sensitive to phosphate inhibition (B-esterases); a second group which supposedly works without serine (A- and C-esterases), although no positive evidence is available on this point.

SUMMARY

1. C-Esterase was separated from other hydrolytic enzymes in hog's kidney extract by fractionation on a diethylaminoethylcellulose column. The non-adsorbable protein contained type I C-esterase, free of B-esterase and an enzyme (DFPase) hydrolysing diisopropyl phosphorofluoridate.

2. In addition, most of the fractions with DFPase activity contained a second type of C-esterase, predominantly in hidden form, i.e. linked to a native inhibitor.

3. Type II C-esterase is liberated from this inhibitor by treatment with *p*-chloromercuribenzoate. It differs from type I C-esterase by its sensitivity towards sulphhydryl inhibitors and by its stronger adsorbability on diethylaminoethylcellulose.

4. Both C-esterases show an abnormal pH-activity curve towards *p*-nitrophenyl acetate. With increasing pH the hydrolytic rate rises steadily. This property is also shared by chymotrypsin.

5. The properties of the two types of purified C-esterase can explain most of the observations made on crude enzyme mixtures.

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The Partial Purification and Properties of Thyroxine Dehalogenase

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In most mammals, dehalogenation is one of the most important metabolic transformations of L-thyroxine, 3:5:3'-tri-iodo-L-thyronine and related substances (Pitt-Rivers & Tata, 1959). The physiological significance of dehalogenation of thyroid hormones remains doubtful although numerous reports have appeared in recent years on the deiodination of thyroid hormones by incubation with slices, homogenates or extracts of various tissues (Albright, Larson & Tust, 1954; Larson, Tomita & Albright, 1955; Sprott & MacLagan, 1955; Béraud & Vannotti, 1956; Tata, Rall & Rawson, 1957; Tata, 1957, 1958*b*; MacLagan & Reid, 1957; Plaskett, 1958; Albright & Larson, 1959; Yamazaki & Slingerland, 1959; Etling & Barker, 1959*a*; Lissitzky, Bénévent, Roques & Roche, 1959). Unfortunately there is little agreement on the nature and products of enzymic deiodination. The major causes of this confusion are: (a) the predominant interest of some investigators in demon-

strating partial deiodination of thyroxine to tri-iodothyronine, (b) variability in techniques of detection and measurement of deiodination, (c) failure to realize the influence of tissue source and method of enzyme preparation used and (d) neglect of non-enzymic processes which can occur simultaneously (Lissitzky, Michel, Roche & Roques, 1956; Tata, 1959*a*; Etling & Barker, 1959*b*; Lein & Michel, 1959).

A series of preliminary investigations were undertaken by the author to overcome the above objections before studying the enzyme system(s) involved in deiodination of the thyroid hormones. These investigations have shown that direct chromatographic analysis with multiple solvent systems is the most reliable method for measurement of deiodination (Tata *et al.* 1957; Tata, 1957, 1958*b*); direct chromatography has also been used by others (Lissitzky, Bénévent, Roques & Roche, 1959; Yamazaki & Slingerland, 1959; Etling & Barker, 1959*a*). It is, also, now possible to distinguish between true enzymic dehalogenation and one

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type of non-enzymic process, the phenomenon of 'instability' (Tata, 1959*b*, 1960*a*). Although liver and kidney deiodinate thyroxine and tri-iodothyronine more actively than any other tissue, both *in vivo* and *in vitro* (Pitt-Rivers & Tata, 1959), skeletal muscle was selected for studies on the dehalogenating enzyme. It was shown that with skeletal muscle, whether as slices, homogenate or soluble extract, deiodination of thyroid hormones was the only reaction involving these hormones that was catalysed (Tata, 1957, 1958*b*). With liver and kidney, complicating side-reactions occur, leading to the formation of metabolites which are themselves substrates for the dehalogenase. With kidney tissue, moreover, the dehalogenating activity is abolished by the loss of tissue integrity (Tomita, Lardy, Larson & Albright, 1957), whereas a 'soluble' preparation of dehalogenase can be obtained from skeletal muscle (Tata, 1958*b*).

The dehalogenating enzyme present in the thyroid gland, first described by Roche and his colleagues (Roche, Michel, Michel & Lissitzky, 1952; Roche, Michel, Michel, Gorbman & Lissitzky, 1953), has high affinity for the iodotyrosines but does not attack thyroxine or tri-iodothyronine. It has been further characterized by Stanbury (1957) and Stanbury & Morris (1958) as a reduced triphosphopyridine nucleotide-dependent enzyme present in the microsomal fractions of thyroid as well as liver tissues. It has been recently shown that the iodotyrosine deiodinase is different from the enzyme in muscle, which is not reduced triphosphopyridine nucleotide-dependent but is activated by Fe^{2+} ions and flavin derivatives (Tata, 1959*c*).

The purpose of this paper is to present a method for the partial purification of the enzyme present in rabbit skeletal muscle and to describe its important properties. It will be shown that thyroxine is the substrate for which the enzyme has the highest affinity and that, besides deiodination, it can also catalyse debromination of brominated analogues. The term 'thyroxine dehalogenase' is therefore proposed for this enzyme.

