Intrathyroidal Iodine Metabolism in the Rat

2. THE IODOTHYRONINES*

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(Received 3 April 1963)

Pitt-Rivers (1962) reported that the ratio of ^{131}I -labelled 3,5,3'-tri-iodothyronine (referred to as 'tri-iodothyronine') to ^{131}I -labelled thyroxine in the rat thyroid gland is higher in the early intervals after a dose of ^{131}I iodide than at isotopic equilibrium. Feuer (1959) had made a similar observation on the ratio of ^{131}I tri-iodothyronine to ^{131}I thyroxine in the plasma of the rat and the rabbit. These results suggest that, if the iodo amino acid pools do not change in size during the experiment, the specific radioactivity of tri-iodothyronine increases more rapidly than that of thyroxine. If this conclusion could be confirmed and if each of the thyroidal iodothyronines is homogeneous with respect to turnover, the hypothesis that tri-iodothyronine biosynthesis involves thyroxine as a precursor (Gross & Pitt-Rivers, 1953; Plaskett, 1961*a*) would be discredited.

Both Feuer (1959) and Pitt-Rivers (1962) used one-dimensional paper chromatography for separating the thyroid hormones. The same solvent system was used in each case. We have studied the incorporation of ^{131}I into the iodothyronines of the rat thyroid gland by different paper-chromatographic techniques.

METHODS

Animals. Male Wistar albino rats (170–220 g.) were used. They were given a low-iodine diet (Boyd & Oliver, 1960) for 2 weeks before the experiments.

Administration of ^{131}I iodide. A solution of ^{131}I iodide was injected subcutaneously, or, in experiments of very short duration, intravenously. When the interval elapsing between injection and killing was 1 hr. or longer a dose of $10\mu\text{C}$ was used. For experiments of shorter duration 10 – $100\mu\text{C}$ was used. In some experiments in which high levels of radioactivity were required to permit radioautography of iodothyronines on two-dimensional chromatograms, the dose was 1 mC. In such experiments the interval between injection and killing was 5–15 min., the thyroid gland uptake did not exceed 1% of the dose and the radiation dose to thyroid tissue was below 40 rads.

Preparation of thyroid-gland hydrolysates. The thyroid gland was dissected out rapidly immediately after death of the animal, homogenized and hydrolysed enzymically as described by Plaskett, Barnaby & Lloyd (1963).

Paper chromatography. Feuer (1959) and Pitt-Rivers (1962) both employed the solvent system butan-1-ol-dioxan-2*N*- NH_3 (4:1:5, by vol.). In the present work the resolution of thyroxine and tri-iodothyronine in this system was studied, and it was found that the separation is incomplete (Fig. 2); quantitative determination of the two substances is therefore difficult. Similarly, incomplete separation is shown in the published chromatogram scans of Feuer (1959) and Albright, Larson & Deiss (1953). Pitt-Rivers (1962) did not publish any chromatogram scans, but detected carrier spots of thyroxine and tri-iodothyronine with ninhydrin and excised the coloured regions of the paper for determination of total radioactivity. This technique disregards the overlapping character of the peaks so that the radioactivity situated between the peaks, where the concentration of the carrier is below the sensitivity limit of the ninhydrin stain, would have been lost. In the present work butan-1-ol-dioxan-2*N*- NH_3 (4:1:5, by vol.) was used initially. Subsequently the systems butan-1-ol-9*N*- NH_3 (1:1, v/v), butan-1-ol-pentan-1-ol-2*N*- NH_3 (1:1:2, by vol.) and water-saturated collidine-aq. NH_3 (sp.gr. 0.88) were used. Some chromatograms were developed in butan-1-ol-2*N*-acetic acid (1:1, v/v) to separate the iodothyrosines. Two-dimensional chromatograms were also prepared, with butan-1-ol-dioxan-2*N*- NH_3 (4:1:5, by vol.) for the first development and butan-1-ol-9*N*- NH_3 (1:1, v/v) for the second development.

Radioactive spots were identified by reference to the position of carrier compounds. Iodide was detected with a spray of aq. 1% (w/v) palladium chloride, and iodo amino acids with ninhydrin or ultraviolet-absorption techniques.

