composition of the buffer and temperature had slight or no effect.

3. The initial reaction of the loss of iodophenol radioactivity was associated with ionization of the phenolic hydroxyl group in these compounds. The cause of the re-formation of the iodinated phenol was not discovered.

4. Although paper-chromatographic analysis indicated the formation of a radioactive substance of an R_F similar to that of iodide, during the initial reaction, results of other tests were incompatible with a mechanism of deiodination. With L-thyroxine, simultaneous chromatographic and spectrophotometric analysis showed a true increase in the concentration of the original substance during the reincorporation of ^{131}I into thyroxine.

5. The hitherto unreported ultraviolet-absorption maxima at 231 and 227 mμ for L-thyroxine and 3:5:3′-tri-iodo-L-thyronine respectively have been described.

I am indebted to Dr R. V. Pitt-Rivers, F.R.S., for help and advice throughout the investigations. I thank Dr W. L. Money, Sloan-Kettering Institute for Cancer Research, New York, N.Y., for providing me with labelled L-thyroxine and 3:5:3′-tri-iodo-L-thyronine of high specific radioactivity.

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A New Aspect of the Interaction Between Thyroxine and Proteins

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In the preceding paper details have been given about a hitherto undescribed property of thyroxine and some related iodophenols (Tata, 1959). This unusual property consists of a reversible loss of the ^{131}I-labelled iodophenol (as judged by its radioactivity) which occurs when the phenolic hydroxyl group undergoes ionization; such an effect is commonly observed during dilution of a solution of the ^{131}I-labelled compound in a small volume of organic solvent with a large volume of aqueous buffer. In view of the implication of such a reaction as a confusing factor in the study of enzymic deiodination of thyroid hormones, an examination of the temporary instability of the labelled iodophenol in the presence of biological material appeared desirable. The first results of such an examination revealed that the reversible loss of labelled thyroxine, as judged by its radioactivity, failed to occur if blood serum was present in the aqueous diluent. The spontaneous reaction was absent even when the serum was present at a fivefold dilution, and on further dilution the extent of inhibition was a function of the serum concentration. Serum behaved as an agent which masks the dissociation of the phenolic hydroxyl group of thyroxine in that it brought about an instantaneous re-formation of radioactive thyroxine if it was added to the aqueous medium after the initial loss of ^{131}I-labelled iodophenol had already taken place.

This effect of serum on the stability of thyroxine presented a new question: is it in any way related to the well-known interaction between certain serum proteins and thyroxine? Up to now the interaction has been studied as a selective binding of thyroxine to certain serum proteins as seen by co-precipitation (Taurog & Chaikoff, 1948), retention during dialysis (Trevorrow, 1939) and electrophoretic mobility (Gordon, Gross, O'Connor & Pitt-Rivers, 1952). Electrophoretic analysis has been most extensively used in characterizing serum 'thyroxine-binding protein' (TBP) and in determining the properties of binding of thyroxine by serum proteins (Robbins & Rall, 1957). Thus the

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problem presented in the above question was attacked by a simultaneous electrophoretic study of binding of thyroxine by proteins and their action of 'stabilization' of thyroxine in the process of ionization of its phenolic hydroxyl group (the term 'stabilization' as used in this context refers only to the inhibition of the transient loss and re-formation of thyroxine and bears no reference to the long-term instability of thyroxine, observed on storage). In this way, the study of different human serum and rabbit tissue proteins revealed that the 'stabilization' effect of any fraction was indeed intimately related to its thyroxine-binding ability and the extent of inhibition of the lability of the iodophenol was a direct function of the concentration and 'affinity' of any given protein for binding thyroxine. The following is a detailed account of this new aspect of the interaction between proteins and thyroxine, and some practical applications of this effect in the biochemistry of thyroid hormones have also been proposed.

