Iodinated Phospholipids and the *in vitro* Iodination of Proteins of Dog Thyroid Gland

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Slices of dog thyroid gland were incubated with liposomes consisting of ^{125}I -labelled phosphatidylcholine (the iodine was covalently linked to unsaturated fatty acyl chains). The ^{125}I label of ^{125}I -labelled liposomes was incorporated into thyroid protein and/or thyroglobulin at a higher rate than was the ^{131}I label of either Na^{131}I or $^{131}\text{I}_2$. The iodine was shown to be protein-bound by the co-migration of the labelled iodine with protein under conditions where free iodine, iodide and lipid-bound iodine were removed from protein. The uptake of iodine from the iodinated phospholipid was probably due to phospholipid exchange between the iodinated liposomes and the thyroid cell membrane, since (a) ^{14}C -labelled phospholipid was metabolized to $^{14}\text{CO}_2$ and (b) many lipids in the tissue slice became ^{14}C -labelled. A very strong inhibition of iodide 'uptake' from Na^{131}I , caused by thiosulphate, produced only a minor inhibition of the incorporation of ^{125}I from ^{125}I -labelled liposomes into thyroid protein and/or thyroglobulin. This implies that free iodide may not necessarily be formed from the iodinated phospholipids before their entrance or utilization in the cell. Synthetic polytyrosine polypeptide suspensions showed some iodination by ^{131}I -labelled liposomes. In tissues with low tyrosine contents, such as liver and kidney, only a trace uptake was observed. Salivary gland showed some uptake. Endoplasmic reticulum of thyroid gland showed a higher iodine uptake than that of the corresponding plasma membranes. These experiments, together with the demonstration of the diet-dependent presence of iodinated phospholipids in dog thyroid, leads us to suggest that iodination of the membrane phospholipids of thyroid cells may be directly or indirectly involved at some stage in the synthesis of thyroglobulin, or exists as a scavenger mechanism, to re-utilize and/or recover released iodine from unstable compounds inside the thyroid cell.

We have demonstrated by nuclear magnetic resonance the presence of an iodinated methine proton peak at $\delta = 4.5$ p.p.m. for covalently bound iodine in the fatty acyl moieties of the phospholipids of dog thyroid glands (Zanger & Rabinowitz, 1975; Rabinowitz *et al.*, 1976). The amount of bound iodine was related to the dietary iodine intake. The present investigation was undertaken to determine whether these iodinated phospholipids have some role in the 'uptake' of iodine *in vitro* by thyroid proteins and/or thyroglobulins, and also to ascertain if this iodine 'uptake' by the proteins is iodination and not an artifact.

The results show that iodinated phospholipid liposomes contribute iodine for iodination of thyroid proteins and/or thyroglobulin and to synthetic polytyrosine polypeptides and that they do so at higher rates than do free iodide or iodine. Evidence was also found that suggests that iodinated phospholipids were better iodinating agents in several other situations than was iodine or iodide.

Methods

Biological materials

Male beagle dogs approx. 15–20 months old were used in these studies. The animals were kept on a low-iodine diet (ICN, Cleveland, OH, U.S.A.). The thyroid gland was surgically removed under anaesthesia and kept in Krebs–Ringer phosphate buffer, pH 7.2 (De Luca & Cohen, 1964) at 0°C with an O_2 gas phase. Immediately after removal of the gland, thin slices (0.5 mm) were prepared by use of a Stadie–Riggs microtome. After incubation, the slices were thoroughly washed (with 3×25 ml of the Krebs–Ringer phosphate buffer) and assayed for lipids, protein and iodine. The controls used were preboiled thyroid slices (3 min at 100°C).

Incubation

Tissue slices were incubated in a Dubnoff shaker in beakers or in conical flasks with an inner cup so that $^{14}\text{CO}_2$ formed from the ^{14}C -labelled precursors

could be absorbed in Hyamine solution and radioassayed at various time intervals (Rabinowitz & Hercker, 1974). After removal, the slices were immediately placed into 100ml of ice-cold 10% (w/v) trichloroacetic acid and washed with 100ml of chloroform/methanol (1:1, v/v), then with an additional 100ml of ice-cold 10% trichloroacetic acid.

