Radioautographic Studies of the Initial Site of Formation of Protein-Bound Iodine in the Rat Thyroid Gland

By C. J. CROFT AND ROSALIND PITT-RIVERS

National Institute for Medical Research, Mill Hill, London N.W.7, U.K.

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1. Predominantly cellular labelling was observed in radioautographs of rat thyroid glands fixed by perfusion from the aorta at intervals between 5 and 55s after [125 I]iodide administration via the aorta. 2. When perfusion was delayed for 2min, or if immersion fixation was used, the labelling was predominantly over the peripheral portion of the follicular lumen, in agreement with the observations of other investigators. 3. The findings support the concept that the initial site of binding of iodine to protein is intracellular, but the nature of this protein has not been established.

Radioautographic studies of the site of iodination of thyroid protein *in vivo* (Wollman & Wodinsky, 1955) have shown that the first site at which labelled iodoprotein is readily demonstrable is at the periphery of the lumen of the thyroid follicle. Electron-microscopic studies (Stein & Gross, 1964; Ibrahim & Budd, 1965; Fujita, 1969) have also shown that the initial radioautographic reaction occurs predominantly at the colloid-cell boundary overlying the microvilli of the cells. In one of these studies (Ibrahim & Budd, 1965) grains observed over cells were present at two to three times the background, but all authors have emphasized that the early cytoplasmic labelling is not associated with particular intracellular organelles. Nevertheless there is evidence (Kondo & Ui, 1966; Hosoya & Morrison, 1967) that the enzyme involved in the organic binding of iodine (thyroid peroxidase), is associated with a subcellular microsomal fraction.

As Wollman & Wodinsky (1955) have pointed out, it is possible that iodoprotein may be formed inside the cell, but that failure to demonstrate it at this site could be due to its rapid movement and accumulation in the lumen of the follicle. These workers studied the radioautographic distribution of 131 I-labelled iodoprotein at intervals between 11s and 60min after [131 I]iodide administration. Even at 11s after intravenous injection they found that the label was over the peripheral portion of the luminal colloid. Nevertheless they still considered that a rapid transfer of iodoprotein from the cell to the lumen, though improbable, might occur during the time-interval before fixation. Ibrahim & Budd (1965) found that in thin sections prepared for electron microscopy there was not sufficient label to determine the distribution of radioactivity when glands were fixed at less

than 3min after [125 I]iodide administration. In light-microscopic radioautography the high concentration of radioactivity in the luminal colloid makes the detection of smaller concentrations of radioactivity in the cells difficult, because grains seen over the cells could be caused by cross-firing of radioactivity from the colloid.

The present studies using light-microscopic radioautography were undertaken to investigate further the possible cellular localization of iodoprotein in the brief period before accumulation of large amounts of label in the colloid. To avert delays associated with removal of the thyroid before immersion in fixative and with gradual penetration of fixative into the gland, a technique for rapid fixation by arterial perfusion from the aorta was developed. The effect of this method of fixation on the distribution of radioactive protein-bound iodine was studied by comparison with immersion-fixed material.

MATERIALS AND METHODS

Male hooded rats (150-170g) were fed on diet 41B (Bruce, 1958) and tap water *ad libitum*. A modification of the method of retrograde perfusion from the abdominal aorta described by Maunsbach (1966) was used. Animals were anaesthetized with ether, the jugular veins dissected and ligatures placed around them, care being taken to ensure that there was no obstruction to the flow of blood. The abdominal aorta was exposed, tied off at the bifurcation and a cannula tied into it in a retrograde direction. When the abdominal aorta was to be cannulated, the cannula consisted of a steel tube (1mm external diam., 0.5mm internal diam.) and was passed 5mm into the aorta. When the thoracic aorta was to be cannulated, a polythene tube of similar dimensions (Portex no. 8/40/140; Portland Plastics Ltd., Hythe, Kent, U.K.) was passed 5.6cm upwards from the abdominal aorta. Carrier-free