EXPERIMENTAL

Substrates. All enzyme incubations were performed with radioactive substrates labelled in the *ortho* position to the phenolic OH group. The following ^{131}I -labelled compounds were either obtained from Abbott Laboratories, Oak Ridge, Tenn., U.S.A., or prepared by radio-iodination of the corresponding 3:5-di-iodothyronine derivative or of tyrosine: L-thyroxine (L- T_4), D-thyroxine (D- T_4), DL-thyroxine, 3:5:3'-tri-iodo-L-thyronine (T_3), 3:5:3':5'-tetraiodothyroacetic acid, 3:5:3'-tri-iodothyroacetic acid, 3-iodo-L-tyrosine (MIT) and 3:5-di-iodo-L-tyrosine (DIT). The specific radioactivity of the iodothyronine derivatives was 15–24 mc/ μmole (Abbott Laboratories) or 4–7 mc/ μmole

(this laboratory) and that of the iodotyrosines 5–9 mc/ μmole . Radioactive 3-bromo-L-tyrosine, 3:5-dibromo-L-tyrosine, 3:5:3':5'-tetrabromo-DL-thyronine and 3:5:3'-tribromo-DL-thyronine were prepared by the radiobromination (^{82}Br) of L-tyrosine or 3:5-dibromo-DL-thyronine (3.5 g. atoms of Br/mole of amino acid) to give a specific radioactivity of 0.8–1.4 mc/ μmole . The mono- and disubstituted radioactive compounds were separated by preparative paper chromatography. ^{131}I and ^{82}Br were obtained from A.E.R.E., Harwell. The unlabelled compounds were the products of Glaxo Laboratories, Warner-Chilcott Laboratories or synthesized by Dr R. Pitt-Rivers, F.R.S. Stock solutions, at suitable concentrations of all compounds, were prepared in 10% (v/v) propane-1:2-diol in water.

Buffers. All buffer solutions were prepared according to Gomori (1955) in demineralized water. The buffer used for tissue extraction was 2-amino-2-hydroxymethylpropane-1:3-diol (tris)-HCl-KCl, pH 7.1, I 0.22 (I was 0.10 and 0.12 with respect to tris-HCl buffer and KCl respectively).

Enzyme preparations. All procedures, unless otherwise stated, were performed at 0–2°.

(a) Crude extract. Muscle was removed from the leg, thigh and back of 8–12-month-old rabbits killed by a blow on the neck. Care was taken to avoid contamination of tissue fragments with blood. Tissue (550 g.) was suspended in 2 l. of tris-HCl-KCl buffer, pH 7.1, I 0.22, and finely chopped with scissors. The suspension was homogenized in a MSE Ato-Mix blender and the homogenate centrifuged at 20 000 g for 20 min.; the sediment was discarded.

(b) Decrease in ionic strength. The crude supernatant was dialysed against 20 vol. of tris-HCl-KCl buffers of increasing dilutions until a value for I of 0.015 was reached. The precipitate formed was discarded by centrifuging at 7000 g for 10 min., leaving a slightly turbid supernatant.

(c) Heat treatment. The extract of I 0.015 was brought to 45° with stirring and maintained at that temperature $\pm 1^\circ$ for 5 min., when a copious precipitate was formed. The suspension was chilled in ice and centrifuged at 7000 g for 10 min. to yield a clear supernatant that was enzymically active.

(d) First ammonium sulphate precipitation. To the above-mentioned solution sufficient saturated $(\text{NH}_4)_2\text{SO}_4$, adjusted to pH 8.0 with NH_3 , was slowly added to 48% saturation. The suspension was left to stand overnight before collecting the active precipitate by centrifuging. The precipitate was suspended in 120–150 ml. of tris-HCl-KCl buffer, pH 7.1, I 0.20, stirred vigorously and dialysed first against 100 vol. of diluted buffer (I 0.015) and then against running water for 18 hr. The insoluble material was removed by centrifuging at 20 000 g for 15 min.

(e) Adsorption on calcium phosphate gel. One-year-old calcium phosphate gel (19 mg./ml.), prepared according to Keilin & Hartree (1938) (50 ml.), was stirred into 100 ml. of the supernatant from the last step. After 30 min. the suspension was centrifuged, the supernatant discarded and the precipitate was washed and centrifuged three times with 30 ml. of 0.15 M-phosphate buffer, pH 7.0. The washings were discarded and the precipitate was stirred into 30 ml. of 0.55 M-phosphate buffer, pH 7.0, before being centrifuged at 5000 g for 10 min. This was repeated twice, the three washings were pooled and dialysed against running water for 18 hr.

(f) Second ammonium sulphate precipitation. To 120 ml. of the above-mentioned dialysed solution was added sufficient saturated $(\text{NH}_4)_2\text{SO}_4$ solution, pH 8.0, to give 42% saturation. After 15 hr. the precipitate was recovered by centrifuging, suspended in tris-HCl-KCl buffer (*I* 0.10) and dialysed first against running water for 12 hr. and then for 10 hr. against demineralized water. The slightly opalescent solution was clarified by centrifuging at 20 000 *g* for 20 min. The resulting supernatant represented the purified thyroxine dehalogenase used for most studies of enzyme properties reported below.

Storage of enzyme. The active preparation was usually stored in solution in water or 0.05 M-tris-HCl buffer, pH 7.1, at 0–2° without freezing. The concentration was adjusted to 10–15 mg. of protein/ml. In some cases the enzyme was stored as a freeze-dried preparation, although this procedure caused some loss of enzyme activity.

Enzyme assay. The enzyme was incubated aerobically at 37°, with shaking, in a 25 ml. conical flask, the final volume of contents being 2.5–3.0 ml. The substrate in 10% propane-1,2-diol solution was added last; the concentration of the substrate was so arranged that the final concentration of the organic solvent would not exceed 0.02% (v/v) in the presence of the enzyme. The course of enzymic dehalogenation was followed by chromatographic analysis of samples withdrawn periodically during incubation (Tata *et al.* 1957). The distribution of radioactivity in different fractions on chromatograms was automatically recorded and calculated by an integration device or by planimetry of zones of radioactivity as follows:

$$\mu\text{m-moles of } T_4 \text{ dehalogenated} = \frac{\text{area under } T_4 \times \mu\text{m-moles of } T_4 \text{ added}}{\text{area under all peaks}}$$

Corrections were made for the amount of contaminating radioactive iodide or bromide originally present.

It was assumed that substrate was absent, or present in negligible amounts only, in the enzyme preparation and that isotopic exchange did not occur. One enzyme unit was tentatively defined as the amount of enzyme necessary to catalyse the dehalogenation of 1 $\mu\text{m-mole}$ of L-thyroxine/10 min.