Degradation of ^{131}I -labelled tri-iodothyronine. Pitt-Rivers (1962) suggested that the specific radioactivity of tri-iodothyronine increased more rapidly than that of thyroxine because the specific radioactivity of monoiodotyrosine, a possible immediate precursor of tri-iodothyronine, was observed to exceed that of di-iodotyrosine, the thyroxine precursor. If this is a correct explanation then the 3'-iodine atom of tri-iodothyronine would be expected to have a higher specific radioactivity than the 3- and 5-iodine atoms. To test this view ^{131}I tri-iodothyronine was isolated from the thyroids by butan-1-ol-dioxan-2*N*- NH_3 chromatography and submitted to degradation by the diazo-coupling technique (Plaskett, 1961*a*).

Coupling of tri-iodothyronine with *m*-nitrobenzene-diazonium chloride eliminates iodine from the 3'-position in the form of inorganic iodide. Only a little over 50% of the 3'-iodine is eliminated and it is therefore likely that some of the coupling also occurs in the untenantated 5'-position. Most of the iodine in positions 3 and 5 fails to react, pre-

* Part 1: Plaskett, Barnaby & Lloyd (1963).

Fig. 1. Distribution of radioactivity on chromatograms of rat-thyroid hydrolysates developed in butan-1-ol-dioxan-2N-NH₃: (a) 10 min. after the injection of [¹²⁵I]iodide; (b) 2½ hr. after the injection of [¹²⁵I]iodide; (c) 24 hr. after the injection of [¹²⁵I]iodide. DIT, Di-iodotyrosine; MIT, monoiodotyrosine; I⁻, iodide; T4, thyroxine; T3, tri-iodothyronine; O, origin; SF, solvent front.

sumably because the phenyl ether does not activate the *ortho*-position so well as a phenolic hydroxyl group.

The released iodide remains in the supernatant after precipitation of the azo compounds with acid and centrifuging. The precipitate contains 80.5% of the total iodine. When the reaction is performed with [3,5-¹³¹I]₂tri-iodothyronine 96.7% of the radioactivity appears in the precipitate. When [3'-¹³¹I]tri-iodothyronine is used 48.6% appears in the precipitate. Uniformly labelled tri-iodothyronine shows 80.5% precipitation of radioactivity.

The percentage precipitation of radioactivity is therefore a measure of the distribution of ¹³¹I in the molecule.

Fig. 2. Distribution of radioactivity on chromatograms of rat-thyroid hydrolysates developed in butan-1-ol-dioxan-2N-NH₃ in a detailed scan of the iodothyronine region: (a) 10 min. after the injection of [¹²⁵I]iodide; (b) 2½ hr. after the injection of [¹²⁵I]iodide. T4, Thyroxine; T3, tri-iodothyronine.

Table 1. *Apparent amounts of [^{131}I]tri-iodothyronine and [^{131}I]thyroxine in rat thyroid glands*

The apparent amounts were measured by one-dimensional chromatographic analysis in the solvent system butan-1-ol-dioxan-2N-NH₃ (4:1:5, by vol.).

No. of rats	Time after injection of [^{131}I]iodide	Percentage of total thyroid gland ^{131}I		Ratio of [^{131}I]tri-iodothyronine to [^{131}I]thyroxine
		[^{131}I]Thyroxine	[^{131}I]Tri-iodothyronine	
6	10 min.	0.65	0.27	0.41
2	20 min.	1.19	0.41	0.34
6	1 hr.	2.26	0.72	0.32
6	2½ hr.	3.87	1.20	0.31
6	4 hr.	5.51	1.58	0.29
6	6 hr.	7.62	1.86	0.24
6	8 hr.	9.66	2.13	0.22
4	24 hr.	16.07	3.03	0.19

EXPERIMENTAL AND RESULTS

The uptake of radioactivity in the thyroid gland reached a maximum of 18–20 % of the dose about 8 hr. after the injection and fell to about 10 % at 24 hr.

Results of chromatography in one dimension. [^{131}I]Thyroxine and [^{131}I]tri-iodothyronine were first estimated by one-dimensional chromatography in butan-1-ol-dioxan-2N-ammonia at various intervals from 10 min. to 24 hr. after the injection. The results are given in Table 1. In the early intervals after the dose the ratio of [^{131}I]tri-iodothyronine to [^{131}I]thyroxine was higher than at 24 hr. The results are in closer agreement with those of Pitt-Rivers (1962) than with those of Feuer (1959). Pitt-Rivers found that the value of this ratio was always less than unity, but Feuer, working with plasma, reported higher values for [^{131}I]tri-iodothyronine than for [^{131}I]thyroxine in the early intervals after the injection. In the present study 0.41 was the highest ratio recorded.

