8. Both enzymes are strongly inhibited by Ag^+ and Hg^{2+} ions in low concentrations; Cu^{2+} ions are powerful inhibitors of α -mannosidase activity and moderate inhibitors of β -galactosidase activity.

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The Biological Action of Substances Related to Thyroxine

11. ACETIC ACID ANALOGUES OF THYROXINE AND TRI-IODOTHYRONINE*

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The acetic acid analogues of thyroxine [3:5-di-iodo-4-(4-hydroxy-3:5-di-iodophenoxy)phenylacetic acid (tetraiodothyroacetic acid)] and tri-iodothyronine [3:5-di-iodo-4-(4-hydroxy-3-iodophenoxy)phenylacetic acid (tri-iodothyroacetic acid)] have been shown by Pitt-Rivers (1953) to possess thyroxine-like activity in rats. Thibault & Pitt-Rivers (1955) subsequently demonstrated that both compounds produced an immediate rise in the oxygen consumption of rat-kidney slices and in the basal metabolic rates of thyroidectomized rats. Immediate effects upon the glycolysis and respiration of cells have been reported (Heimberg, Park, Isaacs & Pitt-Rivers, 1955; Alexander & Bisset, 1958), but the immediate response of whole animals has not yet been confirmed by other workers. The possible physiological importance of tetra- and tri-iodothyroacetic acid, however, has been indicated by the chromatographic demonstration of their formation from thyroxine and tri-iodothyronine respectively in rat-kidney preparations (Roche, Michel, Jouan & Wolf, 1955; Albright, Larson, Tomita & Lardy, 1956). In clinical trials both compounds were shown to relieve the symptoms of myxoedema (Lerman & Pitt-Rivers, 1955; Trotter, 1955; Goolden, 1956) and there was some indication that they had a relatively greater effect upon the serum cholesterol than upon the basal metabolic rate.

Studies of the distribution of tri-iodothyroacetic acid in the tissues and excreta of the rat have been made by Roche, Michel, Jouan & Wolf (1956a) and by Larson & Albright (1958), who found that after the administration of small physiological doses the amount excreted as urinary iodide is less than when tri-iodothyronine was similarly used. In view of the possible clinical use of tri- and tetra-iodothyroacetic acid it seemed desirable to extend these studies by comparing the rates of excretion of both compounds with the corresponding iodothyronines. As deiodination processes have been shown to be important factors in the metabolism of the iodothyronines (Wilkinson, Sprott, Bowden & Maclagan, 1954; Wilkinson & Maclagan, 1954), the effect of the deiodination inhibitor, butyl 4-hydroxy-3:5-di-iodobenzoate (Sheahan, Wilkinson & Maclagan, 1951), has also been investigated in rats treated with the acetic acid compounds.

A preliminary report of some of the results has already appeared (Wilkinson, 1958).

MATERIALS AND METHODS

¹³¹I-labelled tri-iodothyroacetic acid. 3:5-Di-iodo-4-(4-hydroxyphenoxy)phenylacetic acid (11.1 mg.) (prepared by the method of Wilkinson, 1956) was dissolved at 0° in 2 ml. of methanol-aq. NH_3 soln. (sp.gr. 0.88) (1:1, v/v). A solution of I_2 (5.7 mg.) and Na^{131}I (5 mc) in aq. 50% methanol (4 ml.) was added gradually over 30 min. After setting

* Part 10: Wilkinson & Feetham (1955).

aside for 1 hr. the mixture was evaporated under reduced pressure to about 2 ml., the temperature being kept at 0° throughout. The solution was then acidified with HCl and set aside at 0° for a further 1.5 hr., after which the product was collected by centrifuging for 5 min. at 2000 rev./min. The supernatant was removed and the residue washed three times with water (8 ml.). The wet residue was recrystallized from aq. methanol and dried at 20°/1 mm.Hg over CaCl₂. Yield: 6.9 mg. The product was shown to be pure by paper chromatography and radioautography. The specific activity was usually in the range 50–100 μ C/mg.

¹³¹I-labelled tetraiodothyroacetic acid. This was prepared similarly by treating a solution of tri-iodothyroacetic acid (11.2 mg.) in 1 ml. of methanol-aq. NH₃ soln. (sp.gr. 0.88) (1:1, v/v) with a solution of I₂ (4.5 mg.) and Na¹³¹I (5 mC) in aq. 50% methanol (2 ml.). The specific activity of the product was 69 μ C/mg. For later experiments materials of higher specific activity (e.g. 1 μ C/ μ g.) were prepared by the method of Roche, Michel, Jouan & Wolf (1956b), in which the product was separated from inorganic iodide by paper electrophoresis. ¹³¹I-labelled thyroxine (specific activity 1 mC/mg.) was purchased from The Radiochemical Centre, Amersham, Bucks. and was diluted with sufficient non-radioactive thyroxine to give convenient counting rates in the animal experiments. ¹³¹I-labelled tri-iodothyronine (specific activity 60 μ C/mg.) was prepared by the method of Wilkinson & MacLagan (1954).

