The Effects of Detergents on Autolysis of ^{131}I -Labelled Rat Thyroid Homogenates

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It has been shown by Pitt-Rivers (1963) that the rate of release of ^{131}I -labelled iodotyrosines from thyroglobulin during autolysis of rat thyroid homogenates at pH 3.6 is more rapid, the shorter the time of labelling, between 30 min. and 48 hr. In subsequent experiments, the rate of release of ^{131}I -labelled iodotyrosines was found to be slower during autolysis of glands labelled for 5 and 10 min. than for 30 min. This was thought to be due, at the shortest times of labelling, to an increase in the amount of particulate [^{131}I]iodoprotein, which might be more resistant to thyroid protease than is thyroglobulin. In the present experiments, studies have been made of the hydrolysis of whole thyroid homogenates, particulate iodoprotein and soluble iodoprotein labelled with ^{131}I for 10 min. to 18 hr. The effects of various detergents on the rates of hydrolysis of soluble and particulate iodoprotein were also studied for the following reasons. (1) Both anionic and cationic detergents liberate sulphhydryl groups from proteins; one of the former, Duponol, has been shown to release a higher proportion of sulphhydryl groups from egg albumin than are released by other methods of denaturation such as heat or treatment with urea (see Putnam, 1948). Such an effect might produce a further uncoiling of the thyroglobulin molecule. (2) Detergents are known to solubilize or suspend precipitated proteins (see McBain, 1942; Putnam, 1948); such a process might assist proteolysis of thyroglobulin, which is precipitated at pH 3.6, and also that of the particulate protein. It was not thought that detergents would activate the thyroid protease(s) since the latter are water-soluble. The actions of three detergents, anionic (sodium dodecyl sulphate), cationic (dodecyltrimethylammonium chloride), neutral (Triton X-100), and of urea on the rates of hydrolysis of soluble and particulate thyroidal iodoproteins labelled for different times have been compared.

METHODS

Male hooded rats weighing about 200 g. were maintained as described by Pitt-Rivers (1963). The animals were injected intraperitoneally with carrier-free ^{131}I (25–200 μC in aq. 0.9% NaCl) and killed by exsanguination under chloro-

form anaesthesia, 10 min., 30 min., 2 hr. and 18 hr. after the dose. In most experiments, two thyroid glands were homogenized in all-glass homogenizers in 0.3 ml. of 0.2M-acetate buffer, pH 3.6. The homogenates were kept at 2° overnight and then gently shaken in stoppered tubes at 35°. Samples were removed for chromatographic analysis at about 2 hr. intervals up to 9 or 10 hr. and again at about 24 hr. The chromatograms were developed as described by Pitt-Rivers (1963). The degree of autolysis was measured by estimating the amount of 'origin' material (iodoprotein) on the chromatograms in a strip counter.

Particulate iodoprotein was separated from soluble iodoprotein essentially by the method of DeGroot & Davis (1962). Two rat thyroids were homogenized in 1 ml. of aq. 0.9% NaCl containing 100 μg . of thiouracil/ml. The suspension was centrifuged at 70g for 15 min. at 2°; the supernatant was transferred to Perspex tubes and centrifuged at 85000g for 1 hr. The precipitated material was used as particulate iodoprotein; it was washed three times by rinsing with 1 ml. of aq. 0.9% NaCl without disturbing the precipitate. Hydrolysis of particulate iodoprotein was carried out with the homogenate of two unlabelled rat thyroids in 0.3 ml. of acetate buffer, pH 3.6, with or without detergent.

In the experiments on autolysis of soluble [^{131}I]iodoprotein, the thyroids of six rats were homogenized in 0.9 ml. of aq. 0.9% NaCl containing 100 μg . of thiouracil/ml., and centrifuged at 85000g for 1 hr. The precipitate was discarded; portions of the supernatant, which contained thyroglobulin and proteases, were diluted with an equal volume of acetate buffer, with or without detergent.