A similar series of chromatograms developed in butan-1-ol-pentan-1-ol-2N-ammonia gave results which differed from those in Table 1 and which were more erratic. The fact that chromatographic analysis of identical samples in different solvent systems can yield substantially different results was taken as an indication that in at least one of the solvent systems the iodothyronine peaks were being contaminated by material of a different identity. Clearly, if such material runs with tri-iodothyronine in the butan-1-ol-dioxan-2N-ammonia system, the results of Feuer (1959) and Pitt-Rivers (1962) may have a technical rather than a biochemical explanation.

Diazo coupling. Evidence supporting the view that tri-iodothyronine was not the only labelled substance present on the area of the chromatogram indicated by the tri-iodothyronine carrier was obtained from the degradation studies employing the diazo-coupling reaction (see the Methods

section). In this reaction precipitation values lower than 80.5 % indicate preferential labelling of the iodine atom at position 3'.

In the present work values lower than this were obtained, especially in the early intervals after ^{131}I administration, and would normally have been regarded as evidence for non-uniform labelling (Table 2). However, when the incorporation of ^{131}I into the thyroidal mono- and di-iodotyrosine was studied as a function of time, it was found that their relative specific radioactivities remained equal during the entire period of the experiment. This unexpected finding, which has already been reported (Plaskett *et al.* 1963), precludes the only theoretically probable pathway by which non-uniformly labelled tri-iodothyronine might be produced.

Presence of an unknown compound ('unknown I'). One hypothesis compatible with these facts was that the tri-iodothyronine was uniformly labelled, but was contaminated with another radioactive substance of similar R_F which readily passed into the soluble fraction after diazo coupling. On the basis of this hypothesis the values in Table 2 indicate that the contaminant made up a relatively larger part of the radioactivity of the tri-iodothyronine region of the chromatogram when the interval between injection and killing was short. This finding is readily comprehensible by reference to Fig. 1, which shows that as this interval is shortened the iodothyronines represent a progressively smaller percentage of the total radioactivity of the hydrolysate. Therefore, at the very short time-intervals gross errors could be caused in the estimation of [^{131}I]iodothyronines by very slight contamination of the iodothyronine spot by materials with a faster turnover. This view is supported in Fig. 2, which shows a detailed scan of the iodothyronine region of two of the chromatograms. In a hydrolysate 2½ hr. after the injection the tri-iodothyronine peak is symmetrical and seems to be largely homogeneous. In a hydrolysate

10 min. after the injection the tri-iodothyronine peak is elongated and irregular. Although count rates were low the irregularities cannot be entirely accounted for as random fluctuations of count rate.

Separation of unknown I. In view of this evidence that the radioactive material of the tri-iodothyronine peak is heterogeneous, attempts were made to

Table 2. *Apparent distribution of ^{131}I on the two rings in tri-iodothyronine*

Samples were obtained by chromatographing thyroid hydrolysates on paper in the solvent system butan-1-ol-dioxan-2*N*- NH_3 (4:1:5, by vol.) and eluting the peak corresponding to marker tri-iodothyronine.

Time after injection of ^{131}I iodide	Percentage precipitation of radioactivity after diazo coupling (random labelling = 80.5%)	Apparent percentage of the total radio-activity of the 'tri-iodothyronine' on the 3 and 5 positions (random labelling = 66.3%)
10 min.	59.4	22
1 hr.	73.3	51
2½ hr.	76.3	57
4 hr.	76.3	57
6 hr.	78.5	62
8 hr.	75.8	57
12 hr.	76.5	58
24 hr.	79.2	64

Fig. 3. The radioactive material that runs with tri-iodothyronine carrier in butan-1-ol-dioxan-2*N*- NH_3 was developed in a second dimension in butan-1-ol-9*N*- NH_3 . The Figure shows the resulting distribution of radioactivity. T3, Tri-iodothyronine; U₁, unknown I; O, origin; SF, solvent front.

separate a contaminating compound. Hydrolysates were prepared from the thyroids of rats 10 min. after the injection and were chromatographed in butan-1-ol-dioxan-2*N*-ammonia. The radioactivity corresponding in position to marker tri-iodothyronine was eluted from the paper with ethanol-2*N*-ammonia (1:1, v/v) and developed in a different solvent on another chromatogram. Several solvent systems were tested in this way for ability to separate tri-iodothyronine from a contaminating compound. The most effective system was butan-1-ol-9*N*-ammonia.