Excretion studies

Solutions for injection. ¹³¹I-labelled tri- or tetra-iodothyroacetic acid (1 mg.) was dissolved in sterile aq. 50% propylene glycol (0.5 ml.) containing diethanolamine (0.05 g.) and the volume made up to 10 ml. with sterile water. The solution was then diluted with sterile 0.9% NaCl soln. so that the required dose was contained in 1 ml. Butyl 4-hydroxy-3:5-di-iodobenzoate (BHDB; 1 g.) was dissolved in 50% propylene glycol (5 ml.) containing diethanolamine (0.3 g.). The volume was made up to 20 ml. with water.

Animals. Male albino rats (100–120 g.) were fed on cubed stock diet and tap water containing 10 mg. of KI/l., both *ad lib*. Groups of three were injected subcutaneously with tri- or tetra-iodothyroacetic acid and kept in metabolism cages, from which the urine and faeces were collected daily. The excreta were treated and the radioactivity was measured as described by Wilkinson *et al.* (1954). In experiments involving the administration of BHDB this was injected subcutaneously (25 mg./rat) on the day preceding the tri- or tetra-iodothyroacetic acid injection and on each subsequent day during the experiment.

Measurements of oxygen consumption

Mice weighing 16 $\pm$ 4 g. were randomized into groups of eight. Two groups were injected subcutaneously with tri-iodothyroacetic acid (2 or 5 mg./kg. in 0.02 N-Na₂CO₃) and two with the tetra-iodo compound (2 or 5 mg./kg. in 0.02 N-Na₂CO₃). Two further groups received the medium only and served as controls. Measurements of oxygen consumption were made at intervals of 3–5 hr. over the next 2 days by the closed-circuit method of MacLagan & Sheahan (1950) as modified by Sheahan *et al.* (1951). All results were expressed as a percentage of the controls and their statistical significance was analysed by Student's *t* test.

In order to measure the effect of BHDB on the oxygen consumption of mice treated with tri- or tetra-iodothyroacetic acid, two groups of eight mice were given BHDB (50 mg./kg.) in 0.2 ml. of arachis oil by intraperitoneal injection at 10.30 a.m. and again at 5 p.m. on day 0. These and two further groups of eight mice received single injections of tri- (or tetra-)iodothyroacetic acid (5 mg./kg.) in 0.2 ml. of 0.02 N-Na₂CO₃ at 12.30 p.m. on day 0. The tri- (or tetra-)iodothyroacetic acid controls were given arachis oil at the same times as the BHDB-treated groups received their injections. Two untreated control groups were injected with the media only. Measurements of oxygen consumption were commenced at 11 a.m. on day 1, after which the BHDB-treated animals were given a third injection at 5 p.m. on day 1, and these and the tri- (or tetra-)iodothyroacetic acid controls were treated with a second dose of tri- (or tetra-)iodothyroacetic acid at 4 p.m. on day 1. Measurements of oxygen consumption were repeated on day 2. Since BHDB had not been administered previously in arachis oil, it was confirmed, by a similar experiment in which thyroxine was used in place of tri-iodothyroacetic acid, that it exerted an antithyroxine effect when injected in this medium.

RESULTS

Excretion of tri- and tetra-iodothyroacetic acid in rats

The urinary radioactivity of rats treated with ¹³¹I-labelled tri- or tetra-iodothyroacetic acid was shown by paper chromatography and radioautography to consist almost exclusively of iodide. This was confirmed by the fact that addition of serum to the urine followed by protein precipitation with trichloroacetic acid did not significantly reduce the counting rate. Like thyroxine and tri-iodothyronine the acetic acid analogues were co-precipitated with protein. By contrast the faecal radioactivity was substantially removed by co-precipitation with protein and thus appeared to consist of unchanged tri- or tetra-iodothyroacetic acid or conjugates of these. In order to prevent losses of urinary radioiodide due to uptake by the thyroid gland, the animals were given small amounts of iodide in their drinking water.