EXPERIMENTAL

The following proteins or protein mixtures were studied for their interaction with L-thyroxine or structurally related compounds: whole normal human serum, human serum albumin (Lister Institute), γ-globulin (Lister Institute), β-globulin (Fraction G-2, Lister Institute), β_1-globulin (Siderophilin, Protein Foundation Inc.), 'thyroxine-binding protein' in the form of Cohn fraction IV-6 of human serum (Freinkel, Dowling & Ingbar, 1955), human serum pre-albumin prepared by Schultze, Schönenberger & Schwick (1956) and rabbit skeletal muscle cellular thyroxine-binding protein fraction. The last fraction was prepared by a method similar to that described for rat skeletal muscle (Tata, 1958); the extract was then purified by electrophoresis on a cellulose column in 0·05M-tris (2-amino-2-hydroxymethylpropane-1:3-diol) buffer according to the method described by Flodin & Porath (1954) and the fraction of the eluate with maximal thyroxine-binding capacity was retained. Except for β_1-globulin (siderophilin), all protein samples were dissolved in the smallest volume of 0·05–0·1M-NaCl, dialysed against a large volume of 0·025M-NaCl and lyophilized. The freeze-dried preparations were used as such by weighing out the required amount for the individual reaction vessel. β_1-Globulin was dialysed against 0·05M-ethylenediaminetetra-acetate and stored as a 15% (w/v) stock solution.

L-Thyroxine, labelled with ^{131}I in the 3′ and 5′ positions, was stored, frozen in solution in 15% propane-1:2-diol. In some experiments, ^{131}I-labelled 3:5:3′-tri-iodo-L-thyronine, 3:5-di-iodo-L-tyrosine, 3-iodo-L-tyrosine and 3:5-di-iodo-*p*-cresol were also used. Radioactive thyroxine and tri-iodothyronine were obtained from Abbott Laboratories, Oak Ridge, Tenn., U.S.A.; the other substances were prepared in our laboratory by radio-iodination.

Buffers used were prepared according to Gomori (1955), except for those of extremely high or low pH, for which appropriate dilutions of HCl and NaOH were used. When proteins were added, the pH of the medium was measured again after the addition of proteins. Water dimineralized by ion-exchange was used throughout the work.

The techniques of observing and measuring the loss and reincorporation of ^{131}I in thyroxine or other iodophenol and the material used were identical with those described in the preceding paper (Tata, 1959). The temperature in all experiments was 37°. The binding of thyroxine to proteins was measured by electrophoresis on paper and in some cases supplemented by the measurement of ^{131}I in the protein precipitate obtained with 12·5% trichloroacetic acid. Electrophoresis was carried out either in 0·075M-veronal buffer (Block, Durrum & Zweig, 1955) or in the same buffer used for the incubation. The fraction of total radiothyroxine migrating with the protein fraction studied was determined by measuring the areas of distribution of ^{131}I on the electrophoresis diagram. The fraction of radioactivity that remained immobile on electrophoresis was judged to be the fraction of unbound or 'free' thyroxine.

A deiodinase from rabbit skeletal muscle was used to illustrate the effect of serum proteins on the enzymic deiodination of thyroxine. The skeletal muscle enzyme was obtained from an extract of low ionic strength (I, 0·02) by fractionation with 48–56% saturated $[NH_4]_2SO_4$, followed by heat treatment (43° for 2·5 min.) and adsorption on calcium phosphate gel (Tata, unpublished work).

RESULTS

Fig. 1 illustrates the effect of inclusion of serum proteins in the aqueous medium into which is introduced a small volume of ^{131}I-labelled L-thyroxine, dissolved in 20% propane-1:2-diol. In the protein-free buffer of pH 7·5, about 30% of

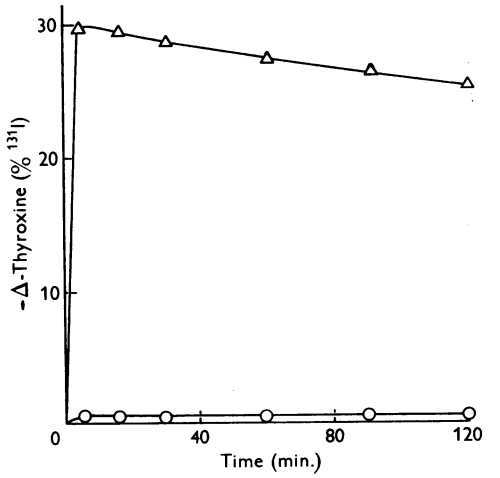

Fig. 1. Inhibitory effect of the presence of human serum in the aqueous buffer on the spontaneous disappearance of a part of radioactivity in the thyroxine fraction, as judged by chromatographic analysis. At time 0 min., 0·05 μg. of ^{131}I-labelled L-thyroxine in 0·04 ml. of 20% propane-1:2-diol was added to 2·8 ml. of the aqueous medium. ○, Buffer (tris maleate, pH 7·5) + human serum (diluted 1:4); △, buffer alone.

radioactivity in the thyroxine fraction had disappeared instantaneously after mixing, followed by a slow re-formation of radioactive thyroxine. But thyroxine failed to exhibit this property in the presence of human serum proteins at the same pH. In this particular case, the final concentration of serum proteins corresponded to a 1:4 dilution of whole serum. A quantitative relationship was observed between the final concentration of serum proteins and the extent to which the reaction was inhibited and this relationship is discussed below.

