under widely different conditions and had entirely failed. Moreover the essential feature of the process of Ludwig and von Mutzenbecher appeared to be that the iodination was carried out under conditions which might be expected to favour oxidative side-reactions, namely, in dilute alkaline solution at a slightly elevated temperature; in this respect their experiments contrasted with most modern work on the iodination of proteins, which, as we have seen, was directed towards quantitative iodination of the tyrosine. Assuming the hypothetical coupling of two molecules of di-iodotyrosine to give one of thyroxine to be an oxidative process, it seemed reasonable to suppose that hypiodite, formed on addition of iodine to the alkaline solution, might be the oxidising agent responsible. There were, however, certain features of the reaction which were difficult to understand on the simple hypothesis just outlined. For instance, the proportion of iodine added appeared to be fairly critical and was less than that which gave products with maximal iodine content; such products in fact showed little or no physiological activity and failed to yield any thyroxine. Moreover, if the picture which has been given above were correct, it might have been supposed that the yield of thyroxine obtainable from the iodinated product would bear a direct relationship to the tyrosine content of the original protein; this, however, was not the case, silk fibroin, for instance, which contains a very high proportion of tyrosine, failing to give any active product. The reaction is thus obviously a complex one in which the structure of the protein is involved. The work of Ludwig and von Mutzenbecher has considerable practical importance, since it offers the possibility of the cheap and easy preparation in large quantities of products having the specific physiological properties of thyroid gland. This being so, it is not surprising that the reaction has been studied extensively both in this country (unpublished work in my own laboratory) and in America (Reineke and Turner, 1942); the large amount of work which has been carried out has, however, done little but confirm the original observation, extend it to other proteins and define more sharply the conditions which must be observed if optimal yields of active material are to be obtained; the essential chemistry of the process remains obscure. The establishment of the possibility of forming thyroxine by iodination of proteins, interesting as it is, does not therefore take us much further in the detailed explanation of the process.

About a year after the publication of the work on iodinated proteins, however, another paper appeared from von Mutzenbecher (1939) in which he described the formation of thyroxine during prolonged incubation of di-iodotyrosine in slightly alkaline solution. The proportion of thyroxine thus formed was so minute that a faint suspicion attached to the observation in so far as it applied to di-iodotyrosine derived from natural tyrosine; before long, however, this doubt was removed by the repetition of the experiment in America (Block, 1940) with di-iodotyrosine of synthetic origin, the yield of thyroxine being similar to that obtained by von Mutzenbecher. This new observation of von Mutzenbecher seemed to open up fresh possibilities for the study of the chemical transformation of di-iodotyrosine into thyroxine. In von Mutzenbecher's experi-

ment the formation of thyroxine was accompanied by liberation of a significant amount of iodine, which, at the alkaline reaction employed, must have formed some hypoiodite; here again, therefore, as in the protein iodination experiments, we have the suggestion that the oxidising action of hypoiodite may be concerned in the transformation. This suggestion was taken up by T. B. Johnson (Johnson and Tewkesbury, 1942) in America, who propounded a theory of the mechanism of coupling of two molecules of di-iodotyrosine into the details of which I shall go later; he also claimed that the addition of hypoiodite to the solution of di-iodotyrosine slightly increased the yield of thyroxine, but he gave no actual figures in support of this claim.

Assuming the truth of the suggestion that hypoiodite was indeed the effective agent both in the formation of thyroxine in iodinated proteins and in the direct conversion of di-iodotyrosine into thyroxine, it seemed to us that a study of the action of other mild oxidising agents in bringing about the latter reaction might be profitable. Johnson's theory was based on the Pummerer oxidation of phenols in which the oxidising agent employed was potassium ferricyanide; the obvious first step, therefore, was to examine the action of the latter on di-iodotyrosine; in none of a number of experiments, however, was any indication obtained of the formation of thyroxine; extensive disruption of the di-iodotyrosine occurred under the mildest conditions which could be devised.

