Intracellular and extracellular sites of iodination in dispersed hog thyroid cells

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Iodination and hormone synthesis has been studied in isolated hog thyroid cells in suspension. We characterized three iodination processes by use of pharmacological agents. (1) Intracellular iodination dependent on active iodide transport, which was inhibited by NaClO_4 or ouabain, but not by catalase. This iodination was linear for 6 h with an apparent K_m for iodide of $1.5 \mu\text{M}$, was stimulated by thyrotropin or N^6O^2 -dibutyryl adenosine 3':5'-cyclic monophosphate, yielded mostly iodinated thyroglobulin and was efficient for tetraiodothyronine synthesis. (2) Extracellular iodination, which was sensitive to catalase, but not to NaClO_4 or ouabain. This iodination plateaued after 2 h and the apparent K_m was $16.5 \mu\text{M}$. This process was insensitive to thyrotropin and dibutyryl cyclic AMP. The major products were iodoprotein other than thyroglobulin and iodolipid and the yield of tetraiodothyronine was low. (3) Intracellular iodination from passively diffused iodide, which was not sensitive to inhibitors. Other characteristics of passive intracellular iodination were intermediate between active intracellular iodination and extracellular iodination. The fact that the three processes are inhibited by similar concentrations of methimazole, and that their apparent K_m values, when corrected for the concentrating effect of iodide trapping, are all of the same order as the K_m of purified thyroid peroxidases, suggest that although their locations are different, the enzymic systems involved are identical. These results show that, besides an extracellular site of iodination, dispersed thyroid cells possess an intracellular site of iodination with biochemical characteristics of physiological relevance.

It is generally thought that all the reactants involved in the iodination process in the thyroid, i.e. thyroglobulin, peroxidase, H_2O_2 and iodide, could be present both inside the cells and outside at the apical surface. Attempts to localize the site(s) of iodination in intact thyroid tissue by using histochemical and autoradiographic techniques have yielded divergent results. In most reports (Wollman & Wodinsky, 1955; Strum & Karnovsky, 1970; Tice & Wollman, 1974; Ekholm & Wollman, 1975; see Ekholm & Wollman, 1975, for additional references), the iodination reaction was found to take place on the outer side of the thyroid cell apical plasma membrane. But, in some other reports (Leblond & Gross, 1948; Pitt-Rivers *et al.*, 1964; Croft & Pitt-Rivers, 1970; Edwards & Morrison, 1976), the labelled products of the iodination reaction were observed inside the cells.

In the present study, the problem was re-examined from a biochemical point of view by using dispersed thyroid cells. Freed of follicular structure, such cells are known to retain the main properties of the intact thyroid tissue: iodide uptake (Tong *et al.*, 1962; Rodesch & Dumont, 1967; Wilson *et al.*, 1968; Knopp *et al.*, 1970; Sherwin & Tong, 1974), thyroglobulin iodination (Nunez *et al.*, 1965; Wilson *et al.*, 1968), hormone synthesis (Tong *et al.*, 1962; Rodesch & Dumont, 1967; Wilson *et al.*, 1968; Rousset *et al.*, 1976) and hormone secretion (Rousset *et al.*, 1976).

In this system *in vitro*, it has generally been assumed that iodination takes place inside the cells. However, Rodesch *et al.* (1968) suggested the existence of two distinct iodinating systems in isolated-cell preparations, one inside and the other outside the cells. Developing this line of reasoning, we propose a theoretical model involving three pathways of iodination depending on the source of

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iodide: (1) intracellular iodination functioning from actively trapped iodide, (2) intracellular iodination functioning from iodide that passively enters the cells and (3) extracellular iodination using iodide of the incubation medium. The latter pathway could presumably reflect the iodinating activity of the outer side of the apical plasma membrane. To test the model, we used the properties that active iodide transport can be blocked by perchlorate ion and that H_2O_2 can be degraded by catalase, which is thought not to enter cells.

Experimental model

The three hypothetical iodination pathways, for brevity termed 'active intracellular iodination', 'passive intracellular iodination' and 'extracellular iodination', were differentiated by using the experimental approach depicted in Fig. 1. A model was built using four different incubation conditions (A, B, C and D). The type-A incubation was used to determine the total iodination under the basal conditions and the iodinating activity measured was taken as the sum of the three partial iodinations: active intracellular iodination + passive intracellular iodination + extracellular iodination. In the type-B incubation, it was assumed that catalase blocked extracellular iodination (Rodesch *et al.*, 1968) and therefore that the remaining iodinating activity could be assigned to active intracellular iodination + passive intracellular iodination. In the type-C incubation, it was postulated that perchlorate inhibited active intracellular iodination as a consequence of its blocking effect on active iodide uptake and that the residual iodinating activity could represent the sum of passive intracellular iodination + extracellular iodination. In the type-D incubation only passive intracellular iodination should then take place since the two other iodination pathways would be blocked in the presence of catalase and perchlorate. This incubation protocol was used to determine each of the three iodinating activities as follows: (a) measurements under the conditions of incubation D gave the value of passive

intracellular iodination; (b) values of active intracellular iodination and extracellular iodination could be obtained by subtracting the value of passive intracellular iodination from the iodination values measured in incubations B and C respectively.

The validity of this experimental model was directly checked by comparing the total iodination measured in the type-A incubations (experimental iodination) with the calculated iodination: the sum of the three iodinating activities determined according to (a) and (b).