A similar 'stabilization' of thyroxine in the presence of serum proteins was observed at higher but not at lower pH values. A summary of the effect of total human serum proteins on the spontaneous loss of radiothyroxine in the first 5 min. is presented in Fig. 2. It was found that the addition of serum proteins partially or totally failed to suppress the initial transient loss of ^{131}I from thyroxine at pH values lower than 6·0. Although the reaction of spontaneous ^{131}I disappearance is less intense between pH 3·0 and 6·0 than in more alkaline media, the hump seen in the lower curve in Fig. 2 is very significant. The absence of the serum effect was most marked at pH 4·5, which is in the neighbourhood of the isoelectric point of many serum proteins.

Addition of serum proteins to a solution of labelled thyroxine 30 min. after dilution, to give the equivalent of serum diluted 1:4, caused the immediate reappearance of all the ^{131}I in thyroxine. A typical result is illustrated in Fig. 3. The final protein concentration determined the magnitude of the effect. An example of this is given in the second case in Fig. 3, in which only a partial immediate re-formation of thyroxine was produced when the final serum protein concentration corresponded to a 1:10 dilution. The remainder of ^{131}I then reappeared as thyroxine at the same slow rate seen in the control protein-free samples.

From these results it can be concluded that (*a*) the spontaneous and reversible breakdown of thyroxine cannot occur under physiological conditions in the presence of blood and (*b*) the net effect of serum is the same as that found under conditions that tend to suppress the ionization of the phenolic hydroxyl group of thyroxine (Tata, 1959).

Relationship between thyroxine-binding and 'stabilization' of thyroxine by proteins

In establishing this relationship, advantage was taken of the fact that the binding of thyroxine is localized in well-defined protein fractions of the

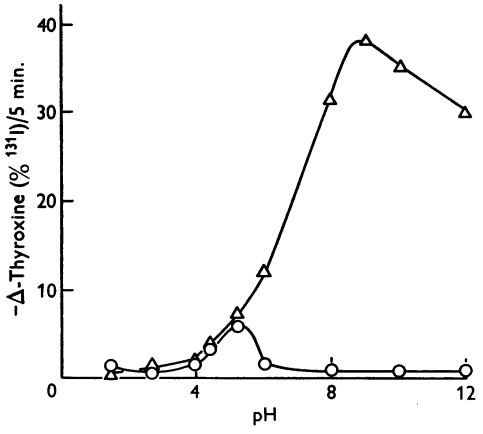

Fig. 2. Effect of H^+ ion concentration on the inhibition by human serum (diluted fourfold) of the spontaneous loss of radioactivity in the thyroxine fraction caused by mixing a solution of the labelled iodophenol in 20% propane-1:2-diol with aqueous buffers. The change in the radioactivity in the thyroxine fraction in chromatograms was recorded 5 min. after the mixing. Final concentration of thyroxine, 8–20 μmM. ○, Human serum + buffer; △, buffer alone. Buffers used: up to pH 2·5, acetic acid; pH 3·0–5·0, citrate; pH 5·0–7·0, phosphate; pH 7·0–9·0, tris maleate or tris HCl; pH 9·0–12·0, carbonate or NaOH.

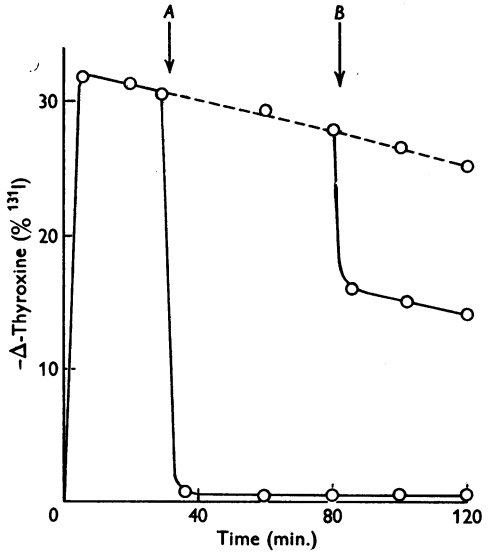

Fig. 3. Accelerated re-formation of radioactive thyroxine in chromatograms as a result of the addition of human serum after the initial disappearance of thyroxine ^{131}I had occurred in the usual manner. At the point indicated by vertical arrow *A*, sufficient human serum was added to obtain a final fourfold dilution; at the point of vertical arrow *B*, in another reaction vessel, the amount of human serum added gave a final 1:10 dilution. The dashed line represents the slow reappearance of thyroxine radioactivity in the serum-free control. Final concentration of ^{131}I-labelled L-thyroxine, 7 μmM; 0·04 ml. of thyroxine solution in 20% propanediol was mixed with 2·5 ml. of tris maleate buffer, pH 7·4.

