The 'Transient Instability' of Thyroxine and its Biochemical Applications

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Tata (1959*a, b*, 1960) reported that, when a propylene glycol solution of a ^{131}I -labelled iodophenol is diluted about 100-fold in aqueous media of the appropriate pH and in the presence of bright light, and a sample is taken shortly thereafter and applied to paper for chromatographic analysis, a considerable proportion of the radioactivity is found to move with the R_F of iodide in two solvent systems. This radioactivity, however, decreases and that corresponding to the iodophenol then progressively increases again as samples are taken from the mixture and applied to paper at later intervals. For this reason Tata regarded this phenomenon as a 'transient' or 'self-reversible' alteration of iodophenols, occurring after their dilution from an organic to an aqueous medium. He concluded that an apparent and not a true deiodination of the iodophenol was involved, since it was not possible to demonstrate by analytical procedures other than paper chromatography the presence of ^{131}I iodide in the mixtures in amounts exceeding those contaminating the original propylene glycol solution of the labelled iodophenol. Tata (1959*a*) showed that light and ionization of the phenolic hydroxyl group were the necessary conditions leading to the observation of this phenomenon. In the absence of light or at a pH much lower than the pK of the iodophenol no alterations were observed: the pH values of the half-maximal rate of the 'transient instability' of a given iodophenol coincided with the pK of the phenolic hydroxyl group of the compound. An important observation was that proteins which showed binding affinity for the iodophenols 'stabilized' the latter when included in the aqueous media at a pH above the corresponding pK , presumably because the binding of the iodophenol to the protein masked or inhibited the ionization of the phenolic hydroxyl group. Increasing concentration, thyroxine-binding affinity and capacity of a given protein resulted in an increasing stabilization of the hormone. This finding led Tata to several conclusions about thyroxine-protein interactions, and to the description of a simple technique for the evaluation of thyroxine-binding powers of purified proteins and protein mixtures (Tata, 1959*b*, 1960): the intensity with which increasing amounts of the proteins inhibit the alterations of the iodophenol on

dilution into an alkaline aqueous buffer are determined by paper chromatography and compared.

The technique described by Tata (1959*b*, 1960) would have considerable advantages over the electrophoretic procedures usually employed (Robbins & Rall, 1957). However, though Galton & Ingbar (1961) have reported a 'stabilizing' effect of serotonin and analogues on the 'self-reversible' instability of thyroxine with this technique, in other Laboratories the observations described by Tata (1959*a, b*, 1960) have not been reproduced.

Taurog (1962) reported that, after dilution of ^{131}I thyroxine from a propylene glycol solution with an aqueous buffer at alkaline pH and paper-chromatographic analysis of samples taken from the mixture, much of the radioactivity is found in the spot corresponding to iodide only when 15–20 min. elapses between application of the sample to the paper and the chromatographic separation. When samples were applied to paper and transferred immediately to the chromatography jar, the proportion of ^{131}I found as iodide corresponded to that demonstrated in the original undiluted solution by other analytical procedures. Dilution of ^{131}I thyroxine into human plasma instead of the buffer, or addition of large amounts of stable thyroxine carrier to the spots, considerably reduced the deiodination of thyroxine on paper left to dry for a long interval before chromatography. Taurog (1962) suggests that part of the abnormal behaviour of iodophenols observed by Tata (1959*a, b*, 1960) might have been due to a true deiodination occurring after the sample had been applied to paper. Taurog (1962), contrary to Tata (1959*a*), found that light does not play an important role in the phenomenon.

The results reported in the present paper agree generally with the findings summarized by Taurog (1962), though it is shown that the deiodination of thyroxine on paper is due to the combined action of light and air. Several ways in which this oxidation may be avoided are summarized. The observations described by Tata (1959*a, b*, 1960) are easily reproduced once it is realized that the alterations of the iodophenols he demonstrated by paper chromatography had not taken place in solution but were due to their actual deiodination on the paper

exposed to light and air. The experimental conditions necessary to reproduce the technique described by Tata for the evaluation of the binding of iodophenols by proteins and the situations where it cannot be validly applied are also given.

