and does not modify the conclusion that acetylcholinesterase is present.

Of those tested here the only preparation splitting triacetin vigorously was that of the erythrocytes. This was to be expected, since acetylcholinesterase from erythrocytes does hydrolyse triacetin; furthermore, the preparation was not free from ali-esterases (Whittaker, 1951). Augustinsson (1949) has obtained a considerable activity with this substrate in preparations from both electric organ of electric eel (*Electrophorus electricus*) and muscles of guppy (*Lebistes reticulatus*). Acetylcholinesterase preparations from different fishes thus seem to differ in this respect.

An esterase is considered to be a cholinesterase if it is inhibited by 10^{-5} M-physostigmine (Augustinsson, 1957). This is true for the acetylcholinesterase activity of the goldfish preparation, as well as for those of electric ray and erythrocytes (Fig. 6). B & W 284C51 has been used as a selective inhibitor of acetylcholinesterase by several authors (e.g. Austin & Berry, 1953; Holmstedt, 1957). Recently, Tammelin (1958b) has shown methylethoxyphosphorylthiocholine to be a selective inhibitor of acetylcholinesterase. On the other hand, mipafox and DFP are known to be selective inhibitors of butyrylcholinesterase, their pI_{50} values [$-\log$ (concn. of inhibitor)] being between 7 and 8 (Holmstedt, 1957).

The results given in Figs. 7 and 8 show conformity between the three preparations tested. This is particularly clear for the two selective inhibitors of acetylcholinesterase. Also when treated with the selective inhibitors of butyrylcholinesterase the three preparations behave rather similarly. None is inhibited at significantly lower concentrations of the inhibitors than any of the others, the pI_{50} values being about 4 (mipafox) and 7 (DFP), instead of 7 and 8 as for butyrylcholin-

esterase. It thus seems reasonably well established that the extract from body muscles of goldfish contained acetylcholinesterase activity. Its substrate and inhibitor relations agree with those of the recognized acetylcholinesterase preparations from the electric organ of the electric ray and from erythrocytes.

SUMMARY

An extract from body muscles of goldfish (*Carassius auratus*) has been shown to contain acetylcholinesterase with the same substrate and inhibitor relationships as acetylcholinesterase preparations from human erythrocytes and from electric organ of electric ray (*Torpedo ocellata*).

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An Unusual Property of Thyroxine and Other Iodophenols

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In the course of the work on enzymic deiodination of thyroid hormones, L-thyroxine and 3:5:3'-triodo-L-thyronine, it was necessary to determine whether, and if so to what extent, spontaneous deiodination might occur. In some of the preliminary control experiments, which consisted of incubating thyroxine in enzyme-free aqueous

buffer solutions, a surprising observation was made: when a solution of ^{131}I -labelled thyroxine in an aqueous organic solvent was diluted with a relatively large volume of aqueous buffer, there occurred virtually instantaneously a loss of radioactivity originally present in thyroxine. But later, mild shaking of the solution, without any other treatment whatsoever, produced a gradual re-appearance of the lost radioactivity as thyroxine.

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This unusual and hitherto undescribed property of thyroxine was thought of interest and the phenomenon was further studied with thyroxine and other related iodophenols. In this paper the influence of various conditions, many identical with those used for studying the deiodinating enzyme (Tata, Rall & Rawson, 1957), on the reversible disappearance of radioactive thyroxine and related iodophenols, is described.

EXPERIMENTAL

The properties of the following iodinated phenols were studied after labelling them with ^{131}I : L-thyroxine, 3,5,3'-tri-iodo-L-thyronine, 3,5-di-iodo-L-tyrosine, 3-iodo-L-tyrosine, 3-iodo-5-bromo-L-tyrosine and 3,5-di-iodo-*p*-cresol. The compounds were labelled in the iodine atoms *ortho* to the phenolic group by substitution with radioiodine (see Taurog & Chaikoff, 1957) to give a specific radioactivity of 0.5–3.0 mc/ μmole . Thyroxine and tri-iodothyronine of higher specific activities were labelled by an exchange reaction and were obtained from Abbott Laboratories, Oak Ridge, Tenn., U.S.A. At least 90% of the total radioactivity was present as the iodinated phenol in each sample. All radioactive compounds were either stored in the dry state at 0° or frozen in 20–50% (v/v) aqueous propane-1:2-diol or butan-1-ol solutions.