Alternatively, two-dimensional chromatograms were prepared as described in the Methods section.

A typical result is shown in Fig. 3. In this example unknown I contained about 34% of the radioactivity which corresponded in position with the tri-iodothyronine marker in one-dimensional development. The exact value of the percentage varied widely, however, because the contaminant spot sometimes occupied a position on the chromatogram which was almost co-extensive with the tri-iodothyronine spot, but sometimes ran with a significantly higher R_f . In the latter instance a larger proportion of the contaminating radioactivity was separated from the tri-iodothyronine. To obtain an accurate determination of the amounts of the two substances present, it was necessary to locate the spots on the two-dimensional chromatograms by radioautography before scanning for radioactivity. For this purpose doses of 1 mc were necessary. At 10 min. after the injection the uptake of radioactivity in the thyroid gland is only about 0.5% of the dose, and only about 0.25% of the thyroïdal radioactivity is in the form of tri-iodothyronine. A dose of 1 mc therefore gave a thyroïdal concentration of [^{131}I]tri-iodothyronine of approx. 0.0006 $\mu\text{C}/\text{mg.}$ of fresh tissue. This was the lowest concentration that could be readily detected by the relatively insensitive technique of radioautography. After locating the spots by this method the amounts of labelled thyroxine, tri-iodothyronine and unknown I were determined by scanning the relevant areas of the paper. The results are given in Table 3, which also shows the results from similar chromatograms of thyroid hydrolysates 24 hr. after the injection.

Tri-iodothyronine:thyroxine ratio. The fact that unknown I and tri-iodothyronine have similar R_f values in butan-1-ol-dioxan-2*N*-ammonia indicates that previous measurements that depended solely on one-dimensional development in this system gave artificially high values for the amount of [^{131}I]tri-iodothyronine. Table 3 shows that the quantity of labelled unknown I relative to that of labelled tri-iodothyronine is much greater in the early intervals after the dose than at 24 hr. Unknown I is therefore a compound with a shorter turnover

Table 3. *Determination of ^{131}I -labelled thyroxine, tri-iodothyronine and unknown I in thyroid hydrolysates by two-dimensional chromatography*

Values were normalized to thyroxine = 1.0. Each value represents the mean of eight measurements.

Time after injection of ^{131}I iodide	Thyroxine	Tri-iodothyronine (relative to thyroxine = 1.0)	Unknown I (relative to thyroxine = 1.0)
5-15 min.	1.0	0.18 (0.14-0.24)	0.11 (0.06-0.13)
24 hr.	1.0	0.19 (0.16-0.23)	0.014 (0.005-0.020)

Table 4. *Quantitative determination and chromatographic properties of four unknown ^{131}I -labelled compounds detected by two-dimensional chromatography*

The solvent systems were: (1) butan-1-ol-dioxan-2N-NH₃; (2) butan-1-ol-9N-NH₃; (3) water-saturated collidine-aq. NH₃ (sp.gr. 0.88). Known compounds are included in the Table for comparison.

Substance	R_F in solvent (1)	R_F in solvent (2)	R_F in solvent (3)	Percentage of total ^{131}I	
				5-15 min. after injection of ^{131}I iodide	24 hr. after injection of ^{131}I iodide
Unknown I	0.69	0.69	—	0.07	0.22
Unknown II	0.66	0.66	—	0.01	—
Unknown III	0.52	0.39	0.27	—	0.48
Unknown IV	0.83	0.87	—	0.05	—
Thyroxine	0.55	0.42	—	0.65	16.1
3,5,3'-Tri-iodothyronine	0.67	0.45	—	0.12	3.1
3,3',5'-Tri-iodothyronine	0.52	0.39	0.25	None detected	None detected
4-Hydroxy-3,5-di-iodo-benzaldehyde	0.83	0.87	—	—	—

time than tri-iodothyronine, and its presence could explain the rather high initial ratio of ^{131}I tri-iodothyronine to ^{131}I thyroxine reported by Pitt-Rivers (1962). When the ratios of ^{131}I tri-iodothyronine to ^{131}I thyroxine were measured in the present work by two-dimensional chromatography during the period 5-15 min. after the dose they were not significantly different from the 24 hr. value (Table 3).