From potassium ferricyanide we turned to hydrogen peroxide, a reagent which, as is well known, frequently resembles biological oxidation systems in the reactions which it brings about. At first we employed the hydrogen peroxide in slightly alkaline solution at 37°; the mixture was incubated for various times and worked up for thyroxine by the method used by von Mutzenbecher. In this way we were able to observe at least an acceleration of the formation of thyroxine, although the yield remained minute and of the same order as that which resulted from the more prolonged incubation of an alkaline solution of di-iodotyrosine without added oxidising agent. We next found, somewhat to our surprise, that the yields of thyroxine were significantly improved by employing more drastic conditions of oxidation with larger amounts of peroxide at a higher temperature. We also found that the reaction was influenced by the pH of the solutions, rough orientating experiments indicating an optimum at about pH 10.

So long as the experiments were carried out in aqueous solution, the crude thyroxine being isolated at the end of the reaction by precipitation with acid, the yields remained very small. The dark colour of the oxidised solution and the large proportion of tarry by-products formed indicated that extensive oxidative destruction was taking place, and indeed it appeared likely that much of the thyroxine which might be formed would undergo secondary oxidation under the conditions employed. We therefore adopted a technique which we hoped would protect the thyroxine by removing it as it was formed from further contact with the oxidising agent. It was observed some years ago by Leland and Foster (1932) that, when an alkaline solution of a mixture of thyroxine and di-iodotyrosine was shaken with butyl alcohol, 92% of the thyroxine passed into the alcohol whilst over 97% of the di-iodotyrosine remained in the aqueous phase; the experiments of Leland and Foster were actually carried out with solutions of *N*- or 2*N*-sodium hydroxide, but the distribution coefficients presumably depend upon the relative solubilities of the salts of the two amino-acids and do in fact bear a similar relation to one another at pH 9–10 (the pH of our oxidation), although the separation is not so sharp as in more strongly alkaline solutions. It seemed therefore that, if the solution of di-iodotyrosine being oxidised were to be shaken with butyl alcohol, the thyroxine should pass into the latter and there be relatively protected. This process has indeed met with some success in our hands and has led to significantly increased yields of thyroxine.

In a typical experiment of this kind 0.05 g.-mol. of di-iodotyrosine was dissolved in 95 c.c. of *N*-sodium hydroxide to give a solution of about pH 10; 100 c.c. of butyl alcohol were added and the mixture was heated on the steam-bath; 20 vol. hydrogen peroxide was then added in 10 c.c. portions with constant shaking and after each five additions the butyl alcohol was separated and replaced with fresh solvent; the reaction was kept alkaline to phenolphthalein by addition of alkali as necessary. In all some thirty additions of hydrogen peroxide were made over 5–6 hours, representing a very large excess (8 atomic equivalents), but naturally under the conditions of the experiment much of the peroxide decomposed immediately. The thyroxine was isolated from the butyl alcohol extracts by a method which need not be described in detail. The pure thyroxine ultimately obtained amounted to 319 mg., representing a gross yield of 1.36% and a net yield of 3.4% after allowing for the unchanged di-iodotyrosine which could be recovered. This compares with a gross yield of 0.23% obtained by von Mutzenbecher; his net yield is not known, since figures are not given for the recovery of di-iodotyrosine.

It is not to be supposed that the experimental conditions which I have described necessarily represent the best that can be found for the conversion of di-iodotyrosine into thyroxine; indeed it seems improbable that this should be so. It is, however, clear that oxidation with hydrogen peroxide brings about with much greater rapidity and in much greater yield the same transformation which occurs on incubating di-iodotyrosine in alkaline solution. One point of interest which may be mentioned at this stage is that the thyroxine which is formed from di-iodotyrosine (itself prepared from natural *l*-tyrosine) is laevorotatory and therefore identical with natural thyroxine isolated from the thyroid by enzymic hydrolysis. This is the piece of direct chemical evidence to which I referred earlier, finally proving the configurative relationship of natural thyroxine and natural tyrosine.

