Further Studies on the Ceric Sulphate-Arsenious Acid Reaction for the Detection of Various Analogues of Thyroxine

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This study was occasioned by the discovery that 4'-deoxyiodothyronines yielded very poor spots of decolorization with the ceric sulphate-arsenious acid procedure (Bowden, MacLagan & Wilkinson, 1955) for detecting spots of iodothyronines on paper chromatograms. Since the application was intended to follow the metabolism of methyl-substituted 3,5-di-iodothyronines, our attention was particularly directed towards conditions leading to reactivity of iodine atoms adjacent to the ether oxygen atom, i.e. in the 3- and 5-positions. Bowden *et al.* (1955) had emphasized the importance of a phenolic hydroxy group *ortho* to the iodine atom and they did not specifically comment on their own demonstration of the lability of 3-iodine and 5-iodine atoms.

EXPERIMENTAL

Double- or triple-distilled water was used throughout. The 10% (w/v) ceric sulphate was made by stirring 50 g. of $\text{Ce}(\text{SO}_4)_2 \cdot 4\text{H}_2\text{O}$ into 500 ml. of 2N- H_2SO_4 previously cooled to 5°. The cloudy mixture became clear during prolonged mechanical stirring in a cold bath (glass rod or Teflon-coated magnetic stirring-bar). If necessary, it was completely clarified by decantation or by filtration through a sintered-glass funnel. It was stored cold.

For the 5% (w/v) arsenite solution, 25 g. of NaAsO_2 was dissolved in 500 ml. of water with warming. The solution was kept at room temperature to minimize precipitation. If very rapid decolorization occurred with a given set of reagents, the arsenite reagent was the one most likely to be contaminated. If As_2O_3 had to be used, for reasons of purity, 19 g. was dissolved with warming in 25 ml. of 5N-NaOH and diluted to 500 ml. In this instance, the strength of the H_2SO_4 used to dissolve the ceric sulphate had to be increased to 2.25N.

Thyroxine analogues were dissolved in methanol containing 5% (v/v) of aq. NH_3 solution (sp.gr. 0.88). Serial dilutions were made from a stock solution so that 10 μl . volumes containing widely differing quantities of iodothyronine could be applied consistently to Whatman no. 1 chromatography paper in four approximately equal portions, allowing drying in a gentle stream of air. A piece of the same type of paper 1 cm. larger in both dimensions

was soaked with a fresh mixture of equal parts of 10% ceric sulphate and 5% arsenious acid. This paper was laid on a clean glass plate. The smaller paper carrying the spots was then laid on top, starting from one edge to enable optimum centring and elimination of air bubbles. A second piece of glass was placed on top and pressure applied until the solution was uniformly distributed. After 15 min. at laboratory temperature (a reading of which was taken for reference purposes), the average diameter of each spot was quickly measured.

After most of this work was completed, it was found possible to simplify the procedure by spraying the paper with the arsenite solution, allowing it to dry, and spraying with the acid ceric sulphate solution. After allowing the appropriate length of time for decolorization, the reaction could be stopped by spraying with 5% (w/v) *o*-phenylenediamine in acetone or methanol (Fletcher & Stanley, 1956). Because of the toxicity of the reagents, a fume cupboard had to be used, with absorbent paper hung behind the frame supporting the papers being sprayed.

In the initial survey, 0.01–10 μg . of organic I was applied per spot, but it soon became apparent that amounts above 1 μg . were excessive with reactive compounds, namely iodothyronines, whereas amounts as high as 5 μg . were needed for minimal decolorization by 4'-deoxy-3,5-iodothyronines. Two ranges were therefore employed: 10, 40, 160 and 320 μg . of I for reactive compounds, and 0.32, 0.64, 1.28, 5.12 μg . of I for poorly reactive compounds. Since 3,5-di-iodothyronine was considered as a key compound, reference spots of this material were always included in every series of substances studied.

RESULTS

Fig. 1 compares the relationship between quantities of organic iodine and the diameter of the spots produced, as directly measured. Fig. 2 shows the logarithm of iodine concentration plotted against the area of the spot of decolorization. There is strong theoretical background for this latter relationship (cf. Block, Durrum & Zweig, 1958). On this basis, the data on spot size were used to determine liberation of iodine from each compound, and this was further expressed in terms of percentage of iodine produced from 3,5-di-iodothyronine run on the same paper as an internal reference standard.