EXPERIMENTAL

[¹³¹I]Thyroxine labelled in the iodine atoms of the phenolic ring was obtained from Abbott Laboratories (North Chicago, U.S.A.) in a 50% propylene glycol solution. Small volumes (10–30 μ l.) were diluted in the presence of light with 0.5–1.0 ml. of aqueous buffers of the pH indicated in each instance. After 5–10 min. a sample (15–30 μ l.) of the mixture was applied to Whatman no. 1 or 3MM filter-paper strips and dried as quickly as possible with hot air. The strips were then either chromatographed immediately in butan-1-ol-ethanol-aq. 2N-ammonia (5:1:2, by vol.) or left on a bench or windowsill for several hours before submitting them to paper chromatography. Stable thyroxine (30 μ g.) and iodide (10 μ g.) were added immediately before the strips were placed in the chromatographic tanks, once it had been found that this procedure did not alter results and later facilitated the identification of labelled compounds.

Whenever it is indicated that the experiments were carried out in the dark it is meant that, after dilution of the labelled hormone with the buffer in the presence of light, the rest of the procedure, namely application of the samples to the paper, drying and chromatography, was carried out in a dark-room. Diffuse red light was admitted for pipetting.

Paper electrophoresis was used in some instances for the determination of the intensity of deiodination. The samples of the mixtures containing [¹³¹I]thyroxine were either applied to the moist strips and electrophoresis was started immediately or applied to strips illuminated a few hours before the separation. The latter was carried out with a sodium veronal-sodium acetate-sodium chloride buffer, molal ratio 2:1:1 and *I* 0.1, adjusted to pH 8.6 with hydrochloric acid. A potential difference of 125 v was applied during 60–70 min.

The location of the [¹³¹I] moving as iodide or as thyroxine after chromatography or electrophoresis was determined by radioautography, by staining the iodide with palladium chloride and the phenol with diazotized sulphanilic acid or by scanning the strips automatically with two sensitive Geiger-Müller tubes. The proportions of [¹³¹I] in each compound were determined by counting the corresponding spots in a well-type scintillation counter or by planimetry of the areas resulting from the automatic scanning.

In most instances the [¹³¹I]thyroxine was diluted with 0.1M-citric acid-sodium phosphate buffer, pH 7.4. For other experiments at values between pH 3 and 8, citric acid-sodium phosphate buffers were used; for pH 9, 0.1M-sodium veronal-hydrochloric acid buffer and, for pH 11, 0.1M-sodium carbonate-sodium bicarbonate buffer were used.

The illumination procedure has been standardized in some experiments by placing the chromatographic paper strips on the glass lid of a box containing four parallel 20 w Philips daylight fluorescent tubes 12 cm. from the lid and illuminating for 2 hr. The procedure is carried out in the dark-room to avoid intrusion of other sources of illumination.

RESULTS

Samples of [¹³¹I]thyroxine, diluted in 0.5 ml. of buffer, pH 7.4, in the manner described above, were applied to Whatman 3MM filter paper and either kept in the dark or illuminated for 1 hr. on the windowsill. The spots were then eluted with methanol-aq. 2N-ammonia (3:1, v/v) and the eluates were submitted immediately to paper chromatography, paper electrophoresis and precipitation with trichloroacetic acid after addition of bovine plasma. The percentage of the total [¹³¹I] behaving as thyroxine with these analytical procedures was 91, 96 and 91% respectively for eluates of samples kept in the dark, and 70, 75 and 75% respectively for eluates of samples exposed to light. The rest of the radioactivity behaved as iodide.

When samples of a similar solution of [¹³¹I]-thyroxine in buffer, pH 7.4, were applied to Whatman no. 1 filter paper and kept in the dark or exposed to light for several hours, both in air or an oxygen-free nitrogen atmosphere, deiodination of the hormone, as assessed by paper chromatography, occurs only when the paper is exposed to light and air. In this case 40% of the total [¹³¹I] behaved as iodide, whereas with samples exposed to light in a nitrogen atmosphere, or kept in the dark in air or nitrogen, the percentage of the total [¹³¹I] was 5.6 and 6% respectively. This proportion of radioactivity corresponded to that contaminating the original solution of [¹³¹I]thyroxine in propylene glycol.

The effect of the nature and duration of illumination for the deiodination of trace amounts of [¹³¹I]-thyroxine on paper is shown in Fig. 1. The amount of [¹³¹I]iodide found on paper chromatograms kept in the dark during the whole procedure coincides with that contaminating the solution, as determined by several other analytical methods.