Materials and methods

Materials

Trypsin was purchased from GIBCO (Grand Island, NY, U.S.A.) and Earle's salt solution and calf serum were from B.D. Mérieux (Lyon, France). Porcine thyrotropin was obtained from Organon (Paris, France), Pronase from Calbiochem (San Diego, CA, U.S.A.), catalase (39 000 units/mg) from Serva (Heidelberg, Germany), chlorpromazine from Specia (Paris, France), 3,5-dinitrotyrosine from Nutritional Biochemicals (Cleveland, OH, U.S.A.), ouabain, puromycin, cycloheximide, methimazole and $N^6O^{2'}$ -dibutyryl adenosine 3':5'-cyclic monophosphate from Sigma (St. Louis, MO, U.S.A.). ^{131}I was provided by CEA (Saclay, France).

Methods

Incubation of dispersed thyroid cells. Thyroid glands from adult hogs were collected on ice at the local slaughterhouse and processed within 2 h. Thyroid cells were obtained by a discontinuous trypsin-treatment procedure as described previously (Rousset *et al.*, 1976). Dispersed thyroid cells were used within 1 h of the end of the preparation. Cells $[(1-5) \times 10^7]$, equivalent to 10–50 μ l packed cell volume, were incubated in Earle's balanced salt solution, pH 7.4, in the presence of 0.050 μ M-iodide labelled with 1–5 μ Ci of $Na^{131}I$ (sp. radioactivity 10 Ci/mg) for 30 min to 6 h. Incubations were performed in a total volume of 3 ml in 27 ml plastic vials, at 37°C with air as the gas phase and under constant shaking (60 cycles/min).

Measurement of iodide uptake. Iodide trapping, expressed in terms of the cell ^{131}I /medium ^{131}I ratio, i.e. the ratio between intracellular and medium labelled-iodide concentration, was determined in the presence of 2 mM-methimazole by the method of Rodesch & Dumont (1967).

Measurement of organic iodine formation. This was assessed by measurements of acid-insoluble ^{131}I . Cells were pelleted by centrifugation at 80–100 g for 10 min at 4°C. The pellet was re-suspended in 0.1 ml of 0.05 M-Tris/HCl, pH 8.6, containing 0.05 M-methimazole. Cells were disrupted by freezing and thawing. Cell homogenates were then supplemented with 0.1 ml of 0.2 mM-NaI and 0.1 ml of 1% (w/v)

Incubation	Condition	Iodination type
A	Nothing added	Passive intracellular iodination + active intracellular iodination + extracellular iodination
B	Catalase	Passive intracellular iodination active intracellular iodination + 0
C	$NaClO_4$	Passive intracellular iodination + 0 + extracellular iodination
D	Catalase + $NaClO_4$	Passive intracellular iodination + 0 + 0

Fig. 1. Incubation protocol of thyroid cells

bovine serum albumin and precipitated with 5% (w/v) trichloroacetic acid. The pellet formed after centrifugation at 1500g for 10min was washed twice with 5% trichloroacetic acid and the radioactivity of the acid-insoluble material was measured in a well-type Packard autogamma counter. The counting efficiency was about 40%. Net total counts that were less than three times the background counts were considered to be non-significant.

Measurement of lipid and protein iodination. ^{131}I incorporated into proteins and lipids was measured respectively as the radioactivity of iodoamino acids (monoiodotyrosine + di-iodotyrosine + tri-iodothyronine + tetra-iodothyronine) and the radioactivity at the solvent front on paper chromatograms of cell homogenates treated with Pronase (Rousset *et al.*, 1976). In some experiments, radioiodinated lipidic material of cell homogenates was directly extracted with 3 ml of methanol/chloroform (1:2, v/v). Proteins were precipitated by addition of 1 ml of water and after centrifugation (1500g for 10min) the organic phase was counted for radioactivity. When extracted material was chromatographed in organic solvents (Rousset *et al.*, 1976) more than 90% of the radioactivity was found at the solvent front.

Measurement of thyroid hormone synthesis. Iodide incorporation into thyroid hormones (mainly tetra-iodothyronine) after 6h of incubation was measured as reported previously (Rousset *et al.*, 1976). Briefly, cell pellets were treated with 0.5% (w/v) Pronase in 0.05 M-Tris/HCl, pH 8.6, containing 0.05 M-methimazole for 16h at 37°C in the presence of penicillin (100 units/ml) and streptomycin (50 µg/ml). After centrifugation at 1500g for 10min, hydrolysate supernatants and washing liquids of the particulate material were chromatographed on an anion-exchange column (2 cm × 1 cm) (Oxford Laboratory, Athy, Ireland). ^{131}I -labelled hormonal fraction was eluted from the column with 60% acetic acid.

^{131}I incorporation in acid-insoluble material, iodoproteins, iodolipids and iodothyronines was completely inhibited when thyroid cells had been incubated in the presence of 1 mM-methimazole.

Density-gradient centrifugation. For centrifugation on sucrose density gradients (5–20%, w/v), cell pellets were disrupted in a Teflon/glass homogenizer in 0.01 M-potassium phosphate buffer, pH 7.0, containing 0.15 M-NaCl and 2 mM-methimazole. The 3000g supernatant was dialysed at 4°C for 16h against the same buffer. Dialysis residues were centrifuged at 200 000g (r_{av} 11.2 cm) for 15h at 4°C in a Beckman L2-65B ultracentrifuge with an SW 40 rotor. Fractions were collected from the bottom to the top of the gradient. Rat [^{125}I]thyroglobulin radioiodinated *in vivo* was used as a 19S marker (Rousset *et al.*, 1976).