The various buffers used were prepared according to Gomori (1955) in demineralized water (doubly glass-distilled water was also suitable). In general, the technique consisted in rapidly introducing 0.02–0.06 ml. of a solution of the radioactive iodinated compound, dissolved in 15–50% (v/v) aqueous propane-1:2-diol or butan-1-ol, into 2.5–3.0 ml. of the buffer; the diluted solution was then gently shaken at constant temperature in the presence of air. The usual temperature was 37°, although the effect of variation of temperature was also studied. At intervals of 5 min. to several hours after the mixing, portions (0.005–0.02 ml.) of the diluted iodophenol solution were withdrawn by capillary tubes for paper-chromatographic or electrophoretic analysis. For shorter time-intervals up to 30 sec., the mixing was done in haematological pipettes from which samples could be directly delivered for chromatography. The source of light used throughout the work was provided by two 40 w Osram Natural (84–23) fluorescent tubes placed at 100–150 cm. from the reaction vessels. Diffused red light was used to determine the effect of light on the reaction. Polythene apparatus replaced glassware in experiments for studying the influence of glass.

^{131}I -labelled constituents present in each sample were separated by chromatography on paper and the distribution of radioactivity was measured by automatic scanning and recording of chromatogram strips. The results were expressed as percentage of total ^{131}I remaining in the iodophenol fraction at any given time, after correction for the contaminant radioactivity present in the original solution in organic solvent. Quantitative chromatographic analysis, as used here, entails an error of ± 5 –10% and figures given in the results represent average values derived from 2–6 determinations. The usual chromatographic solvent used was butan-1-ol-dioxan (4:1, v/v) saturated with 2N-NH₃; other solvent systems used are described elsewhere (Tata *et al.* 1957). Paper-electrophoretic analysis at 30–40 v/cm.

for 10–20 min. was performed on Whatman paper no. 3MM, with the same buffer as that used for dilution of the compound.

The ultraviolet-absorption spectra of thyroxine and tri-iodothyronine were determined in 0.1M-Na₂CO₃ or 0.05M-NaOH in 1 or 4 cm. quartz cells in a Hilger Uvispek spectrophotometer. The apparent ionization constants of the phenolic hydroxyl groups of the iodinated phenols were determined by the spectrophotometric method of measuring the molar extinction at different H⁺ ion concentrations, as described by Gemmill (1955).

In some experiments the stability of iodine atoms was studied spectrophotometrically simultaneously with chromatographic analysis. For this purpose, the reaction was carried out in the absorption cell of the spectrophotometer by introducing the ^{131}I -labelled compound present in propane-1:2-diol into the cell containing the buffer and measuring the change in u.v. absorption at the wavelength maxima as a function of time; this change was compared with the change in distribution of radioactivity in samples withdrawn at different time intervals.

RESULTS

Some typical recordings of the distribution of radioactivity in chromatograms, prepared at different time intervals after the addition of ^{131}I -labelled thyroxine in 20% propane-1:2-diol to tris

Fig. 1. Distribution of radioactivity on chromatograms of samples withdrawn at different time intervals after the mixing of 0.018 μg . of ^{131}I -labelled L-thyroxine dissolved in 0.05 ml. of 20% propane-1:2-diol with 2.5 ml. of tris-HCl buffer at pH 7.6. Time of withdrawal of samples: A, before mixing; B, 10 min., C, 180 min., D, 300 min., after mixing. Chromatographic solvent: butan-1-ol-dioxan-ammonia; the peak with low R_f resembles that of iodide, while the other peak represents thyroxine. The number within or above each peak is the percentage of radioactivity distributed in each peak.