The effect of the pH of the buffer with which the labelled thyroxine is diluted is shown in Fig. 2. When samples from these mixtures were applied to Whatman no. 1 paper and submitted immediately to paper electrophoresis, no appreciable deiodination of the hormone was found. When the samples were applied to paper strips and exposed to a standardized light-source for 2 hr. before starting the electrophoretic procedure, deiodination of [¹³¹I]-thyroxine increased with the pH. The pH value corresponding to the half-maximal rate of deiodination corresponds to the pK of the phenolic hydroxyl groups given by Tata (1959a) for thyroxine. Ionization of the phenolic hydroxyl group is therefore an important factor for the deiodination of trace amounts of thyroxine on paper by light and air. The same pH curve was obtained when the experiment was done with paper chromatography as the analytical procedure.

The effect of the addition of increasing amounts of human and duck plasma to the buffer, pH 7.4, with which the [131 I]thyroxine was diluted, is shown in Fig. 3. When the dilution was carried out in the presence of light and the samples were applied to paper in the dark, there was no deiodination of the hormone, as indicated above. When, however, the samples were illuminated after application to the paper, the resulting deiodination of [131 I]thyroxine decreased as the proportion of plasma in the mixture increased. Human plasma had a more intense effect than duck plasma. When the results were plotted against the actual concentration of protein in the medium there was hardly any difference between the protecting effect of human and duck

plasma, since the former had 7.4 g. of protein/100 ml. and the latter had 2.8 g. of protein/100 ml. Qualitatively the same results were obtained when the experiment was done in a slightly different manner (Fig. 3B): 20 μ l. samples of the mixtures of protein and buffer were applied to paper strips and immediately afterwards 20 μ l. of [131 I]thyroxine previously diluted with the aqueous buffer was added to the same spots and illuminated.

Plasma proteins are not the only substances that protect thyroxine from the oxidative action of light and air. For example, addition of suitable amounts of a reducing agent, such as propylthiouracil, to the paper in suitable amounts stabilizes the hormone. About 150 μ g. of this drug must be applied to the spot to ensure complete protection of trace amounts of thyroxine from the effect of exposure to light and air for several hours. This amount is much higher than that which would be delivered to the paper if the solution containing the hormone were made about 1 mM with respect to the reducing agent. The latter was the procedure employed by Tata (1959a), who found no influence of reducing agents on the 'transient instability' of thyroxine. If stable thyroxine is also added to the spot containing trace amounts of labelled hormone, the amount of reducing agent necessary to ensure complete protection for several hours decreases considerably. We have found that the addition of 40 μ g. of stable thyroxine and 20 μ g. of propylthiouracil before or immediately after the application of the sample containing

Fig. 1. Filter-paper strips to which trace amounts of thyroxine in solution in citric acid-sodium phosphate buffer, pH 7.4, had been applied were kept in the dark (●), or illuminated on the windowsill (▲) or over a standardized light-source (○) described in the Experimental section, for different periods. Stable iodide and thyroxine carriers, and propylthiouracil were applied to the spots at the end of these times and the samples were submitted to paper chromatography.

Fig. 2. Effect of pH of the buffer used for diluting the [131 I]-thyroxine on the intensity of deiodination of samples applied to filter paper and either submitted immediately to electrophoresis (●) or exposed to light and air (○) for 2 hr.

Fig. 3. Effect of human (●) and duck (○) plasma on the intensity of the deiodination of [131 I]thyroxine exposed to light and air on filter paper. The decrease in the [131 I]iodide found after paper chromatography, calculated from the maximal deiodination obtained with the sample containing no protein, is expressed as a percentage of thyroxine 'stabilized'. The amount of [131 I]iodide originally contaminating the [131 I]thyroxine solution was determined by working in the dark and corresponded to 100% 'stabilization'. (A) [131 I]Thyroxine was diluted with mixtures of buffer, pH 7.4, and increasing amounts of plasma; samples were applied to paper, illuminated and chromatographed. (B) Samples of mixtures of the buffer, pH 7.4, and increasing amounts of plasma were applied as spots to filter paper. A few minutes later samples of a solution of [131 I]thyroxine in the buffer, pH 7.4, were delivered to the same spots, which were then illuminated and later chromatographed.

the labelled hormone protects thyroxine from deiodination and permits good staining with diazotized sulphanilic acid. Higher amounts of propylthiouracil interfere with the latter reaction.

