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Biochem. J. (1963) **87**, 334

The Adsorption of Corticosteroids to Particulate Preparations of Rat Liver

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(Received 22 October 1962)

Previous experiments have shown that plasma cortisol entered rat liver by diffusion and was rapidly metabolized (Berliner, Keller & Dougherty, 1961; Bellamy, Phillips, Jones & Leonard, 1962). It was also found that some unchanged cortisol was adsorbed to the particulate fraction of the tissue (Bellamy *et al.* 1962). There is a possibility that particle-bound steroids are resistant to the action of enzymes and that the persistence of small amounts of intracellular adsorbed cortisol is responsible for some of the long-term biochemical changes that follow injections of cortisol. In view of the evidence that liver may be a target organ for corticosteroids (Chiu, 1950; Winternitz, Dintzis & Long, 1957; Goodlad & Munro, 1959; Ashmore, 1960; Long, Smith & Fry, 1960; Harding, Rosen & Nichol, 1961; Goldstein, Stella & Knox, 1962) the interactions of cortisol and corticosterone with the particulate fractions of this tissue have been examined in more detail.

EXPERIMENTAL

Extraction and estimation of ^{14}C -labelled steroids. The materials and methods used have been described by Bellamy *et al.* (1962).

Fractionation of liver homogenates. Liver from a freshly killed rat (1.5 g.) was homogenized in 15 ml. of 0.9% NaCl and made up to 30 ml. with 0.9% NaCl. The homogenate (5 ml.) was centrifuged at 600g for 2 min. and the supernatant solution pipetted off. The sedimented tissue (fraction 1) was washed three times by resuspension in 0.9% NaCl (4 ml.) and the washings were added to the first supernatant solution. The combined solutions were centrifuged at 8000g for 2 min. and the supernatant

solution was pipetted off. The sedimented tissue (fraction 2) was washed three times as fraction 1 above. The supernatant solution and washings were finally centrifuged at 25000g for 5 min. to sediment all the particulate matter (fraction 1A). In some experiments 0.25M-sucrose solution was used in place of 0.9% NaCl. All the operations were carried out with the temperature as close to 0° as possible, a refrigerated centrifuge (MSE) being used.

Extraction and chromatography of tritiated steroids. About 1 g. wet wt. of tissue was cut into small pieces and refluxed for 2 hr. with 100 ml. of chloroform-methanol mixture (2:1, v/v) containing 50 µg. of unlabelled cortisol, cortisone and corticosterone. The extract was evaporated to dryness on a rotary flash evaporator, dissolved in 100 ml. of water and extracted three times with 100 ml. of chloroform. The combined chloroform extracts were evaporated to dryness as described above and dissolved in a mixture of 5 ml. of light petroleum (b.p. 40–60°) and 5 ml. of ethyl acetate. The solution was put through a column of 2 g. of silica gel prepared according to Bush & Sandberg (1953). The column was washed successively with 20 ml. of ethyl acetate-light petroleum (1:1, v/v), followed by 9.9 ml. of this mixture containing 0.1 ml. of methanol. The corticosteroids were eluted with a mixture of 6.0 ml. of ethyl acetate-light petroleum (1:1, v/v) and 4.0 ml. of methanol.

The solvent was evaporated off, the residue dissolved in 0.2 ml. of ethyl acetate and spotted $\frac{3}{4}$ in. from the end of a strip (1 in. $\times$ 8 in.) of Whatman no. 1 filter paper. The paper strip was sprayed lightly with the bottom phase of the B5 solvent system (Bush, 1952) and the chromatogram developed with the upper phase ascending the paper. Unlabelled cortisol, cortisone and corticosterone were located on the paper by ultraviolet absorption. The strip was then cut into half-inch pieces, which were left in contact with 5 ml. of ethanol overnight. After removal of the pieces of paper the ethanol was evaporated off and the residue was dissolved in phosphor solution and the radioactivity determined (Bellamy *et al.* 1962).