Enzymic dehalogenation was distinguished from an apparently similar but spontaneous reaction (Tata, 1959*a*) by a test based on the phenomenon of 'stabilization' effect of thyroxine-binding proteins (Tata, 1959*b*, 1960*a*). This test was performed by the addition of 25–30 mg. of dried whole human-blood plasma to the incubation mixture 1 min. before the last sample was withdrawn for chromatography. Corrections were applied for the fraction of

radioactivity which 'reappeared' in the substrate fraction. The addition of plasma proteins, with thyroxine, also served to stop the enzyme reaction.

Chromatography. Ascending chromatography on Whatman no. 1 paper was used for enzyme assay and identification of the products formed. Whatman no. 3 paper was used for preparation of labelled compounds. Two solvent systems were used: (a) butan-1-ol-acetic acid-water (78:10:12, by vol.); (b) butan-1-ol-dioxan (80:20,v/v), previously shaken with aq. 2 N-NH₃ solution, was used to detect the possible formation of tri-iodothyronine from thyroxine. The short half-life (35.9 hr.) of ⁸²Br necessitated the acceleration of paper chromatography by centrifugal force for ⁸²Br-labelled compounds. An apparatus recently developed in this laboratory was used for this purpose according to techniques described elsewhere (Tata & Hemmings, 1960).

Electrophoresis. The analysis of tissue proteins or serum proteins was performed by horizontal-paper electrophoresis in veronal-acetate-KCl buffer, pH 7.4, *I* 0.1 (Kotter, Lenz & Prändl, 1955), or 0.05 M-veronal buffer, pH 8.6 (Block, Durum & Zweig, 1955). The purification of enzyme was also followed by starch-gel electrophoresis in borate buffer, pH 8.8, according to Smithies (1955). Paper electrophoresis was used for studying protein-binding of thyroxine according to methods described by Robbins & Rall (1957). Preparative electrophoresis on columns of cellulose (treated with acid and ethanol) was performed according to Flodin & Porath (1954) for the isolation of a cellular thyroxine-binding fraction from a crude extract of rabbit muscle.

Protein-nitrogen determinations. The protein content was measured by the determination of nitrogen in trichloroacetic acid-insoluble material according to Lang (1958).

RESULTS

Partial purification of the enzyme

The progressive enrichment of thyroxine dehalogenase in a typical procedure of purification is summarized in Table 1. It is essential to prepare the tissue homogenate in a medium of relatively high ionic strength (*I* 0.22) in order to obtain a 'soluble' enzyme extract, although in the later stages of purification the enzyme was soluble in water. The enzyme could be 'salted out' with ammonium sulphate only in a broad zone of 32–50% saturation. For most studies reported below, preparations of 60–90-fold purification were used.

Table 1. *Purification of thyroxine dehalogenase from rabbit skeletal muscle*

All enzyme assays were performed in the presence of 10 μM -flavin mononucleotide and 6 μM -Fe²⁺ ion in tris-HCl buffer, pH 7.1, over 15 min. of incubation.

Step	Volume (ml.)	Dehalogenase (units)	Protein (mg.)	Yield (%)	Purity (units/mg. of protein)	Purification
1. Homogenate	2 250	11 000	56 000	100.0	0.19	1
2. Supernatant (<i>I</i> 0.22)	1 650	9 960	22 500	90.4	0.40	2.1
3. Decrease to <i>I</i> 0.015	1 610	9 200	13 300	83.6	0.68	3.6
4. Supernatant after heat-treatment	1 595	8 950	9 160	79.1	0.95	5.0
5. Ppt. from 48% $(\text{NH}_4)_2\text{SO}_4$	210	6 150	2 030	55.9	2.93	15.4
6. Eluate from calcium phosphate gel	150	3 280	265	29.8	11.99	63.1
7. Ppt. from 42% $(\text{NH}_4)_2\text{SO}_4$	80	2 950	213	26.9	13.68	72.0

Cruder preparations were used in some experiments where enrichment of enzyme was not essential.

Electrophoresis indicated that even the final enzyme preparation of Table 1 was quite heterogeneous in terms of proteins present. The 10-12 skeletal-muscle proteins present in the crude extract were especially well revealed by starch-gel electrophoresis, although only four or five zones could be detected by paper electrophoresis. The method of purification used here has, however, resulted in the removal of several (five or six) inactive proteins, as was seen from a comparison of the patterns of starch-gel electrophoresis.

Stability of the enzyme

Even under the best conditions the enzyme could not be preserved for more than 2-3 weeks without substantial loss of activity. The best preparation was obtained in a solution of 10-15 mg. of protein/ml. of 0.025 M-tris or phosphate buffers, pH 7.1, at 0-2° and without freezing. Under these conditions about 25 % of the activity was lost in 4 days and 50 % in 12 days of storage. Six alternate operations of freezing and thawing of the solution destroyed all enzymic activity. Freeze-drying of a salt-free solution resulted in the loss of 30-60 % of the activity. The addition of glutathione or cysteine and storage under nitrogen offered little advantage.

Properties of thyroxine dehalogenase

The relationship between enzyme concentration and initial velocity of deiodination of thyroxine is illustrated in Fig. 1. As explained by Tata (1960*a*), the non-linearity of the uncorrected curve with small amounts of enzyme was due to the phenomenon of 'instability' of thyroxine (Tata, 1959*a*), which was responsible for the apparently higher values of deiodination as measured by chromatography. When larger amounts of enzyme were used a 'stabilization' of thyroxine could have resulted from the presence of proteins in the enzyme preparation.

The stimulating effect of Fe^{2+} ions and flavin derivatives on thyroxine dehalogenase and its inhibition by metal-complexing agents, flavin antagonists, mercurials and SH-group poisons have already been described (Tata, 1959*c*). In Fig. 2 it is shown that the activation of the enzyme by flavin mononucleotide (FMN) and Fe^{2+} ions appears to be additive to some extent. However, as it will be shown later, there was an important difference in the action of the two factors when the products of dehalogenation were studied. Similar results were observed when FMN was replaced by flavinadenine dinucleotide (FAD) or riboflavin. No metal ions were found to replace Fe^{2+} ions.