From Table 1 it is apparent that only about one-tenth of the iodine in 3,5-di-iodothyronine was

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liberated under these circumstances; for instance, equivalent decolorization was obtained from 4 μ mg. of potassium iodide iodine and 40 μ mg. of 3,5-di-iodothyronine iodine. Table 1 also shows that the iodine atoms in 3-monoiodothyronine, 3,5-di-iodothyronine, 3- and 3'-iodothyronine, 3,3' and 3',5'-di-iodothyronine, 3,3',5- and 3,3',5'-tri-iodothyronine, and thyroxine were all equivalent to those of 3,5-di-iodothyronine in reactivity. Further, alteration of the alanine side chain of 3,5-di-iodothyronine by acetylation or replacing it by C₁, C₂ or C₃ simple fatty acid residues (Table 2) did not alter the reaction.

The presence of alkyl substituents in various positions on the outer phenolic ring is seen from

Fig. 1. Decolorization of ceric sulphate solution by increasing amounts of 3,5-di-iodothyronine previously applied as discrete spots to Whatman no. 1 chromatography paper. The quantities of 3,5-di-iodothyronine, shown as μ mg. of I, were each applied as 10 μ l. of a methanol-ammonia solution, in four approximately equal portions separated by air drying. Application of ceric sulphate-arsenious acid reagent is described in the text. Each point shown is the mean of 20 individual spots, average diameters being measured after 15 min. of incubation at a room temperature of 18–22°.

Fig. 2. Decolorization of ceric sulphate solution by 3,5-di-iodothyronine. The same results as in Fig. 1 are plotted as the areas (mm.²) of spots of decolorization against the logarithms of 3,5-di-iodothyronine iodine.

Table 3 to be essentially without effect on the deiodination of 3,5-di-iodothyronine as long as there remained a free hydroxyl group at the 4'-position. However, the more drastic alteration of moving the 4'-phenolic hydroxyl group to 3'- or 2'-position diminished deiodination, although the results are

Table 1. *Deiodination of iodothyronines and iodothyronines as measured by the decolorization of the ceric sulphate-arsenious acid reagent*

Each compound was dissolved in 5% (v/v) of aq. NH₃ (sp.gr. 0.88) in methanol (except for KI, in which case water was used) and diluted so that quantities equivalent to 10–230 μ mg. of I (1–32 μ mg. of KI iodine) were contained in 10 μ l. of solution. This volume was applied to Whatman no. 1 chromatography paper in four approximately equal portions separated by air drying. Application of ceric sulphate-arsenious acid reagent is described in the text. After 15 min. of incubation at a room temperature of 18–22°, the average diameter of each spot was measured for calculation of area. Each compound was applied in at least three of the quantities of 10, 40, 160, 320 μ mg. of I, in duplicate. As a reference, 3,5-di-iodothyronine was always present in the above range.

Compound	Deiodination (% of that of 3,5-di-iodo- thyronine)
Potassium iodide	996
3-Monoiodothyronine	115
3,5-Di-iodothyronine	108
3-Monoiodothyronine	95
3'-Monoiodothyronine	101
3,5-Di-iodothyronine	100
3,3'-Di-iodothyronine	97
3,3',5-Tri-iodothyronine	113
3,3',5'-Tri-iodothyronine	107
Thyroxine	90
N-Acetylthyroxine	95

Table 2. *Effect of modification of the side chain and of substitution on the aromatic ring on the deiodination of iodothyronines as measured by the decolorization of the ceric sulphate-arsenious acid reagent*

The conditions were as described for Table 1.

Compound			Deiodination (% of that of 3,5-di-iodo- thyronine)
5'	3'		
H	H	$\text{CH}_2 \cdot \text{CH}(\text{NH} \cdot \text{CO} \cdot \text{CH}_3) \cdot \text{CO}_2\text{H}$	95
H	H	CO_2H	94
H	H	$\text{CH}_2 \cdot \text{CO}_2\text{H}$	109
H	H	$\text{CH}_2 \cdot \text{CH}_2 \cdot \text{CO}_2\text{H}$	101
H	I	$\text{CH}_2 \cdot \text{CO}_2\text{H}$	104
I	I	$\text{CH}_2 \cdot \text{CO}_2\text{H}$	101

not adequate to differentiate between the 2'- and 3'-locations. When the phenolic function was blocked by formation of the methyl ether or was removed altogether (Table 4), the resulting reactivity was less than 1% of that of 3,5-di-iodothyronine. Substitution of a 4'-methyl group for the 4'-hydroxyl group in 3,5-di-iodothyronine permitted no return of deiodination, but the presence of a 4'-amino group was adequate for full activity.

