Intrathyroidal Iodine Metabolism in the Rat

THE IODOTYROSINES

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(Received 7 November 1962)

It is usual to regard thyroxine biosynthesis as occurring in five stages: (i) accumulation of iodide by the thyroid gland; (ii) oxidation of iodide to iodine; (iii) iodination of tyrosine, yielding monoiodotyrosine; (iv) iodination of monoiodotyrosine, yielding di-iodotyrosine; (v) coupling of two molecules of di-iodotyrosine. Stages (iii), (iv) and (v) are generally supposed to take place within the thyroglobulin molecule. Monoiodotyrosine, di-iodotyrosine and thyroxine are presumed to be released as free amino acids by the complete proteolysis of thyroglobulin (Roche, Deltour, Michel & Lissitzky, 1950). Mono- and di-iodotyrosine do not leave the thyroid gland, but are deiodinated; the iodine that they contain reacts with thyroglobulin again; thyroxine is released into the blood.

Stage (iv), the subject of the present study, has always seemed a logically necessary step. If it occurs, however, it should be possible to demonstrate a precursor-product relationship between the two iodotyrosines by isotopic-labelling experiments. The rates at which the mono- and di-iodotyrosine pools of the rat thyroid become radioactively labelled after a single dose of ^{131}I have been studied in several Laboratories, with conflicting findings. Michel (1956), Taugog, Tong & Chaikoff (1958), Pitt-Rivers & Tata (1959) and Pitt-Rivers (1962) report that, in the early time-intervals after the dose, labelled monoiodotyrosine is large in amount relative to labelled di-iodotyrosine. For example, Pitt-Rivers (1962) found that the ratio, R , mono[^{131}I]iodotyrosine to di-[^{131}I]iodotyrosine, is considerably greater than unity 30 min. after the dose, but gradually diminishes, approaching 0.33 at 24 hr. In all these reports the early phase of changing R values presumably reflects changing specific radioactivities of the two iodoamino acid pools, consistent with a precursor-product relationship between mono- and di-iodotyrosine.

In contrast with this, Bois & Larsson (1958) found R to be constant in the rat thyroid from 30 min. to 7 days after giving [^{131}I]iodide, and De Groot & Davis (1961) have reported R to be constant from 2 min. to 24 hr. Kobayashi & Gorbman (1960) encountered a similar situation in the chick.

In experiments with rats Verain, Verain-Pinoy & Pons (1961) found R to vary within the range 0.7–1.7, with no clear trend towards higher values in the early times after giving the dose.

Recently results have been obtained intermediate between these two classes of conflicting findings. R. Pitt-Rivers (personal communication) and Stole (1962) have found R values in the rat which diminish only to approximately two-thirds of their original value between the times of the earliest and latest measurements.

All authors who have investigated the amount of labelled iodothyronines simultaneously with measurement of R agree that in the first few hours after administering [^{131}I]iodide to the rat the radioactivity of the iodothyronines is a progressively increasing fraction of the total radioactivity of the gland. If it can be confirmed that R can remain constant at a time when the percentage of labelled iodothyronines is increasing, current views on the mechanism of thyroid-hormone biosynthesis would have to be revised. For this reason, and because the high specific activity of monoiodotyrosine in the early time-intervals after a dose is being made the basis of a theory of the origin of one of the iodide pools of the rat thyroid (Wollman, 1962), it was decided to try to resolve the existing conflict of experimental findings on the R values. From the present work it was concluded that in the rats used in this Laboratory R is constant from 1 min. to 24 hr. after administration of [^{131}I]iodide, having a mean value of 0.9–1.0. Attempts made to duplicate the finding of progressively decreasing R reported from other Laboratories failed. Three hypotheses to explain the conflicting findings in different Laboratories were considered: (a) the existence of gross metabolic differences between rats of different strains; (b) the feeding with diets containing different concentrations of iodine; (c) the feeding in some Laboratories with diets containing traces of goitrogens.

METHODS

Experimental animals. Unless otherwise stated the animals used were male Wistar albino rats (170–220 g.) that had been given a low-iodine diet (Boyd & Oliver, 1960)

for 2 weeks. In some experiments, designed to confirm reports of R values that change with time, rats from other Laboratories were used. Male Porton rats were obtained from Burroughs Wellcome and Co., Dartford, Kent; male Sprague-Dawley rats were obtained from the Department of Neuropathology, The Maudsley Hospital, London, and male hooded rats were obtained from the Department of Surgical Science, University of Edinburgh. In these cases low-iodine diet was not given, the animals being used within 24 hr. of arrival.