(2-amino-2-hydroxymethylpropane-1:3-diol) buffer at pH 7-8, are shown in Fig. 1. At time zero, which shows the analysis of radioactive thyroxine as present in propane-1:2-diol before mixing with the aqueous medium, more than 90% of ^{131}I was present in the thyroxine fraction; 5 min. after the mixing, about 40% of the original thyroxine radioactivity had disappeared and was found at a position on the chromatogram similar to that occupied by iodide ion. It is shown below that the transient product formed was not iodide, as was suggested by its chromatographic properties. With the passage of time, as shown in Fig. 1, analysis of samples withdrawn from the same reaction vessel revealed that the lost radioactivity began to reappear spontaneously in the thyroxine fraction until almost the original pattern was obtained. During this period of time no intervention, except the withdrawal of small samples for chromatography, was involved. This phenomenon is not simply a dilution effect, because no change occurred when 0.02-0.06 ml. of the same thyroxine solution was introduced into 2.5 ml. of the original solvent (20% propane-1:2-diol).

Fig. 2. Spontaneous disappearance and reincorporation of radioactivity in the thyroxine fraction as a function of time after the mixing (at time 0 min.) of ^{131}I -labelled thyroxine, dissolved in 18% propane-1:2-diol, with aqueous buffers of different pH values. Propanediol solutions (0.05 ml.) were added to 2.5 ml. of buffer and the final concentration of thyroxine was 10^{-7}M . The pH of the buffer is shown by the figure at the end of each curve. Buffers: pH 4.0, 0.05M-citrate; pH 7.2, 0.05M-tris-maleate-NaOH; pH 9.4, 0.05M- Na_2CO_3 - NaHCO_3 ; for pH 1.5 and 13.0, 0.08N-HCl and 0.1N-NaOH respectively were used. The term '-Δ-thyroxine' is used in this Figure (and in all subsequent Figures) to express the change in the percentage loss of ^{131}I in the thyroxine fraction in chromatograms of samples withdrawn after the mixing of the radioactive material.

The rate and extent of both the initial loss of radioactivity from thyroxine and its reappearance varied according to the pH of the aqueous medium. Fig. 2 depicts the influence of H^+ -ion concentration on the reaction with L-thyroxine.

The first points represent the initial breakdown of radioactive thyroxine in buffers of different pH values on the basis of difference in thyroxine ^{131}I on chromatograms before (time 0) and after mixing. The initial reaction was very rapid; in some experiments the maximum loss of radioactivity had occurred 20 sec. after the mixing. Thereafter, the reaction had in effect reversed and the downward-sloping lines in Fig. 2 represent the reincorporation of radioactivity in the thyroxine fraction on further standing. The time taken for the reappearance of the original distribution of ^{131}I in the thyroxine fraction varied according to the pH of the aqueous dilution medium: about 100 min. at pH 3.0-4.0; 250-350 min. at pH 6.0-8.0 and 150-220 min. at pH 12.0-14.0. Both the forward and backward processes were influenced by the pH of the buffer, but to a different extent.

Qualitatively similar results were observed with other radio-iodinated compounds studied. Results showing the effect of pH on the initial loss of

Fig. 3. Effect of H^+ ion concentration on the extent of disappearance of radioactivity, as seen chromatographically, from the various ^{131}I -labelled iodophenol fractions in the first 5 min. after the mixing has taken place. All substances were originally dissolved in 20% propane-1:2-diol, and 0.04 ml. of propanediol solution was added to 2.5 ml. of buffer. The nature of the buffer used had no effect. Final concentrations: 0.085 μM for thyroxine and tri-iodothyronine; 0.3 μM for other iodophenols. $\circ$, L-Thyroxine; $\triangle$, 3,5-di-iodo-p-cresol; $\square$, 3,5:3'-tri-iodo-L-thyroxine; $\bullet$, 3,5-di-iodo-L-tyrosine; $\blacktriangle$, 3-iodo-L-tyrosine. The effect, with thyroxine, of replacement of all glassware by polythene apparatus is shown by the dashed line ($\circ$).