Fig. 1 shows the result of a typical experiment in which the urinary excretion after the administration of equimolecular doses of tri- and tetra-iodothyroacetic acid were compared. The latter compound was deiodinated to a lesser extent than the former, a finding which proved to be independent of the dose administered. The percentage of the dose excreted as urinary iodide within 72 hr. of administration of various doses of thyroxine, tri-iodothyronine, tri- and tetra-iodothyroacetic acid is shown in Fig. 2. Both acetic acid analogues underwent deiodination to a markedly less extent than the iodothyronines, that of tetraiodothyroacetic acid being significantly less (*P* < 0.01) than that of tri-iodothyroacetic acid.

The faecal output in these experiments is shown in Fig. 3. Except with thyroxine, the percentage

excreted via the intestine also bore no relation to the dose. The rate of excretion of tetraiodothyroacetic acid was significantly lower than those of the iodothyronines, whereas tri-iodothyroacetic acid was excreted more rapidly than any of the other compounds by this route. The combined urinary and faecal excretion (Fig. 4) clearly illustrates the comparatively slow overall rate of excretion of tetraiodothyroacetic acid: only about 50 % of the dose was accounted for in 3 days. Some experiments were therefore continued for a total of 7 days, but even at the end of this time the overall excretion was only 66 %.

Fig. 1. Excretion of urinary [^{131}I]iodide after subcutaneous injection of equimolecular doses of ^{131}I -labelled tri- and tetra-iodothyroacetic acid in rats. Each point represents the combined excretion of a group of three rats: $\circ$, each rat was given tri-iodoacetic acid ($8.3 \mu\text{g.}$); $\bullet$, each rat was given tetraiodoacetic acid ($10 \mu\text{g.}$).

Fig. 2. Rates of excretion of urinary [^{131}I]iodide during the 72 hr. after the subcutaneous injection of ^{131}I -labelled tri- and tetra-iodothyroacetic acid, tri-iodothyronine and thyroxine. Each point represents the mean of two or four observations with the combined excretion of groups of three rats: $\circ$, rats treated with tri-iodothyroacetic acid; $\bullet$, rats treated with tetraiodothyroacetic acid; $\square$, rats treated with tri-iodothyronine; $\blacksquare$, rats treated with thyroxine.

Effect of butyl 4-hydroxy-3:5-di-iodobenzoate on deiodination of tri- and tetra-iodothyroacetic acid

The excretion of urinary iodide after the administration of either tri- or tetra-iodothyroacetic acid in rats was markedly reduced by the simultaneous administration of BHDB. Typical results are illustrated in Figs. 5 and 6, but the same effect was observed at several different dose levels.

The effect of BHDB on the radioactivity excreted in the faeces during the 72 hr. immediately after the administration of ^{131}I -labelled tri- and tetra-iodothyroacetic acid, tri-iodothyronine and thyroxine is shown in Table 1. In all four cases an increase occurred, but although this was negligible after the iodothyronines it was especially marked when tetraiodothyroacetic acid was given.

Fig. 3. Rates of excretion of ^{131}I -labelled tri- and tetra-iodothyroacetic acid, tri-iodothyronine and thyroxine in the faeces of rats during the 72 hr. immediately after subcutaneous injection. Each point represents the mean of two or four observations with groups of three rats. See Fig. 2 for explanation of symbols.

Fig. 4. Combined urinary and faecal excretion of ^{131}I -labelled tri- and tetra-iodothyroacetic acid, tri-iodothyronine and thyroxine in rats during the 72 hr. immediately after subcutaneous injection. Each point represents the mean of two or four observations with groups of three rats. See Fig. 2 for explanation of symbols.

Table 1. *Effect of butyl 4-hydroxy-3:5-di-iodobenzoate on the faecal radioactivity of rats treated with ¹³¹I-labelled tri- and tetra-iodothyroacetic acid, tri-iodothyronine and thyroxine*

Compound	Dose (μg./rat)	Percentage of dose appearing in faeces in 72 hr.		
		Rats injected with medium only	Rats injected with BHDB	Mean increase
Tri-iodothyroacetic acid	5	63.7	72.8	19.3
		49.2	78.7	
Tetraiodothyroacetic acid	2	38.3	75.2	34.9
		39.4	72.3	
Tri-iodothyronine	6	65.8	60.3	7.1
		49.8	69.5	
Thyroxine	4	34.5	54.6	8.9
		38.5	36.1

Fig. 5. *Effect of BHDB on the excretion of urinary [¹³¹I]-iodide in rats injected with ¹³¹I-labelled tri-iodothyroacetic acid (5 μg./rat). Each point represents the combined excretion of a group of three rats: ○, given tri-iodothyroacetic acid alone and, ●, in addition given BHDB (25 mg./rat/day).*