Preparation of iodinated phosphatidylcholine

Phosphatidylcholines obtained from animal sources (Supelco, Supelco Park, Bellefonte, PA, U.S.A.) or the total phospholipids obtained from dog thyroids were purified by chromatography (Rabinowitz *et al.*, 1971) and the pure recovered materials iodinated (Chase & Rabinowitz, 1972) with unlabelled I_2 , ^{131}I or ^{125}I (New England Nuclear, Boston, MA, U.S.A.). After iodination the solution was rechromatographed on a thin-layer plate of silica gel H with a developing system of chloroform/methanol/acetic acid/water (40:24:5:3, by vol.). The phospholipid layer has R_F 0.32–0.50 (Rabinowitz *et al.*, 1971) and was detected at the edge of the plate by exposure to I_2 vapour. After removal of the appropriate area the iodinated phospholipids were extracted from the silica gel with diethyl ether. The labelled iodinated phospholipids recovered were then checked for their labelled-iodine content by radioassay (Rabinowitz & Hercker, 1974) and for their lipid content by charring (Marsh & Weinstein, 1966). The unlabelled iodinated phospholipids were assayed for their chemical iodine content by the Williamson method (Taurog & Chaikoff, 1946). In addition, the presence of an iodinated methine proton at δ 4.5 p.p.m. was confirmed by nuclear-magnetic-resonance spectroscopy (Zanger & Rabinowitz, 1975; Rabinowitz *et al.*, 1976). The analysis revealed that 15–22% of available double bonds of the acyl component of the phosphatidylcholines had been iodinated. Total phospholipids obtained from pooled thyroids were also used in similar iodination procedures; for these 12–17% of the available unsaturated fatty acid moieties of the phospholipids were iodinated.

Chemical assays

The content of the thyroid slices was assayed for thyroglobulin (Bouchilloux *et al.*, 1964) and for the total protein content (biuret method; Itzhaki & Gill, 1964). Since linearity (Bouchilloux *et al.*, 1964) was found between total protein and thyroglobulin content, the results are equally related to either of these parameters in the determination of specific radioactivity. Lipid separations and assays were performed on the recovered tissue slices or the incubation solutions by standard methods (Rabinowitz *et al.*, 1971).

[^{131}I]- or [^{125}I]-iodinated-protein assays

The trichloroacetic acid-precipitated proteins obtained from thyroid slices after incubation with ^{131}I - or ^{125}I -labelled liposomes were washed as described above and dried in a desiccator at 0°C. Portions (1–2mg) of the homogenized protein powder were placed for 2–3 h in 10ml of 1% SDS*. The insoluble materials were separated by centrifugation (1000 g for 20 min).

(a) *Chromatography.* Samples of the stock solution of known protein content (biuret assay) containing 50–150 μ g of ^{131}I - or ^{125}I -iodinated protein were layered on top of columns (1.5 cm $\times$ 9 cm) of agarose A-1.5m (Bio-Rad Corp., Richmond, CA, U.S.A.). The eluent was 0.1% SDS in 0.1 M-sodium phosphate, pH 7.4 (Marsh, 1976). The column was kept at room temperature (23°C) at a constant pressure of 25 cm of water. The elution rate was 6ml/h; 3 ml fractions were collected. The total radioactivity recovered (92–98% of the radioactivity loaded) was found in several protein fractions. No radioactivity was found in a non-protein fraction.

(b) *SDS/polyacrylamide-gel electrophoresis.* Several runs were performed by the method of Weber & Osborn (1969) and by the method of Swaney *et al.* (1974). Portions of the stock solution containing 100–150 μ g of [^{131}I]- or [^{125}I]-iodinated protein (biuret assay) were placed on the SDS/polyacrylamide gel. The total radioactivity recovered in the protein fractions was 90–95% of the radioactivity loaded. No radioactivity was found in any portion not associated with protein.