As has already been noted, the oxidation of di-iodotyrosine with hydrogen peroxide is accompanied by extensive decomposition with formation of tarry by-products; oxidative disruption of the di-iodotyrosine

is also indicated by the formation of acid which occurs during the reaction. A search has been started among the by-products in the hope that a compound might be found which would throw light on the course which the reaction follows; however, in spite of the fact that several pure substances have been isolated in minute yield, none has yet been identified which is useful for this purpose. Anything further therefore which may be said about the chemistry of the conversion of di-iodotyrosine into thyroxine must be of a theoretical and speculative nature; I am encouraged to enter on such speculation not only by the intrinsic chemical interest of the reaction but by the terms of reference of the Pedler Lecture which state that "the lecture should indicate directions in which further work is required."

Mention has already been made of the theory propounded by T. B. Johnson. This is based on the work of Pummerer on the oxidation of phenols in alkaline solution with potassium ferricyanide. Pummerer's earlier experiments were carried out for the most part with naphthol derivatives. He observed (Pummerer, 1919; Pummerer and Cherbuliez, 1919) that the dehydrogenation of a compound such as 1-methyl-2-naphthol led to an intermediate which had the empirical formula of di-(1-methylnaphthyl) peroxide; this intermediate did not, however, behave like a peroxide and was later formulated on good evidence as having the structure (I). Structures of type (I) were assumed by Pummerer to be formed from the interaction of free radicals (Pummerer and Frankfurter, 1914; Pummerer and Cherbuliez, 1914), into which they themselves indeed readily dissociated; in the more complicated cases the free radicals showed exceptional stability. The best explanation of the formation of structure (I) and of other reactions observed was given by the assumption that the radicals



were of two types, one, as we should now say, having the odd electron on the carbon atom carrying the methyl group and the other having the odd electron on the oxygen atom. Interaction of the two different radicals would give (I) or its analogue, whilst interaction of two radicals of the first type would give a dinaphthyl such as was already known to arise from the oxidation of naphthols under certain conditions.

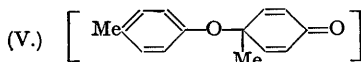
In the case of β -naphthol itself the intermediate of type (I) is able to stabilise itself by internal rearrangement and the product of dehydrogenation is 2-hydroxy-1:2'-dinaphthyl ether (II). It may be noted, in view of what is to be said later, that in a phenol carrying substituents in the *o*-positions, similar stabilisation with ether formation may be expected to occur in the *p*-position if this is free.

That peroxide formation does in fact occur during the dehydrogenation reaction was demonstrated by Pummerer (Pummerer and Rieche, 1926), when he isolated a mixed peroxide by oxidation of hydroxy-naphthylene oxide; it was only in this instance, however, that a peroxide could be obtained and it was extremely unstable; Pummerer regarded the peroxide as the initial intermediate in the production of compounds of type (I); it could equally well be the product of reaction of two free radicals having the odd electron on the oxygen atom.

The example taken by Johnson for an analogy in considering the conversion of di-iodotyrosine into thyroxine is the oxidation of *p*-cresol. In Pummerer's hands (Pummerer *et al.*, 1922, 1925) this led to the formation of the ketotetrahydrobenzofuran derivative (III) and to the di-*p*-cresol (IV) as main products:



In the light of his earlier work the formation of (III) was explained as proceeding through the intermediate compound (V):

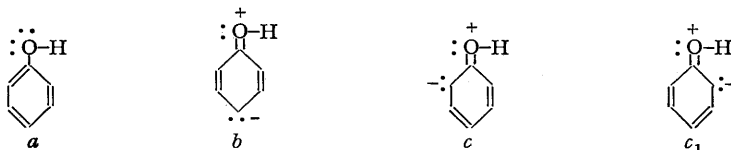


Johnson points out that, if a similar course of reactions is assumed for the oxidation of di-iodotyrosine, the rearrangement of the intermediate quinonoid compound to the stable tetrahydrobenzofuran structure cannot take place, being prevented by the presence of the iodine atoms *ortho* to the phenolic group. If, therefore, an intermediate product of this type is formed from di-iodotyrosine, the molecule may stabilise itself in one of two ways, both of which involve the loss of the side chain attached to the same carbon atom as the ether oxygen; this side chain will split off as a dehydroalanine residue, which either may be hydrolysed to pyruvic acid and ammonia or may add the elements of water to give serine; Johnson states that he was able to identify pyruvic acid and ammonia among the oxidation products and he therefore favours the first mechanism. The identification of the pyruvic acid in Johnson's experiments was not, however, very conclusive, and plausible as his theory is it cannot therefore be said to rest on a very secure foundation. Further evidence which was foreshadowed in his communication has not yet been forthcoming.