RESULTS

The binding of cortisol and its metabolites to cell particles. Previous experiments have shown that cortisol enters the liver by diffusion and that both the unchanged hormone and its metabolites are adsorbed to intracellular sites. It was also found that cortisol added to liver homogenates was adsorbed to the particulate fraction (fraction 1A; Bellamy *et al.* 1962).

To obtain more precise information on the localization of the particle-bound steroids, this fraction was divided into three subfractions based on the sedimentation properties of the particulate matter. The first fraction (fraction 1) contained no intact cells and consisted mainly of cell debris, red cells and nuclei; the second (fraction 2) contained particles of mitochondrial size (1–3 μ diam.) and the third fraction contained particles that were just visible with the light-microscope. When this fractionation scheme was applied to the homogenate of liver obtained from a rat that had been injected with [^{14}C]cortisol (Table 1), over 50 % of the particle-bound radioactivity was found in fraction 1. The radioactivity per unit weight of this fraction was lower than in the combined fractions (fraction 1A). Fraction 3 contained about 30 % of the particle-bound radioactivity and the concentration of ^{14}C was nearly twice as high as in fraction 1A. Fraction 2 was apparently intermediate in its properties between fractions 1 and 3. About 75 % of the radioactivity of the homogenate was found in the supernatant solution. From these results it is not possible to say that any one type of cell particle had the special property of binding steroids. This conclusion is supported by the distribution of [^{14}C]cortisol added to a liver homogenate from an untreated rat (Table 1). The experiment, which was carried out at 4° to reduce the metabolism of the added cortisol (Bellamy *et al.* 1962), showed that the distribution of the hormone between the various cell fractions followed almost

the same pattern as that of the ^{14}C from a rat injected with cortisol. The difference in concentration of ^{14}C between the supernatant fraction and the cell particles followed an adsorption isotherm (Bellamy *et al.* 1962); therefore the proportion of the total ^{14}C found in the supernatant solution depended on the degree of dilution of the homogenate. It is likely that in the intact tissue almost all of the radioactive cortisol was bound.

It has been found in previous experiments (Berliner *et al.* 1961; Bellamy *et al.* 1962) that over 90 % of the radioactivity found in rat liver 30 min. after injection of cortisol is due to the presence of cortisol metabolites. These compounds were found to pass largely into the water phase when partitioned between chloroform and water. They may be separated in this way from unchanged cortisol (non-polar), which enters the chloroform phase (Braunsberg & James, 1961). When this simple two-phase system (Bellamy *et al.* 1962) was used to separate the radioactivity extracted from liver with chloroform-methanol most of the polar metabolites were found in the liver supernatant fraction, whereas the non-polar compounds occurred mainly in the cell particles (Table 2). Furthermore, the polar compounds that were found in the particulate fraction were more easily removed by washing this fraction with 0.9 % sodium chloride (Table 2). About 60 % of the residual radioactivity found in the non-polar fraction after washing the particles was due to unchanged cortisol and about 15 % was accounted for by ^{14}C that appeared in the cortisone area of paper chromatograms (see also Ulrich, 1959; De Venuto & Westphal, 1961). The remaining ^{14}C -labelled compounds were not identified. These findings indicate that cortisol is more strongly adsorbed to cell particles than its polar metabolites and that most of the radioactivity in the particulate fraction was due to the presence of unchanged hormone. The results are in agreement with those of Ulrich (1959), who found that after cortisol was incubated with rat liver at 37.5° (when a large

Table 1. *Distribution of radioactivity in rat liver after the injection or addition of [^{14}C]cortisol*

A 300 g. male rat (A) was injected intravenously with [^{14}C]cortisol (5.10×10^5 counts/min.) and killed 30 min. later. The liver was homogenized in 0.9 % NaCl as described in the text. The liver from a second rat (B) was treated in the same way except that the tissue was homogenized in 0.9 % NaCl containing [^{14}C]cortisol (12,000 counts/min./ml.). A sample of each homogenate (5 ml.) was fractionated as described in the text (1 $\mu\text{g.}$ of cortisol = 96 800 counts/min.).