serum; a comparison of the influence of thyroxine-binding and non-thyroxine-binding proteins on 'stabilization' of thyroxine hence proved useful. For this purpose, electrophoretic analysis was simultaneously performed on the same sample withdrawn for chromatographic determination of the distribution of ^{131}I.

Results obtained from such studies revealed that the thyroxine-binding property of proteins was indeed closely related to their 'stabilizing' action on thyroxine. This is illustrated in Fig. 4 for some human serum protein fractions. In the experiments illustrated, the final protein concentration in any fraction was adjusted to about 30% of what was estimated to be its concentration in normal human serum. It is thus seen that the extent to which thyroxine was 'stabilized' always very nearly corresponded to the fraction of thyroxine bound to the proteins. β- and γ-Globulin fractions which are feeble in binding thyroxine failed to affect significantly the spontaneous reaction; on the other hand, strong inhibition was exhibited by the 'thyroxine-binding protein' (Cohn fraction IV-6, in this case) and human serum albumin. Also the pre-albumin fraction, about which nothing was known at the time of this work as regards thyroxine-binding, showed the most intense stabilizing effect and a correspondingly intense thyroxine-binding power at concentrations much lower than for other proteins. More recently, Rich & Bearn (1958) and S. H. Ingbar (personal communication) have also found the pre-albumin fraction to be the most effective thyroxine-binding protein. Among the other protein fractions also tested in this manner, but not reported in Fig. 4, were human-serum β_1-globulin or metal-binding protein (siderophilin) and a preparation of cellular thyroxine-binding protein from rabbit skeletal muscle. The former had only a insignificant capacity for binding thyroxine or inhibiting the spontaneous disappearance of thyroxine, whereas the tissue extract occupied a position midway between human γ-globulin and serum albumin fractions.

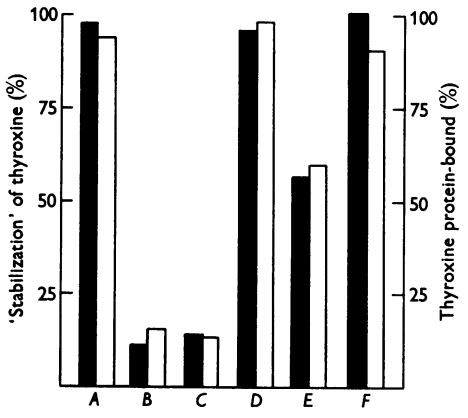

Fig. 4. Relationship between the 'stabilization' effect (black bars) and thyroxine-binding (open bars) by human serum and some of its protein fractions. The 'stabilization' effect was determined by measuring the percentage inhibition of initial loss of radioactivity, in the first 5 min. after mixing, in the thyroxine fraction (chromatographically) in the presence of proteins as compared to the protein-free buffer (0·05M-tris maleate, pH 7·5). The percentage of thyroxine protein-bound was determined by paper electrophoresis carried out on the same samples. Protein fractions and their concentrations: *A*, Whole human serum, 28·0 mg./ml.; *B*, γ-globulin, 5 mg./ml.; *C*, β-globulin (Lister fraction G-2), 5 mg./ml.; *D*, Cohn fraction IV-6, 5 mg./ml.; *E*, human serum albumin, 15 mg./ml.; *F*, pre-albumin, 2·5 mg./ml. Final concentration of thyroxine, 2·5 μmM. 0·04 ml. of propanediol containing thyroxine was mixed with 2·5 ml. of buffer.