[125 I]iodide with added reducing agent (The Radiochemical Centre, Amersham, Bucks, U.K.) was diluted with 0.5 ml of Dulbecco's phosphate-buffered physiological saline, pH 7.4 Dulbecco & Vogt, 1954, and injected through the cannula into the aorta. After various time-intervals the ligatures around the jugular veins were tied and the veins cut on the side of the ligature away from the heart. Fixative was then injected rapidly through the cannula by using 5 ml syringes. The dose of [125 I]iodide was 100 or 200 μ Ci per rat. Time-intervals are shown in Table 1. In most studies 0.25 ml of a 1% solution of Trypan Blue in distilled water was added to each 5 ml of fixative (see below) to show, by the blue colour of the thyroid, that the perfusion had been successful. In experiments in which fixation was required at intervals of less than 30 s after [125 I]iodide injection it was necessary to tie and cut the jugular veins before administration of the [125 I]iodide. Invariably the animals' respiration and circulation continued for the short time-intervals involved. Thyroidal uptake of iodide is known to be somewhat depressed by ether anaesthesia (Oyama, 1959) and also by severe blood loss (Money, 1955). However, there was sufficient uptake of [125 I]iodide for clear-cut radioautographic localization. In some experiments the fixative was chilled in ice-water. In rat 15 the fixative was preceded at 10 s by an injection of 3 ml of Dulbecco's physiological saline containing 1.0 mg of KI, and in rat 14 at 7.5 s by 5 ml of cold Dulbecco's physiological saline. After perfusion of fixative the thyroid glands were dissected and placed in 20 ml of fixative.

In two experiments immersion fixation was used. In each case [125 I]iodide was administered by injection into a cannula in the abdominal aorta, and the jugular veins were tied and cut as in the perfusion studies. The perfusion was omitted, and after the appropriate time-interval the glands were removed and placed in fixative.

Histological and radioautographic techniques. After 3 h the fixative was replaced with ethanol and the thyroids were trimmed for histological sectioning. The tissues were then cleared with chloroform and embedded in paraffin wax. Serial 3 μ m sections were mounted on gelatinized slides, dewaxed and taken to water and washed overnight in running tap water to ensure removal of unbound [125 I]iodide. Radioautographs were prepared with Kodak AR10 stripping film and were processed by the procedures of Curran & Clark (1961), electrostatic flashing being avoided by the method of Mazia & Bucher (1960). They were exposed for 3, 7 and 10 days and stained with Neutral Red. Each slide for radioautography carried a control section from the thyroid of a normal rat that had not been treated with [125 I]iodide and that had been fixed and processed in a manner identical with that used for the experimental specimens. In this way artifacts that might confuse radioautographic interpretation were avoided. The source of radioactivity in the radioautographs was determined by the grain-density distribution method as applied to stripping-film radioautographs (Rogers, 1967).

Parallel sections were stained with haematoxylin and eosin to assess microanatomical preservation. Wollman (1965) has pointed out that displacement of colloid as an artifact, as well as tangential cutting of thyroid follicles, can result in colloid overlapping portions of cells. To demonstrate the extent of these phenomena sections were

stained with Schiff-periodic acid reagent. In addition, some radioautographs were restained with Schiff-periodic acid reagent so that the edge of the colloid could be accurately determined. This resulted in removal of the Neutral Red stain and specific staining of the colloid.

In preliminary tests aimed at selecting the most suitable fixative for this investigation thyroids from untreated (non-radioactive) rats were fixed in the following fluids: ethanol; Carnoy's fixative (60 ml of ethanol, 30 ml of chloroform, 4 ml of acetic acid); Clarke's fixative (75 ml of ethanol, 25 ml of acetic acid); formalin-alcohol-acetic acid (5 ml of concentrated formaldehyde, 45 ml of 90% ethanol, 2 ml of acetic acid); 10% neutral formalin-0.9% NaCl (1:9). Full radioautographic procedures were performed on sections from these glands to test for chemical fogging of the photographic emulsion (positive chemography) attributable to the fixative (Rogers, 1967).

RESULTS

Selection of fixative. Both of the fixative solutions that contained formalin showed fogging of the photographic emulsion (positive chemography), particularly prominent over the colloid, when non-radioactive control material was examined. Clarke's fixative gave good microanatomical preservation, and when perfused showed less frequent and usually only slight separation of the colloid from the cells. There was no evidence of positive chemography, and it was selected for all the radioautographic experiments. Carnoy's fixative and ethanol showed no advantage over Clarke's fixative.