Microscopic examinations. Dispersed thyroid cell suspensions were examined through phase-contrast optics to check the degree of cell dispersion. Suspensions of freshly dispersed thyroid cells were composed of single rounded cells and groups of 2–10 cells. The number and the size of cell clusters depend on the density of the cell population. Of the cells 50–60% appeared completely dissociated when the concentration was about 3×10^7 cells/ml. Cell aggregates resulted in part from the interaction between completely isolated cells and in part from incomplete dissociation. Approx. 95% of the thyroid cells appeared viable at the beginning of incubation. Cell lysis increased during incubation as a function of time, but did not exceed 10% after 6h (Rousset *et al.*, 1976).

For electron-microscope examination, cells were fixed with 2% (v/v) glutaraldehyde in 0.1 M-cacodylate buffer, pH 7.4, for 30min, washed in the same buffer containing 0.2 M-sucrose, and post-fixed for 30min with 1% (w/v) OsO_4 in 0.15 M-cacodylate buffer, pH 7.4. Cell pellets were washed and embedded in 1.5% (w/v) agarose. Small fragments were dehydrated and embedded in epon resin. Electron micrographs showed that cells have lost their polarity. Some microvilli distributed over the whole cell membrane could be observed both on single cells or on cells included in clusters (Rousset, 1978). Other aspects of the cellular integrity were similar to those reported by others (Neve *et al.*, 1968; Tixier-Vidal *et al.*, 1969).

Results presentation. In most experiments, thyroid-cell incubations were performed in triplicate and the mean and S.E.M. were calculated. Because of the complexity of the experimental approach and the result calculation and because of the biological variability of the material collected at the slaughterhouse, data of a representative experiment (out of three or more) or data of a group of independent experiments are reported. When the object was to determine the value of a quantity characteristic of the biological material used, results were expressed as the mean $\pm$ S.E.M. for n experiments and Student's t test was used for statistical analysis.

Results

Validity of the experimental conditions

The experimental model was based on the assumptions that (1) perchlorate ion suppresses active iodide uptake by dispersed thyroid cells and (2) catalase inhibits a fraction of the total iodinating activity without entering the cells and without altering iodide transport.

Perchlorate (2mM) decreased the 2h cellular ^{131}I /medium ^{131}I ratio to a value very close to 1 (Table 1). A cellular/medium ^{131}I ratio of 1 is expected to be reached, in the absence of active

Table 1. *Effect of the various agents used on ^{131}I uptake by dispersed thyroid cells*

Cells were incubated for 2 h in the presence of $0.05\text{ }\mu\text{M}$ -iodide and 2 mM -methimazole. Results are means $\pm$ s.e.m. for triplicate incubations.

Expt.	Conditions	Cellular/medium ^{131}I ratio
I	Control	7.9 ± 1.3
	NaClO_4 (2 mM)	1.1 ± 0.2
	Dimethyl sulphoxide (0.3%)	8.2 ± 1.5
	Ouabain (1 mM) in dimethylsulphoxide (0.3%)	1.7 ± 0.2
II	Control	8.5 ± 0.5
	NaClO_4 (2 mM)	1.2 ± 0.1
	Catalase (0.2 mg/ml)	8.0 ± 0.2
	NaClO_4 (2 mM) + catalase (0.2 mg/ml)	1.0 ± 0.1
III	Control	10.7 ± 0.4
	Thyrotropin (60 m-units/ml)	10.2 ± 0.5
	NaClO_4 (2 mM)	1.3 ± 0.2
	Thyrotropin (60 m-units/ml) + NaClO_4 (2 mM)	1.0 ± 0.2
IV	Control	9.0 ± 0.2
	Chlorpromazine (0.5 mM)	1.5 ± 0.2
	3,5-Dinitro-L-tyrosine (1 mM)	9.0 ± 0.1

iodide transport, by a simple diffusion process. Iodide transport was also inhibited by 1 mM -ouabain in 0.3% (v/v) dimethyl sulphoxide and by 0.5 mM -chlorpromazine. Catalase (0.2 mg/ml) did not affect the active iodide uptake, nor did it alter the diffusion of iodide into the cells (measured in the presence of perchlorate). In a 2 h incubation period, thyrotropin did not alter the cellular/medium ^{131}I ratio.

Preliminary experiments have shown that organic iodine formation by dispersed thyroid cells was sensitive to catalase in a concentration-related manner. At 0.2 mg/ml , the inhibitory effect of catalase was nearly maximum. Since higher concentrations did not produce any further statistically significant inhibition (at $P < 0.05$) (results from three concentration-response curves), the concentration of catalase of 0.2 mg/ml was used in all experiments reported in the present paper. It is noteworthy that albumin at the same concentration had no effect on organic iodine formation. Given its large molecular weight ($240\,000$), catalase probably does not enter the cells. This was checked in our experimental conditions, by using the inhibition of iodination as a marker. When thyroid cells were preincubated with 0.2 mg of catalase/ml, then washed and further incubated with ^{131}I for 2 h, acid-insoluble ^{131}I was not different from that observed in control cells (results not shown).

Acid-insoluble ^{131}I measured in the incubation media under different experimental conditions represented less than 5% of the total (cell + medium) acid-insoluble ^{131}I . Since organic iodine formation in the medium could result, at least in part, from cell lysis, values of medium acid-insoluble ^{131}I were not taken into account in our study. Therefore, reported

values of iodination represented acid-insoluble ^{131}I measured in cell pellets.