Administration of [^{131}I]iodide. Carrier-free Na^{131}I was injected subcutaneously or, in some experiments that involved killing the animal shortly after the dose, intravenously. The injected radioactivity was normally within the range 7–15 μC . For experiments of short duration this was increased to 20–100 μC ; in these, uptake was low and the experiment so short (1 min.–2 hr.) that the thyroid received only low doses of radiation (<40 rads) in spite of the large quantities of ^{131}I injected.

Hydrolysis of thyroids. Thyroids were removed immediately after the animals were killed and were hydrolysed enzymically by a procedure similar to that described by Tong & Chaikoff (1958). Each thyroid was homogenized in 1.0 ml. of a buffer containing 0.11M-NaCl, 0.04M-tris, 4 mM-MnSO₄ and 0.4% of thiouracil. Viokase (pancreatin manufactured by the VioBin Corp., Monticello, Ill., U.S.A.) (33 mg.) was added in suspension in a further 0.66 ml. of the same buffer. Toluene was added and the mixture incubated at 37° for approximately 42 hr. Afterwards 1.66 ml. of methanol-conc. aq. NH₃ (1:1, v/v) was added and the resulting suspension centrifuged. The supernatant liquid contained 96–98% of the total radioactivity. The volume of the insoluble residue was approximately 3% of the total volume of the centrifuged homogenate, and it thus seemed likely that the radioactivity of this residue was due simply to inclusion of some of the liquid hydrolysate. Most of the radioactivity of the residue could, in fact, be removed by a single wash with methanol-conc. aq. NH₃ (1:1).

Chromatograms made from hydrolysates prepared in this way usually revealed less than 1% of the radioactivity as 'origin material' and about 6–10% as inorganic iodide. These results are slightly better than those obtained by Tong & Chaikoff (1958). These authors lost 5% of the total radioactivity of the thyroid by centrifuging the thyroid homogenate before hydrolysis, and detected 5–10% of 'origin material'.

A comparison of chromatograms run before and after the enzymic hydrolysis of a thyroid homogenate revealed that 4.5–5.0% of the radioactive iodine originally present as thyroglobulin is released as iodide during the hydrolysis procedure.

Chromatography. Samples of the centrifuged hydrolysate (0.2 ml.) or samples of the unhydrolysed homogenate (0.1 ml.) were applied as a 1.25 in. band on Whatman no. 3MM paper and developed by the descending method in the solvent system butan-1-ol-2N-acetic acid (1:1).

Assay of radioactivity. After development the chromatograms were cut transversely into segments 1 cm. or 0.5 cm. in width. The radioactivity of each segment was determined separately by placing between two mica end-window Geiger-Müller tubes (20th Century Electronics, type EW2C), having an end-window thickness of 1.5 mg./cm.². This counting technique gave high efficiency in counting ^{131}I (about 40% with Whatman no. 3MM paper) and was

used with an anticoincidence shield to reduce the background to 3.6 counts/min.

Completeness of chromatographic separation. It seemed desirable initially to check the resolution of mono- and di-iodotyrosine in the system butan-1-ol-2N-acetic acid. The completeness of chromatographic separations are often judged by reference to radioautographs that show separate spots when viewed by eye, or by reference to chromatograms stained with diazotized sulphanilic acid, ceric sulphate or ninhydrin. In these cases the spots may appear to be completely separated simply because the eye is too insensitive to detect the darkening of the film or the colour reaction that takes place between the spots. Similarly, when radioactive scanning is employed, counting statistics and collimation are frequently too poor to permit any conclusions about the level of radioactivity between the peaks. L. G. Plaskett, C. F. Barnaby & G. I. Lloyd (unpublished work) have shown that paper-chromatographic separations of thyroxine and tri-iodothyronine present difficulties of this type, and that serious errors can arise from failing to recognize them. In the present work counting systems were used that would have permitted the accurate measurement of the radioactivity of the interpeak region, even if this had dropped to less than 1% of the peak heights recorded at the centre of the mono- and di-iodotyrosine spots. Typical results are illustrated in Fig. 1*a* and *b*, in which the level of radioactivity between the peaks is respectively 6.8 and 3.9% of the height of the lower of the iodotyrosine peaks. The chromatographic separation was apparently better than these figures would indicate, since subdivision of the chromatogram segments into 0.5 cm. pieces and re-scanning reduced the value 3.9 to 2.4 (Fig. 2).