Fig. 1. Relationship between enzyme concentration and the initial rate (first 10 min.) of deiodination of L-thyroxine ($5.0 \mu\text{mole}$) by thyroxine dehalogenase of rabbit skeletal muscle, purification 72-fold. Each sample (2.5 ml.) was incubated in the presence of $10 \mu\text{M-Fe}^{2+}$ ion and $20 \mu\text{M}$ -flavin mononucleotide. Corrected values (—) are derived from samples to which 20 mg. of whole human plasma proteins were added 1 min. before withdrawal of a portion for chromatographic analysis. ---, Uncorrected values.

Fig. 2. Activation of thyroxine dehalogenase by Fe^{2+} ions and flavin mononucleotide (FMN). Each incubation vessel contained 12 mg. of enzyme, purification 72-fold, and $4.5 \mu\text{m-moles}$ of L- T_4 as substrate in 2.7 ml. of tris-HCl buffer, pH 7.0. Additions: ○, none; □, $0.1 \mu\text{mole}$ of FMN; △, $0.034 \mu\text{g.}$ ion of Fe^{2+} ; ●, $0.1 \mu\text{mole}$ of FMN and $0.034 \mu\text{g.}$ ion of Fe^{2+} .

Among other factors affecting the enzymic reaction it was found that: (1) molecular oxygen was not necessary, (2) the dehalogenation could be catalysed under nitrogen, (3) addition of hydrogen peroxide or catalase had no effect, indicating that dehalogenation was not a peroxidative mechanism, and (4) addition of pyruvate did not modify the reaction.

The inhibition of thyroxine dehalogenase with mercuric chloride presented a peculiar pattern since the degree of inhibition was not only determined by the amount of inhibitor added but also by whether it was added before or after FMN (Table 2). There

Table 2. *Influence of flavin mononucleotide on the inhibition of thyroxine dehalogenase by mercuric chloride*

The final volume was 2.7 ml.; 15 mg. of enzyme of specific activity 42, 6.0 μ m-moles of L-T₄, tris-HCl buffer, pH 7.1, and 0.10 μ g. ion of Fe²⁺ were added to each sample.

First addition	Amount (μ moles)	Second addition	Amount (μ moles)	Inhibition (%)
FMN	0.10	None	—	0.0
FMN	0.10	HgCl ₂	0.01	46.5
FMN	0.10	HgCl ₂	2.5	94.7
HgCl ₂	2.0	None	—	96.0
HgCl ₂	2.0	FMN	0.10	86.5
HgCl ₂	2.0	FMN	1.0	72.3
HgCl ₂	2.0	FMN	10.0	61.1

Fig. 3. Influence of pH on the initial velocity of deiodination of L-thyroxine (L-T₄) and 3:5:3'-tri-iodo-L-thyronine (L-T₃) by thyroxine dehalogenase (80-fold purification). Each vessel contained: 10 mg. of enzyme, 4.5 μ m-moles of L-T₄ or L-T₃, 0.04 μ g. ion of Fe²⁺ and 0.1 μ mole of FMN in 2.65 ml. of buffer. ○, L-T₄; △, L-T₃. Buffers: pH 3.0–4.5, citrate; pH 4.5–5.5, acetate; pH 5.6–7.5, phosphate; pH 7.5–8.5, tris-HCl; pH 8.5–10.0, carbonate.

was slightly, but significantly, less inhibition if the flavin cofactor was added after Hg²⁺ ions, and the addition of increasing amounts of FMN led to some reactivation of the enzyme. The reactivation did not exceed 40–50 % of the activity in the control sample and could not be achieved by the addition of Fe²⁺ ions. No explanation is available for this phenomenon; somewhat similar behaviour has also been described by Frisell & Hellerman (1957) for kidney D-amino acid oxidase, also a flavin-dependent enzyme.

A study of the influence of pH of the medium on the initial velocity of reaction showed that the pH optima for thyroxine and tri-iodothyronine were not identical (Fig. 3). Similar increments of 0.8–1.0 unit in the pH optimum were observed for monohalogenated thyronine or tyrosine derivatives as compared with the dihalogenated derivatives. Fig. 3 shows also that, at nearly equimolar concentrations, thyroxine was deiodinated more rapidly than tri-iodothyronine at any pH value.

In Fig. 4 is shown the relationship between substrate concentration and initial rate of deiodination of thyroxine, tri-iodothyronine and di-iodotyrosine. The use of higher substrate concentrations was limited by the low solubility of thyroxine and related compounds. The *K_m* values (Michaelis constant) deduced from Fig. 4 were hence not accurate. It was, however, easier to derive a graphical value of *K_m* by plotting the results according to the method of Lineweaver & Burk (1934). The results of such measurements are

Fig. 4. Effect of substrate concentration on the initial rate of deiodination. Each vessel contained 10 mg. of enzyme (152 units), 0.2 μ mole of FMN, 0.1 μ g. ion of Fe²⁺ in 3.0 ml. of tris-HCl buffer, pH 7.1. Substrates: ○, L-thyroxine; △, 3:5:3'-tri-iodo-L-thyronine; □, 3:5-di-iodo-L-tyrosine.

summarized in Table 3. The presence in the enzyme preparation of a protein with strong binding affinity for any of the substrates could modify the K_m values shown in Table 3 and hence these are tentative until a pure enzyme preparation is available. However, with the same preparation, the skeletal-muscle enzyme exhibits an affinity about four times as high for thyroxine as for tri-iodothyronine and an affinity 15–20 times as high as for di-iodotyrosine. The enzyme was almost equally active in deiodinating L- or D-thyroxine and was remarkably active in catalysing the de-

bromination of brominated thyronines. This is therefore one of the rare examples of enzymic debromination described.