Other unknown compounds. In addition to unknown I three other unidentified substances (unknowns II, III and IV) were detected on the two-dimensional chromatograms. Their chromatographic properties and details of the quantities that were detected are given in Table 4. In the first (butan-1-ol-dioxan-2N-ammonia) development unknown II ran with R_F similar to those of tri-iodothyronine and unknown I; it was an extremely minor component. Unknown III ran with R_F very slightly lower than that of thyroxine in both systems, and the radioautographs showed it to be incompletely separated from the thyroxine spot. That it was distinct from thyroxine, and not merely the result of an artificially misshapen spot, was established by eluting and re-running with marker thyroxine. The chromatographic properties of unknown III suggested that it might be an iodothyronine with fewer substituents than thyroxine on the α -aromatic ring. One such compound that has been reported to be present in the thyroid gland

Fig. 4. Unknown III was eluted from one of the two-dimensional chromatograms and re-developed in the system collidine-water-ammonia. The Figure shows the resulting distribution of radioactivity. M, Marker 3,3',5'-tri-iodothyronine. The radioactivity of chromatogram segments not included in the Figure was below the limit of detection.

is 3,3',5'-tri-iodothyronine. Unknown III was run with marker 3,3',5'-tri-iodothyronine in three different solvent systems, and the results obtained in two of the systems showed complete correspondence of the marker with the radioactive zone (Table 4). The water-saturated collidine-aq. ammonia (sp.gr. 0.88) system, however, produced some separation (Fig. 4). No identification of unknown III has yet been possible, but these results indicate that

3,3',5'-tri-iodothyronine, which was stated by Roche & Michel (1956) to constitute up to 2% of the total iodine of the rat thyroid gland, was not present in significant quantities in the present experiments.

Unknown IV had a high R_f in both of the solvent systems used for the two-dimensional chromatography and was well separated from all the known radioactive components. Its chromatographic properties in these two systems were the same as those of 4-hydroxy 3,5-di-iodobenzaldehyde (Table 4).

DISCUSSION

Pathway of tri-iodothyronine biosynthesis: theoretical considerations. Two mechanisms of tri-iodothyronine biosynthesis have been proposed. The first of these [eqn. (1) of Scheme 1] involves thyroxine as a precursor. Roche, Michel, Michel & Lissitzky (1952) expressed the view that this mechanism was improbable because the thyroidal deiodinases, which readily degrade iodotyrosines, were found not to act on thyroxine. However, the finding of Tata (1959) that these deiodinases will act on thyroxine when they are freed from blood proteins re-opened the possibility that thyroxine deiodination is a pathway of tri-iodothyronine formation. But, if all the tri-iodothyronine were formed by this route, isotopic experiments would reveal a precursor-product relationship between thyroxine and tri-iodothyronine. Present evidence, including that of the present paper (Table 3), indicates that no such relationship exists.

The second possible route of tri-iodothyronine formation [eqn. (2) of Scheme 1] involves the coupling of one monoiodotyrosyl and one di-iodotyrosyl residue in a reaction analogous to thyroxine biosynthesis. If this is the main pathway, then, in conditions in which the two iodotyrosines have different specific radioactivities, the iodine atoms on the two aromatic rings of the newly formed tri-iodothyronine will also have different specific radioactivities (Plaskett, 1961*a*). It is

easily possible to predict the extent of this difference for the case of short intervals after the dose, when the rates of loss of radioactivity from the iodothyronine pools are very low compared with the rates of influx. If the specific radioactivity of the iodine in di-iodotyrosine at a given time is x and the specific radioactivity of the iodine in monoiodotyrosine is y , then the specific radioactivity of the iodine in newly formed tri-iodothyronine at that time will be $(2x + y)/3$ and the specific radioactivity of the iodine in newly formed thyroxine will be x . If it is assumed that the turnover times of the thyroidal tri-iodothyronine and thyroxine pools are equal, then the ratio between the rates of increase of the specific activities of the two pools will be $(2x + y)/3 : x$. The ratio between the specific radioactivities of the two pools will be a similar function of the integrated values of x and y from the time of the injection to the time of death.

Therefore, provided that the necessary assumption about turnover times can be justified, it is theoretically possible to establish the route of tri-iodothyronine biosynthesis by measuring the specific radioactivities of tri-iodothyronine and thyroxine as functions of time. Experiments in which the specific radioactivities of the iodine on the two aromatic rings of highly purified tri-iodothyronine are determined separately would offer even more conclusive evidence. In the present work these crucial experiments were not possible because, unlike other workers (Michel, 1956; Taurog, Tong & Chaikoff, 1958; Pitt-Rivers, 1962), we have not been able to produce conditions in which the values of x and y were different.