These results were considered to reveal a separation adequate for the present work.

The front-running peak (Fig. 1*a* and *b*) is composed mainly of iodothyronines. The activity of this peak has been used as an indication of the total iodothyronines present in thyroid hydrolysates by several workers, e.g. Yamada & Seichijo (1962). In the present experiments the activity of this peak has been redeveloped in a second dimension with butan-1-ol-dioxan-aq. 2N-NH₃ (4:1:5) or butan-1-ol-pentan-1-ol-aq. 2N-NH₃ (1:1:2). Thyroxine and tri-iodothyronine were the principal substances present. Although there were minor contaminants, the radioactivity of the front-running peak in butan-1-ol-2N-acetic acid gives a useful measure of the total iodothyronines present. The values are rather high, however, compared with the sum of the thyroxine and tri-iodothyronine peaks obtained by separations in other solvents.

RESULTS

Uptake of ^{131}I . The uptake of ^{131}I in the thyroid gland rose to a maximum of 18–20% of the dose about 8 hr. after injection, and fell to about 10% at 24 hr.

Labelling of the iodoamino acids. The distribution of label between the different iodoamino acids is set out in Table 1. During the period under study the percentage of the total thyroidal radio-iodine present as iodothyronines increased. After an initial rise the percentage of iodotyrosines dimin-

(a)

(b)

Fig. 1. (a) Distribution of radioactivity on a chromatogram of a hydrolysate of rat thyroid 1 hr. after injection of [^{131}I]iodide. (b) As in (a) but 4 hr. after injection of [^{131}I]iodide. R_F values: I^- ion, 0.34; moniodotyrosine, 0.63; di-iodotyrosine, 0.77; iodothyronines, 0.96.

ished slightly with time, presumably because these compounds undergo conversion into iodothyronines. The ratio of labelled moniodotyrosine to labelled di-iodotyrosine did not change significantly during the period 1 min.–24 hr. after the dose. This result contrasts very sharply with that obtained by Pitt-Rivers (1962). From graphs drawn by this author it is possible to derive values of R 1.6 at 30 min., 0.6 at 4 hr. and 0.35 at 48 hr. This changing ratio reflects a four- to five-fold change in the ratio of the specific activities of these compounds between 30 min. and 48 hr. For strict comparison, the findings in the present work have been converted into 'relative specific radioactivities' (Fig. 3), calculated by the method described

Fig. 2. Distribution of radioactivity on a chromatogram of a hydrolysate of rat thyroid 4 hr. after injection of [^{131}I]iodide: detailed scanning of the iodotyrosine region.

Table 1. *Distribution of radioactive iodine among the fractions of the thyroidal iodine in the rat*Results were obtained from 1 min. to 24 hr. after administration of [^{131}I]iodide.

Time after injection of ^{131}I	No. of rats	Percentage of total radioactivity					<i>R</i>
		Origin material	I^- ion	Iodo- thyronines	Monoiodo- tyrosine	Di-iodo- tyrosine	
1 min.	1	1.0	33.6	0.6	28.2	31.2	0.90
5 min.	3	0.8	22.5	1.7	37.0	35.8	1.03
10 min.	7	0.6	13.1	2.0	39.1	42.1	0.92
15 min.	3	1.0	13.2	3.2	38.2	41.7	0.92
20 min.	1	0.5	8.4	3.0	46.5	39.2	1.19
1 hr.	7	0.6	6.6	4.7	41.4	41.5	1.00
2½ hr.	6	0.8	5.7	7.2	40.1	43.4	0.92
4 hr.	7	0.8	6.0	10.0	41.0	39.0	1.05
6 hr.	6	0.7	6.0	10.5	37.7	42.1	0.90
8 hr.	6	1.0	5.7	15.8	35.6	38.4	0.93
12 hr.	5	1.0	5.3	10.0	33.6	47.3	0.75
24 hr.	2	0.9	10.6	21.9	28.9	33.0	0.88

Fig. 3. Estimated relative specific activities of monoiodotyrosine, diiodotyrosine and iodothyronines in rat thyroids 10 min.–8 hr. after injection of [^{131}I]iodide. □, Monoiodotyrosine; △, diiodotyrosine; ○, iodothyronines.

by Pitt-Rivers (1962). The values for mono- and di-iodotyrosine are very close, and the fluctuations that occur appear to be random. There is no justification for drawing separate curves for the two substances.