Verification of the model

In a series of ten experiments, the calculated total iodination was compared with the experimental total iodination (Fig. 2). The ratio between experimental acid-insoluble ^{131}I and calculated acid-insoluble ^{131}I was equal to 0.97 (slope of the line calculated by the least-squares method) either in control or in thyrotropin-treated cells. Similar results were obtained when ouabain [1 mM in 0.3% (v/v) dimethyl sulphoxide] was used instead of perchlorate to block active iodide transport. Since the sum of partial acid-insoluble ^{131}I (passive intracellular iodination + active intracellular iodination + extracellular iodination) differs from experimental acid-insoluble ^{131}I by less than 5% (order of magnitude of experimental errors), the theoretical model based on three ways of iodination accounts for the iodination in dispersed thyroid cells. The validity of our model was further examined over the range $0.05\text{--}100\text{ }\mu\text{M}$ -iodide (Fig. 2). Whatever the medium iodide concentration, calculated iodination was in very good agreement with experimental total iodination.

Quantitative analysis

In the basal conditions (2 h of incubation, $0.05\text{ }\mu\text{M}$ -iodide), the amount of ^{131}I incorporated into acid-insoluble material per $100\text{ }\mu\text{l}$ of cells (packed cell volume equivalent to 10^8 cells) was $7.2 \pm 1.0\%$ of the total ^{131}I (mean $\pm$ s.e.m., $n = 17$). Therefore, at nearly physiological iodide concentration ($0.05\text{ }\mu\text{M}$), 10^8 hog thyroid cells converted about 0.7 ng of iodide/h, into an organic form. As previously

reported for the tetra-iodothyronine secretion (Rousset *et al.*, 1976), the thyroid cell activity was variable from one cell preparation to another. The coefficient of variation for the parameter iodide organification was 0.60.

The relative contribution of passive intracellular iodination, active intracellular iodination and extracellular iodination to the formation of acid-insoluble ^{131}I is shown in Fig. 3. When the cells were incubated for 2 h in the presence of $0.05\text{ }\mu\text{M}$ -iodide, passive intracellular iodination, active intracellular iodination and extracellular iodination accounted respectively for 20.4 ± 1.6 , 26.0 ± 3.4 and $53.7 \pm 3.3\%$ of the total acid-insoluble ^{131}I (mean $\pm$ S.E.M., $n = 10$).

Differential modulation of the three iodinating activities

Chlorpromazine (0.5 mM), an inhibitor of iodide uptake, induced a nearly complete inhibition of active intracellular iodination but decreased extracellular iodination by only 10%. In contrast, bovine serum albumin (1 mg/ml) decreased extracellular iodination by 57%, but decreased active intracellular iodination by less than 10% (Table 2). This finding supports the extracellular location of extra-

cellular iodination. Indeed, bovine serum albumin in the incubation medium could serve as a substrate and compete with the regular substrate(s) of an iodinating system located on the outside of the cells with, as a consequence, a decrease in the iodination of the regular substrate. It should be noted that bovine serum albumin does not enter the thyroid cells *in vitro*. The cell to medium concentration ratio measured with ^{125}I -labelled albumin was always ≤ 1 (B. Rousset, unpublished work).

Decreasing the incubation temperature from 37 to 22°C resulted in a 85% decrease of active intracellular iodination, whereas extracellular iodination was unchanged. The inhibition of active intracellular iodination observed at 22°C could be related, at least in part, to a decrease in iodide uptake (Rousset, 1978). In the three experimental conditions mentioned above, passive intracellular iodination had an intermediary behaviour between active intracellular iodination and extracellular iodination.

Results in Table 2 show that passive intracellular iodination, active intracellular iodination and extracellular iodination can vary independently.

Since proteolysis of thyroglobulin and consequently deiodination of iodotyrosines normally

Fig. 2. Relationship between experimental and calculated acid-insoluble ^{131}I in dispersed thyroid cells

Calculated total iodination corresponds to the sum of partial iodinating activities (passive intracellular iodination + active intracellular iodination + extracellular iodination) measured as indicated in the Experimental model section. Cells were incubated for 2 h in the presence of $0.05\text{ }\mu\text{M}$ -iodide (O, ●) or various iodide concentrations ranging from 0.1 to $100\text{ }\mu\text{M}$ (Δ) with (●) or without (O, Δ) thyrotropin (60 m-units/ml). Each point represents the mean value for triplicate incubations. Results were obtained with ten different cell preparations.

Fig. 3. Relative contribution of passive intracellular iodination (open bar), active intracellular iodination (filled bar) and extracellular iodination (hatched bar) to the formation of acid-insoluble ^{131}I

Thyroid cells were incubated for 2 h in the presence of $0.05\text{ }\mu\text{M}$ -iodide. Passive intracellular iodination, active intracellular iodination and extracellular iodination were determined as indicated in the Experimental model section. Each point represents the value obtained in an individual experiment. The mean value for the ten determinations is indicated as a column. Results reported here correspond to those plotted in Fig. 3 (O).

Table 2. *Differential modifications of the three iodinating activities in various experimental conditions*

Cells were incubated for 2 h in the presence of $0.05 \mu\text{M}$ -iodide. Experimental and control cells were incubated in triplicate as described in the Experimental model section. The percentage inhibition of acid-insoluble ^{131}I formation was expressed as the ratio (here written for the inhibition of passive intracellular iodination by chlorpromazine) $\{[\text{passive intracellular iodination (control)} - \text{passive intracellular iodination (chlorpromazine)}] / \text{passive intracellular iodination (control)}\} \times 100$. Results are means for two or three determinations.