Fraction	Total ^{14}C in fraction (counts/min.)		Wet wt. of pellet (g.)		$10^{-3} \times$ Concn. of ^{14}C (counts/min./g. wet wt.)	
	A	B	A	B	A	B
1A	375	13 740	0.2220	0.2574	1.69	53.4
1	218	9 450	0.1808	0.2135	1.21	44.3
2	98	3 696	0.0445	0.0568	2.20	65.1
3	109	6 259	0.0376	0.0642	2.90	97.5
4	1 105	42 200	—	—	—	—

proportion of hormone had been metabolized) the particulate fraction of the homogenate contained nearly 50 % less radioactivity than when the steroid was added at 0°.

The binding of corticosterone and its metabolites to cell particles. It is established that corticosterone is the principal corticosteroid in rat plasma (Bush, 1953; van der Vies, 1961). For this reason, when radioactive corticosterone became available, some

of the experiments previously carried out with [^{14}C]cortisol (Bellamy *et al.* 1962) were repeated with [^{14}C]corticosterone. Corticosterone was taken up by liver slices at a faster rate than cortisol (Fig. 1; compare with Fig. 3 of Bellamy *et al.* 1962), but the other characteristics of the uptake process were the same as for cortisol (Bellamy *et al.* 1962).

Corticosterone was also firmly bound to the particulate fraction of liver homogenates (Table 3). To investigate the strength of binding, the fraction was washed three times with 0.9 % sodium chloride and with sodium chloride solution containing non-radioactive corticosterone. The results show (Table 3) that most of the particle-bound radioactive steroid exchanged very slowly with the unlabelled hormone. After three washes with 0.9 % sodium chloride, which contained the highest concentration of unlabelled steroid, the concentration of [^{14}C]corticosterone in the water of the sedimented pellet was about 40 % lower than that of the non-radioactive hormone in the supernatant solution. When this washed pellet was incubated for 2 hr. at 4° in 0.9 % sodium chloride containing 10 $\mu\text{g.}$ of unlabelled corticosterone/100 ml., the particle-bound radioactivity was found to have decreased by about 80 %. The amount of bound radioactivity that remained in the particles would be expected from the known adsorption isotherm for the distribution of corticosterone between the sedimented tissue and the supernatant solution (D. Bellamy, unpublished work). When the above-mentioned incubation was carried out at 37° over 99 % of the particle-bound radioactivity passed into the medium. These results suggest that particle-bound corticosterone is slow to reach adsorption equilibrium at low temperatures. A similar conclusion may be drawn from the experiments of Ulrich (1959), which showed that a substantial amount of cortisol could not be removed

Table 2. *Distribution of polar and non-polar compounds in the liver of a rat injected with [^{14}C]cortisol*

An homogenate was prepared from the liver of a rat 60 min. after the injection of [^{14}C]cortisol, as in Table 1, and centrifuged at 25000g for 5 min. The radioactivity in the pellet of sedimented tissue (fraction 1A) was separated into water-soluble and chloroform-soluble fractions as described by Bellamy *et al.* (1962) and compared with the radioactivity in the supernatant solution (fraction 4). The results are expressed as counts/min./g. of water (concn.) and as percentage of total counts in liver (%).

	Radioactivity in fractions			
	Water-soluble		Chloroform-soluble	
	Concn.	%	Concn.	%
Fraction 1A				
Unwashed	472	3.8	2400	18.4
Washed three times with 0.9 % NaCl	220	1.8	1605	12.3
Fraction 4	432	70.7	43.2	7.1

Fig. 1. Uptake of [^{14}C]corticosterone by liver slices. Liver slices (approx. 100 mg. each) were incubated at 4° in a phosphate saline solution (medium II of Krebs, 1950; 10 ml./slice) containing [^{14}C]corticosterone, with air as gas phase. At intervals three slices were removed and the ^{14}C content was determined as described in the text. Content of ^{14}C in medium (counts/min./ml.): ●, 700; ○, 350; ■, 175.