All the above discussion is subject to the condition that iodination of tri-iodothyronine to yield thyroxine (as proposed by Feuer, 1959) does not occur. It is necessary to make this reservation because if this iodination reaction took place it would influence the specific radioactivity-time relations in a complex manner. In the early intervals after a dose of ^{131}I it would tend to increase the amount of [^{131}I]tri-iodothyronine relative to that

Scheme 1. Possible reactions in the final stages of thyroid-hormone biosynthesis as they have been proposed in the literature (see especially Feuer, 1959; Plaskett, 1961*a*; Pitt-Rivers, 1962; Gross, 1962).

of [^{131}I]thyroxine both by shortening the turnover time of tri-iodothyronine and by establishing a precursor-product relationship between the two thyroid hormones. On the other hand, the introduction, during the iodination step, of iodine from the very radioactive iodine pool of the thyroid would tend to produce a heavier early labelling of thyroxine. It is difficult to forecast the eventual outcome of these opposing actions. The present authors conclude, however, that iodination of tri-iodothyronine does not occur, because it would be expected to lead to the formation of thyroxine preferentially labelled at positions 3' and 5'. Plaskett (1961*b*) showed that no such preferential labelling exists. An idea of the way in which the untenanted 5'-position in tri-iodothyronine may escape iodination can be derived from the two-compartment mechanism of thyroid-hormone biosynthesis proposed by Plaskett *et al.* (1963). Iodination and coupling probably occur in different fractions of thyroglobulin. If these fractions are spatially, as well as functionally, separated, then no tri-iodothyronine would be present in the region of the follicle where iodination of tyrosyl residues occurs. Since iodination probably takes place in a region situated at the periphery of the follicles (Levenson, 1960), this explanation seems a reasonable one. Tri-iodothyronine would, of course, have to pass through the peripheral 'zone of iodination' in transit from the follicle into the blood, and on this basis one might speculate that tri-iodothyronine, though not a precursor of the thyroidal thyroxine, could be a precursor of the circulating thyroxine.

Pathway of tri-iodothyronine biosynthesis: the evidence of the present work. The present work shows that, as stated by Feuer (1959) and Pitt-Rivers (1962), the results of chromatography in butan-1-ol-dioxan-2*N*-ammonia suggest more rapid incorporation of radioactivity into tri-iodothyronine than into thyroxine. However, this phenomenon is a peculiarity of one solvent system and is an artifact caused by coincidence of the tri-iodothyronine spot with that of unknown I, a compound with faster turnover. Pitt-Rivers's (1962) suggestion, that in her work an initially higher specific radioactivity of tri-iodothyronine resulted from the initially higher specific radioactivity of its likely precursor mono-iodotyrosine, is not supported, since Pitt-Rivers's chromatographic results have been produced (Table 1) in conditions in which the specific radioactivities of the iodotyrosines were always equal.

The evidence presented here is nevertheless compatible with the view that much of the thyroidal tri-iodothyronine is formed by the coupling of mono- and di-iodotyrosine. The finding that the ratio of [^{131}I]tri-iodothyronine to [^{131}I]thyroxine is the same at 5-15 min. and at 24 hr. after the dose

suggests that the specific radioactivities of the two hormones increase in parallel. Therefore neither of the thyroid hormones is a major precursor of the other. If tri-iodothyronine is formed by the coupling of mono- and di-iodotyrosine, then the specific radioactivity of newly formed tri-iodothyronine would not be expected to differ from that of newly formed thyroxine when the values of x and y are identical. Clearly, this prediction is in agreement with the present findings. However, present evidence does not preclude the formation of minor quantities of tri-iodothyronine from thyroxine.

Evidence that tri-iodothyronine is formed by the coupling of mono- and di-iodotyrosine might be strengthened if the experiments of Pitt-Rivers (1962) could be repeated with an improved chromatographic technique, or if the present work could be repeated in conditions in which the values of x and y are different.

Diazo-coupling results. The fact that in the present work diazo-coupling results with tri-iodothyronine were invalidated by the presence of an impurity in no way invalidates determinations by Plaskett (1961*a*) of the distribution of [^{131}I] in biosynthetically labelled [^{131}I]tri-iodothyronine. Plaskett (1961*a*) employed three solvent systems for purification of the tri-iodothyronine, including butan-1-ol-9*N*-ammonia.