Conditions	Inhibition of acid-insoluble ^{131}I (%)		
	Passive intracellular iodination	Active intracellular iodination	Extracellular iodination
Chlorpromazine (0.5 mM)	60.0	97.2	10.4
Bovine serum albumin (1.0 mg/ml)	30.5	6.0	57.0
Incubation temperature (22°C)	52.4	85.2	0.3
3,5-Dinitro-L-tyrosine (1 mM)	8.0	0	0

occurs in the dispersed thyroid cell system (Rousset *et al.*, 1976), we have examined the possibility that iodide deriving from deiodination of iodotyrosines might interfere with the organification processes of labelled iodide. When cells were incubated for 2 h in the presence of 1 mM-dinitrotyrosine to prevent deiodination, none of the three iodinating activities was significantly altered (Table 2).

Whatever the changes in the incubation conditions (addition of chlorpromazine, bovine serum albumin, dinitrotyrosine, change in the temperature of incubation) the ratio between calculated iodination (passive intracellular iodination + active intracellular iodination + extracellular iodination) and experimental total iodination was within the range 0.95–1.05.

Inhibition by methimazole

Each of the three iodinating activities was completely inhibited by 0.1 mM-methimazole. The methimazole -concentration - iodination - inhibition curves were similar for passive intracellular iodination, active intracellular iodination and extracellular iodination (Fig. 4). A 50% inhibition of each iodinating process was achieved in the presence of $0.5\text{--}0.8 \mu\text{M}$ -methimazole.

Effect of thyrotropin

Thyrotropin (60 m-units/ml) increased active intracellular iodination 2.5-fold (mean for seven experiments), but did not alter passive intracellular iodination and extracellular iodination (Fig. 5a). The stimulatory effect of thyrotropin on active intracellular iodination was concentration-dependent in the range $0.1\text{--}60 \text{ m-units/ml}$ (Fig. 5b). N^6O^2 -Dibutyryl adenosine $3':5'$ -cyclic monophosphate ($0.1\text{--}2 \text{ mM}$), as well as thyrotropin, stimulated active intracellular iodination, but did not affect passive intracellular iodination or extracellular iodination (results not shown).

Fig. 4. *Inhibition by methimazole of iodide organification in dispersed thyroid cells*

Cells were incubated for 2 h in the presence of $0.05 \mu\text{M}$ -iodide with various concentrations of methimazole. Iodide organification was measured as acid-insoluble ^{131}I . Passive intracellular iodination (O), active intracellular iodination (●) and extracellular iodination (Δ) were determined as described in the Experimental model section. The values obtained in the presence of methimazole are expressed as a percentage of control values (without methimazole). Each point represents the mean for triplicate incubations of a representative experiment.

Kinetics of the iodination processes

For analysis of the time course of acid-insoluble ^{131}I formation, thyroid cells were incubated in the

Fig. 5. *Stimulatory effect of thyrotropin on iodide organification in dispersed thyroid cells*

Cells were incubated for 2 h in the presence of $0.05\text{ }\mu\text{M}$ -iodide with or without thyrotropin. Iodide organification was measured as acid-insoluble ^{131}I . Passive intracellular iodination, active intracellular iodination and extracellular iodination were determined as indicated in the Experimental model section. (a) shows the effect of thyrotropin (60 m-units/ml) on each iodinating activity. Columns and vertical lines represent means $\pm$ S.E.M. for seven determinations. (b) shows the effect of increasing thyrotropin concentration on active intracellular iodination. The results of three experiments are reported. Each point and vertical bar represents the mean $\pm$ S.E.M. for triplicate incubations. In (a), the open bar shows passive intracellular iodination, the filled bar shows active intracellular iodination and the hatched bar shows extracellular iodination.

Fig. 6. *Time course of acid-insoluble ^{131}I by passive intracellular iodination (O), active intracellular iodination (●) and extracellular iodination (Δ) in dispersed thyroid cells (a) and time course of thyrotropin stimulation on active intracellular iodination (b)*

Cells were incubated in the presence of $3.3\text{ }\mu\text{M}$ -iodide without or with thyrotropin (60 m-units/ml). Passive intracellular iodination, active intracellular iodination and extracellular iodination were determined as indicated in the Experimental model section. Each point represents the mean for duplicate incubations of a representative experiment. Symbols in (b): ▲, thyrotropin stimulation; ●, control.

presence of $3.3\text{ }\mu\text{M}$ -iodide, a concentration within the range of the apparent K_m values of the three iodinating activities (see below). ^{131}I incorporation into acid-insoluble material by passive intracellular iodination, active intracellular iodination or extracellular iodination increased linearly during the first hour of incubation. Whereas the iodination rate of control or thyrotropin-stimulated active intracellular iodination remained constant for up to 6 h, acid-insoluble ^{131}I formation by extracellular iodination plateaued after 2 h of incubation (Fig. 6). These differences in iodination rates could be related to some differences in the nature and the availability of the substrates (see below). A statistically significant thyrotropin stimulation of active intracellular iodination could be observed after 30 min of incubation ($P \leq 0.05$).

Iodide-saturating concentrations of the three iodinating systems

Fig. 7 shows the relationship between organic iodine formation and medium iodide concentration in the dispersed thyroid cell system. Acid-insoluble iodine was calculated from the specific radioactivity of medium iodide. Each type of iodination was a saturable process and had a different saturation concentration. An important consequence of this finding is that the relative contribution of each iodinating process to the total acid-insoluble-iodine formation depends on the medium concentration. At physiological iodide concentration ($0.05\text{ }\mu\text{M}$), active intracellular iodination and extracellular iodination account for 26 and 54% of the total acid-insoluble iodine respectively (Fig. 3). At a concentration of $\geq 3.0\text{ }\mu\text{M}$, active intracellular iodination become a minor iodinating system (about 5%), whereas extracellular iodination was responsible for more than 80% of the total iodination. Double-reciprocal plots of acid-insoluble-iodine formation rate against medium iodide concentration were linear. Half-maximal velocities of passive intracellular iodination, active intracellular iodination and extracellular iodination were reached when medium iodide concentrations (apparent K_m) were 9.5 ± 1.5 , 1.5 ± 0.3 and $16.5 \pm 2.2\text{ }\mu\text{M}$ (mean $\pm$ S.E.M., $n = 5$) respectively. Fig. 7(a) shows that the stimulatory effect of thyrotropin on active intracellular iodination was accounted for by an increase in the maximal velocity of the iodination process.