The following detergents were used: sodium dodecyl sulphate (British Drug Houses Ltd.), 0.02M and 0.004M; dodecyltrimethylammonium chloride (F. W. Berk and Co. Ltd.), and Triton X-100 (Rohm and Haas, Philadelphia, Pa., U.S.A.). The effect of 3M- and 1M-urea on autolysis of the homogenates was also examined.

RESULTS

The rates of autolysis (disappearance of 'origin' material) of whole thyroid homogenates labelled with ^{131}I for 10 min. to 18 hr. are shown in Fig. 1. Since the amounts of radio-iodide at t_0 varied considerably according to the times of labelling, the [^{131}I]iodoprotein has been taken as 100% at t_0 and all subsequent values have been corrected accordingly. The free iodotyrosines of the thyroid, which are present only in minute amounts (Pitt-Rivers & Cavalieri, 1963) and which were not detectable on chromatograms at t_0 , have been ignored.

As found by Pitt-Rivers (1963), the rate of autolysis was greater when the glands were labelled for 30 min. than for 18 hr. The amount of origin material present after 24 hr. autolysis ranged from 72% with the glands labelled for 18 hr. to less than 50% with the glands labelled for 30 min. Alpers, Robbins & Rall (1955) labelled rat thyroids with ^{131}I for 48 hr. and allowed extracts to autolyse for 24 hr.; they obtained a range of labelled origin

material (unhydrolysed) varying from 58 to 76% of the total radioactivity. It should be mentioned that in the present experiments the autolysis curves were not always linear beyond the 10 hr. point, probably owing to deterioration of the enzymes; this might account for the range of values for origin material obtained by Alpers *et al.* (1955). Very little further autolysis was observed after 48 hr. in our experiments.

Fig. 1. Semi-log plot of the rate of disappearance of 'origin' material during autolysis of soluble iodoprotein. Glands were labelled for: 10 min., ■; 30 min., ●; 2 hr., ▼; 18 hr., ▲.

Fig. 2. Semi-log plot of the percentage increase in the rate of disappearance of 'origin' material during autolysis of soluble iodoprotein in the presence of 0.01 M-dodecyltrimethylammonium chloride. Glands were labelled for: 10 min., ■; 30 min., ●; 2 hr., ▼; 18 hr., ▲.

Effect of detergents

Sodium dodecyl sulphate. Autolysis of glands labelled with ^{131}I for 18 hr. was completely inhibited in the presence of 0.02 M-sodium dodecyl sulphate; at 0.004 M it was without effect. This detergent has been shown to inactivate pepsin at pH 4 (see Putnam, 1948) and may inactivate the thyroid protease by the same mechanism. The action of sodium dodecyl sulphate on the substrates at pH 3.6 was not investigated.

Dodecyltrimethylammonium chloride. Autolyses of whole thyroid homogenates labelled for 10 min., 30 min., 2 hr. and 18 hr. were carried out in the presence of 0.01 M-dodecyltrimethylammonium chloride; control autolyses were run at the same time. Curves similar to those in Fig. 1 were drawn; in all the experiments, the compound was found to increase the rate of autolysis, but to different degrees, according to the time of labelling. Data from these curves were used to calculate the percentage increases in autolysis in the treated compared with the control homogenates. The results are shown in Fig. 2; the most prolonged effect of dodecyltrimethylammonium chloride was found on autolysis of thyroid homogenates labelled for 18 hr.; the most transient effect was on homogenates labelled for 30 min.

Particulate [^{131}I]iodoprotein. Thyroids were labelled with ^{131}I for 10 min., 30 min., 2 hr. and 18 hr. and the particulate iodoprotein was hydrolysed by homogenates of unlabelled thyroids with and without dodecyltrimethylammonium chloride. The rates of hydrolysis of the control and treated hydrolyses are shown in Fig. 3. It is evident that the particulate iodoprotein is not heterogeneous as regards ease of hydrolysis, as is the whole homogenate; it is also evident that the detergent has the same effect on the rate of hydrolysis of particulate iodoprotein, whatever the time of labelling.