Radioactivity assays

These were carried out with an Inter technique multi-channel gamma spectrometer capable of distinguishing ^{131}I from ^{125}I (one part in 10000). Assays were repeated at different time intervals to permit decay of the isotope with a shorter half-life, and radioassays were repeated. It was thus possible to determine accurately, up to $\pm 0.1\%$, the contribution of one iodine isotope in the channel of the other. Counting was carried out to a statistical error of $\pm 0.01\%$. For ^{14}C radioassays a Packard Tri-Carb beta spectrometer was used (Chase & Rabinowitz, 1972).

Liposomes

To 100ml of Krebs–Ringer phosphate buffer was added the various phosphatidylcholine preparations, or other lipids (1–3mg in 0.5ml of ethanol); these were then sonicated (Bronson Ultrasonic Vibrator; setting 5, 7 A d.c.) for 5 min until a homogeneous clear suspension was obtained. By electron microscopy the average particle diameter was estimated to be about 0.05 μ m. The polytyrosine used was pur-

* Abbreviation: SDS, sodium dodecyl sulphate.

chased from Pilot Chemicals (Watertown, MA, U.S.A.) and dispersed with the liposomes by additional sonication.

Excessive sonication. When the iodinated liposomes were sonicated for excessive times (20–25 min), there was obvious breakdown of materials. A small increase in sonication time of only a few minutes did not appear to change the average particle diameter materially. Sonication for 10 min caused production of somewhat smaller particles.

Incubation of thyroid slices with iodinated U-¹⁴C-labelled phosphatidylcholines

Dog thyroid slices were incubated at different temperatures, and ¹⁴CO₂ produced was measured at various times. The solutions and the slices were assayed for their [¹⁴C]lipid content (Rabinowitz *et al.*, 1971).

Lugol preparation

This strong iodine solution was prepared by the addition of 50 g of iodine to 100 g of NaI in 1 litre of water. The material was diluted to give 1.0 μM and 0.1 μM concentrations. The iodine content was assayed by the procedure described in U.S. Pharmacopoeia XIX (1971), p. 259, which consisted of titration with potassium arsenite with starch as the indicator.

Results and Discussion

Slices of dog thyroid were incubated for up to 45 min at different temperatures with liposomes made of U-¹⁴C-labelled phosphatidylcholine; ¹⁴CO₂ was released with a maximum yield at about 20 min (Fig. 1). The reaction was temperature-dependent, with the highest ¹⁴CO₂ release at 43°C. Pre-iodination of the phospholipid of the ¹⁴C-labelled liposomes did not alter the rate or amount of ¹⁴CO₂ released. Preboiled slices were incubated as controls; no significant ¹⁴CO₂ was formed.

These experiments suggest that both iodinated and non-iodinated phospholipid liposomes are capable of entering thyroid cells. These experiments, however, do not shed light on the mechanism of phospholipid uptake. It is likely, as indicated from the work of Zilversmit & Hughes (1976), that phospholipid exchange with the cell membrane may be the first step, although endocytosis and fusion cannot be ruled out. Intact ¹⁴C-labelled phosphatidylcholine as well as ¹⁴C-labelled fatty acids, glycerides and other lipids were identified in the thyroid slices after incubation with the ¹⁴C-labelled liposomes. This temperature-dependent activity is shown in Fig. 2. Maximal ¹⁴C incorporation occurred at 43°C, the temperature of greatest ¹⁴CO₂ release.

When ¹²⁵I-labelled liposomes were incubated with thyroid slices at different temperatures (Fig. 3),

Fig. 1. Release of ¹⁴CO₂ by thyroid slices incubated with iodinated U-¹⁴C-labelled phosphatidylcholine liposomes

Each incubation mixture contained 1 g of freshly prepared thyroid slices and 2 mg dry wt. of ¹⁴C-labelled liposomes containing 200 μg of unlabelled iodine and 10⁴ d.p.m. of U-¹⁴C-labelled phosphatidylcholine. The incubations were carried out in 100 ml of Krebs–Ringer buffer. Each flask contained an inner cup containing 10 ml of 2% (v/v) Hyamine solution. The ¹⁴CO₂ released from the buffer was trapped by the Hyamine solution and was sampled at different time intervals. Incubations at different temperatures were performed: ●, 43°C; ■, 37°C; ▲, 21°C; ○, 12°C. □, Slices preboiled at 100°C and then incubated at 37°C. Vertical bars represent the S.E.M. for experiments repeated at least three times. Data points without bars represent single assay determinations.