Table 3. *Effect of unlabelled corticosterone on particle-bound [^{14}C]corticosterone*

An homogenate of rat liver was prepared in 0.9 % NaCl containing [^{14}C]corticosterone as described in Table 1. Six samples (5 ml.) were centrifuged to isolate fraction 1A. The supernatant solution was discarded and the radioactivity of each pellet was determined after washing three times on the centrifuge with 5 ml. of 0.9 % NaCl containing varying amounts of non-radioactive corticosterone. The unwashed tissue pellet contained 11 063 counts/min.

Corticosterone in washing solution ($\mu\text{g./100 ml.}$)	Radioactivity of sedimented tissue (counts/min.)
Nil	6138
5	5571
10	5563
20	5129
40	5285

Table 4. *Distribution of [¹⁴C]corticosterone in rat-liver homogenate*

A male rat was anaesthetized with ether and the tissues were perfused through the portal vein with 0.9% NaCl containing 500 units of heparin/ml. The liver was homogenized in 0.9% NaCl containing corticosterone, as in Table 1, and the various particle fractions were isolated as described in the text. The distribution of ¹⁴C in the perfused liver (P) is compared with that in a normal liver (N).

Fraction	Total ¹⁴ C in fraction (counts/min.)		Wet wt. of sedimented tissue (g.)		10 ⁻³ × Concn. of ¹⁴ C (counts/min./g. wet wt. of pellet)	
	N	P	N	P	N	P
1A	5586	6603	0.1212	0.1562	46.1	42.3
1	2307	3974	0.0762	0.1220	30.3	32.6
2	556	477	0.0228	0.0166	24.4	28.7
3	1275	1053	0.0267	0.0212	47.8	49.7

Table 5. *Comparison of tissue radioactivity after the intravenous injection of [¹⁴C]corticosteroids*

Equivalent amounts of radioactive cortisol and corticosterone (96 800 counts/min./μg.) were dissolved in 1 ml. of rat blood and injected into the saphenous vein of 300 g. male rats under ether anaesthesia. Thirty minutes later the animals were killed and the ¹⁴C content of tissues and plasma was determined as described in the text.

Steroid injected	...	...	Corticosterone (counts/min./g. wet wt.)		Cortisol (counts/min./g. wet wt.)	
			Tissue	Plasma	Tissue	Plasma
		Tissue				
Small intestine		15500	3.41	6460	1.78	
Liver		7830	1.72	13170	3.63	
Kidney		5603	1.23	6610	1.82	
Submaxillary gland		3065	0.67	2321	0.64	
Brain		1740	0.38	258	0.07	
Skeletal muscle		1742	0.38	1510	0.42	
Spleen		1343	0.30	11520	3.17	
Plasma		4550	—	3633	—	

from rat-liver particles by washing twice with 0.25M-sucrose. At higher temperatures it is possible that metabolism of the free hormone increased the rate of release of the adsorbed hormone, preventing the establishment of adsorption equilibrium, or that corticosterone had a lower affinity for the adsorption sites.

The distribution of both cortisol and corticosterone between the three particulate fractions of liver was found to be almost identical (Table 4; cf. Table 1 and Westphal, 1961). Perfusion of the liver before homogenization, which removed most of the red cells, made no difference to either the amount of particle-bound corticosterone or the distribution pattern. From other work on the location of radioactive steroids in fractionated tissues it appears that the observed distribution is influenced by the electrolyte composition of the medium (Harding & Samuels, 1962). In the present experiments most of the adsorbed corticosteroids in 0.9% sodium chloride homogenates sedimented with the cell debris and nuclei. If the fractionation procedure was carried out in 0.25M-sucrose, however, the steroid was not adsorbed to the cell particles so strongly. It was also found that about 49% of the bound hormone now occurred in the light-particle fraction and 20% in fraction 1, giving

a steroid distribution similar to that reported by Ulrich (1959) for fractionated sucrose homogenates containing [¹⁴C]cortisol.