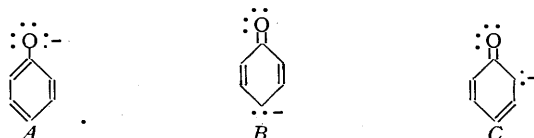
Whilst it remains probable, therefore, that the mechanism proposed by Johnson gives a generally correct

picture of the course of events in the transformation of di-iodotyrosine into thyroxine, it seems worth while to analyse the reaction somewhat more closely, and particularly to see whether useful indications may be obtained by application of accepted theories of resonance structure in so far as they relate to phenols and phenoxide ions. We may first consider this in relation to the Pummerer reaction.

The large magnitude of the acid dissociation constant of a phenol compared with that of an aliphatic alcohol is attributed to resonance of the normal structure *a* with the three structures *b*, *c*, and *c*₁:

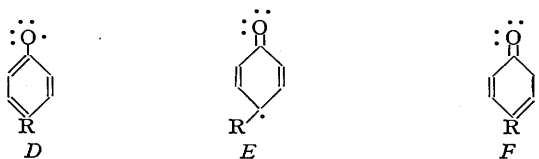


For the purpose of the subsequent argument *c* and *c*₁ are identical and will be treated as one. In the case of the phenols themselves the contributions of structures *b* and *c* to the normal state of the molecule are small,

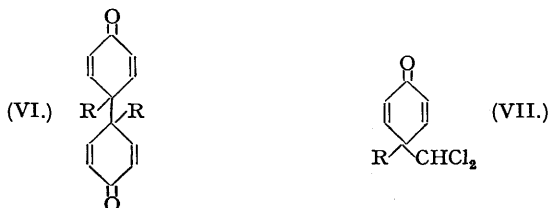


but in the corresponding phenoxide ions, *A*, *B*, and *C*, the contributions of *B* and *C* are greater, since the resonance forms of the ions differ only in the position of the negative charge, whilst the resonance forms of the phenols have separated charges and are therefore less stable. This point should be borne in mind in relation to the fact already stated that the transformation of di-iodotyrosine into thyroxine occurs most readily at a pH at which the phenolic group will be completely dissociated. The same conditions obtain in Pummerer's oxidation of *p*-cresol, which is carried out in sodium carbonate solution and must therefore involve principally the phenoxide ion; on the other hand, in the peroxidase oxidation of *p*-cresol recently reported by Westerfeld and Lowe (1942), which takes place at pH 6.5 and leads to the same products as those obtained by Pummerer, the reactant must be the phenol itself. Since the reaction with which we are chiefly concerned, not only as it is conducted in the test-tube but as it must occur *in vivo*, undoubtedly involves phenoxide ions, for the rest of this discussion we shall proceed as if we were dealing with dissociated phenolic groups.

If now we assume the oxidation of a *p*-substituted phenoxide ion to consist in the removal of one electron from the lone pair on the oxygen of form *A* and from the *p*- and *o*-carbons respectively of forms *B* and *C*, we get three corresponding free radicals *D*, *E*, and *F*, and we may consider the probabilities of reaction between these forms and the nature of the products which would arise therefrom.



Self-interaction of *D* would lead to a diphenyl peroxide structure; even if this were isolable, we may judge from Pummerer's work that it would be very unstable. Self-interaction of *E* would lead to a dicyclic ketone (VI); no compounds of this precise type are known, although there are no steric factors which would preclude their formation. The nearest analogy seems to be cyclic ketones of type (VII) which were obtained by Auwers and Keil (1902) as by-products in the Reimer-Tiemann reaction applied to *p*-alkylated phenols.