It was at first suspected that the conflict of results could be attributed to the use by different authors of rats of different strains. Attempts were made to reproduce the findings of Pitt-Rivers (1962) by working with rats of several strains. Injections of [^{131}I]iodide were given and the animals killed after 10 min.–2 hr. From the data of Pitt-Rivers, *R* would be expected to change during this period from a value well above 1.6 (found by this author at 30 min.) to 0.8 at 2 hr. The values actually obtained are recorded in Table 2. As with the Wistar albino rats, the ratio of the radioactivity

Table 2. *Experiments with rats from other Laboratories*

R values were determined at different times after a dose of [^{131}I]iodide. Single rats were used for each determination.

Time after dose	<i>R</i>		
	Hooded rats	Sprague-Dawley rats	Porton rats
10 min.	0.88	0.89	1.05
30 min.	0.87	0.67	1.15
1 hr.	—	0.75	—
2 hr.	0.87	0.70	1.19

in mono- and di-iodotyrosine does not change markedly with time.

Secondly, it was suspected that the iodine content of the diet, by controlling turnover rates, might affect the time required for the mono- and di-iodotyrosine pools to become isotopically equilibrated. Rats were therefore kept on sodium iodide-supplemented drinking water for 14 days before administration of [^{131}I]iodide, or were injected with sodium iodide solution before or simultaneously with the [^{131}I]iodide dose. The rats that received injections of sodium iodide were killed 24 hr. after receiving [^{131}I]iodide (Table 3). The sodium iodide carrier dose, which was administered with the radioactive dose, was 30 μg . of I^- ion, as in the uptake studies of Perlman, Chaikoff & Morton (1941). In accord with the findings of Leloup & Lachiver (1955), Querido, Schut & Terpstra (1957) and Bois & Larsson (1958), iodide administration was found to diminish the value of *R* at 24 hr. However, when iodide-treated rats were injected with [^{131}I]iodide and killed at intervals of 20 min., 1 hr. and 4 hr. afterwards (Table 3), *R* was again diminished as at 24 hr., and showed no tendency to change markedly with time. This result is in accord

Table 3. *Effect of iodide administration on R values in the rat thyroid gland*

Time after ^{131}I	Iodide treatment	R	No. of rats
20 min.	None (control)	1.19	1
1 hr.		0.94	1
4 hr.		0.97	1
20 min.	3 $\mu\text{g.}$ of I^- ions/ml. (in drinking water)	0.73	1
1 hr.		0.95	1
4 hr.		0.96	1
20 min.	10 $\mu\text{g.}$ of I^- ions/ml. (in drinking water)	0.64	1
1 hr.		0.70	1
4 hr.		0.58	1
24 hr.	None (control)	0.88	2
24 hr.	30 $\mu\text{g.}$ of I^- ions injected with ^{131}I	0.59	2
24 hr.	500 $\mu\text{g.}$ of I^- ions injected 48 hr. before ^{131}I , and 30 $\mu\text{g.}$ of I^- ions injected with ^{131}I	0.44	1

with the detailed studies on the effects of high-iodine diets carried out by Bois & Larsson (1958).

The current disagreements about the kinetics of labelling of the iodotyrosines might be attributed to goitrogens present in some commercially available rat foods. The diet used in this study has been tested for goitrogen activity by Dr G. S. Boyd and his collaborators and was free from goitrogens. If diets used in other Laboratories contained goitrogens of the thiouracil type this would be expected to lead to very high R values, as reported by Slingerland *et al.* (1959) in propylthiouracil-treated rats. However, the kinetics of labelling of the thyroidal iodine pools do not appear to have been studied in detail in goitrogen-treated rats. In the present work rats were given a diet containing 0.05 % of thiouracil for 4 days. [^{131}I]Iodide was then injected and the animals were killed at 10 min., 30 min. or 2 hr. after the dose. R values 6–13 were found, and there was no tendency for this to be higher in the early intervals after the dose. For this reason, and because R values obtained at isotopic equilibrium by Pitt-Rivers (1962) and by Taurog *et al.* (1958) were low, it seems unlikely that the findings of these authors were caused by an effect of dietary goitrogen in slowing down the equilibration of the mono- and di-iodotyrosine pools.