Fig. 2. ¹⁴C recovered in total lipids (phospholipids, triacylglycerols, fatty acids etc.) of thyroid slices at different time intervals after incubation with iodinated U-¹⁴C-labelled-phosphatidylcholine liposomes

Each incubation mixture contained 1 g of freshly prepared thyroid slices and 2 mg dry wt. of ¹⁴C-labelled liposomes containing 10⁴ d.p.m. of U-¹⁴C-labelled phosphatidylcholine and 200 μg of unlabelled iodine. The incubations were carried out in 100 ml of Krebs–Ringer buffer. At the stated time intervals the thyroid slices were removed, washed with buffer and extracted for 18 h with chloroform/methanol (2:1, v/v) to obtain their total lipid content. Incubations at different temperatures were performed: ●, 43°C; ■, 37°C; ▲, 21°C; ○, 12°C. □, Slices preboiled at 100°C and then incubated at 37°C. Vertical bars represent the S.E.M. for experiments repeated at least three times. Data points without bars represent single assay determinations.

Fig. 3. ^{125}I recovered in the proteins of thyroid slices incubated with ^{125}I -labelled liposomes at different temperatures

Each incubation mixture contained 1 g of freshly prepared thyroid slices and 2 mg dry wt. of ^{125}I -labelled liposomes containing $200\text{ }\mu\text{g}$ of $^{125}\text{I}_2$ (10^6 d.p.m.). The incubations were carried out in 100 ml of Krebs-Ringer buffer. At stated time intervals the thyroid slices were removed, washed and the trichloroacetic acid-precipitable protein was isolated and purified as described. Incubations at different temperatures were performed: $\bullet$, 43°C ; $\blacksquare$, 37°C ; $\blacktriangle$, 21°C ; $\circ$, 12°C . $\square$, Slices preboiled at 100°C and then incubated at 37°C . Vertical bars represent the S.E.M. for experiments repeated at least three times. Data points without bars represent single assay determinations.

maximal iodination of thyroid proteins (and/or thyroglobulin) was reached in 20 min at 43°C . At 37°C the highest yield was reached after 35 min, and at 12°C the high-yield plateau was not reached even after 45 min. Preboiled control slices showed only trace incorporation after 30–45 min.

To ascertain if the trichloroacetic acid precipitates of iodinated proteins were free of other labelled materials, many of the samples were subjected to (a) column chromatography (SDS with agarose) and (b) SDS/polyacrylamide gel electrophoresis. SDS/agarose column chromatography (six different specimens were tested) showed radioactivity (^{131}I or ^{125}I) only in the fractions containing proteins. Recovery of label was 92–98% of the radioactivity loaded, and that of protein was 80–89%. The labelled iodine was found in several protein fractions, although there were protein fractions that contained no radioactivity. By the use of thyroglobulin standards (Sigma, Chemical Co., St. Louis, MO, U.S.A.), with the same procedure it was estimated that most of the soluble proteins corresponded to the thyroglobulin protein fractions. SDS/polyacrylamide-gel electrophoresis (four different specimens were tested) showed that radioactivity was only associated with the protein bands. Some of the protein bands did not contain radioactivity. It was possible to recover 90–95% of the radioactivity loaded. Many of the protein bands corresponded to the bands obtained from the thyroglobulin standard. There was good proportionality (45–55%) between total protein applied and the protein content of bands corresponding to thyroglobulin.