Qualitative analysis of [^{131}I]iodo compounds generated by each iodinating system

Results summarized in Table 3 show that ^{131}I was incorporated into iodoproteins and iodolipids. The sum of protein-bound ^{131}I (measured as ^{131}I -labelled amino acids) + lipid-bound ^{131}I (measured as ^{131}I soluble in organic solvents) was in good agreement with acid-insoluble ^{131}I . From Expts. A and B of

Fig. 7. Effect of increasing medium-iodide concentration on the rate of acid-insoluble iodine formation by passive intracellular iodination ($\circ$), active intracellular iodination ($\bullet$) and extracellular iodination (Δ) in non-stimulated cells (b) and by active intracellular iodination in basal ($\bullet$) and thyrotropin-treated cells (Δ) (a)

Cells were incubated for 1 h without or with thyrotropin (60 m-units/ml). Iodine incorporated into acid-insoluble material was calculated from the specific radioactivity of iodide in the incubation medium. Passive intracellular iodination, active intracellular iodination and extracellular iodination were determined as indicated in the Experimental model section. Each point represents the mean for triplicate incubations of a representative experiment.

Table 3, it was calculated that acid-insoluble ^{131}I differed from protein-bound ^{131}I + lipid-bound ^{131}I by less than $\pm 10\%$, using both partial and total iodination values. This finding indicates that iodolipids are precipitated along with iodoproteins by the trichloroacetic acid-precipitation procedure. The precipitation of lipidic material can be explained either by a biological interaction between cell proteins and iodinated lipidic material or by a binding of iodolipids to albumin, added as carrier protein for the precipitation procedure. When thyroid cells were incubated for 2 h in the presence of $0.05\text{ }\mu\text{M}$ -iodide, 30–40% of organic iodine was

found in iodolipids. Similar results had been reported by others (Rodesch & Dumont, 1967).

From the values shown in Table 3, passive intracellular iodination, active intracellular iodination and extracellular iodination accounted for

18.2, 8.7 and 73.0% of the total [^{131}I]iodolipid formation. The incorporation of [^{131}I]iodide into lipids is therefore mainly attributable to extracellular iodination.

Active intracellular iodination appeared to be the

Table 3. *Relative contribution of passive intracellular iodination, active intracellular iodination and extracellular iodination to the formation of [^{131}I]iodoproteins and [^{131}I]iodolipids*

Cells were incubated for 2 h in the presence of $0.05\text{ }\mu\text{M}$ -iodide. Radioiodide incorporated in iodoproteins and iodolipids was measured respectively as the radioactivity of ^{131}I -labelled amino acids and the radioactivity at the solvent front of paper chromatograms of cell homogenates previously submitted to Pronase proteolysis. In Expt. C, radioiodinated lipids were measured after chloroform/methanol extraction. Each series of triplicate incubations was performed in duplicate. One set served for the determination of acid-insoluble ^{131}I and the second set was used for the chromatographic analysis or the chloroform/methanol extraction. Passive intracellular iodination, active intracellular iodination and extracellular iodination were determined as described in the Experimental model section. Results are presented as absolute values (c.p.m./vial) and percentages of total radioactivity values (in parentheses). Absolute values are expressed as means $\pm$ S.E.M. for triplicate incubations.

Expt.	Parameters	Radioactivity (c.p.m./vial)			Total
		Passive intracellular iodination	Active intracellular iodination	Extracellular iodination	
A	Acid-insoluble ^{131}I	5539 ± 167 (13.7)	7597 ± 394 (18.8)	27187 ± 344 (67.4)	40323
	^{131}I -labelled amino acids	4156 ± 119 (15.0)	6344 ± 477 (22.9)	17162 ± 506 (62.0)	27662
	^{131}I at the solvent front	1934 ± 60 (14.3)	854 ± 38 (6.3)	10720 ± 115 (79.4)	13508
B	Acid-insoluble ^{131}I	7547 ± 369 (16.9)	12979 ± 1878 (29.1)	24022 ± 671 (53.9)	44548
	^{131}I -labelled amino acids	4436 ± 84 (14.9)	9814 ± 1101 (33.0)	15450 ± 597 (52.0)	29700
	^{131}I at the solvent front	3480 ± 216 (19.9)	1986 ± 408 (11.3)	12074 ± 1096 (68.8)	17540
C	Acid-insoluble ^{131}I	8320 ± 251 (20.6)	10490 ± 527 (26.0)	21588 ± 508 (53.4)	40398
	Chloroform/methanol-extractable ^{131}I	3135 ± 142 (20.5)	1315 ± 223 (8.6)	10842 ± 605 (70.9)	15292

Table 4. *Relative contribution of passive intracellular iodination, active intracellular iodination and extracellular iodination to the formation of tetra- ^{131}I iodothyronine*

Cells were incubated for 6 h in the presence of $0.05\text{ }\mu\text{M}$ -iodide, with or without thyrotropin (60 m-units/ml). Each series of triplicate incubations was performed in duplicate; one set served for the determination of acid-insoluble ^{131}I and the second set was used for extraction of tetra- ^{131}I iodothyronine by anion-exchange chromatography after Pronase proteolysis. Passive intracellular iodination, active intracellular iodination and extracellular iodination were determined as indicated in the Experimental model section. Results are expressed as means $\pm$ S.E.M. for triplicate incubations of a representative experiment.