Neither Pummerer nor Westerfeld and Lowe isolated a compound of type (VI) from the oxidation of *p*-cresol; this may be explained either by the impossibility of formation of such a compound or by its polymerisation, if formed, to give unrecognisable products. The same general considerations apply to interaction between *E* and *F*. Self-interaction of *F* can occur and would lead, where R = CH₃, to 2 : 2'-dihydroxy-5 : 5'-dimethyldiphenyl, a compound which, as we have already seen, was obtained both by Pummerer and by Westerfeld and Lowe. Repetition of this interaction would lead to the corresponding terphenyl derivative, which has

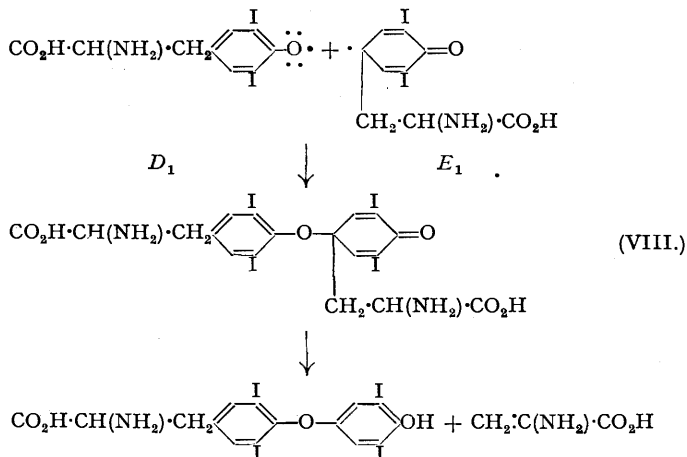
also been identified by Westerfeld and Lowe among the products of oxidation of *p*-cresol by peroxidase, and was isolated but not identified by Pummerer.

Interaction of *D* and *E* would give the intermediate compound which (where R = CH₃) rearranges to the ketonic tetrahydrobenzofuran obtained by Pummerer as already described.

Interaction of *D* and *F* should lead to an isomeric ketonic tetrahydrobenzofuran which has not been isolated.

It is thus seen that, of the three compounds which would be predicted on this theory as arising from the oxidation of *p*-cresol, two have actually been obtained.

If we now transfer these ideas to a phenol which is not only substituted in the *p*-position but also carries substituents in both *o*-positions, *i.e.*, to such a phenol as di-iodotyrosine, we find that the probabilities of interaction of the various forms are more limited. In addition to the limitations already noted, self-interaction in the *o*-position will be inhibited by the presence of the iodine atoms, so that the formation of a diphenyl is not to be expected. We are left, therefore, with interaction between forms *D*₁ and *E*₁ as the most probable



course of events and this is precisely the reaction postulated by Johnson, leading to the intermediate compound (VIII): this, as already pointed out, being unable to undergo the rearrangement to a tetrahydrobenzofuran because of the presence of the iodine atoms *ortho* to the phenolic group, can only rearrange itself in another way, namely, by loss of one side chain as dehydroalanine, leading to ammonia and pyruvic acid or to serine.

Theoretical reasoning therefore not only demonstrates a possible mechanism for the oxidative coupling of two molecules of di-iodotyrosine to give thyroxine, but suggests that such a reaction is rendered more likely by the presence of the iodine atoms in the former compound. Objective evidence in favour of the reaction mechanism which has been postulated might be more readily forthcoming if conditions for the oxidation could be devised more favourable than any which we have yet discovered. Such objective evidence of an important character would be provided by the unequivocal recognition of the lost side chain in the form either of serine or of pyruvic acid; under the conditions of our experiment, however, both of these products, if formed, would be largely destroyed, and up to the present we have not been able to demonstrate their presence.

The excuse for the speculation in which I have indulged in the latter part of this lecture is that, in my view, it indicates the directions in which we may profitably seek for the explanation of the mechanism of a somewhat obscure reaction, which is interesting not only to the organic chemist but also to the biologist who desires to understand the details of an important biosynthetic process.

The development of the theoretical arguments in this lecture owes much to discussion with my colleague, Dr. A. Neuberger, to whom I wish to express my gratitude.

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