Fig. 6. *Effect of BHDB on the excretion of urinary [¹³¹I]-iodide in rats injected with ¹³¹I-labelled tetraiodothyroacetic acid (10 μg./rat). Each point represents the combined excretion of a group of three rats: ○, given tetra-iodothyroacetic acid alone and, ●, in addition given BHDB (25 mg./rat/day).*

Effects of tri- and tetra-iodothyroacetic acid on oxygen consumption of mice

Fig. 7 shows the effect of tri- and tetra-iodothyroacetic acid on the oxygen consumption of mice. No effects were observed 4 hr. after the sub-

Fig. 7. *Effect of tri- and tetra-iodothyroacetic acid upon the oxygen consumption of mice. Each point represents the mean of three readings with each of two groups of eight mice (i.e. six observations): ○, mice injected subcutaneously with tri-iodothyroacetic acid (5 mg./kg.); ●, mice injected subcutaneously with tetraiodothyroacetic acid (5 mg./kg.).*

cutaneous injection of 5 mg./kg., but significant elevation occurred 10 hr. after administration with tri-iodothyroacetic acid but not until 24 hr. with tetra-iodothyroacetic acid. The effect was maximal at about 48 hr. with both substances, but had completely disappeared at 72 hr. Similar results were obtained with doses of 2 mg./kg., but at most time intervals the increase in the oxygen consumption was not statistically significant.

No inhibitory effect was exerted when BHDB was administered at the same time as the iodothyroacetic acids. Thus when expressed as a percentage of the control values the mean oxygen consumption of mice treated with tri-iodothyroacetic acid (5 mg./kg.), 24 hr. after injection, was 123 and 132 in two experiments; the groups treated with BHDB gave figures of 123 and 131 respectively. However, with tetra-iodothyroacetic acid some slight enhancement of the effect was seen, but this was of doubtful significance. The mean oxygen consumption of mice treated with tetra-iodothyroacetic acid was 129 % of the control values in each of two experiments; BHDB-treated animals gave corresponding figures of 138 % ($P < 0.05$) and 136 % ($P > 0.05$).

DISCUSSION

The finding that the urinary radioactivity of rats treated with ^{131}I -labelled tri- or tetra-iodothyroacetic acid was almost entirely inorganic iodide is consistent with the results of Roche, Michel, Closon & Michel (1957), who, in thyroidectomized rats, found that the principal urinary excretion product of the metabolism of tri-iodothyroacetic acid was inorganic iodide, which, however, was accompanied by several unidentified substances and small amounts of the unaltered acid. During the present study at least 97 % of the urinary radioactivity was accounted for as iodide, and no attempt was therefore made to detect these minor metabolites.

The observation that both acetic acid analogues undergo deiodination *in vivo* to a markedly less extent than the corresponding iodothyronines confirms the finding of Maclagan & Reid (1957), who showed that they were very much less readily attacked by the deiodinating system in rat-liver preparations than tri-iodothyronine and thyroxine. These low rates of deiodination were further depressed when BHDB, the antithyroxine compound, was given at the same time. However, the fact that BHDB had little effect on the oxygen consumption of mice that was enhanced by the treatment with tri-iodothyroacetic acid indicates that deiodination probably does not play an important part in the stimulation of respiration by this substance. The slight enhancement of the tetra-iodothyroacetic acid effect by BHDB might be interpreted as indicating that deiodination ordinarily interferes with the respiration-stimulating action of this compound, but the significance of the results obtained is doubtful.

These effects are in marked contrast with those obtained with thyroxine and tri-iodothyronine (Maclagan, Sprott & Wilkinson, 1952), for BHDB antagonized the action of the tetraiodo compound and enhanced that of tri-iodothyronine. It seems that, if the acetic acid compounds appear in the pathway by which thyroxine is degraded, as suggested by Pitt-Rivers (1953), they occur at some stage beyond that at which deiodination has any physiological significance.

The remarkably slow overall rate of excretion of tetraiodothyroacetic acid in comparison with those of tri-iodothyroacetic acid and the iodothyronines was independent of the amount injected, in the range 2–40 $\mu\text{g.}/\text{rat}$. The difference in rates of excretion suggested that tetraiodothyroacetic acid might have a more prolonged effect on oxygen consumption than the tri-iodo derivative, but no such effect could be demonstrated. A possible explanation of the slow rate of excretion of the tetraiodo compound has been suggested by Dr J. R.