Unknown compounds. Of the four unknown compounds reported above, only one ran consistently with one of the marker compounds, namely unknown IV, which may be 4-hydroxy-3,5-di-iodobenzaldehyde. One would expect small quantities of this material in thyroid hydrolysates, since Pitt-Rivers (1948) showed that it was formed as a result of incubating di-iodotyrosine solutions at an alkaline pH.

SUMMARY

1. Incorporation of [^{131}I] into iodothyronines in the rat thyroid gland has been studied by two-dimensional paper chromatography.

2. In contrast with the results obtained in this Laboratory and elsewhere by one-dimensional chromatography, it was found that the ratio of [^{131}I]tri-iodothyronine to [^{131}I]thyroxine did not diminish as the time elapsing between administration of [^{131}I] and the death of the animal was lengthened. The ratio was approximately the same 5-15 min. after the injection as at 24 hr.

3. The reason for the discrepancy between one-dimensional and two-dimensional chromatographic analysis was that one-dimensional analysis failed to separate tri-iodothyronine from an unknown iodine-containing compound with faster turnover.

4. A total of four unknown iodine-containing compounds was detected in the thyroid hydrolysates.

5. These results are compatible with the view that much of the thyroidal tri-iodothyronine is formed by the coupling of mono- and di-iodotyrosine. The formation of minor quantities of tri-iodothyronine by deiodination of thyroxine is not precluded.

The authors thank Miss Anne Underwood and Mr B. Shanks for skilled technical assistance.

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Biochem. J. (1963) **89**, 486

The Mechanism of Formation of Thymidine 5'-Triphosphate by Enzymes from Landschutz Ascites-Tumour Cells

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(Received 6 May 1963)

The deoxyribonucleoside 5'-triphosphates of adenine, guanine, cytosine and thymine have been shown to be utilized in the synthesis of polydeoxyribonucleotides by enzymes from Ehrlich and Landschutz ascites-tumour cells (Smellie *et al.* 1960), and the presence of the kinases responsible for the formation of the four deoxyribonucleoside 5'-triphosphates in extracts of ascites-tumour cells has been demonstrated (Keir & Smellie, 1959).

The ability of enzyme preparations from ascites-tumour cells to catalyse the formation of TTP from TdR or TMP is very limited (Keir & Smellie, 1959; Gray *et al.* 1960; Ives, Morse & Potter, 1962), particularly in comparison with their capacity to form dATP, dGTP and dCTP (Keir & Smellie, 1959; Gray *et al.* 1960). Similarly, though extracts of non-proliferating tissues contain active enzymes catalysing the formation of dATP, dGTP and dCTP from the corresponding deoxyribonucleoside 5'-monophosphates, the activities of the kinases responsible for the formation of TTP are exceedingly low (Gray *et al.* 1960).

Fractionation experiments on thymidine kinase and thymidylate kinase led Weissman, Smellie & Paul (1960) to suggest that the formation of TTP from TdR occurred by the stepwise addition of orthophosphate, forming successively TMP, TDP and TTP thus:

However, from experiments on the time-course of phosphorylation of TdR, Bianchi, Butler, Crathorn & Shooter (1961) have suggested that the mechanism involves the addition of orthophosphate to TdR to yield TMP followed by the addition of pyrophosphate to yield TTP directly. TDP observed in the reaction products was considered to arise from the degradation of TTP after prolonged incubation. This mechanism may be illustrated as follows:

The purpose of the present investigation was to distinguish between these two mechanisms, and evidence is presented to show that TDP is an intermediate in the formation of TTP by the enzyme preparations from Landschutz ascites-tumour cells. A preliminary report of this work has already appeared (Grav & Smellie, 1962).

METHODS

Abbreviations. The following abbreviations are used: TdR, thymidine; TMP, thymidine 5'-monophosphate, except where necessary specially to distinguish thymidine 5'-monophosphate from thymidine 3-monophosphate where the abbreviations TMP-5' and TMP-3' are used; TDP, thymidine 5'-diphosphate; TTP, thymidine 5'-triphosphate; [α - ^{32}P]TTP and [$\beta\gamma$ - ^{32}P]TTP, thymidine 5'-triphosphate labelled with ^{32}P on the ester phosphate and the terminal pyrophosphate respectively; [α - ^{32}P]TDP and