The relative K_m values for the dehalogenase are, to a large extent, similar to the relative affinities with which serum thyroxine-binding protein binds thyroxine and related compounds (Robbins & Rall, 1957; Tata, 1960*b*). It was therefore decided to investigate the influence of thyroxine-binding protein added to the enzyme. The term 'thyroxine-binding protein' is used in this paper to denote the human-serum protein fraction which migrates in the α_1 - α_2 -globulin zone on paper electrophoresis. An experiment in which whole serum was added in different amounts indicated an interference with enzymic dehalogenation which was most intense for thyroxine. As seen in Fig. 5, there was a progressive inhibition of the deiodination of thyroxine which resulted in a virtually total loss of enzyme activity when the serum was present in a final six- to seven-fold dilution. In order to decide whether this inhibitory action of serum was actually due to the binding of thyroxine by thyroxine-binding protein and albumin, experiments were performed in which every sample withdrawn from the incubation vessel was submitted to analysis by both chromatography and electrophoresis. The former determined the degree of inhibition of dehalogenase and the latter revealed the fraction of radioactive

Table 3. *Apparent Michaelis constants of thyroxine dehalogenase for some iodinated and brominated analogues of L-thyroxine*

Conditions were identical with those in Fig. 4.

Substrate	Michaelis constant (K_m) (μ M)
L-Thyroxine	1.7
D-Thyroxine	2.4
DL-Thyroxine	2.2
3:5:3'-Tri-iodo-L-thyronine	7.4
3:5:3':5'-Tetraiodothyroacetic acid	7.9
3:5:3'-Tri-iodothyroacetic acid	13.2
3:5-Di-iodo-L-tyrosine	30
3-Iodo-L-tyrosine	50
3:5:3':5'-Tetrabromo-DL-thyronine	9.2
3:5:3'-Tribromo-DL-thyronine	45
3:5-Dibromo-L-tyrosine	98
3-Bromo-L-tyrosine	130

Fig. 5. Inhibitory effect of human serum on the deiodination of L-thyroxine by thyroxine dehalogenase, 61-fold purification. Each flask contained tris-HCl buffer, pH 7.2, 15 mg. of enzyme, 1.0 μ M-mole of L- T_4 , 0.04 μ g. ion of Fe^{2+} , 0.1 μ Mole of FMN. Amounts of human serum: A, none; B, 0.07 ml.; C, 0.1 ml.; D, 0.275 ml.; E, 0.5 ml. Final volume was 2.75 ml., made up with the buffer.

Fig. 6. Simultaneous determination of the fraction of substrate (L-thyroxine) deiodinated by thyroxine dehalogenase (○) and the fraction bound to human-serum proteins (thyroxine-binding protein and albumin) (□), when different amounts of human serum were added to the enzyme. Conditions were the same as in Fig. 5. Enzymic deiodination was determined by paper chromatography and paper electrophoresis was used for the determination of the percentage of L-thyroxine bound to serum proteins.

thyroxine bound to serum thyroxine-binding protein and albumin (it was easy to distinguish these proteins from rabbit-skeletal-muscle proteins). When the results of the two analyses were plotted, as shown in Fig. 6, it was found that for any amount of serum added the percentage inhibition of enzyme activity was nearly equal to the fraction of thyroxine bound to serum proteins. Other factors present in the serum contributed little to the inhibition. The inhibition could be reversed at any point by addition of sufficient thyroxine and it was concluded that the enzyme could only attack free or 'unbound' substrate.

Further proof of the above conclusion was provided by two more observations. (a) When isolated serum-protein fractions or polyvinyl pyrrolidone of different thyroxine-binding powers were added to the enzyme, the additives with higher thyroxine-binding powers were found to be the more potent inhibitors. Thus as seen in Table 4, Cohn fraction IV-4 of human plasma, an enriched thyroxine-binding fraction [thyroxine-binding protein isolated electrophoretically from Cohn fraction IV-9 (J. R. Tata, unpublished work)] and polyvinyl pyrrolidone, all known to bind thyroxine firmly (Tata, 1959*b*, 1960*a*), inhibited the enzyme at quite low concentrations. On the other hand γ -globulin, which binds thyroxine feebly, is a poor inhibitor; human-serum albumin and thyroxine-binding fraction extracted from rabbit skeletal muscle (free from dehalogenase activity and rabbit-serum thyroxine-binding protein and albumin) occupied intermediate positions. (b) Whole human serum, Cohn fractions IV-4 and IV-9, or isolated thyroxine-binding protein inhibited the dehalogenation of thyroxine more than that of any other

iodo- or bromo-thyronine. Their effect on the dehalogenation of halogenated tyrosines was barely perceptible under similar conditions.

Products of enzymic dehalogenation

A typical pattern of distribution of ^{131}I on a chromatogram, run during the deiodination of radioactive thyroxine, is seen in Fig. 7*a*. The major product formed was iodide; however, a significant fraction of the original radioactivity of thyroxine was found in a substance of R_F 0 in different chromatographic solvent systems [Figs. 7 (*a*), (*b*)]. This material, after elution from preparative chromatograms, was found to be iodinated protein(s) present in the enzyme preparation, as shown by the release of 3- ^{131}I iodotyrosine and a trace of 3:5-di-iodotyrosine after tryptic digestion [Fig. 7 (*c*)]. Radioactive tri-iodothyronine was found in traces in only six out of about 1500 chromatograms, as a

Table 4. Effect of whole human serum, its protein fractions, rabbit skeletal muscle thyroxine-binding protein (C-TBP) and polyvinyl pyrrolidone on enzymic deiodination of L-thyroxine

Enzyme (15 mg.) of 82-fold purification in 2.85 ml. of tris-HCl buffer, pH 7.1, with $10\ \mu\text{M}$ -FMN, $5\ \mu\text{M}$ - Fe^{2+} ion and $2.4\ \mu\text{M}$ -moles of L- T_4 . Inhibition was calculated after 15 min. of incubation.

Substance added	Amount (mg./ml.)	Inhibition of T_4 dehalogenase (%)
Whole serum	15.0*	74.5
Cohn fraction IV-4	5.0	88.0
Cohn fraction IV-9	5.0	90.5
TBP-33†	2.0	85.8
Albumin	15.0	70.0
γ -Globulin	15.0	18.6
C-TBP	10.0	57.3
Polyvinyl pyrrolidone	10.0	81.0

* Expressed as amount of serum protein in final dilution.