DISCUSSION

It is theoretically possible that in some rats, including all those we have studied, the iodide, monoiodotyrosine and di-iodotyrosine pools of the thyroid are all brought rapidly into equilibrium by high activities of thyroidal proteolytic enzymes and iodotyrosine diiodinase. However, this explanation would necessitate extremely rapid turnover of all the thyroglobulin in the gland, which is probably unlikely in view of the very large amount of thyroglobulin present. Rather more direct evidence against rapid equilibration by this

mechanism can be found in the progressively increasing content of labelled iodothyronines in the thyroid (Table 1). If there were rapid complete turnover of all the thyroglobulin, then labelled thyroxine and tri-iodothyronine could not accumulate over a period of several hours as they have been shown to do. Both hormones are released as free amino acids when thyroglobulin is degraded and are consequently secreted from the gland (Taurog, Wheat & Chaikoff, 1956; Ackerman & Arons, 1958).

The current theories of thyroid-hormone formation must be modified to take account of the fact that R values are, or can be, constant during a period in which the thyroidal content of [^{131}I]iodothyronines is increasing. Some possible modifications are set out below.

(1) The present finding of almost constant R values would be compatible with the view that all formation of iodotyrosines in the thyroid gland is a reaction between iodine and free tyrosine. The iodide and free iodotyrosine pools of the gland could become isotopically equilibrated very quickly; free mono- and di-iodotyrosine of equal specific activity would then be available for incorporation into thyroglobulin. This possibility has been rejected, however, because it is not easily reconciled with the studies on iodination *in vitro* by Taurog, Potter & Chaikoff (1955) and by Tong, Taurog & Chaikoff (1957), and because in the present work preliminary experiments on the chromatography of unhydrolysed thyroid homogenates have failed to show any significant change in the ratio of labelled free iodotyrosines to labelled protein-bound iodotyrosines from 10 min. to 2 hr. after giving [^{131}I]iodide. Labelled free iodotyrosines made up 0.3 % of the total radioactivity of the thyroid gland.

(2) A constant R value could also result if particular tyrosyl residues in thyroglobulin are monoiodinated or di-iodinated, depending upon

their position in the protein, or upon local environmental conditions, such as the availability of a second iodine molecule at the moment when the first reacts. According to this hypothesis, once monoiodination had occurred, a second iodination would not be possible. This seems to have been the view of De Groot & Davis (1961), who speak of 'considerable specificity in the extent of iodination of tyrosyl groups in thyroglobulin'.

This hypothesis is not favoured, however, because, as is clear from the literature cited, *R* values are sometimes constant and sometimes not. It is unlikely that the rats studied in different Laboratories differ from one another in the mechanism of the fundamental process of iodination.

(3) The thyroidal mono- and di-iodotyrosine may be heterogeneous with respect to turnover because the proteolysis of thyroglobulin is incomplete or selective. For example, the scheme of thyroid-hormone biosynthesis set out in Fig. 4 would permit monoiodotyrosine to act as a precursor of di-iodotyrosine, even under conditions in which no precursor-product relationship is demonstrable isotopically. Radioactive iodine entering the thyroid gland as I^- ions could undergo oxidation to iodine and iodinate newly synthesized iodine-free thyroglobulin, yielding thyroglobulin molecules which contain iodotyrosines (iodoprotein pool 1). From this point two reactions could take place. Either coupling could occur within the thyroglobulin molecule, yielding iodothyronine-containing protein, which would enter the main thyroglobulin iodine pool of the gland (iodoprotein pool 2), or, if coupling fails to take place, the thyroglobulin could be completely degraded to

amino acids, the iodine being reconverted into iodide by iodotyrosine deiodinase.

If reactions (1), (2), (4), (5) and (9) are rapid by comparison with reactions (3), (6) and (7), then there would be a more rapid turnover of the iodotyrosine-containing thyroglobulin than of the thyroglobulin containing both iodotyrosines and iodothyronines. The condition necessary for high initial *R* values, namely that labelled di-iodotyrosine is derived largely from monoiodotyrosine synthesized before the radioactive dose was given, is not then fulfilled. Iodoprotein pool 1 is postulated to be a small pool with a very fast turnover. Incorporation of the iodotyrosines from iodoprotein pool 1 into iodoprotein pool 2 is assumed to be slow, iodoprotein pool 2 being large with slow turnover. Reaction (3) would result in the gradual accumulation in iodoprotein pool 2 of labelled monoiodotyrosine, di-iodotyrosine and iodothyronines having roughly equal, though increasing, specific activities.