To compare the iodination of thyroid proteins (and/or thyroglobulin) by Na^{131}I or $^{131}\text{I}_2$ with that by ^{125}I -labelled liposomes, the double-labelling experiments shown in Fig. 4 ($^{131}\text{I}_2$) and Fig. 5 (Na^{131}I) were carried out. The temperature was lowered to 21°C to allow more accurate measurements of early incorporation rates. Even though iodine was present in the same amount from both sources, the incorporation of ^{125}I was always faster than that of ^{131}I , yet the

Fig. 4. ^{125}I and ^{131}I recovered in the protein of thyroid slices in the presence of ^{125}I -labelled liposomes and $^{131}\text{I}_2$

Each incubation mixture contained 1 g of freshly prepared thyroid slices and 2 mg dry wt. of ^{125}I -labelled liposomes containing $200\text{ }\mu\text{g}$ of $^{125}\text{I}_2$ (10^6 d.p.m.) in 100 ml of Krebs-Ringer buffer. In addition the solution contained 0.2 mg of $^{131}\text{I}_2$ (10^6 d.p.m.), added from a solution of iodine in acetone, total amount of acetone less than $100\text{ }\mu\text{l}$. The flasks were incubated at 21°C . At the time prescribed the slices were removed, washed and the trichloroacetic acid-precipitable protein was isolated and purified as described. $\blacksquare$, $^{125}\text{I}_2$; $\square$, ^{131}I . Vertical bars represent the S.E.M. for experiments repeated at least three times. Data points without bars represent single assay determinations.

Fig. 5. ^{125}I and ^{131}I recovered in the protein of thyroid slices in the presence of ^{125}I -labelled liposomes and ^{131}I iodide

Each incubation mixture contained 1 g of freshly prepared thyroid slices and 2 mg dry wt. of ^{125}I -labelled liposomes containing $200\text{ }\mu\text{g}$ of iodine (10^6 d.p.m.) in 100 ml of Krebs-Ringer buffer. In addition the solution contained 0.2 mg (10^6 d.p.m.) of Na^{131}I . The individual flasks were incubated at 21°C . At the time prescribed the slices were removed, washed and the trichloroacetic acid-precipitable protein was isolated and purified as described. $\blacksquare$, ^{125}I ; $\square$, ^{131}I . Vertical bars represent the S.E.M. for experiments repeated at least three times. Data points without bars represent single assay determinations.

total amount incorporated of each iodine isotope after 45 min was about the same, regardless of the source of the label. These two experiments were repeated by using ^{131}I -labelled liposomes and Na^{125}I and $^{125}\text{I}_2$ (reversed labelling), with the same results; the liposomal iodine always entered first. A time-lag was noticed in the incorporation of iodide compared with that of iodine. This delay may reflect the time required for iodide oxidation from I^- to I^+ . This I^+ may then yield protein-bound iodine via iodinated phospholipid, or possibly by another mechanism.

In Fig. 6 the effect of thiosulphate on iodine uptake by protein from simultaneously present Na^{131}I and ^{125}I -labelled liposomes was studied. The uptake from Na^{131}I was almost completely inhibited, even at very low thiosulphate concentrations, whereas the uptake from ^{125}I -labelled liposomes was only decreased. This may indicate that in the transfer of iodine from the iodinated liposome to protein, no free iodide or iodine is liberated into the medium.

To determine if iodine exchange occurs between the iodine of ^{125}I -labelled liposomes and iodide or iodine in solution before or during the iodination of thyroid slices, the experiment shown in Fig. 7 was performed. Lugol in high concentrations ($1.0\mu\text{M}$) diluted the specific radioactivity of the ^{125}I present in the system and thus maximum ^{125}I uptake did not take place. At lower concentrations ($0.1\mu\text{M}$) the effect was less severe. Iodinated liposomes recovered from this experiment had a lower $^{125}\text{I}/\text{P}$ ratio after incubation than before. This indicates that at 21°C after 45 min, iodine exchange had taken place between

Fig. 7. ^{125}I recovered in protein of thyroid slices in the presence of ^{125}I -labelled liposomes and non-radioactive Lugol

Each incubation mixture contained 1 g of freshly prepared thyroid slices and 2 mg dry wt. of ^{125}I -labelled liposomes containing $200\mu\text{g}$ of iodine (10^6 d.p.m.). The incubations were carried out at 21°C in 100 ml of Krebs-Ringer buffer. ■, No Lugol; ●, $0.1\mu\text{M}$ -Lugol (unlabelled); ▲, $1.0\mu\text{M}$ -Lugol (unlabelled); □, slices preboiled at 100°C and then incubated at 21°C . After incubation the tissue slices were removed, washed and the trichloroacetic acid-precipitated protein was purified as described. Vertical bars represent the S.E.M. for experiments repeated at least three times. Data points without bars represent single assay determinations.

iodinated liposomes and the iodine/iodide present in solution.