† Thyroxine-binding protein extracted from Cohn fraction IV-9 (unpublished work).

Fig. 7. Distribution of radioactivity (^{131}I) on chromatograms. (a) Sample withdrawn after 10 min. of enzymic deiodination of ^{131}I -labelled L-thyroxine under conditions shown in curve (O) of Fig. 2; chromatographic solvent, butan-1-ol-dioxan- NH_3 . (b) Elution and re-chromatography of the substance of R_F 0 in chromatogram (a); solvent, butan-1-ol-acetic acid-water. (c) Chromatography of the products of tryptic hydrolysis (with crude pancreatin) of the substance of R_F 0; solvent, butan-1-ol-acetic acid-water. The position of markers is shown by solid lines above each recording. MIT, 3-iodotyrosine; DIT, 3:5-di-iodotyrosine; I, iodide. Vertical arrows indicate points of application of samples.

product of deiodination of ^{131}I -labelled thyroxine. No other major or consistent metabolite of thyroxine or other compounds was detected except for a radioactive substance of R_f 0.88–0.92 (in both acid and alkaline butan-1-ol) when labelled thyroxine was incubated in the presence of mercuric chloride. The nature of the material is not known and its formation is probably not due to enzyme action.

As seen in Fig. 8, about 20–25 % of the total ^{131}I was found in the form of iodinated protein at any time interval during the deiodination of thyroxine. It is important to distinguish between true incorporation of iodine into protein molecules as 'protein-bound ^{131}I ' in these experiments from thyroxine bound to proteins in the electrophoresis experiments mentioned earlier. The formation of protein-bound ^{131}I was also observed during the deiodination of tri-iodothyronine and other iodinated compounds, but the radiobromination of proteins could not be firmly established during the debromination of brominated compounds. With the latter, the major product was the radioactive bromide.

The formation of protein-bound ^{131}I was influenced by the presence of flavin (Fig. 9). Thus although Fe^{2+} ions in the absence of FMN (or FAD) could stimulate deiodination of thyroxine more than if the flavin was added without the metal ions, there was a virtual absence of formation of protein-bound ^{131}I if the enzyme was only activated by Fe^{2+} ions. On the other hand, incu-

bation in the presence of FMN without Fe^{2+} ions resulted in the incorporation of nearly 40 % of the radioactivity into proteins, whereas the overall dehalogenation of thyroxine proceeded at a relatively slower rate. The dependence of formation of protein-bound ^{131}I on flavin was slightly influenced by the presence or absence of light. Little ^{131}I was incorporated into protein(s) when flavin was added after most of the substrate had been deiodinated by the Fe^{2+} ion-activated enzyme.

Species and organ distribution of thyroxine dehalogenase

The enzyme was found in a variety of non-thyroidal tissues from every species of animal studied (Table 5). All extracts were prepared according to the method described for the first step for the rabbit-muscle enzyme. It is likely that more than one mechanism operated for the deiodination of thyroxine in tissues such as liver and kidney. The deiodination, due to an enzyme of the type of thyroxine dehalogenase in rabbit muscle, was therefore estimated from the control incubation in the presence of mM-atetrin and 0.5 mM-8-hydroxyquinoline, which have been shown to inhibit the rabbit-muscle enzyme (Tata, 1959c). The values in Table 5 are only approximate since with liver and kidney the products formed by the action of other enzymes on thyroxine could also have been substrates for the dehalogenase. A further complication was that the contamination of

Fig. 8. Distribution of radioactivity in the substrate and the products formed during deiodination of ^{131}I -labelled L-thyroxine. Thyroxine dehalogenase (12 mg., 72-fold purification), 6.5 μm -moles of L-T₄, 0.05 μg . ion of Fe^{2+} , 0.1 μm ole of FMN in 2.7 ml. of tris-HCl buffer, pH 7.0. O, Thyroxine; Δ , iodide; $\square$, iodinated protein.

Fig. 9. Effect of activators when added separately or together on the nature of radioactive products formed at the end of incubation for 60 min. of ^{131}I -labelled L-thyroxine with the enzyme. Conditions are as in Fig. 8. $\square$, Thyroxine; $\blacksquare$, iodide; $\boxtimes$, iodinated protein. A, 0.1 μm ole of FMN alone; B, 0.05 μg . ion of Fe^{2+} alone; C, 0.1 μm ole of FMN and 0.05 μg . ion of Fe^{2+} added together.

extracts of more vascular tissues such as liver and kidney with serum proteins would cause an inhibition of the enzyme due to thyroxine-binding, as shown above, and hence tend to give lower values. Allowing for these considerations, liver and kidney possessed more thyroxine dehalogenase than skeletal muscle in any species. Also the muscles of both sea-water fish (plaice) and fresh-water fish (trout) were relatively richer in thyroxine dehalogenase than was the mammalian tissue.

DISCUSSION

The enzyme properties described above and those described by Tata (1959c) show clearly that the thyroxine dehalogenase of skeletal muscle is different from the iodotyrosine-deiodinating systems in the thyroid and liver described elsewhere (Roche *et al.* 1952, 1953; Tong, Taurog & Chaikoff, 1954; Stanbury, 1957; Stanbury & Morris, 1958;

Lissitzky, Bénévnt, Roques & Roche, 1959; J. Wolff, personal communication). The principal points of difference are summarized in Table 6. The differences in cofactors required and the preference shown for the different substrates are striking; the exactly opposite order of affinity for iodothyronines and iodotyrosines exhibited by the two types of enzymes is an interesting phenomenon. It should, however, be pointed out, as the work of Tong *et al.* (1954) and Nakano & Danowski (1959) on the deiodination of iodotyrosine by liver and thyroid preparations has shown, that the deiodination of iodotyrosines might involve different mechanisms or different enzymes in the two tissues. Thyroxine dehalogenase does not possess the same properties as either the 'N' or 'R' systems of Spratt & MacLagan (1955). It may, however, be present in preparations deiodinating thyroxine described by others (MacLagan & Reid, 1957; Plaskett, 1958; Yamazaki & Slingerland, 1959; J. B. Stanbury personal communication), although the data available for comparison are limited and some properties are not identical.