If the pH of the medium and concentration of thyroxine were held constant, the only variable was the concentration of the protein. Thus the values for the percentage inhibition of ^{131}I disappearance and the fraction of thyroxine that was protein-bound both decreased to the same extent as the concentration of serum or protein fraction. The same was also true for the accelerated re-formation of thyroxine caused by the addition of thyroxine-binding proteins after the initial loss of ^{131}I had occurred. For example, in the experiment illustrated in Fig. 3, when samples were analysed for thyroxine-binding by electrophoresis, it was found that in the mixture to which the serum was added to obtain a final 1:4 dilution, over 90% of the thyroxine was bound to the protein. But in the sample containing the 1:10 dilution of serum, only about 50% of the thyroxine was bound to any protein, which corresponded to the extent of accelerated re-formation of thyroxine.

A more systematic study of the effect of protein concentration on the relationship between 'stabilization' of thyroxine and the protein-binding was then carried out with different protein fractions and some of the results are summarized in Fig. 5. All measurements were made using the same lowest possible levels of labelled thyroxine (0·003 μM) and the H^+ ion concentration was adjusted as near as possible to the physiological pH.

Besides providing further evidence for the identification of binding with stabilization, the results bring out the following characteristics: (1) As protein concentration fell, the values of inhibition of transient loss of thyroxine and the extent of binding to protein were found to fall off until both the functions were lost. Except at very low concentrations, the rate of fall was identical in both cases. Thus the extent to which the instability of thyroxine was inhibited by any binding protein

was directly proportional to the fraction of total thyroxine that was bound to the protein. (2) The rates at which the 'stabilization' effect and protein-binding diminished with decreasing concentrations of the proteins were different for the different proteins. (3) The minimal concentration of the protein fraction necessary to bring about binding or 'stabilization' depended on the protein studied. It was in increasing order as follows: human serum pre-albumin, Cohn fraction IV-6, human serum albumin, rabbit skeletal muscle thyroxine-binding protein fraction and γ-globulin (not shown in Fig. 5). (4) As seen by criteria of thyroxine-binding, studied by electrophoresis, or precipitation with trichloroacetic acid, and the inhibition of transient disappearance of thyroxine, human serum pre-albumin as isolated by Schultze *et al.* (1956) was found to exhibit the most intense interaction with thyroxine. This was valid both as regards the amount of protein required to obtain a given degree of binding or 'stabilization' and the narrow range of concentration observed for zero and maximum interaction.

At very low protein concentrations the issue is confused by competitive binding of thyroxine to the paper used for electrophoresis.

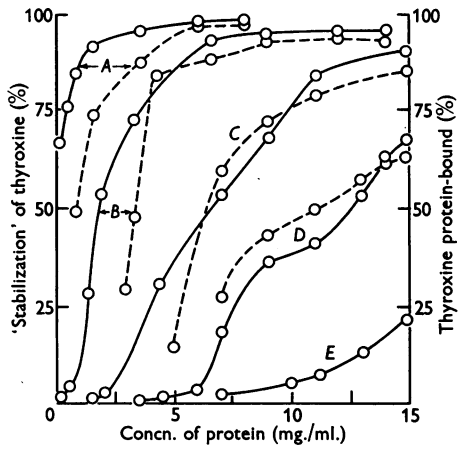

Fig. 5. Effect of variation of protein concentration on two aspects of the interaction between thyroxine-binding protein fractions and thyroxine, measured simultaneously. The 'stabilization' of thyroxine and the fraction of protein-bound thyroxine were determined as mentioned in Fig. 4, and all other experimental conditions were identical. O—O, Percentage of thyroxine 'stabilized'; O- - -O, percentage of thyroxine protein-bound; *A*, human serum pre-albumin; *B*, Cohn fraction IV-6; *C*, human serum albumin; *D*, rabbit skeletal muscle thyroxine-binding protein extract; *E*, human serum γ-globulin. It was not possible to determine the percentage of thyroxine bound to γ-globulin because of low electrophoretic mobilities of thyroxine and this protein fraction.

Interaction with tri-iodo-L-thyronine and iodinated tyrosines

In general, the same holds true for tri-iodo-L-thyronine as for L-thyroxine in the experiments described below, except that the extent of transient instability of tri-iodothyronine is small compared with that of thyroxine (Tata, 1959) which means that the values obtained for the 'stabilization' effect of proteins are less accurate. But all experiments with tri-iodothyronine confirm the previously described finding that the affinity of whole serum for this hormone is about one-third of that shown towards thyroxine (Robbins & Rall, 1955; Larson & Albright, 1955). The same is true for the 'stabilization' and binding effects with Cohn fraction IV-6 and serum pre-albumin, with which the lower affinity was even more marked. With the human serum-albumin fraction, however, the affinity for tri-iodothyronine was only slightly lower than for thyroxine.