labelled iodine or the forward reaction are summarized in Fig. 3. The two major points of interest in the curves are: (1) Although identical conditions were employed for equimolar concentrations of the various iodophenols, the extent to which the initial breakdown occurred varied greatly from one compound to another. The following relationship for the magnitude of the reaction is obtained: thyroxine > di-iodo-*p*-cresol > tri-iodothyronine > di-iodotyrosine > monoiodotyrosine. This relationship is strictly valid for the forward reaction only and not for the reincorporation of radioactivity. The reaction with 5-bromo-3-iodotyrosine was too feeble to justify a comparison. (2) The optimum pH at which the very rapid loss of ^{131}I occurred was found to be different for the different iodophenols.

With all the *o*-iodophenols, the transient product formed occupied very nearly the same chromatographic position as that of iodide (R_f 0.25–0.30) in the butan-1-ol-dioxan-ammonia solvent system. Under similar conditions the R_f values of *o*-iodophenols here studied were as follows: L-thyroxine, 0.48; 3:5:3'-tri-iodo-L-thyronine, 0.64; 3:5-di-iodo-*p*-cresol, 0.89; 3:5-di-iodo-L-tyrosine, 0.10; 3-iodo-L-tyrosine, 0.19.

The dips observed in the pH curves at high pH values for thyroxine and di-iodo-*p*-cresol in Fig. 3 are caused by interference by some factor in glass produced under increasingly alkaline conditions. Thus, with thyroxine, the pH curve was found to attain a plateau, as shown by the dotted line, when polythene apparatus replaced all glassware.

Significance of the ionization of the phenolic group

In attempts to find the cause of this unusual property, the ionization of the phenolic hydroxyl group was implicated as being the most likely one, because this was the only common dissociable group in all the above compounds and the reaction occurred only during the passage of the iodophenol from the organic solvent into the aqueous medium. Results of the following two experiments supported the idea that the ionization of the phenolic

hydroxyl group was indeed responsible for the self-reversible change in distribution of ^{131}I : (1) The pH at which the initial disappearance of the compound proceeded at half the maximal rate was found to correspond closely to the dissociation constant of the phenolic hydroxyl group of each iodophenol. Such a relationship is shown in Table 1. The values of pH for half maximal rate

Fig. 4. Reversible effect of change in pH of the aqueous medium after the mixing has taken place. ^{131}I -Labelled thyroxine ($0.54\text{ }\mu\text{g.}$), dissolved in 0.06 ml. of 20% propanediol, was added to 2.5 ml. of 0.1N-NaOH or 0.1N-HCl , at time 0, and the loss or gain of radioactivity in thyroxine fraction determined chromatographically. (a) Aqueous medium was 0.1N-NaOH . At 30 min. after mixing (shown by vertical arrow) enough N-HCl was added to lower the pH to 0.5. Subsequent analysis revealed rapid reappearance of all thyroxine radioactivity; control to which no acid was added is shown as the dashed line. (b) 0.1N-HCl was the aqueous medium. At 30 min. after mixing (shown by vertical arrow) enough N-NaOH was added to raise the pH to 13.3. The change in pH is followed by instantaneous disappearance and slow reappearance of radioactive thyroxine. The dashed line indicates the control to which no alkali was added.