Radioautographic appearances (see Table 1). (a) **Immersion fixation.** Radioautographs of thyroid glands that had been immersion-fixed at 3 min, and at 55 s after injection of [125 I]iodide into the abdominal aorta (rats 1 and 2), showed extensive labelling of all follicles. In most follicles there was a wide band of label extending from the edge of the colloid to near the centre of the lumen. In addition, there were many grains over the epithelial cells, but the concentration was less than that over the adjacent colloid. Such labelling could therefore have been due to cross-firing of radioactivity from a source in the colloid, or at the cell-colloid border. In regions of follicles where the colloid had separated as an artifact from the cells the radioautographic image over the cells was invariably less than that over cells of the same follicle adjacent to unretracted colloid. The grains were, however, present in high concentration over the edge of the colloid that had separated from the cells. This confirms the observations of other investigators (Wollman & Wodinsky, 1955) and shows that where colloid retraction artifacts occur the radioautographic image over the cells is thereby decreased. Some follicles cut tangentially (so that the luminal portions of the cells were visible but no colloid could be identified) were distinctly labelled.

EXPLANATION OF PLATE I

(a) Stripping-film radioautograph of thyroid fixed by perfusion 12.5s after [125 I]iodide injection into the thoracic aorta (Rat 14, exposure time 10 days). The grains over the cells are in focus. Neutral Red stain was used. Magnification $\times 1176$. (b) Same field as (a), but the grains over the colloid are in focus. Neutral Red stain was used. Magnification $\times 1176$. (c) Same field as (a) and (b), but the colloid has been stained with Schiff-periodic acid reagent (no counterstain), during which the Neutral Red was removed. The grains over the colloid are in focus. It should be noted that the grains are more concentrated over the cells than over the colloid. Phase-contrast microscopy was used. Magnification $\times 1176$.

EXPLANATION OF PLATE 2

(a) Complete follicle from the same radioautograph as Plate 1(a)–(c). The grains over the cells are in focus. Neutral Red stain was used. Magnification $\times 1015$. (b) Same field as (a), but the grains over the colloid are in focus. Neutral Red stain was used. Magnification $\times 1015$. (c) Same field as (b), but the colloid has been stained with Schiff-periodic acid reagent. The grains over the cells are in focus. It should be noted that over more than half the follicle there is a greater density of grains over the cells than the adjacent colloid. Phase-contrast microscopy was used. Magnification $\times 1015$.

Table 1. *Summary of radioautographic observations*

Rat no.	Method of fixation	Time-interval before fixation(s)	Radioautographic appearance
1, 2	Immersion	180, 55	Grain density over colloid invariably greater than over adjacent cells, in agreement with observations of other investigators
3	Perfusion from abdominal aorta	120	
4-8	Perfusion from abdominal aorta	28-45	Very weak labelling; in some follicles grain density probably greater over cells than the adjacent colloid
9-14	Perfusion from thoracic aorta	5-55	Many follicles have greater grain density over cells than the adjacent colloid
15*	Perfusion from thoracic aorta	17	

* 'Chase' with KI.

Here too, however, because of the strong labelling of the colloid in these specimens, it cannot be excluded that this apparent cellular labelling was due to traces of colloid present in the section even though they were not microscopically demonstrable. Such labelling is not therefore acceptable as evidence that the source of radioactivity was in the cell cytoplasm. It was concluded that in immersion-fixed thyroids intracellular iodoprotein cannot be convincingly demonstrated at the time-intervals studied.

(b) Perfusion fixation via the abdominal aorta. The most prominent feature of the glands fixed by perfusion after [125 I]iodide administration into the abdominal aorta was that separation of the colloid as an artifact from the cells, with the related shift of the radioautographic image, was less prominent than in the immersion-fixed material. It was noted, in addition, that the intensity of labelling was less and seemed to be more closely related to the cells. At 2 min after [125 I]iodide injection (rat 3) there were no regions in which the concentration of grains over the cells was greater than that over the adjacent colloid. But between 28 and 45 s grain counts showed that some follicles had more grains over the cells than over the adjacent colloid. However, because of the very weak labelling in these experiments it was not possible to estimate the grain densities accurately, and therefore the source of radioactivity could not be determined with certainty. Nevertheless the presence of more grains over the cells than the colloid was a finding essentially different from that obtained in the immersion-fixed material, and also from that obtained in rat 3, in which perfusion was performed 2 min after [125 I]iodide administration.