Conditions	Parameters	Radioactivity (c.p.m./vial)		
		Passive intracellular iodination	Active intracellular iodination	Extracellular iodination
Unstimulated cells	Tetra- ^{131}I iodothyronine (a)	103 ± 21	1799 ± 23	219 ± 27
	Acid-insoluble ^{131}I (b)	4293 ± 196	8900 ± 813	11760 ± 267
	$[(a)/(b)] \times 10$	0.24	2.02	0.19
Thyrotropin-stimulated cells	Tetra- ^{131}I iodothyronine (c)	98 ± 12	7712 ± 493	204 ± 21
	Acid-insoluble ^{131}I (d)	4423 ± 125	28271 ± 605	10199 ± 120
	$[(c)/(d)] \times 10$	0.22	2.73	0.20

Fig. 8. Sedimentation-velocity pattern on a sucrose gradient (5–20%) of [^{125}I]iodo compounds synthesized in dispersed thyroid cells

Cells were incubated in the presence of $0.05\text{ }\mu\text{M}$ -iodide for 2 h. The concentrations of catalase and perchlorate were 0.2 mg/ml and 2 mM respectively., Control; —, catalase; ---, catalase + NaClO_4 .

most specific iodinating system as far as iodination of a protein substrate is concerned. On average, 80% of [^{125}I]iodide organified by active intracellular iodination was found in the protein-bound iodine fraction.

Active intracellular iodination was responsible for more than 85% of [^{125}I] incorporation in tetra-iodothyronine (Table 4). The efficiency of each iodination process for hormone synthesis was compared by using the ratio tetra-[^{125}I]iodothyronine/acid-insoluble [^{125}I]. It appeared that active intracellular iodination was about ten times more efficient for tetra-iodothyronine synthesis than were passive intracellular iodination or extracellular iodination. Only active-intracellular-iodination-mediated tetra-[^{125}I]iodothyronine synthesis was increased when cells were incubated in the presence of thyrotropin.

The centrifugation analysis on a sucrose gradient revealed that dispersed thyroid cells incorporated iodide into 4–8S moieties, 17–19S thyroglobulin and particulate material found at the bottom of the tube (Fig. 8). In the presence of catalase, [^{125}I] incorporation into 4–8S species no longer occurred, whereas iodination of the 17–19S fraction was not affected. When cells were incubated with catalase and perchlorate, the 17–19S fraction disappeared. Therefore, extracellular iodination is involved in the iodination of the 4–8S fraction, whereas active

Fig. 9. Effect of puromycin (hatched columns) or cycloheximide (filled columns) on the acid-insoluble- ^{125}I formation by passive intracellular iodination (a), active intracellular iodination (b) and extracellular iodination (c)

Cells were preincubated with 0.5 mM -puromycin or cycloheximide for 30 min then incubated with [^{125}I]iodide ($0.05\text{ }\mu\text{M}$) for 2 h. Passive intracellular iodination, active intracellular iodination and extracellular iodination were determined as indicated in the Experimental model section. Each column and vertical bar represents the mean $\pm$ S.E.M. for triplicate incubations.

intracellular iodination preferentially iodates 17–19S thyroglobulin.

To determine whether active intracellular iodination iodinated pre-existing or newly synthesized thyroglobulin, the experiment shown in Fig. 9 was conducted. Puromycin or cycloheximide at 0.5 mM concentration induced a 50–70% decrease in active intracellular iodination (Fig. 9). No significant changes in passive intracellular iodination or extracellular iodination were observed. The thyrotropin stimulatory effect on active intracellular iodination was decreased neither by puromycin nor by cycloheximide (results not shown). In separate experiments, these agents were tested for their possible inhibitory effect on active iodide uptake. In agreement with previous reports (Wilson *et al.*, 1968; Knopp *et al.*, 1970), we found that the basal cellular/medium [^{125}I] ratios were decreased at the most by 10–15% by either of the two protein-synthesis inhibitors.

Discussion

Experimental data are in agreement with theoretical model involving three iodination processes in the dispersed thyroid cell system. It must be emphasized that the sum of the partial iodinating activities, i.e. passive intracellular iodination + active intracellular iodination + extra-

cellular iodination, fitted very well with the total experimental iodination, whatever the incubation conditions, i.e. duration and temperature of incubation, and whatever the iodide concentration or the amount of thyrotropin stimulation.

In the dispersed thyroid cell system, organic iodine formation involves three different pathways: a perchlorate-sensitive iodination, a catalase-sensitive iodination and a perchlorate- and catalase-resistant iodination.

Since the perchlorate-sensitive iodination is also ouabain- and chlorpromazine-sensitive and since all these agents inhibited active iodide transport, probably by a different mechanism, it is concluded that the perchlorate inhibitory effect is related to the inhibition of iodide entry into the cells and therefore that the perchlorate-inhibited iodination process is intracellular.