Table 1. Relationship between pH-dependence of forward reaction and the apparent pK values for the phenolic hydroxyl group of various iodophenols

Compound	pH of $\frac{1}{2}$ maximal initial ^{131}I disappearance	pK of phenolic OH (u.v. absorption)
L-Thyroxine	6.4	6.6
3:5:3'-Tri-iodo-L-thyronine	8.1	8.4
3:5-Di-iodo- <i>p</i> -cresol	4.9–5.8*	6.0
3:5-Di-iodo-L-tyrosine	6.6	6.5
3-Iodo-L-tyrosine	8.7	8.7

* It was not possible to reproduce values in a narrow range for this compound.

were derived from the pH curves in Fig. 3, while those of the apparent pK of the phenolic hydroxyl group were obtained by measurements of u.v.-absorption spectra of the different iodophenols. (2) The direct relationship between the degree of ionization of the phenolic hydroxyl group and the instability of the compounds was enhanced by the reversibility of both the forward and backward reactions brought about by a change in pH after the mixing of organic and aqueous media had taken place. Examples of two opposite effects obtained with thyroxine are illustrated in Fig. 4.

In Fig. 4*a*, it is seen that the suppression of the ionization of the phenolic-hydroxyl group caused by lowering the pH from 13.0 to 0.5 was accompanied by an instantaneous reappearance of the original amount of radiothyroxine. This effect was not due to the presence of salt formed by the neutralization of the alkali, because the addition of an equivalent amount of NaCl, without a diminution in the pH, had no effect. In Fig. 4*b*, it is seen that the sudden ionization of the phenolic group by making the medium alkaline was accompanied by the disappearance of thyroxine ^{131}I , followed by recombination in the usual manner. This feature of reversibility caused by change in pH was shared by all the other compounds.

Effect of other variables and light

The inclusion in the aqueous medium of organic solvents in which these iodophenols are highly soluble resulted in an inhibition of the initial

reaction as exemplified in Table 2 for radiothyroxine. The extent of inhibition depended upon the organic solvent used and the final concentration at which it was present. It is possible that more than one phenomenon is involved in the inhibitory effect of the above solvents because the effect of dimethylformamide cannot be reconciled with that of other solvents, solely on the basis of a change in the dielectric constant of the medium.

The concentration of the iodophenol, temperature, the composition of the buffer and the ionic strength of the aqueous medium all had minor effects on the rate and extent of the reaction, which are not observed in enzymic deiodination. The variation in concentration of these iodinated compounds was limited, except at very high or low pH, by their extreme insolubility in water. However, in most cases, approximately the same fraction of radioactivity was found to disappear after the passage of the substance from organic to aqueous medium, over a wide range of concentration. But the concentration of the organic solvent in which the iodophenol was dissolved was important; in general, the extent of reaction occurring after mixing was proportional to the concentration of propane-1:2-diol or butan-1-ol. Variation of temperature over the range 7–57° had little effect. At most, a variation of 10° resulted in a few cases in only a 5–10% difference in reaction rates. Interchange of citrate, acetate, tris maleate, tris hydrochloride, phosphate and carbonate buffers at over-lapping pH values resulted in no change in the pH curves shown in Fig. 3.

Table 2. *Effect of inclusion of organic solvents in the aqueous buffer on the extent of initial ^{131}I disappearance*

Aqueous buffer: 0.05 M-tris, pH 7.8; original thyroxine solution in 25% propane-1:2-diol; final thyroxine concentration 0.095 μM .

Organic solvent	Final concn. (% v/v)	– Δ -L-Thyroxine (% ^{131}I /5 min.)	Inhibition (%)
None	—	48.3	—
Methanol	30	25.0	48.3
	70	3.6	92.5
Ethanol	30	21.7	55.0
	60	2.0	95.8
Propan-1-ol	30	24.0	50.4
	60	4.6	90.5
Butan-1-ol	10	28.5	41.0
	25	10.5	78.2
	40	3.0	93.8
Acetone	30	30.5	36.5
	70	2.8	94.2
Propane-1:2-diol	10	24.0	50.4
	20	8.6	82.2
	30	0.0	100.0
<i>NN'</i> -Dimethylformamide	10	30.0	36.8
	20	16.5	65.8
	30	4.5	90.7

In contrast with the slight effects of the above variables, the reaction was found to be extremely photosensitive. This photosensitivity is illustrated in Fig. 5, for thyroxine.