Table 5 shows the results with a series of analogues carrying substituted naphthyl rings in place

of the outer benzene ring. When a 4'-hydroxyl group was present, deiodination at the 3,5-positions was equivalent to that in 3,5-di-iodothyronine. When the hydroxyl group was absent or was shifted to the 5'-naphthyl position, deiodination dropped to less than 1%.

Table 6 presents a further series of changes involving loss of the oxygen bridge normally present in the diphenyl ether. No deiodination was detectable in the diphenyl analogues of 3,5-di-iodothyroformic acid or 3,5-di-iodothyroacetic acid,

Table 3. *Effect of the substitution on the phenolic benzene on the deiodination of iodothyronines as measured by the decolorization of the ceric sulphate-arsenious acid reagent*

The conditions were as described for Table 1, except for the O-methyl ether of thyroxine (compound 11), in which case 0.64, 1.28 and 5.12 $\mu\text{g.}$ of I were present, because of the low reactivity of this compound.

Compound no.	Compound					Deiodination (% of that of 3,5-di-iodothyronine)
	4'	5'	3'	2'		
1	OH	H	H	CH ₃		120
2	OH	H	CH ₃	H		93
3	OH	H	CH ₃	CH ₃		110
4	OH	CH ₃	H	CH ₃		125
5	OH	CH ₃	CH ₃	H		98
6	OH	CH ₃	CH ₃	CH(CH ₃)·CH ₃		102
7	H	H	H	OH		66
8	H	H	OH	H		45
9	H	I	I	OH		79
10	I	OH	H	I		59
11	O·CH ₃	I	I	H		0.8

Table 4. *Effect of the absence of the 4'-oxygen atom on the deiodination of iodothyronine analogues as measured by the decolorization of the ceric sulphate-arsenious acid reagent*

The conditions were as described for Table 1, except that compounds 1-10 were studied at levels of 0.64, 1.28 and 5.12 $\mu\text{g.}$ of I, because of diminished reactivity.

Compound no.	Compound					Deiodination (% of that of 3,5-di-iodothyronine)
	4'	5'	3'	2'	R	
1	H	H	H	H	CH ₂ ·CH(NH ₂)·CO ₂ H	0.3
2	H	H	H	H	CO ₂ H	0.4
3	H	H	H	H	CH ₂ ·CO ₂ H	0.3
4	H	H	CH ₃	H	CH ₂ ·CH(NH ₂)·CO ₂ H	0.6
5	H	H	CF ₃	H	CH ₂ ·CH(NH ₂)·CO ₂ H	0.5
6	CH ₃	H	H	H	CH ₂ ·CH(NH ₂)·CO ₂ H	0.7
7	CH ₂ ·CH(NH ₂)·CO ₂ H	H	H	H	CH ₂ ·CH(NH ₂)·CO ₂ H	0.7
8	H	H	CH ₃	CH ₃	CH ₂ ·CH(NH ₂)·CO ₂ H	0.7
9	H	CH ₃	H	CH ₃	CH ₂ ·CH(NH ₂)·CO ₂ H	0.7
10	H	CH ₃	CH ₃	H	CH ₂ ·CH(NH ₂)·CO ₂ H	0.3
11	NH ₂	H	H	H	CH ₂ ·CH(NH ₂)·CO ₂ H	87
12	NH ₂	H	I	H	CH ₂ ·CH(NH ₂)·CO ₂ H	99
13	NH ₂	H	CH ₃	H	CH ₂ ·CH(NH ₂)·CO ₂ H	99

Table 5. *Effect of replacing the phenolic benzene of 3,5-di-iodothyronine by substituted naphthalenes on the deiodination as measured by the decolorization of the ceric sulphate-arsenious acid reagent*

The conditions were as described for Table 1, except that compounds 2-5 were studied at levels of 0.64, 128 and 5.12 μg . of I because of diminished reactivity.