Tata (personal communication), who in electrophoretic studies found it to be more firmly bound to serum protein than tri-iodothyroacetic acid. Alternatively, it may be chemically converted in the body into some inactive substance which is only slowly excreted, a suggestion which is supported by the greater chemical reactivity of this compound (Wilkinson, 1956). The acceleration of the excretion of tetraiodothyroacetic acid by BHDB may be brought about by interference with the formation of this hypothetical inactive compound. Whatever may be the cause of this acceleration of faecal excretion it can be shown, by calculation, that it is not sufficiently great to be solely responsible for the fall in urinary iodide after the administration of BHDB. This view is supported by the studies of Maclagan & Reid (1957), who found that BHDB interfered with the deiodination of both iodothyroacetic acids by liver preparations *in vitro*.

SUMMARY

1. The metabolism of the ^{131}I -labelled acetic acid analogues of thyroxine and tri-iodothyronine has been compared in the rat with that of the corresponding iodothyronines.
2. The urinary radioactivity was shown to consist almost exclusively of iodide and the urinary excretion could therefore be regarded as a measure of the extent of deiodination in all four cases.
3. The acetic acid analogues underwent deiodination to a much smaller extent than the iodothyronines. The overall rate of excretion of tetraiodothyroacetic acid was markedly less than that of any of the other compounds.
4. Both acetic acid analogues increased the rate of oxygen consumption of mice, but no effect was observed until 10 hr. after administration. The duration of this effect was about the same in each case.
5. The antithyroxine compound, butyl 4-hydroxy-3:4-di-iodobenzoate, reduced the degree of deiodination of both acetic acid analogues, but did not diminish the increase in the oxygen consumption produced by these substances in mice.

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A Method for the Estimation of Dehydroepiandrosterone in Urine

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The evidence indicating the origin of dehydroepiandrosterone (3 β -hydroxyandrost-5-en-17-one) in the adrenal cortex (cf. Lieberman, 1956) has been strengthened by the isolation of this steroid from an adrenal tumour by Plantin, Diezfasusy & Birke (1957). The work of Landau, Knowlton, Lugibihl & Munson (1951), Bitman & Cohen (1951), Dingemanse, Huis in't Veld & Hartogh-Katz (1952) and Ronzoni (1952) suggested that dehydroepiandrosterone was one of the major 17-oxo steroids in urine. Accordingly its estimation in urine should provide a method of measuring one aspect of adrenal function.

Many methods for the estimation of dehydroepiandrosterone or of the β -fraction of urine extracts, of which dehydroepiandrosterone was thought to be the only steroid component, have been described. The earlier methods in which dehydroepiandrosterone was purified via the digitonide and estimated by the Zimmermann reaction (Talbot, Butler, MacLachlan & Jones, 1940; Baumann & Metzger, 1940; Frame, 1944; Salter, Cahen & Sappington, 1946; Dobriner, 1953) or polarographically (Butt, Henly & Morris, 1948), or methods in which dehydroepiandrosterone after purification by means of the Girard separation is estimated by the Allen, Hayward & Pinto (1950) reaction (Ronzoni, 1952) or the Pettenkofer reaction (Munson, Jones, McCall & Gallagher, 1948; Landau *et al.* 1951; Bray & Merivale, 1953; Prunty, 1953), or the methods of Talbot, Ryan & Wolfe (1943), Bitman & Cohen (1949, 1951) and Jensen & Totterman (1952), in which dehydroepiandro-

sterone is liberated from its conjugate by heating under neutral or slightly acid conditions, lack specificity since no chromatographic separation is used. Fotherby (1958) showed that substances other than dehydroepiandrosterone, which give positive Pettenkofer and Zimmermann reactions and are precipitated with digitonin, occur in the ketonic fractions of urine extracts. Moreover, most of the earlier methods employ acid hydrolysis at elevated temperatures, which results in the production of artifacts and thus may not be quantitative. In few cases has the sensitivity of the method or the accuracy based upon the amount of added steroid recovered been adequately determined. These remarks also apply to the method described by Cohen & Oneson (1953), in which the steroid sulphates are hydrolysed with dioxan and trichloroacetic acid. Fotherby (unpublished work) found that while pure potassium dehydroepiandrosterone sulphate is readily hydrolysed by this method the recovery of steroid when the conjugate is added to urine or to urine extracts is poor, presumably owing to the presence of inhibitors of the reaction in the urine extract.

Recently methods for the determination of the major 17-oxo steroids, including dehydroepiandrosterone, present in urine have been developed, in which either column chromatography (Dingemanse *et al.* 1952; Kellie & Wade, 1957) or paper chromatography (Brooks, 1958; Bush, 1959) is employed, but these methods are lengthy.

It therefore seemed desirable to have a method for the estimation of dehydroepiandrosterone in