This reaction scheme, requiring preferential proteolysis of iodothyronine-free thyroglobulin, seems superficially to conflict with the report of Alpers, Robbins & Rall (1955) that digestion of rat thyroglobulin with thyroidal proteolytic enzymes for 24 hr. released iodoamino acids in roughly the proportions in which they are present in thyroglobulin. These authors found that after incubation 15% of the iodine originally present as thyroglobulin was di-iodotyrosine, 9% of it was monoiodotyrosine and 6% was thyroxine. Moreover, Mayberry & Astwood (1960) have found a preferential release of iodothyronines from the thyroids of propylthiouracil-treated rats. These reports can be readily reconciled with the reaction scheme set out in Fig. 4 on the basis of the assump-

Fig. 4. Postulated scheme of reactions in thyroid-hormone biosynthesis: (1) Reaction catalysed by iodide oxidase. (2) Iodination of tyrosyl residues. (3) Coupling of iodotyrosyl residues. (4) Proteolysis. (5) Reaction catalysed by iodotyrosine deiodinase. (6) Proteolysis. (7) Secretion of thyroxine. (8) Uptake of blood iodide. (9) Protein synthesis.

tion that iodoprotein pool 2 is very large in comparison with iodoprotein pool 1. The studies that have been cited on the proteolysis of thyroglobulin would then, in effect, be studies on the proteolysis of iodoprotein pool 2.

The scheme set out in Fig. 4 is compatible with the reports of the heterogeneity of thyroglobulin (e.g. Ingbar, Askonas & Work, 1959; Ui, Tarutani, Kondo & Tamura, 1961) and receives support from the work of Shulman & Stanley (1961), who isolated iodothyronine-rich and iodothyronine-poor fractions of hog thyroglobulin by chromatography on diethylaminoethylcellulose. Further, Halmi & Pitt-Rivers (1962) have obtained kinetic data which are best explained on the assumption that the iodotyrosines of the rat thyroid are heterogeneous with respect to turnover.

This hypothesis can be readily reconciled both with the pattern of changing *R* values described by Michel (1956), Taurog *et al.* (1958) and Pitt-Rivers (1962) and with the constant *R* values first noted by Bois & Larsson (1958). In Fig. 4 an increase in size and a decrease in the turnover rate of iodoprotein pool 1 could lead to the appearance of an isotopically demonstrable precursor-product relationship between the two iodotyrosines.

The testing of this hypothesis clearly depends upon studying the kinetics of labelling of the various thyroglobulin fractions that it is now possible to isolate.

Fig. 4 could be, at best, only an over-simplification of a very complex process. If the hypothesis is acceptable, reaction (3) must induce a change that renders the molecule less liable to proteolysis. Unless selectivity is achieved on the basis of a very precise enzyme specificity, one must suppose that the coupling reaction causes a fairly drastic change, either structural or conformational, in the protein molecule. Indeed, if pyruvic acid derivatives are produced in the coupling reaction, as suggested by the work of Pitt-Rivers & James (1958), peptide-bond cleavage would be a consequence of coupling. Further, since the thyroxine content of thyroglobulin is about 2 moles/mole of protein (Robbins & Rall, 1960), there must be two or more of these events in each protein molecule.

During our preparation of this paper Stolc (1962) proposed a two-compartment mechanism of hormone synthesis with several points of similarity to that which is described here.

SUMMARY

1. Estimations have been made of the ^{131}I incorporated into monoiodotyrosine, di-iodotyrosine and iodothyronines in the rat thyroid gland at intervals from 1 min. to 24 hr. after injection of [^{131}I]iodide.

2. In confirmation of work in other Laboratories, the percentage of the total radioactivity in the iodothyronines increased during the period studied, from amounts that could barely be measured at 1 min. to about 20% at 24 hr.

3. The ratio of labelled monoiodotyrosine to labelled di-iodotyrosine remained almost constant during this period.

4. Attempts have been made to duplicate the finding reported in the literature of ratios of mono- to di- ^{131}I iodotyrosine which changed with time, by working with rats of several strains. All these experiments have resulted in approximately constant ratios.