(c) Perfusion fixation via the thoracic aorta. Because of the low radioactivity of specimens that were fixed by perfusion at 28-45 s after [125 I]iodide

injection into the abdominal aorta, experiments were undertaken in which the thoracic aorta was cannulated so that the injected [125 I]iodide would pass directly into the thyroid arteries. The radioautographs showed much stronger labelling after intervals between 5 and 20 s than was obtained in abdominal cannulation experiments after 28-45 s. Moreover, many follicles showed extensive regions in which there was clearly more label over the cells than the adjacent colloid (Plates 1 and 2). In other parts of the same follicles the label was more prominent over the colloid than the adjacent cells. Frequently such colloidal labelling was at, or close to, regions in which the colloid had separated as an artifact from the cells. In many of the small follicles, also, the label over the colloid was more prominent than that over the cells.

In rat 15 carrier iodide was perfused with two objects: to inhibit further iodination and to dilute out the [125 I]iodide. The radioautographs also showed a similar degree of cellular labelling. This indicated that cellular radioactivity was protein-bound before perfusion with fixative.

Wollman (1965) has suggested that apparent cellular labelling can be caused by colloid overlying cells as an artifact due to displacement, or to sections being cut close to the edge of the lumen. Examination of serial sections showed that the cellular labelling was present in follicles cut at their maximum diameters (i.e. equatorially) so that overlapping of cells due to tangential cutting was excluded. Additional sections in which the colloid had been stained with Schiff-periodic acid reagent showed that displacement of colloid over the surface of cells was infrequent, irregular, and not sufficient to account for the radioautographic labelling of cells. Confirmation was obtained by restaining of the radioautographs with Schiff-periodic acid reagent (Plates 1c and 2c).

DISCUSSION

The present studies are in agreement with the observations of a number of workers (Wollman & Wodinsky, 1955; Stein & Gross, 1964; Ibrahim & Budd, 1965; Nadler, 1965a; Fujita, 1969) that in immersion-fixed material the radioautographic reaction is seen predominantly over the luminal colloid. However, at 5–55 s after [125 I]iodide administration, and by using perfusion fixation, extensive labelling over the cells has been observed.

Wollman (1965) suggested that the cellular labelling reported in an earlier study (Pitt-Rivers, Niven & Young, 1964), in which immersion fixation was used, could have been due to 'overflow' of colloid on to the cut surface of the cells, or to overlapping of cells by colloid due to tangential cutting. Staining of the colloid with Schiff-periodic acid reagent as recommended by Wollman (1965) showed that the observations of cellular labelling in the present study cannot be due to these phenomena. Nevertheless we were impressed with the number of grains seen over the colloid, and the distance into the colloid at which they are present. This is particularly prominent in follicles cut close to their edge. To some extent colloidal labelling can be explained by cross-firing of radiation from a source in the cells, particularly as the photographic emulsion over the colloid receives irradiation from the cells all around it. However, this is not an explanation for the colloidal labelling adjacent to regions of separation as an artifact of the colloid from the cells. In such regions the artifact may be caused by a delay in fixation, during which the iodoprotein could be displaced from the cells into the colloid.

The distribution of cellular labelling observed in the present studies supports the concept that the initial site of binding of iodine is predominantly at an intracellular site. The later concentration of label at the periphery of the lumen could be due either to rapid secretion and accumulation, or to binding by the luminal colloid itself. The hypothesis (Nadler, 1965b) that initial binding occurs predominantly in the colloid is not supported by these observations. Such a process could not explain the appearances in follicles where there are more grains over the cells than the colloid. It would be necessary to postulate that iodination occurred in the colloid with rapid reabsorption and concentration in the cells, whereas at longer times the peripheral colloid is well loaded with label.

The question remains whether the cellular labelled protein is thyroglobulin. The nature of the protein-

bound [125 I]iodine in cells and colloid has not been demonstrated in this or any previously published study, but it is reasonable to assume that the labelled protein in the follicular lumen is thyroglobulin, since this is the most abundant protein in the colloid. Such an assumption is more difficult to justify with regard to labelled cellular protein and the extremely rapid transfer of thyroglobulin (if such it is) from cells to colloid is not easy to explain. Non-specific iodination of other proteins seems unlikely, since this does not occur to any extent in the normal gland. If the iodinated cellular protein is not thyroglobulin it could nevertheless be associated with thyroglobulin iodination: e.g. as a complex between [125 I]iodide and thyroid peroxidase, the enzyme responsible for the organification of iodide in the thyroid; this problem remains to be elucidated.

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