The partial purification of the enzyme has been based on classical and simple methods of protein fractionation giving preparations sufficiently rich in thyroxine dehalogenase for most studies of its properties. The unusual feature of the purification procedure lies in the first step, where the enzyme could only be extracted in the 'soluble' form by using a medium of relatively high ionic strength. This, together with its solubility in water in the later stages, suggests an initial association with material of low solubility or even with particulate matter.

The higher pH optimum observed for tri-iodothyronine as substrate as compared with thyroxine (Fig. 3) was also encountered with other halogenated mono- and di-substituted phenolic derivatives. The uniform increment of about 1 pH unit for *ortho*-monosubstituted phenols could be due to the fact that the pH optimum depends on the activation of both the active group of the enzyme and the substrate. The ionization of the phenolic hydroxyl group of these compounds may be involved in the activation of the substrate; the partial pK values for the phenolic hydroxyl group are in the region of 6.5 for *ortho*-disubstituted and 8.0 for the *ortho*-monosubstituted thyronines and

Table 5. *Presence of thyroxine dehalogenase in different tissues of a variety of species of animals*

'Supernatant' extracts were prepared in tris-HCl-KCl buffer as for rabbit skeletal muscle. All enzyme assays were performed in the presence of $10\text{ }\mu\text{M}$ -FMN and $6\text{ }\mu\text{M}$ -Fe²⁺ ion; in control experiments $10\text{ }\mu\text{M}$ -atebrin and mm-8-hydroxyquinoline were added; incubation was for 15 min.

Species	Tissue	T ₄ dehalogenase (units/mg. of protein)
Man	Muscle	0.58
	Liver	1.86
Rabbit	Muscle	0.46
	Liver	3.01
	Kidney	2.15
	Brain	0.29
Rat	Muscle	0.68
	Liver	4.12
	Kidney	2.06
	Brain	0.37
Chicken	Muscle	0.70
	Liver	4.55
	Brain	0.52
Frog (<i>Rana pipiens</i>)	Muscle	0.40
	Liver	2.66
Trout (<i>Salmo fario</i>)	Muscle	1.95
	Liver	3.30
Plaice (<i>Pleuronectes platessa</i>)	Muscle	1.70
	Liver	3.72

Table 6. *Differences in the properties of thyroxine dehalogenase in muscle and the iodotyrosine deiodinase of thyroid and liver*

Enzyme	Tissue	Cellular fraction	Cofactor requirement	Relative substrate affinity
Thyroxine dehalogenase	Muscle	'Soluble'	Fe ²⁺ , flavin	T ₄ > T ₃ > DIT > MIT
Iodotyrosine deiodinase	Thyroid Liver	Microsomes	TPNH	MIT > DIT > T ₃ > T ₄

tyrosines. It is relevant to consider that the ionization of the phenolic hydroxyl group has been shown to have a profound influence on some of the physicochemical properties of thyroxine, tri-iodothyronine and related substances (Gemmill, 1955; Tata, 1959a).

The following generalizations may be made from the K_m values in Table 3: (a) thyronine derivatives are dehalogenated more readily than tyrosine derivatives, (b) iodinated derivatives are dehalogenated more rapidly than brominated derivatives, and (c) *ortho*-disubstituted phenols react more rapidly than *ortho*-monosubstituted phenols.

The substrate specificity (Fig. 4 and Table 3) of the enzyme raises some points of physiological significance. The lower affinity of the enzyme for tri-iodothyronine as compared with thyroxine appears at first to be incompatible with the higher rate of deiodination of tri-iodothyronine observed *in vivo* (see Pitt-Rivers & Tata, 1959). This apparent anomaly can be explained by the recent studies of Tata & Shellabarger (1959), who demonstrated that the shorter biological half-life of tri-iodothyronine in mammals could be accounted for by the greater ease with which this hormone can diffuse from blood into tissues. The extremely small amounts of both hormones that would be found in tissues with a relative excess of the dehalogenase means that enzymic dehalogenation is not rate-limiting in the intact animal. Thus the more rapid deiodination of tri-iodothyronine *in vivo* would only be a consequence of its more rapid diffusion into tissue cells. The relatively low affinity of thyroxine dehalogenase for iodotyrosines suggests that it is not the major enzyme involved in the extrathyroidal deiodination of iodotyrosines, especially as a powerful iodotyrosine-deiodinating system of a different nature has been demonstrated in the liver (Roche *et al.* 1952, 1953; Tong *et al.* 1954; Hartmann, 1955; Stanbury, 1957; Stanbury & Morris, 1958). A change in the optical activity of thyroxine did not markedly modify the high affinity of the enzyme for thyroxine. This, at first,

might appear to be at variance with the recent findings of Larson, Tomita & Albright (1959) that D-thyroxine did not undergo deiodination to 3:5:3'-tri-iodothyronine under conditions in which L-thyroxine was partially deiodinated. However, a comparison of their results with those reported here is not justified since no indication is given in their work about the amount of iodide formed from D-thyroxine.

Probably the most interesting property of the enzyme is its resemblance to serum thyroxine-binding protein in its relative affinities for thyroxine and related compounds, as shown in Table 7. Such a resemblance suggests that the binding sites or active centres of the two proteins (one extracellular, the other intracellular) are probably alike. Competition for thyroxine thus exists when the dehalogenase and serum thyroxine-binding proteins are present simultaneously and consequently the enzymic dehalogenation is retarded in the presence of serum (Figs. 5, 6; Table 4). An important feature is the suppression of deiodination of thyroxine by the addition of a protein fraction (C-TBP in Table 4) also extracted from rabbit skeletal muscle and possessing thyroxine-binding properties. This competition of two (or more) proteins present in the same cell for thyroxine recalls the suggestion already made by the author (Tata, 1958a) that the amount of the hormone available for utilization in any tissue is determined by the affinity and capacity of various intracellular components that bind thyroxine. This concept has recently received some support from the work of Lissitzky, Roques & Bénévnt (1959), although their 'supernatant' fraction was likely to contain rabbit-serum thyroxine-binding protein.