It was found that the prior treatment of fraction 1A with ribonuclease made no difference to the adsorption of corticosterone. A similar result was obtained after extraction with hot trichloroacetic acid, which is known to remove ribonucleic acid from tissues (Greengard & Campbell, 1959). Treatment of the particles with chloroform-methanol (2:1, v/v), which extracts a variety of liver lipids (Getz & Bartley, 1961; Getz, Bartley, Stirpe, Notton & Renshaw, 1961), resulted in the loss of two-thirds of the corticosterone-binding capacity of the tissue. The binding capacity could not be restored by adding back the chloroform-soluble fatty material found in the chloroform-methanol extract. The nature of the proteinaceous water-soluble material with the capacity for adsorbing corticosterone (D. Bellamy, unpublished work), which was also extracted from the particles with chloroform-methanol, is under investigation.

From experiments on rats injected with cortisol (Bellamy *et al.* 1962) it appeared that liver has by far the greatest affinity for cortisol and its metabolites *in vivo*. Similar results were obtained after

the injection of [^{14}C]corticosterone (Tables 5 and 7). In contrast with the cortisol-injected animals, however, only a small amount of ^{14}C was found in spleen, and brain appeared to have a higher affinity for corticosterone. To see whether the particulate fraction of all cells had equal ability to adsorb corticosterone, fraction 1A was isolated from a variety of tissue homogenates containing [^{14}C]corticosterone at 4° . The results showed (Table 6) that washed liver particles retained about five times as much corticosterone as particles from other tissues. In particular, the small amount of steroid found in the particulate fraction from the small intestine suggests that most of the ^{14}C found in the intestine *in vivo* was not corticosterone (Table 6; cf. Table 7).

Long-term distribution of corticosterone in the rat. A single injection of cortisol or corticosterone into

starved normal rats brings about an increase in liver glycogen which may remain at an abnormally high concentration for several days (Long *et al.* 1960). In the present work it was found that a subcutaneous injection of [^{14}C] or [^3H]corticosterone (5 mg./animal) produced a maximum increase in the liver glycogen of starved male rats 24 hr. after treatment. At this time there was only a trace of radioactivity in the blood but a considerable amount was found in the tissues (Table 7). Intestine and liver contained the most radioactivity (about 0.2 % of the injected dose). Only a small part of the radioactivity of intestine appeared to be due to non-polar compounds (Table 7), whereas in liver about 40% passed into the chloroform layer when partitioned between chloroform and water. The chloroform-soluble radioactivity of liver was almost equally divided between the corticosterone and cortisol areas of paper chromatograms. In contrast the chloroform fraction from muscle contained more polar material with an R_f similar to that of cortisol.

As far as the distribution of total radioactivity is concerned, these results, although similar to those of Gallagher, Fukushima, Barry & Dobriner (1951), who used radioactive testosterone, may be contrasted with the findings of Hyde (1957), who injected [^{14}C]cortisol. In the latter experiments the liver was observed to be radioactive for a short period only, and ^{14}C was not found in the tissues when a significant increase in glycogen occurred. However, it is likely that the long-term adsorption of a small percentage of the injected cortisol would not be detected in Hyde's (1957) experiments because the specific activity of the hormone was quite low and a relatively insensitive method was used to measure the radioactivity.

Table 6. *Adsorbed corticosterone in particle fractions from different tissues*

Various tissues (0.25 g. wet wt.) were homogenized in 5 ml. of 0.9 % NaCl containing [^{14}C]corticosterone (6000 counts/min./ml.). All the homogenates were centrifuged to isolate fraction 1A and the sedimented tissue was washed three times with 5 ml. of 0.9 % NaCl containing 10 μg . of corticosterone/100 ml.