Serotonin, when added to the solution of labelled thyroxine at a final concentration of about 2.5 mM, also protects the hormone from deiodination on paper, whereas lower concentrations of serotonin afford progressively smaller degrees of protection. Our experiments have not clarified the mode of this action of serotonin. Galton & Ingbar (1961, 1962) have described the inhibiting effect of serotonin on the deiodination of iodothyronines by several other systems.

DISCUSSION

Deiodination of thyroxine in solution during exposure to light has been reported (Lein & Michel, 1959). The present results show that thyroxine is also deiodinated in a dry state on chromatographic paper in the presence of light and air as assessed by chromatography, electrophoresis and precipitation by trichloroacetic acid. This deiodination involves an oxidation potential, as may be concluded from the results reported by Lissitzky, Benevent & Roques (1961), Stanbury (1960) and Yamazaki & Slingerlaand (1959) and may be easily shown by adding [^{131}I]thyroxine to the cathodic and anodic chambers of an electrolytic cell and applying about 2v: deiodination only takes place at the anodic chamber (G. Morreale de Escobar, P. Llorente, T. Jolin & F. Escobar del Rey, unpublished work). In the deiodination of thyroxine on filter paper by air and light the oxidizing agent may be atomic oxygen.

From the results described by Taurog (1962) and the present ones it is clear that considerable errors may be introduced in the chromatographic analysis of samples containing trace amounts of [^{131}I]thyroxine if the findings described above are not taken into consideration. These errors may be avoided or minimized in several ways. The addition of plasma proteins or of carrier stable thyroxine in suitable concentrations protects the iodophenol from deiodination by light and air on paper. The depression of the pH to a few points below the pK of the iodophenol, or the addition of suitable concentrations of serotonin or of a reducing agent, are equally effective. However, it is not always convenient to add one of these substances to the solution or to the spot on the chromatographic paper: their presence may mask the observation of the actual chemical moiety of the iodinated compounds formed during a reaction. The simplest procedure is to apply the samples to paper in the dark and to start the chromatographic or electrophoretic procedure immediately, preferably in a dark-room. In case the onset of the analytical procedure has to be delayed, the strips are kept all the time in the dark.

Avoidance of this source of chromatographic artifacts is specially important in studies on the deiodination of thyroxine by tissues or other systems. The measures described above are valid under the conditions reported in this paper and require to be tested for other experimental situations. We have observed that working in the dark may not avoid further deiodination of thyroxine after the sample has been added to the chromatographic paper, when the deiodinating system under study has a high oxidation potential. The oxidizing action of light and air is then no longer necessary to deiodinate the hormone on paper, since the oxidant is being applied together with thyroxine to the same spot. The presence of an oxidizing agent in the air, for instance, might also deiodinate thyroxine on filter paper in the dark. Failure to realize these facts has caused us considerable trouble in studies on the deiodination of thyroxine by systems such as ferricyanide-ferrocyanide. Further deiodination occurred once the samples were applied to paper, unless suitable reducing agents were added to the same spot. In these cases, working in the dark had not improved results appreciably.

The phenomena described by Tata (1959*a,b*, 1960) have not always been reproduced. This might be due to the fact that in many laboratories stable iodinated carriers and thiouracils or other reducing agents are systematically applied to the chromatographic spot before or immediately after the sample containing the labelled iodophenol (Taurog & Chaikoff, 1957). Once it is realized that the alterations reported by Tata are due to true deiodination of thyroxine resulting from its exposure to light and air after the sample has been applied to paper, and not from its dilution from an organic to an aqueous solvent, both his observations and their biochemical applications are easily reproduced. In this Laboratory we use the technique described by Tata (1959*b*, 1960) for the evaluation of the thyroxine-binding power of purified proteins in the following manner: [^{131}I]thyroxine is diluted with an aqueous buffer, above pH 6, containing increasing amounts of the protein being tested. Samples are then applied to chromatographic strips, dried quickly with hot air, illuminated for 2 hr. at a fixed distance from a standardized light-source and chromatographed after addition of stable iodide, thyroxine and propylthiouracil to the paper. To determine the proportion of [^{131}I]iodide contaminating the original [^{131}I]thyroxine solution in propylene glycol, samples are applied to paper in the dark as described above. Paper electrophoresis is sometimes used instead of chromatography, since the [^{131}I]iodide formed is separated from the hormone in less than 1 hr. Samples of the mixtures of buffer and increasing amounts of the protein may also be added to the paper strips and an alkaline

solution of [^{131}I]thyroxine then applied to the same spots and illuminated.