In diffused red light, the reaction of spontaneous ^{131}I loss was either abolished or drastically reduced in magnitude. In Fig. 5 it is further seen that when the same samples studied in the dark for 1.5 hr. after mixing were then exposed to light (of the same intensity as that used in previous experiments), an instantaneous disappearance and re-formation of radioactive thyroxine was produced. The extent of both the forward and backward reactions was dependent upon the pH in the same manner as in previous experiments.

Actual disappearance and re-formation of thyroxine and the nature of products formed

Before an attempt was made to determine the nature of the radioactive products formed it was necessary to ascertain whether the loss and gain of radio-iodine of the iodophenolic fraction, as judged by the radioactivity of the latter, represented its true disappearance and re-formation. For this purpose it was decided to measure simultaneously the changes in the ultraviolet-absorption spectrum of thyroxine and chromatographic

Fig. 5. Effect of light on the spontaneous disappearance and reappearance of radioactivity in the thyroxine fraction as determined chromatographically. ^{131}I -Labelled L-thyroxine ($0.21\text{ }\mu\text{g.}$) in 0.05 ml. of 20% propane-1:2-diol was mixed with 2.5 ml. of aqueous buffers of different pH values at time 0 min. in 'dark' (diffused red light). 92 min. after the mixing, light, of the usual intensity, was switched on and samples were chromatographically analysed for a further period of 90 min. Figures at the end of curves indicate the pH of the buffers of similar composition as shown in Fig. 2.

distribution of radioactivity by performing the experiment directly in the quartz optical cell of a spectrophotometer. Unfortunately, at the only absorption maximum, at $325\text{ m}\mu$, so far described for thyroxine (Lemmon, Tarpey & Scott, 1950; Lissitzky, 1952; Gemmill, 1955; Lardy, 1955), the extinction was too low to be measured with accuracy at the low concentrations of thyroxine (10^{-8} to 10^{-5} M) used in this work, in spite of a linear relationship between concentration and extinction. However, on further examination it was found that thyroxine showed a second absorption maximum at $231\text{ m}\mu$ and similarly tri-iodothyronine was found to exhibit absorption maxima at 227 and $318\text{ m}\mu$. The absorption spectra of thyroxine and tri-iodothyronine in 0.05 M-NaOH (performed 24 hr. after the solutions were prepared) are shown in Fig. 6.

For both compounds the extinction at shorter wavelengths was found to be higher at all concentrations than that at 318 or $325\text{ m}\mu$. A linear relationship between optical density and concentration, in the range 10^{-6} M to $1.5 \times 10^{-5}\text{ M}$, was also obtained at the shorter wavelengths. Hence, it was possible to perform simultaneous chromatographic and spectrophotometric analyses after the ionization of the phenolic hydroxyl group. Results of one such experiment with thyroxine are summarized in Fig. 7.

Although the absorption at $231\text{ m}\mu$ only is shown in Fig. 7, the full absorption spectrum between 210 and $350\text{ m}\mu$ was measured at successive time intervals. It is seen in this figure that

Fig. 6. Ultraviolet-absorption spectra, in a 1 cm. optical cell, of $30\text{ }\mu\text{M-L-thyroxine}$ and $3:5:3'\text{-tri-iodo-L-thyronine}$ dissolved in 0.05 N-NaOH . Absorption maxima: L-thyroxine, 231 and $325\text{ m}\mu$; $3:5:3'\text{-tri-iodo-L-thyronine}$, 227 and $318\text{ m}\mu$.

the rate of increase in u.v. absorption at 231 m μ corresponds closely to the rate of re-formation of thyroxine as found by chromatography. These results strongly suggest that the disappearance and re-formation of thyroxine radioactivity in chromatograms reflects a true loss and gain of thyroxine.

However, the spectrophotometric studies shed no light on the nature of the transient radioactive product derived from thyroxine, and all attempts to solve this problem by other methods have been unsuccessful. From chromatographic analysis the only radioactive product formed had properties similar to those of iodide. But a consideration of the following results ruled out iodide as the transient radioactive product and showed that the deiodination, as observed chromatographically, was only an apparent one.