With 3-iodo-L- and 3:5-di-iodo-L-tyrosines, although the transient instability of labelled iodophenol is very low, no significant 'stabilization' effect was detected for the different proteins in the concentrations of protein and iodinated compounds used for thyroxine and tri-iodothyronine. Not more than 10% of these amino acids were bound to any protein as seen by electrophoresis and less than 20% as seen by precipitation with trichloroacetic acid, which is in agreement with previous reports on the absence of any significant protein-binding of iodotyrosines (Tong, Taurog & Chaikoff, 1954; Robbins & Rall, 1957).

DISCUSSION

Whole human serum and some of its protein fractions and rabbit skeletal muscle protein(s) will interfere with the spontaneous and self-reversible loss of ^{131}I-labelled thyroxine, as judged by chromatographic analysis, during the ionization of the phenolic hydroxyl group, under conditions described in the preceding paper (Tata, 1959). The forward reaction (disappearance of radioactivity) is inhibited while the backward reaction (re-formation of radioactive thyroxine) is accelerated, hence conferring a 'stability' on thyroxine under conditions in which the phenolic hydroxyl group of the free compound would be partially or wholly ionized.

The mechanism and possible biological function of such an effect remain unknown, but it appears certain that the transient instability, or the loss and re-formation of thyroid hormones, cannot take place in the presence of most animal tissues and body fluids (no animal tissue or fluid has been shown to lack thyroxine-binding proteins). The

most intense interaction between proteins and the iodophenols, i.e. both the 'stabilization' and binding effects, were observed with the iodophenol which exhibits the highest degree of transient instability upon ionization of the phenolic hydroxyl group. Thus the lower degree of transient lability of tri-iodothyronine as compared with that of thyroxine (Tata, 1959) corresponds with the more feeble affinity of thyroxine-binding proteins for tri-iodothyronine. From this comparison it would appear that the degree of dissociation of the phenolic group determines the intensity of the interaction with proteins, as Robbins & Rall (1957) have suggested for the difference in binding affinity for the 3′:5′-dinitro and 3′:5′-dimethyl analogues of thyroxine. However, it is not only a question of the number of substituents *ortho* to the phenolic hydroxyl group or its p*K*, because there is even a more marked difference between thyroxine and diiodotyrosine. Such results also confirm the suggestions previously made (see Robbins & Rall, 1957) that all the major groups of the thyroxine molecule are essential for enabling it to combine more strongly with proteins than do other iodinated compounds.

Determination of thyroxine-binding power of proteins

While the mechanism of this interaction still remains unknown, this property has already been exploited usefully for solving some problems of biochemistry of thyroid hormones. These applications are based on the finding that the 'stabilization' of thyroxine is a direct function of the well-known binding of thyroxine by certain blood and tissue protein fractions. The most useful application so far has been that of studying the binding of thyroid hormones by proteins more quantitatively and under stricter physiological conditions than has been possible by other methods. If the percentage inhibition of the spontaneous loss of ^{131}I from thyroxine is measured at decreasing concentrations of a given protein fraction, then the lines obtained on plotting 'percentage of inhibition' against the 'logarithm of protein concentration' can be used for comparing the thyroxine-binding 'power' of different proteins. The slope of the line denotes the 'affinity' for binding thyroxine and the intercept that of the minimal protein concentration necessary for binding to occur at a fixed concentration of thyroxine. Alternatively, a comparison of the concentration of proteins necessary to obtain a 50% inhibition of the spontaneous loss of ^{131}I can also be used to evaluate the binding 'power' of a protein or protein fractions. The superiority of such a method over the conventional methods in which paper electrophoresis is used for measuring the interaction between proteins and thyroxine rests on the following advantages:

(1) It eliminates the unknown and variable factor of the binding of thyroxine by paper, used as a medium for electrophoresis. Interference by paper becomes substantial at low binding-protein concentrations and hence the non-electrophoretic method is particularly useful in studying thyroxine-binding by fluids of low protein concentration, such as tissue extracts and cerebrospinal and amniotic liquors.

(2) With the new method, only a trace amount of labelled thyroxine or related compound need be added and the relative protein mixture is not altered in comparing the binding potency of different proteins or protein mixtures. This is in contrast with electrophoretic analytical methods in which large amounts of thyroxine have to be added in order to observe the saturation point for a binding protein, or the introduction of different binding proteins in increasing amounts in order to obtain an effect of competition.