The technique described by Tata (1959*b*, 1960) for the evaluation of thyroxine-binding powers of protein preparations may only be used validly for this purpose when other substances, e.g. serotonin, propylthiouracil, that may themselves protect thyroxine from deiodination can be excluded or controlled and evaluated independently.

The experiments described here have been carried out in conditions resembling as closely as possible those described by Tata (1959*a,b*, 1960). We found no alterations of [^{131}I]thyroxine soon after its dilution from an organic to an aqueous solvent of appropriate pH, unless samples were applied to filter paper and left exposed to light before the onset of the chromatographic or electrophoretic separation. Only when aqueous alkaline solutions of labelled thyroxine were kept in very bright light for many hours did the electrophoretic and chromatographic analysis of samples reveal the presence of large proportions of [^{131}I]iodide, although the application of samples to paper and the rest of the procedure had been carried out in the dark. In these cases it could be shown by several different analytical procedures that an actual deiodination and not a 'transient alteration' of the [^{131}I]thyroxine had taken place. These observations confirm the findings of Lein & Michel (1959) and were obtained under experimental conditions different from those used by Tata (1959*a*). The apparent 'self-reversibility' of the phenomenon described by Tata may be understood in view of the present results if it is supposed that the samples taken by him at different intervals after dilution of the iodophenol were applied to the same paper and then left exposed to laboratory light till the onset of chromatography. The first samples applied to the paper would have been exposed to the deiodinating action of light and air for a longer time than the last ones. In our experience the intensity of deiodination of trace amounts of [^{131}I]thyroxine on paper not only depends on the pH of the solution but also on the nature and the duration of the illumination. The impossibility of showing increased amounts of [^{131}I]iodide in the solutions by electrophoresis or by oxidation and extraction with organic solvents, and the lack of an effect of temperature of the solution on the reaction reported by Tata (1959*a*), may be explained by the present results.

The present results support the conclusion that, once the latter explanation is taken into consideration, the conclusions and applications drawn by Tata (1959*a, b*) from his work may be validly applied to the understanding of the conditions leading to the deiodination of iodophenols *in vitro*: an oxidative system, e.g. light and air, deiodinates thyroxine by a mechanism involving the ionization

of the phenolic hydroxyl group. Interactions that mask or inhibit this ionization, e.g. binding to proteins, protect the hormone from deiodination by such a system.

SUMMARY

1. When samples containing trace amounts of [^{131}I]thyroxine are applied to chromatographic paper and exposed to light and air, much of the hormone is deiodinated, as assessed by chromatography, electrophoresis and trichloroacetic acid precipitation of eluates.

2. The degree of deiodination of trace amounts of thyroxine on filter paper depends on the presence of air, the nature and duration of illumination, the ionization of the phenolic hydroxyl group of the hormone and the absence of reducing agents or proteins with high binding affinity for thyroxine.

3. Deiodination of trace amounts of thyroxine on filter paper exposed to light and air may introduce considerable experimental errors. These may be avoided by working in the absence of light, by adding (a) reducing agents to the paper, (b) proteins with thyroxine-binding power (either to the paper or the solution), (c) serotonin or (d) high amounts of stable thyroxine, or by lowering the pH of the solution below the pK of the phenolic hydroxyl group of thyroxine.

4. It is concluded that the observations of Tata (1959*a,b*, 1960), interpreted as a phenomenon of 'transient instability' of iodophenols on dilution from an organic to an aqueous solvent, were probably due to the deiodination of these compounds on chromatographic paper exposed to light and air. The experimental conditions necessary to reproduce systematically Tata's method for the measurement of the thyroxine-binding power of proteins (Tata, 1959*b*, 1960) are also summarized.

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