First, when both electrophoretic and chromatographic analyses were performed simultaneously on the same sample, a constant fraction of radioactivity appeared as iodide at all time intervals in electrophoresis diagrams; this fraction was equal to the percentage of ^{131}I present as iodide in the original organic solvent solution. The reaction product had the same electrophoretic behaviour as the parent iodophenol.

Secondly, only that fraction of ^{131}I present originally as iodide could be extracted from any sample as elementary iodine into CS_2 after acidification in the presence of Fe^{3+} ions or H_2O_2 and addition of ^{127}I as carrier. The acidification necessary for oxidation instantaneously caused the total reappearance of thyroxine or other iodophenol.

Thirdly, the presence of reducing agents such

as thiosulphate, metabisulphite, dithionite and 2-thiouracil in the aqueous medium in concentrations up to 10^{-3} to 10^{-2}M (except for 2-thiouracil, because of low solubility) neither altered the kinetics nor prevented the reincorporation of ^{131}I into thyroxine or other compounds. Fortification of the buffer with increasing amounts of iodide also failed to alter the kinetics of the 'forward' and 'backward' processes.

No partially de-iodinated radioactive products were formed from iodophenols labelled in more than one iodine atom, i.e. thyroxine, di-iodotyrosine and di-iodo-*p*-cresol. With di-iodotyrosine about 2–10 % of the total radioactivity appeared as thyroxine in chromatograms at the end of 1–4 hr. mixing with alkaline buffers at 37°.

The possibility of some kind of a reversible radioactivity-exchange reaction between iodide and the iodophenol was ruled out because, in spite of the addition of varying amounts of stable iodide or the organic solvent, the same fraction of ^{131}I appeared as the transformation product. Besides, the optimum alkaline pH values observed for the property here described are incompatible with the low pH and oxidative conditions essential for exchange reactions, already established for thyroxine, tri-iodothyronine and di-iodotyrosine (Miller, Anderson, Madison & Salley, 1944; Gleason, 1955; Taurog, 1955).

DISCUSSION

A transient disappearance and re-formation of ^{131}I -labelled thyroxine and structurally related iodophenols occurs when their solution in a small volume of organic solvent is diluted with a large volume of aqueous buffer. Although the general mechanism of this unusual reaction remains unknown, it is clear that the initial loss of labelled iodophenol is caused by the ionization of the phenolic hydroxyl group as a result of the passage from the organic to the aqueous medium. This conclusion has been drawn from the close relationship between the pH-dependence of the forward reaction and the *pK* values of phenolic-hydroxyl group for the different iodophenols and from the instantaneous reversibility of the reaction by changes in pH of the aqueous medium. As regards the *pK* values for phenolic hydroxyl groups, the figure of 8.4 reported here for 3:5:3'-tri-iodothyronine agrees better with that of 8.45 obtained by Gemmill (1955) than 9.2 reported by Lardy (1955).

The reaction as presented here might appear to be inconsistent with the second Law of Thermodynamics; this is probably due to the failure to detect an important step in the process and the identification of the transient product. None of the observations on the transient instability of iodophenols, conferred by the dissociation of the

Fig. 7. Simultaneous chromatographic analysis (○) of radioactivity in thyroxine fraction and the change in ultraviolet absorption (Δ) as judged by the extinction 232 m μ (*E*). At time 0 min. ^{131}I -labelled thyroxine in 0.05 ml. of propane-1:2-diol was added to 2.7 ml. of 0.06N-NaOH directly in a 1 cm. optical cell. Final concentration of thyroxine, 25 μM .

phenolic hydroxyl group, was made under steady-state conditions. The initial velocity of the forward reaction is too rapid to be measured with the techniques available, being limited by the time taken for the preparation of chromatograms. The first sample withdrawn represented the point of maximum ^{131}I disappearance, followed by the re-formation of iodophenol and reciprocal diminution in the amount of reaction product.