When freshly iodinated thyroid slices (prepared after incubation with ^{125}I -labelled liposomes after careful washing) were incubated at 21°C for 20 min with either (a) non-iodinated liposomes or (b) ^{131}I -labelled liposomes, the recovered liposomes showed only traces of ^{125}I (0.01%). These experiments suggest that the iodine-carbon bond is firmer in the aromatic ring (of tyrosine) and less likely to exchange iodine with phospholipids.

Incubation for 20 min at 21°C of 20 mg of polytyrosine polypeptide in suspension with 2 mg dry wt. of ^{125}I -labelled liposomes containing $200\mu\text{g}$ of iodine (10^6 d.p.m.) yielded a trichloroacetic acid-precipitable ^{125}I -labelled polypeptide. The amount of protein-bound iodine was approx. 1% of the total iodine present in the system. This suggests that the transfer of iodine from a phospholipid to a protein may under some situations be non-enzymic, although small.

In thyroid slices recovered after incubation with ^{125}I -labelled liposomes, the plasma membrane had a lower content of iodinated methine proton (peak at $\delta = 4.5\text{ p.p.m.}$) than the endoplasmic reticulum of thyroid cells (Rabinowitz *et al.*, 1976) suggesting that the exchange of phospholipids from the plasma membrane to the endoplasmic reticulum was rapid.

^{125}I -Labelled phosphatidylcholine liposomes were as active iodicators as ^{125}I -labelled liposomes made of the total phospholipids from dog thyroid.

Incubation of liver, kidney, parotid or thyroid slices with ^{125}I -labelled liposomes yielded a trichloro-

Fig. 6. ^{125}I and ^{131}I recovered in protein of thyroid slices in the presence of thiosulphate, ^{125}I -labelled liposomes and ^{131}I iodide

Each incubation mixture contained 1 g of freshly prepared thyroid slices and 2 mg dry wt. of ^{125}I -labelled liposomes containing $200\mu\text{g}$ of iodine (10^6 d.p.m.) and 0.2 mg ($1.5 \times 10^6\text{ d.p.m.}$) of Na^{131}I in 100 ml of Krebs-Ringer buffer. Sodium thiosulphate was also present at various concentrations. The incubations were performed at 21°C for 45 min followed by removal of the slices, washing and isolation of the trichloroacetic acid-precipitable protein. ■, ^{125}I ; □, ^{131}I . Vertical bars represent the S.E.M. for experiments repeated at least three times. Data points without bars represent single assay determinations.

acetic acid-precipitable protein fraction. Liver and kidney had no measurable iodine in this precipitate. Parotid-gland trichloroacetic acid precipitate showed low radioactivity, amounting to 1–2% of that in thyroid slices.

These experiments suggest that membrane phospholipids may play a role in the iodination of thyroid proteins. It is not possible to conclude that the phospholipids are obligatory cofactors or intermediates, but if the conversion of iodide into I^+ occurs at the cell membrane, phospholipids may become iodinated.

We have demonstrated the presence of iodinated phospholipids in the dog thyroid. The ready exchange of plasma membranes and internal phospholipids (De Wolf *et al.*, 1976) could easily carry the iodinated phospholipid to the site of iodination of thyroid proteins. The mechanism of uptake of iodine and its addition to protein is not clear, but the present experiments indicate that iodide is not an intermediate.

Many iodinated compounds found in the thyroid are known to be rather unstable and could release iodine intermittently. A scavenger system may be needed in the cell to recover and re-utilize this released iodine. Phospholipids may also serve as a minor storage form of iodine in the fact of fluctuating thyroid and blood contents. The presence of iodinated phospholipids in the thyroid cell suggests that they may have some or all of the above roles.

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