The inhibitory effect of catalase on a definite fraction of the protein + lipid iodination in dispersed thyroid cells seems to be attributable to an extracellular action of catalase. First, catalase did not exhibit any inhibitory effect when cells preincubated with catalase were washed and further incubated in a catalase-free medium. Secondly, the catalase-sensitive iodination was selectively decreased by addition of bovine serum albumin in the incubation medium. Bovine serum albumin that is not trapped by the cells could compete with cell components for the substrate site of an extracellular iodinating enzyme. Although experimental data provide support for an extracellular location of the catalase-sensitive iodination, we must take into account the possibility that catalase might inhibit iodination in broken cells. However, considering the total amount and the kinetics of organic iodine formation by the catalase-sensitive iodination, this appears very unlikely. Indeed, cell lysis, estimated either by Trypan Blue exclusion or by medium-protein measurement (Rousset *et al.*, 1976), increased with the duration of incubation but did not represent more than 10% after 6 h. In contrast, acid-insoluble- ^{131}I formation by the catalase-sensitive iodination was rapid during the first hour of incubation then plateaued and the catalase-sensitive iodination (extracellular iodination) was responsible for 50–80% of the total iodination depending on medium iodide concentration (see Figs. 4, 7 and 8). In addition, if catalase-sensitive iodination proceeded in broken cells, one might expect this iodination reaction to generate the same iodinated compounds as the perchlorate-sensitive iodination. In fact, extracellular iodination preferentially iodinated 4–8S material, whereas active intracellular iodination generated ^{131}I -labelled thyroglobulin (see Fig. 8).

Most probably, catalase-sensitive iodination is located on the outside of the cell membrane. That extracellular iodination was responsible for a large

proportion of the iodolipid formation occurring in the dispersed thyroid cell system would argue in favour of such a location. Indeed, since the incubation medium did not contain any protein, one might expect an extracellular iodination located on plasma membranes to iodinate available substrates such as lipid components of the membrane. The existence of an extracellular iodinating activity in thyroid cell suspension is in keeping with the fact that thyroid peroxidase is a firmly bound membrane component that could still be present after the trypsin-treatment procedure. Another cell membrane component, the hormone receptor, persisted after trypsin treatment, as evidenced by the cell responsiveness to thyrotropin (Rousset *et al.*, 1977).

Intracellular perchlorate-sensitive and extracellular catalase-sensitive iodinating activities were clearly distinct processes; they are differentiated by several biochemical and biological criteria. Quantitatively, extracellular iodination is the predominant iodinating system. But active intracellular iodination is the most specific and the most efficient process as far as the protein substrate and hormone synthesis are concerned (see Table 4). Perchlorate- and catalase-resistant iodination presumably using iodide that diffuses into the cells exhibited intermediary properties. Saturating iodide concentrations expressed in terms of medium-iodide concentration differed for each process of iodination. But, because the iodide concentration of the intracellular pool was obviously higher than medium-iodide concentration, iodide concentrations determining half-maximal velocities cannot be directly compared. Assuming that intracellular iodide concentration was 8–10-fold higher than medium-iodide concentration (the order of magnitude of the cellular/medium ^{131}I ratio measured in methimazole-treated cells) half-maximal saturating iodide concentrations for active intracellular iodination and for passive intracellular iodination and extracellular iodination are in the range 8–18 μM . These data are in keeping with the K_m value for iodide obtained with purified thyroid peroxidase (Taurog, 1970). Together with the similar sensitivity to methimazole, they suggest that the iodinating mechanisms of the three pathways are identical if their location is different.

From a biological point of view, intracellular perchlorate-sensitive iodination exhibited interesting properties. (1) This iodination process was stimulated by thyrotropin. It must be noted that the thyrotropin stimulatory effect was not mediated by an increase in the concentration of the intracellular iodide pool, since thyrotropin had no effect on the iodide-trapping mechanism in a 2 h incubation period. Furthermore, it seems unlikely that an enhancement of protein synthesis could be involved in the thyrotropin effect, because it was unaffected

by puromycin or cycloheximide. More probably, thyrotropin increased the availability of H_2O_2 or acted on the intracellular translocation of the protein substrate thyroglobulin. (2) The preferential protein substrate for active intracellular iodination was thyroglobulin. Accordingly, active intracellular iodination had a high efficiency for tetra-iodothyronine synthesis compared with either passive intracellular iodination or extracellular iodination. Data obtained with protein-synthesis inhibitors suggest that active intracellular iodination iodinated both pre-existing and newly synthesized thyroglobulin (see Fig. 9).

In conclusion, our study provides support for the existence of both intracellular and extracellular sites of iodination in dispersed thyroid cells. (1) The extracellular iodination could be representative of the apical plasma membrane iodinating activity observed in intact thyroid tissue. The fact that this iodination process produced much iodolipid and little hormone in dispersed thyroid cells could be explained by a low supply of extracellular substrate in our system, contrary to what happens in the colloid lumen of follicular organized tissue. Attempts to reconstitute the situation *in vivo* by addition of protein (thyroglobulin or albumin), at high concentration in the incubation medium have been unsuccessful. Protein concentrations higher than 3.0–5.0 mg/ml induced a cell aggregation and altered the capacity of cells to metabolize iodide. (2) Data reported in the present paper show that an efficient iodination can take place inside the follicular cell *in vitro*. Since there is no evidence for a main alteration in the iodine metabolic pathway in dispersed thyroid cells, we suggest that this phenomenon may be operative *in vivo*. Although further studies are obviously required to ascribe a physiological role to an intracellular iodinating system in the normal thyroid gland, it seems reasonable to postulate that such a process could represent an important pathway under certain circumstances characterized by the absence of follicular structures, as in mammalian embryos, in lower vertebrates, in carcinomas or after chronic stimulation. (3) Finally, the present report might reconcile previous work describing either extracellular or intracellular sites of iodination in the thyroid tissue.

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