One of the remarkable features of the reaction is its photosensitivity, demonstrated for thyroxine in Fig. 5. It is not known whether this photosensitivity is related to the long-known instability of alkaline solutions of thyroxine when exposed to light (Kendall & Osterberg, 1919). It must be pointed out that the intensity of light incident on the solutions in these experiments is quite high and much stronger than average daylight. The strong source of light was identical with that used for work on enzymic deiodination for which the present work was originally designed to provide control experiments.

All that can be said of the transient reaction product is that, in spite of chromatographic evidence, it is not iodide. In this respect, it is worth comparing the present reaction with the slow spontaneous deiodination observed in solutions of thyroxine and tri-iodothyronine (Wilkinson, Sprott, Bowden & MacLagan, 1954). In such a breakdown there is true formation of iodide as determined by electrophoresis and oxidation to elementary

iodine; the distinction from the true (non-enzymic) de-iodination was best shown by analysing samples for about 6 days after the mixing of the organic solvent with the aqueous buffer. This is seen in Fig. 8 for a solution of thyroxine. The loss of radioactivity in the thyroxine fraction caused by the slow de-iodination is not reversible, either spontaneously or by acidification as for the transient reaction mediated by ionization of the phenolic hydroxyl group.

It might seem that, by criteria of chromatographic analysis alone, the property here described could cause much confusion in interpreting results of work on enzymic de-iodination of thyroid hormones. (It is common practice to use thyroid hormones dissolved in propan-1:2-diol, butan-1-ol or methanol in studies of their metabolism by paper-chromatographic analysis, both *in vivo* and *in vitro*.) But a consideration of all the principal characteristics of this property, especially the spontaneous reversibility, the accelerated reversal by acids, the pH-dependence, the photosensitivity, independence of concentration, temperature and composition of the buffer and the lack of true iodide formation easily eliminate any doubt about its distinctness from all known biochemical reactions of thyroid hormones and related iodophenols. Further, in an extension of the present investigations it was found that the transient instability of thyroxine and tri-iodothyronine, caused by ionization of the phenolic-hydroxyl group, is most unlikely to occur in the presence of many proteins. Certain serum and tissue proteins, especially those that bind thyroid hormones, are capable of inhibiting the reaction and also of precipitating the reversal if they are added after the mixing of organic and aqueous media. Although enzymic de-iodination is inhibited by thyroxine-binding proteins the reaction cannot be reversed by them, once deiodination has taken place. This newer aspect of interaction between proteins and thyroid hormones is the subject of the next paper (Tata, 1959).

SUMMARY

1. When solutions of ^{131}I -labelled L-thyroxine and related iodophenols (3:5:3'-tri-iodo-L-thyronine, 3:5-di-iodo-L-tyrosine, 3-iodo-L-tyrosine and 3:5-di-iodo-*p*-cresol) in 15–50% aqueous organic solvents were introduced into a large volume of an aqueous buffer, there occurred a very rapid loss of a part of the radioactivity present in the iodophenol. This disappearance was followed by a gradual reincorporation of the radioactivity.

2. The rates of disappearance and re-formation of the radioactive iodophenol were determined by the pH of the aqueous medium and the presence of light. The concentration of the iodophenol, the

Fig. 8. Chromatographic determination of radioactivity in the thyroxine fraction over a long period of time (6 days) after the mixing. At time 0 min. 0.05 ml. of labelled thyroxine was added to 2.5 ml. of 0.05 M-tris HCl buffer at pH 9.5 (final concentration of thyroxine, 0.25 μM). Although the products formed looked chromatographically alike, in the part A of the curve no iodide was detected by other methods, whereas the irreversible formation of iodide in part B of the curve was due to a true deiodination.

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 $\text{m}\mu$ for L-thyroxine and 3:5:3'-tri-iodo-L-thyronine respectively have been described.

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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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