Tissue	Wet wt. of sedimented tissue (g.)	^{14}C in pellet (counts/min.)
Liver	0.1336	3760
Kidney	0.1261	660
Brain	0.1958	533
Intestine	0.1690	456
Spleen	0.1296	410
Muscle	0.1815	333

Table 7. *Amount of radioactivity in tissues 24 hr. after the injection of radioactive corticosterone*

Male rats were injected subcutaneously with 5 mg. of corticosterone containing either ^{14}C (2.8 μC) or ^3H (100 μC). Seven hours later the animals were deprived of food for a 17 hr. period and then killed. The amount of radioactivity in the tissues was determined as described in the text. The total ^{14}C in each tissue is expressed as an equivalent of the amount of corticosterone injected. The tritium extracted with CHCl_3 -methanol is expressed as both total radioactivity and as the amount that was found in the corticosterone area of paper chromatograms.

Tissue	^{14}C Corticosterone			^3H Corticosterone	
	Radioactivity of tissues (counts/min./g. wet wt.)		Corticosterone equivalent (μg /g. wet wt.)	(counts/min./g. wet wt.)	
	Chloroform-soluble	Water-soluble		Total radioactivity	Corticosterone spot
Liver	172	259	1.08	45106	5723
Kidney	70.8	70.4	0.35	16650	—
Spleen	25.6	15.8	0.10	1480	448
Muscle	10.6	4.6	0.03	2140	631
Small intestine	14.2	596	1.53	23114	1047
Heart	—	—	—	2185	—
Brain	—	—	—	1692	1069

DISCUSSION

It is well established that the injection of cortisol or corticosterone into normal or adrenalectomized rats restores liver glycogen that has previously been depleted by starvation (Long, Katzin & Fry, 1940). The hormones appear to have two different effects on glycogen formation. Within 2 hr. after injection it is possible to demonstrate an increase in the conversion of non-protein carbohydrate precursors into liver glycogen (Long *et al.* 1960; Ashmore, 1960). After about 3 hr. a second effect is observed that may last for 48 hr. and appears to involve the increased formation of glycogen precursors from the breakdown of tissue protein (Long *et al.* 1940; Engel, 1951). To account for the formation and long-term persistence of liver glycogen in the normal starved animal, two possibilities may be considered. It could be that the injected steroid initiates a chain of biochemical effects which then proceed autonomously for a while without the intervention of the steroid (Hyde, 1957). On the other hand, it is possible that there is a prolonged hormonal effect because the active steroid molecules remain unchanged at the site of action for a considerable period of time. The latter phenomenon would suggest a catalytic type of action and is more in keeping with what is known about the regulation of enzymic activity.

The results presented in this and a previous paper (Bellamy *et al.* 1962) provide supporting evidence for the second possibility, in that not all of the corticosteroid that enters the cell is immediately metabolized. Initially there may be 20 times as much metabolites as hormone but the polar compounds are less strongly adsorbed to cell structures. In particular, 24 hr. after a single injection of corticosterone, between 15 and 20 % of the radioactivity in liver and muscle appeared to be due to the presence of corticosterone. This suggests that injected hormones may influence metabolism by becoming adsorbed to a part of the cell where they are relatively inaccessible to further enzymic action. The fractionation studies suggest that ribosomes have the greatest affinity for corticosteroids. This conclusion is in agreement with the experiments of Harding & Samuels (1962), which indicate that androstenedione, derived from injected testosterone, was attached to RNA-containing particles in the ventral prostate gland. Since the adsorptive properties of the rat-liver particulate fraction were not altered by the removal of RNA it is likely that in this tissue the corticosteroids were adsorbed to the structural portion of the cell and did not interact with RNA directly.