Soluble [^{131}I]iodoprotein. Thyroid glands were labelled for 10 min. and the soluble iodoprotein was allowed to autolyse with and without dodecyltrimethylammonium chloride. The detergent had no effect on the rate of autolysis.

Triton X-100. The rate of autolysis of whole thyroid homogenates labelled for 2 hr. and 18 hr. was increased by 0.33% Triton X-100, but the effect was only about one-half that produced by

Fig. 3. Semi-log plot of the rate of disappearance of origin material during hydrolysis of particulate iodoprotein with unlabelled thyroid homogenate. C, Control; D, system plus 0.01 M-dodecyltrimethylammonium chloride. Glands were labelled for: 10 min., ■; 30 min., ●; 2 hr., ▼; 18 hr., ▲.

dodecyltrimethylammonium chloride. A higher concentration of Triton X-100 (1 %) had less effect than the lower concentration. Triton X-100 had no effect at all on the rate of autolysis of whole thyroid homogenates labelled for 10 and 30 min., or on particulate or soluble [^{131}I]iodoprotein labelled for 10 min.

Urea. Thyroid homogenates labelled for 19 hr. were autolysed in 3M- and 1M-urea. At the higher concentration, the rate of autolysis was depressed to about one-half of the control value; it was also slightly inhibited by 1M-urea.

DISCUSSION

Edelhoc & Lippoldt (1960) have shown that low concentrations of sodium dodecyl sulphate at neutral or alkaline pH cause the splitting of thyroglobulin into two sub-units and that higher concentrations lead to complete dissociation of the molecules; the changes in viscosity indicated that thyroglobulin behaved as a randomly coiled polyelectrolyte. Steiner & Edelhoc (1961) found that at an alkaline pH, 8M-urea caused a disorganization of the thyroglobulin molecule which exposed a number of tyrosyl radicals in the molecule to iodination *in vitro* that could not be iodinated in the absence of urea (Edelhoc, 1962). At pH 3.6, thyroglobulin is irreversibly denatured (Heidberger & Pedersen, 1935), but the structure of the

denatured molecule is not known. Both sodium dodecyl sulphate and urea inactivated the thyroid protease(s) and no experiments were carried out to discover their effects, if any, on thyroglobulin at an acid pH. The inactivation of thyroid proteases or peptidases or both by urea is of interest in view of the stability of a number of proteolytic enzymes to this denaturing agent (see Christensen, 1952; Anfinsen & Redfield, 1956).

Both dodecyltrimethylammonium chloride and Triton X-100 enhanced the rates of autolysis of homogenates that had been labelled for 2 hr. or more; however, neither detergent had any effect on hydrolysis of soluble iodoprotein that had been labelled for 10 min.; if these detergents potentiate the activity of the protease, the effect should be detectable whatever the time of labelling of the substrate. It is therefore concluded that these detergents were without effect on the enzyme, and exerted their action solely on the substrates. The nature of this action is not known; it might be due to further denaturation of the substrates or to the solubilizing action previously discussed.

Dodecyltrimethylammonium chloride enhanced the rate of autolysis of whole thyroid homogenates at all times of labelling; the effect on homogenates labelled for 18 hr. was the most prolonged, and did not decrease greatly during 24 hr. This would suggest that the detergent had little or no effect on the activity of thyroid proteases or peptidases. It is to be noted that the transient stimulation of autolysis by dodecyltrimethylammonium chloride of whole homogenate labelled for 10 min. must have been entirely due to its effect on particulate [^{131}I]iodoprotein, since it had no effect on autolysis of the soluble [^{131}I]iodoprotein labelled for the same time. This indicates that the [^{131}I]iodoamino acids in thyroglobulin labelled for 10 min. are completely accessible to the proteases, and detergent action is without further effect. The most transient action of dodecyltrimethylammonium chloride was on homogenates labelled for 30 min., which suggests that the [^{131}I]iodoamino acids are still highly accessible to proteolytic activity at this time, and that the proportion of particulate [^{131}I]iodoprotein has diminished.