(3) With the method based on 'stabilization' of thyroxine, the interaction with proteins can be compared at physiological pH. Most workers have used alkaline media for electrophoretic analysis (see Robbins & Rall, 1957) or for equilibrium dialysis (Lein, 1952). A lower H^+-ion concentration is an important factor in the 'opening up' of binding groups of a protein which are not reactive under physiological conditions. This aspect is illustrated by comparing the 'stabilization' effects of proteins at different pH values, as shown in Fig. 6. Whereas almost the maximal 'stabilization'

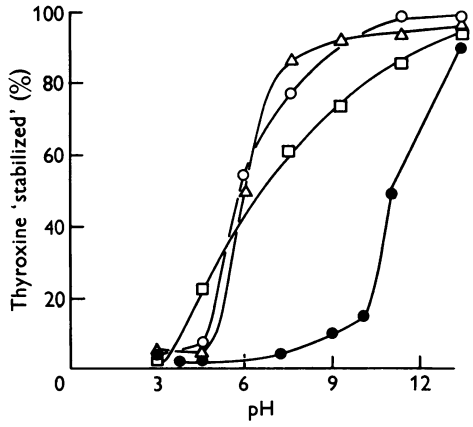

Fig. 6. Influence of pH on the 'stabilization' of thyroxine by human serum and some of its protein fractions. ○, Whole human serum (30 mg./ml.); △, Cohn fraction IV-6 (5 mg./ml.); □, human serum albumin (15 mg./ml.); ●, human serum γ-globulin (7 mg./ml.). The buffers used were the same as for those in Fig. 2; other conditions were identical with those for Fig. 4.

capacity was reached at pH 7·4 for whole serum or its major binding component, Cohn fraction IV-6, an increase in pH above 7·4 was accompanied by a further increase in 'stabilization' effect in the case of the albumin fraction. γ-Globulin, which normally does not bind or 'stabilize' thyroxine, was found to behave as binding proteins do, when the pH value was greater than 9·5. Later, it was confirmed that a similar situation also exists for thyroxine binding as measured by electrophoretic methods with buffers at varied pH.

(4) The method here proposed is useful in cases where electrophoretic separation of the possible binding components is doubtful. An example of this application has been the recent work on the absence in birds of significant thyroxine-binding by an α-globulin fraction (J. R. Tata & C. J. Shellabarger, unpublished work). In chick and duck sera most binding of endogenous and exogenous thyroid hormone was found to occur to albumin and there was no significant difference in the affinity of this fraction for thyroxine and tri-iodothyronine. This last finding can explain the identical biological activities of the two hormones in the chick (Shellabarger, 1955; Newcomer, 1957) and probably also the higher activity of tri-iodothyronine in mammals.

It should, however, be emphasized that the method based on the 'stabilization' of thyroxine is useful only for comparing the binding 'powers' of different proteins or protein mixtures and does not give any indication of the number of binding components in a mixture of proteins. For this purpose, it would be advantageous to use this method in conjunction with methods based on the electrophoretic separation of proteins.

Distinction between true deiodination and self-reversible loss of thyroxine, as judged by paper chromatography

A quite different principle has been used for the distinction between a true enzymic deiodination and the transient deiodination caused by ionization of the phenolic hydroxyl group when thyroxine or another iodophenol passes from an organic to aqueous medium and when the method of analysis is chromatographic. The principle is briefly illustrated in Fig. 7.

The conditions for the deiodinating enzyme were adjusted so that the initial velocity of the reaction approximately corresponded to the rate of loss of radioactive thyroxine in the spontaneous reaction. In both the enzymic and non-enzymic reactions the radioactive product appeared as 'iodide' in paper chromatograms. If human serum or any thyroxine-binding protein fraction was added a few minutes after the start of the enzymic reaction, further disappearance of thyroxine was inhibited and chromatographic analysis at subsequent time intervals showed no change in the amounts of thyroxine and iodide. Electrophoretic analysis revealed that the removal of the substrate by binding of thyroxine to serum proteins was responsible for the inhibition of the 'deiodinase'. However, when human serum was added to the reaction vessel containing the buffer alone, there was an instantaneous reappearance of the original ^{131}I in the thyroxine fraction and the disappearance of what appeared chromatographically as 'iodide', as in the experiments illustrated in Fig. 3. With purified 'deiodinase' solutions of low protein concentration, both reactions might occur simultaneously (based on paper-chromatographic analysis) but the difference in values of the iodide and thyroxine fractions before and after the addition of binding protein will then indicate the extent of true enzymic deiodination. This simple test for