Compound no.	Compound		Deiodination (% of that of 3,5-di-iodothyronine)
	R		
1	4'-Hydroxynaphth-1'-yl		93
2	Naphth-1'-yl		0.8
3	5'-Hydroxynaphth-1'-yl		0.8
4	3'-Hydroxynaphth-2'-yl		0.8
5	7'-Hydroxynaphth-2'-yl		0.6

Table 6. *Effect of absence of the ether oxygen atom on the deiodination of iodothyronine analogues as measured on the decolorization of the ceric sulphate-arsenious acid reagent*

Two different types of compounds are included in this Table, as shown: diphenyl analogues and thio ether analogues. The conditions were as described for Table 1, except that compounds 1, 2 and 7 were studied at levels of 1.28, 2.56, and 5.12 μg . of I and the remainder at 0.04, 0.16, 0.32 and 0.64 μg . of I. With compounds 1, 2 and 7 deiodination was rated as < 0.1% because of the appearance of a pink coloration at the 5.12 μg . level.

Compound no.	Compound			Deiodination (% of that of 3,5-di-iodothyronine)
	5'	3'	R	
1	H	H	CO ₂ H	< 0.1
2	H	H	CH ₂ ·CO ₂ H	< 0.1
3	H	I	CH ₂ ·CO ₂ H	34
4	I	I	CO ₂ H	51
5	I	I	CH ₂ ·CO ₂ H	45
6	I	I	CH ₂ ·CH(NH ₂)·CO ₂ H	56
7	H	H		< 0.1
8	H	I		36
9	I	I		61

although a pink tinge was visible at high concentrations of these compounds. When one iodine atom was present on the hydroxylated outer ring, it exhibited reactivity, as indicated by deiodination equivalent to one-third of the total iodine present. When iodines were present at both 3'- and 5'-positions, both apparently reacted in all three cases of the formic acid, acetic acid and alanine side chains presented in Table 6. Unexpectedly, a similar resistance was encountered in three iodothiothyronines, shown in the lower portion of Table 6.

DISCUSSION

As proposed by Dubravčić (1955), it is most likely that I^- ion is the material actually reacting with Ce^{4+} ion:

Recycling of the iodine formed takes place at the expense of the As^{3+} ion present in excess, leading to the extremely high sensitivity of the overall

reaction process. In the following discussion, it has been assumed that appearance of the colourless Ce^{3+} ion is an indication of iodide formation by deiodination from organic combination. Bowden *et al.* (1955) demonstrated the appearance of iodine after treatment with Ce^{4+} ions of phenols bearing iodine *ortho* to the hydroxyl group, supporting the assumption of deiodination.

In addition to this condition specified by Bowden *et al.* (1955), it is also possible for Ce^{4+} ion to split off iodine in the 3,5-positions, adjacent to the ether oxygen of thyronine, provided there is an hydroxyl group on the outer benzene ring, preferably at the 4'-position. Liberation of iodine from 3,5-di-iodothyronine probably involves prior splitting of the diphenyl ether, resulting in establishment of an ionizable group, such as an hydroxyl group, adjacent to the iodine atoms, since the 4'-hydroxy-diphenyl analogues of 3,5-di-iodothyronine do not release iodine on treatment with ceric sulphate. The series of events resulting in rupture of the diphenyl ether depends on the presence on the outer ring of a group capable of being oxidized by Ce^{4+} ion, since a 4'-amino substituent was as effective as a 4'-hydroxyl group, but both 4'-methyl and 4'-methoxyl groups were without action. One further clue to the oxidative nature of this reaction was supplied by an experiment in which the anaerobic deiodination of 3,5-di-iodothyronine was followed in solution after degassing and replacement with nitrogen under paraffin oil. Under these circumstances of excluding oxygen there was 80% inhibition of deiodination, strongly suggesting that the reaction actually involves the addition of oxygen, as well as shift of electrons.

The slight but distinct difference between the 0.2–0.8% deiodination of 4'-deoxy-3,5-iodothyronine compounds and the lack of measurable deiodination of the diphenyl analogues of the same 3,5-iodothyronines may represent a very weak ability of Ce^{4+} ion to split the ether linkage even in the absence of a 4'-hydroxyl group. The diphenyl compounds apparently cannot be split even to this extent. It is necessary to invoke some other mechanism to explain the absence of reactivity of the thio ether analogue of 3,5-di-iodothyronine. Possibly there is formation of a sulphone type of structure which is resistant to further change.

Since only a single observation after 15 min. was made in the experiments reported in the present paper, no kinetic details can be inferred. It can at least be stated that, in this time period, splitting of the ether is not limiting (i.e. 3,5-di-iodothyronine reacted no less extensively than 3,5-di-iodotyrosine). On the other hand, comparison between potassium iodide and 3,5-di-iodothyronine has been made at various concentrations over a time

range from 5 to 30 min., and in all instances the potassium iodide iodine was approximately 10 times as reactive as the organic iodine. Further, there appeared to be no detectable differences between the behaviour of 3,5- and 3',5'-iodine atoms.