Triton X-100 did not apparently inhibit thyroid proteases, and did stimulate autolysis of homogenates labelled for 2 hr. and 18 hr. This stimulation must be entirely due to its effect on the thyroglobulin molecule, since it had no influence on the hydrolysis of particulate [^{131}I]iodoprotein.

The absence of heterogeneity of particulate [^{131}I]iodoprotein with respect to lability towards thyroid protease at all times of labelling studied merits some comment. It has been questioned whether particulate iodoprotein is related to thyroglobulin (Robbins, Wolff & Rall, 1959a, b; De-

Groot & Carvalho, 1960; Smith, Robbins & Rall, 1961). The present experiments suggest that it is not, since its lability towards thyroid protease, unlike that of labelled thyroglobulin, did not vary with the time of labelling. However, an alternative hypothesis can be suggested: that some of the particulate iodoprotein is a precursor of thyroglobulin, but that it exists in the cells in a constant form and only assumes heterogeneity when assembled in the thyroglobulin molecule.

The nature of proteolytic attack on thyroglobulin in the present experiments is not known. The chromatographic 'origin' material, which was all included as 'unhydrolysed', could have contained fragments ranging from unattacked thyroglobulin of molecular weight 650 000 to polypeptides of molecular weight 3000. O'Donnell, Baldwin & Williams (1958) showed that papain degraded thyroglobulin between pH 4.6 and 6.65 in a 'one by one' type of proteolysis, and they were unable to demonstrate any fragments of high molecular weight. Alpers, Petermann & Rall (1956) on the other hand showed that autolysis at pH 5.2 of a labelled thyroid extract for 35 hr. gave rise to non-diffusible fragments of varied molecular weight. When Pitt-Rivers & Cavalieri (1963) chromatographed diffusates of labelled rat thyroids, they found that a number of radioactive compounds (including peptides; Lissitzky & Haney, 1962) streaked along the chromatograms and interfered with the estimation of the free iodoamino acids. No such confused chromatograms were found in the present work. In view of this, of the good linearity of the curves obtained and of certain considerations about the formation of labile intermediates during proteolysis (Linderstrøm-Lang & Schellman, 1959), it is concluded that in the present experiments thyroglobulin was at no time hydrolysed with the formation of peptide fragments of molecular weight less than 3000; further, that whatever the mode of proteolytic attack it was of a constant nature throughout the 24 hr. autolysis, which permitted comparison of any one curve with another.

The present experiments support the concept of a heterogeneity of thyroglobulin towards thyroid proteases. In the conditions used, the cellular particulate iodoprotein is homogeneous in this respect.

SUMMARY

1. The effects of some detergents and of urea on autolysis of ^{131}I -labelled whole rat thyroid homogenates have been investigated.

2. Sodium dodecyl sulphate and urea inactivated the proteolytic enzymes.

3. Dodecyltrimethylammonium chloride stimulated autolysis of homogenates at all times of labelling between 10 min. and 18 hr. The effect was most prolonged with the homogenate labelled for 18 hr. and most transient with the homogenate labelled for 30 min.

4. Triton X-100 stimulated the autolysis of homogenates labelled for 2 hr. or 18 hr., but not that of homogenates labelled for 10 min. or 30 min. It had no effect on hydrolysis of particulate iodoprotein.

5. Hydrolysis of ^{131}I -labelled particulate iodoprotein by thyroid homogenates has been studied. There was no difference in the rate of disappearance of labelled iodoprotein, whatever the time of labelling.

6. Dodecyltrimethylammonium chloride stimulated hydrolysis of all the preparations of particulate iodoprotein to the same extent.

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