5. Administration of sodium iodide or thiouracil resulted in altered but nevertheless approximately constant ratios.

6. These results are not consistent with a simple precursor-product relationship between the thyroidal mono- and di-iodotyrosine. If di-iodotyrosine is formed from monoiodotyrosine both substances must be heterogeneous with respect to turnover.

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

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Biochem. J. (1963) **87**, 480

The Incorporation of Radioactive Phosphorus in the Leucocyte during the Extrusion of Protein Induced by Staphylococcal Leucocidin

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(Received 7 November 1962)

It has been shown that treatment of the polymorphonuclear leucocyte with leucocidin stimulates the incorporation of radioactive phosphorus into the phospholipids of the cell (Woodin, 1962). The radioactivity of the precursors was not known and so it was uncertain if this represented an increased turnover. The present paper shows that the stimulated incorporation of radioactive phosphorus is found in the nucleotides and other acid-soluble compounds of the leucocidin-treated leucocyte. There is no evidence for a causal connexion between this stimulated incorporation and the extrusion of protein from the granules of the leucocyte.

METHODS

The two components of leucocidin were crystallized as described by Woodin (1960); unless otherwise indicated they were added to cell suspensions to give $2\text{ }\mu\text{g./}10^8$ cells. Polymorphonuclear leucocytes were obtained from peritoneal exudates of rabbits (Woodin, 1962) and will be referred to below as leucocytes. ATP and ADP were obtained from C. F. Boehringer und Soehne, Mannheim, Germany; GTP and GDP were obtained from the Sigma Chemical Co., St Louis, Mo., U.S.A. The solutions used for suspending the cells were Hanks (1948) medium or Krebs-Ringer solutions (Umbreit, Burris & Stauffer, 1957). Carrier-free [^{32}P]orthophosphate, [^{14}C]adenine sulphate and sodium [^{14}C]acetate were obtained from The Radiochemical Centre, Amersham, Bucks. The antileucocidin serum (staphylococcal antitoxin, CPP 76/63) was kindly provided by Miss Mollie Barr, Wellcome Research Laboratories, Beckenham, Kent.

Units. The definition of the L^+ unit and the unit of the antiserum CPP 76/63 is given by Woodin (1960).

Incorporation of ^{32}P into the nucleotides of the leucocyte. Tubes containing 5×10^8 cells in 10 ml. of Hanks solution were incubated for 10 min. at 37° and then leucocidin was added to one tube and ^{32}P (0.05 ml. containing 10^7 counts/min.) was added to both tubes. Incubation was continued for 10 min. and the tubes were cooled in ice. They were centrifuged at 0° for 5 min. at 2000 rev./min. and the supernatant was rejected. The cell pellets were extracted for 2 hr. at room temperature with 20 vol. of chloroform-methanol (2:1, v/v). The suspensions were centrifuged and the supernatants used for the preparation of the phospholipids (see below). The cell residues were washed with ether and dried in a stream of air. The powder was then extracted with 10 ml. of ice-cold HClO_4 (3%, w/v), residual ether and occluded air being removed by evacuating the tubes until the cell residues no longer floated on the surface. Extraction was continued for 30 min. and then the suspensions were centrifuged at 20 000 rev./min. for 20 min. The slightly opalescent supernatant was passed down a column containing 100 mg. of Norit and 100 mg. of Celite (Threlfall, 1957), which was then washed three times with 3 ml. of water. The effluent from the column was collected; it contained the nucleotide-free, acid-soluble fraction. The nucleotides were eluted from the washed Norit-Celite column in 7 ml. of pyridine and freeze-dried. The solid was dissolved in a small volume of water and put on paper for chromatography. After separation as described below the specific activities of the nucleotides were determined.

In a similar experiment, 2×10^8 leucocytes in 12 ml. of Hanks medium were incubated alone or with added leucocidin for 10 min. ^{32}P (0.1 ml.; 2×10^8 counts/min.) was added and incubation at 37° continued for 20 sec. The suspensions of cells were then diluted with 75 ml. of ice-cold Hanks medium and centrifuged at 0° . The nucleotides were isolated as described above and the specific radioactivities determined.

To determine the effect of antitoxin on the stimulated incorporation of ^{32}P into the nucleotides of the leucocidin-treated cell, two tubes each containing 2×10^8 cells in 7 ml.

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