The effect of corticosteroids on the rate of metabolic processes in normal animals increases with the size of the injected dose until a saturation point is

reached, where an increased dose produces no further response (Harding *et al.* 1961; D. Bellamy, unpublished work). This relationship between the dose and the response may be taken to mean that the biochemical system involved has a limited capacity for accepting hormone molecules as catalysts. In the present work, and in other experiments (Levin, Daughaday & Bremer, 1955), hormones were adsorbed to liver according to the tissue isotherm until the bound steroid accounted for more than 0.1 % of the wet weight of tissue. At this steroid concentration, which is far in excess of that required to produce a biochemical response, there was no sign of the adsorption sites becoming saturated, although there was evidence that the concentration ratio of bound to free hormone depended on the free-steroid concentration (Bellamy *et al.* 1962). This renders it unlikely that any one biochemical result can be related to the total amount of adsorbed hormone in a tissue. The results also suggest that the adsorption of steroids to a variety of enzyme systems having different affinity constants could be responsible for the multiplicity of biochemical effects that have been attributed to corticosteroids (Phillips & Bellamy, 1963).

SUMMARY

1. Various cell fractions were isolated from homogenates of rat liver in 0.9 % sodium chloride by differential centrifuging. The distribution of radioactivity derived from injected corticosteroids between the cell fractions was found to be the same as the distribution of [^{14}C]corticosteroids added to homogenates from normal rats.

2. The particle fraction that sedimented at the highest centrifugal force contained the greatest concentration of hormone, but the steroid was not confined to any one fraction of the cell.

3. The steroid hormones were more strongly bound to the cell particles than their polar metabolites. A comparison of the distribution of radioactivity between the particulate fraction of various tissues and the supernatant solution showed that liver had the greatest capacity for adsorbing corticosteroids.

4. Pretreatment of the cell particles, which leads to the removal of ribonucleic acid, did not affect the adsorption of corticosteroids.

5. Twenty-four hours after a single injection of 5 mg. of [^{14}C]corticosterone into normal rats, which brought about an increase in liver glycogen, about 0.1 % of the administered radioactivity was found in the liver as unchanged hormone. The results suggest that the long-term effect of hormonal steroids is linked with their adsorption to proteinaceous cell structures, where they are relatively inaccessible to enzyme action.

The author thanks the Endocrinology Study Section, National Institutes of Health, Bethesda, Md., U.S.A., for the gift of radioactive steroids. The work was largely financed by grants from the Agricultural Research Council.

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Biochem. J. (1963) **87**, 340

Heterogeneity of Rat Thyroglobulin Labelled with ^{131}I *in vivo*

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(Received 19 November 1962)

Halmi & Pitt-Rivers (1962) showed that, at short times after the injection of ^{131}I in rats, the specific-activity curve of the second iodide pool, derived by intrathyroidal deiodination of iodo compounds, rose more steeply than that of any iodo amino acid in rat thyroglobulin (Pitt-Rivers, 1962); they postulated a heterogeneity of thyroglobulin with respect to labelled iodotyrosines, the fraction with a more rapid turnover being the probable precursor of the second iodide pool at short times after the injection of ^{131}I . This did not preclude the possibility that all the iodotyrosine residues, whether labile or not, could be the precursors of the second iodide pool.

Pitt-Rivers & Cavalieri (1963) made quantitative studies of ^{131}I -labelled free iodotyrosines in diffusates of rat thyroids at different times after the

injection of ^{131}I . They found that, up to 4 hr. after the injection, the specific activity of monoiodotyrosine rose more steeply than that of second pool iodide; that of di-iodotyrosine was lower. However, the mean of the sum of the specific activities of the iodotyrosines exceeded that of second pool iodide; it was therefore concluded that both were likely to be a source of this iodide.

In preliminary experiments on the hydrolysis of labelled rat thyroids with crude calf-thyroid protease, Pitt-Rivers & Cavalieri (1963) found that, in glands that had been labelled for 1 or 4 hr., the rate of release of mono[^{131}I]iodotyrosine was rapid for 3–4 hr. and then slowed down to a constant rate up to 10 hr. In the glands labelled for 24 hr., the rate of release of mono[^{131}I]iodotyrosine was constant throughout the experiment. This lent