Other deiodination reactions have received attention. The light-catalysed reversible formation of an iodide-like compound when iodotyrosines and iodothyronine in organic solution were diluted with water is certainly not true deiodination, as discussed by Tata (1959). Galton & Ingbar (1962*a*) have described an irreversible degradation of iodophenolic compounds produced by flavins in the presence of strong illumination. Iodide and an incompletely identified material remaining at the origin of chromatograms and electrophoretograms resulted, but structural requirements for the iodophenols were not established beyond side-chain alterations. This reaction was dependent on the presence of oxygen and was temporarily depressed by the addition of catalase, suggesting involvement of peroxide. The presence of biological material had little effect, in disagreement with previous reports (Lissitzky, 1960; Tata, 1960) on deiodinase activity of mammalian muscle preparations.

Plaskett (1961) reported that a protein-bound iodine fraction appearing during the metabolism of thyroxine by a rat-liver preparation yielded di-iodotyrosine on being heated with alkali. Similar behaviour was seen only with 5'-hydroxy-3,5-di-iodothyronine, suggesting subsequent rupture of the diphenyl ether linkage. Galton & Ingbar (1962*b*) have presented evidence for obligatory deiodination of thyroxine accompanying biological activity of the hormone in anuran metabolism. A similar correlation between metabolic rate and muscle deiodination of thyroxine in the rat was reported by Tata (1961), although Lissitzky (1960) preferred the interpretation that deiodination was a regulatory mechanism rather than an activation. Work in this Laboratory with 2',5'-dimethyl-substituted iodothyronines in contrast with 2',3'-dimethyl-substituted compounds has led us to suggest 5'-deiodination as a preliminary to further target organ effects of thyroxine (Barker, 1962).

SUMMARY

1. A method has been presented for roughly determining the deiodination of thyroxine analogues applied under standard conditions to chromatography paper, by using decolorization of the ceric sulphate–arsenious acid reagent.

2. Deiodination of thyronines iodinated at 3,3',5,5'-positions in any combination appears to be equal to that of mono- and di-iodotyrosine.

3. Alterations in the alanine side chain and the

presence of alkyl substituents on the phenolic ring do not alter deiodination.

4. Removal of the 4'-oxygen atom lowers deiodination of 3,5-di-iodothyronine analogues to less than 1%. Replacement by a 4'-amino group causes a return of activity, but replacement by a 4'-methyl group does not.

5. Substitution of a thio ether sulphur atom for the usual ether oxygen atom or the formation of the diphenyl analogues of 3,5-di-iodothyronine eliminate deiodination.

6. Ceric deiodination of 3,5-iodothyronines is probably initiated by oxidative removal of the outer phenolic ring.

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Nicotinamide Coenzyme Concentrations in Mammary Biopsy Samples from Ketotic Cows

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Tracer experiments in spontaneously ketotic cows have indicated defects in the reductive synthesis of milk fat from acetate and in the oxidation of glucose (Kronfeld, Kleiber & Lucas, 1959; Tombropoulos & Kleiber, 1961). Serum concentrations of metabolites were thought by Bach & Hibbitt (1959) to reflect interference with the citric acid cycle, notably with those reactions in which pyruvate and α -oxoglutarate are converted into citrate and succinate respectively. These impaired oxidative and reductive reactions all involve the nicotinamide-adenine nucleotide coenzymes. For this reason Kronfeld & Kleiber (1959) postulated that the simplest explanation for the concurrence of these metabolic defects in acetonaemia would be a shortage of all forms of these nicotinamide coenzymes. Alternatively, a primary defect in any one of these reactions could lead to defects

in the others: for example, a primary defect in lipogenesis would be expected to lead to the accumulation of reduced forms of the coenzymes and a depletion of oxidized forms, and hence secondary impairment of the oxidative reactions in the pentose and citric acid cycles. A direct approach to possible causal relationships among these defects appeared to be the determination of nicotinamide-adenine nucleotide concentrations in the ketogenic tissue, in this case mammary gland (Kronfeld & Kleiber, 1959). A preliminary report has been presented (Kronfeld & Raggi, 1963).

METHODS

Coenzyme assay. Our fluorimetric assay follows that of Bassham, Birt, Hems & Loening (1959). It depends on the selective destruction of oxidized forms by alkali and of