It is clear from these results that thyroxine dehalogenase is capable of interacting only with free or non-protein-linked substrate. This property can be used in studying the interaction between thyroxine and thyroxine-binding proteins, as explained below.

Table 7. *Relative affinities of thyroxine dehalogenase and human-serum thyroxine-binding protein in interaction with thyroxine and compounds related to it*

Substance	Values for L-thyroxine are taken as 100.	
	Relative affinity of T_4 dehalogenase*	Relative affinity of serum thyroxine-binding protein
L-Thyroxine	100	100
D-Thyroxine	71	80
3:5:3'-Tri-iodo-L-thyronine	22	25-30
3:5-Di-iodo-L-tyrosine	6	<5
3:5:3':5'-Tetrabromo-DL-thyronine	18	20
3:5:3'-Tribromo-DL-thyronine	4	7
3:5-Dibromo-L-tyrosine	2	<3

* Calculated from K_m values in Table 3.

In line with previous reports (Tata *et al.* 1957; Tata, 1957, 1958*b*), 3:5:3'-tri-iodothyronine was not a consistent product of deiodination of thyroxine, even with short incubation periods and low concentrations of the enzyme. This suggests that the enzyme catalyses the simultaneous removal of the two iodine atoms in the *ortho* position to the phenolic hydroxyl group. The failure to detect partial dehalogenation is in contrast with the reports of Larson, Albright and colleagues about the conversion of thyroxine into tri-iodothyronine *in vitro* (Albright *et al.* 1954; Albright & Larson, 1959; Larson *et al.* 1955, 1959). The conversion appears only to be a property of intact kidney slices. The majority of recent publications on the enzymic deiodination of thyroxine, however, also report failure to detect the formation of tri-iodothyronine (Yamazaki & Slingerland, 1959; Lissitzky, Bénévent, Roques & Roche, 1959; Etling & Barker, 1959*a*). On a physiological basis it can be concluded that thyroxine dehalogenase, as studied above, is not involved in the 'activation' of thyroxine to a more potent substance.

Further knowledge of the mechanism of action of the enzyme requires its more complete purification. Also, the experiments recorded in this paper reveal little about the physiological role of thyroxine dehalogenase; studies of this enzyme in health and disease, and in different states of thyroid function and metabolic activity, are desirable.

SUMMARY

1. A method for a 60–90-fold purification of the enzyme, thyroxine dehalogenase from rabbit skeletal muscle, has been described. Enzymic deiodination and debromination of ^{131}I - and ^{82}Br -labelled thyroxine and some iodo- and bromo-thyronines, thyroacetic acids and tyrosines have been described.

2. The enzyme was unstable on storage and was not resistant to freezing and thawing or to freeze-drying.

3. Thyroxine dehalogenase was activated by Fe^{2+} ions and by flavins (flavin mononucleotide, flavinadenine dinucleotide and riboflavin); the activation by these factors appears to be additive, but the activators acting singly lead to formation of different products.

4. Relationship between amount of enzyme and initial velocity of deiodination was linear only if a correction were applied for the 'instability' of thyroxine observed with small amounts of the enzyme.

5. Inhibition of the enzyme by Hg^{2+} ions could be reversed to about 40 % of the original activity by the addition of excess of flavin mononucleotide.

6. The pH optima were around 6.4 for *o*-dihalo-

genated phenols and around 7.3 for *o*-monohalogenated phenols.

7. Of all the compounds tested the enzyme had the highest affinity for thyroxine, which was about four to five times that for tri-iodothyronine and 20 times that for 3:5-di-iodotyrosine. D-Thyroxine was readily deiodinated. K_m values have been given for some iodinated and brominated thyronines, thyroacetic acids and tyrosines. The relative affinities of thyroxine dehalogenase for thyroxine and related compounds closely resemble those of serum thyroxine-binding protein.

8. Addition of blood serum suppressed enzymic dehalogenation of thyroxine. The extent of inhibition thus caused was equal to the fraction of thyroxine bound to serum thyroxine-binding protein and albumin. Thyroxine dehalogenase can attack only free or non-protein-linked substrate. A new method based on this property for studying binding of thyroxine to proteins is proposed.

9. The major product of deiodination was iodide. About 20–25 % of the iodine released was incorporated into proteins present in the enzyme preparation; the formation of iodinated proteins required flavin but not Fe^{2+} ions. 3:5:3'-Tri-iodothyronine was not one of the important products of deiodination of thyroxine.

10. The findings are discussed in the light of enzyme mechanism and some physiological implications.

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The Phospholipids of the Erythrocyte 'Ghosts' of Various Species

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There is abundant biological evidence of an active transport of cations across cell membranes (e.g. Hodgkin, 1951). Active transport is most easily explained by supposing a carrier molecule within the membrane, and an important task in biochemistry is the identification of such a carrier. A number of authors have suggested that one or more of the kephalins would fit the role (e.g. Davies & Krebs, 1952). Solomon, Lionetti & Curran (1956) found that chloroform-methanol extracts of human blood contained a much higher potassium/sodium ratio than was to be expected from the concentration of these ions in the original blood; on shaking the extract with water the ions were released. With purified phospholipid prepara-

tions, phosphatidylserine was particularly effective in taking up potassium relative to sodium. Kirschner (1959) attempted a crude fractionation of the phospholipids of pig erythrocytes. One fraction, which he tentatively identified as phosphatidylserine, contained sodium/potassium in the ratio 1.6/1, although the internal cation of pig erythrocytes is almost entirely potassium. Recently Hoffman, Schulman & Eden (1959) have described a model system in which phosphatidylethanolamine could specifically transport sodium across an artificial membrane.

Vogt (1957) has suggested phosphatidic acid as a possible cation carrier, and this view is supported by Hokin & Hokin (1959), who showed that slices