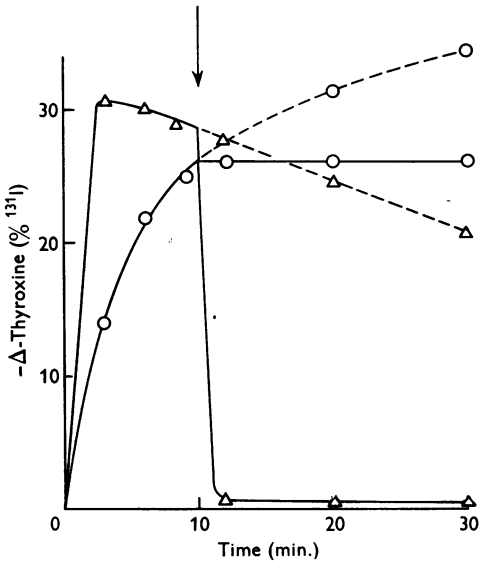

Fig. 7. Distinction between true enzymic deiodination (○—○, ○- - -○) and transient loss and re-formation of thyroxine (△—△, △- - -△) by addition of thyroxine-binding proteins, 10 min. (vertical arrow) after either reaction had started. Sufficient amount of whole serum, Cohn fraction IV-6 or serum albumin was added to bind all free thyroxine. The broken lines show how the two reactions would proceed if no protein were introduced. The transient reversible loss of thyroxine was produced by the mixing of 0·05 ml. of ^{131}I-labelled thyroxine solution in 20% propanediol with 2·5 ml. of tris maleate buffer at pH 7·5. In both this reaction and enzymic deiodination the radioactivity lost from thyroxine fraction appears as iodide in paper chromatograms. Enzyme was prepared in tris maleate buffer, pH 7·5. Final concentration of thyroxine added in both cases, 40 μmM.

differentiating between a true enzymic deiodination and an apparent, spontaneous deiodination has been routinely applied in our laboratory in studies on the purification and properties of the deiodinating enzyme.

The studies here presented raise the question of the importance of the ionization of the phenolic hydroxyl group in problems of thyroid-hormone biochemistry. For example, the function of binding or transport proteins in these studies appears to be the masking of the ionization of the phenolic group of thyroxine which normally, at a physiological pH, would be half-ionized (pK of the phenolic hydroxyl group of thyroxine, 6·4). The question is hence raised whether only the ionized thyroxine molecules are available for penetrating into tissue cells or for combining with some sites on the cell wall. A further question arises as to whether the degree of ionization of the phenolic group of thyroxine is related to the rate of interaction with enzymes controlling cellular oxidation or the control of permeability of membranes with respect to substrates of these enzymes.

SUMMARY

1. The transient instability of thyroxine and related iodophenols described previously (Tata, 1959) was inhibited by the presence of human serum in the aqueous medium, even when the serum was diluted fivefold. If the serum was added after the initial loss of thyroxine was noted, it caused the instantaneous reappearance of the original amount of thyroxine. Thus serum behaved as an agent masking the ionization of the phenolic hydroxyl group of the iodophenol.

2. The following human serum protein fractions were tested for the thyroxine 'stabilization' effect: albumin, γ-globulin, β-globulin, pre-albumin, Cohn fraction IV-6, and β_1-globulin. A rabbit skeletal muscle extract with thyroxine-binding properties was also tested. The effects were observed in decreasing order, with human serum pre-albumin, Cohn fraction IV-6, albumin and rabbit muscle extract; β-, γ- and β_1-globulins had very little, if any, influence on the stability of thyroxine.

3. By simultaneous electrophoretic analysis, it was found that the 'stabilization' effect of any protein was a direct function of the well-known thyroxine-binding properties. By studying the interaction with binding proteins at different dilutions, the fraction of thyroxine 'stabilized' was found to be equal to the protein-bound fraction. Human serum pre-albumin exhibited more intense binding and 'stabilization' properties than any other protein fraction.

4. Although the mechanism of this new aspect of interaction between proteins and thyroxine is unknown, the 'stabilization' effect has been applied in studying some problems of the biochemistry of thyroid hormones. The superiority of the 'stabilization' method over the conventional electrophoretic method for studying thyroxine-binding have been discussed. A new method has been proposed for distinguishing between a true and apparent deiodination of thyroid hormones, when studied by chromatographic analysis. These studies also point out the possible importance of the ionization of the phenolic hydroxyl group in some biochemical problems related to thyroid hormones.

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