Study of fluxes at low concentrations of L-tri-iodothyronine with rat liver cells and their plasma-membrane vesicles

Evidence for the accumulation of the hormone against a gradient

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1. Influx and efflux of L-tri-[¹²⁵I]iodothyronine with isolated rat liver parenchymal cells and their plasma-membrane vesicles were studied by a rapid centrifugation technique. 2. At 23°C and in the concentration range that included the concentration of free L-tri-iodothyronine in rat plasma (3–5 pM) influx into cells was saturable; an apparent K_t value of 8.6 ± 1.6 pM was obtained. 3. At 5 pM-L-tri-[¹²⁵I]iodothyronine in the external medium the ratios of the concentrations inside to outside in cells and plasma-membrane vesicles were 38:1 and 366:1 respectively after 7 s of incubation. At equilibrium (60 s at 23°C) uptake of L-tri-[¹²⁵I]iodothyronine by cells was linear with the hormone concentration, whereas that by plasma-membrane vesicles exhibited an apparent saturation with a K_d value of 6.1 ± 1.3 pM. 4. Efflux of L-tri-[¹²⁵I]iodothyronine from cells equilibrated with the hormone (5–123 pM) was constant up to 21 s; the amount that flowed out was $17.7 \pm 3.8\%$ when cells were equilibrated with 5 pM-hormone. When plasma-membrane vesicles were equilibrated with L-tri-[¹²⁵I]iodothyronine (556–1226 pM) $66.8 \pm 5.8\%$ flowed out after 21 s. 5. From a consideration of the data on efflux from cells and binding of L-tri-[¹²⁵I]iodothyronine to the liver homogenate, as studied by the charcoal-adsorption and equilibrium-dialysis methods, it appears that 18–22% of the hormone exists in the free form in the cell. 6. Vinblastine and colchicine diminished the uptake of L-tri-[¹²⁵I]iodothyronine by cells but not by plasma-membrane vesicles; binding to the cytosol fraction was not affected. Phenylbutazone, 6-n-propyl-2-thiouracil, methimazole and corticosterone diminished the uptake by cells, plasma-membrane vesicles and binding to the cytosol fraction to different extents. 7. These results suggest that at low concentrations of L-tri-[¹²⁵I]iodothyronine rat liver cells and their plasma-membrane vesicles accumulated the hormone against an apparent gradient by a membrane-mediated process. Contribution of cytoplasmic proteins to uptake by plasma-membrane vesicles was negligible. The amount of L-tri-[¹²⁵I]iodothyronine required to achieve half-maximal uptake agrees with that occurring in the free form in the blood, conferring physiological importance to the transporting system in the plasma membrane of the liver cell.

The normal thyroid of the human secretes mainly L-thyroxine and only a small amount of L-tri-iodothyronine (Ingbar & Woeber, 1974). The latter hormone is derived mainly from the monodeiodination of L-thyroxine (Surks *et al.*, 1973; Van Middlesworth, 1974; Chopra, 1978). About 25% of L-tri-iodothyronine present in the liver arises from

the conversion of intracellular L-thyroxine; the major amount of L-tri-iodothyronine, however, is obtained from plasma (Silva & Larsen, 1978; Silva *et al.*, 1978; Larsen *et al.*, 1979; Obregon *et al.*, 1979). Therefore a substantial amount of L-tri-iodothyronine, from non-hepatic sources, enters the liver. The entry of L-tri-iodothyronine into the cell has been shown to be a plasma-membrane-mediated process (Rao *et al.*, 1976; Pliam & Goldfine, 1977;

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Krenning *et al.*, 1978; Holm & Jacquemin, 1979; Eckel *et al.*, 1979; Holm *et al.*, 1980).

The object of the present study was to find out whether L-tri-iodothyronine accumulates in the liver cell and in the plasma-membrane vesicle, against a gradient, at concentrations of the hormone that exist in the free form in female rat plasma, thus providing information on the physiological relevance of the hormone-transporting system. For this purpose the influx and efflux of L-tri-iodothyronine with cells and plasma-membrane vesicles were investigated; together with experiments on binding to the liver homogenate, the amount of free hormone in the cell was determined. The influence of microtubule-assembly inhibitors and other drugs on influx was studied to understand the relationship between membrane-mediated translocation and intracellular binding.

Experimental

Chemicals

L-Tri-[125 I]iodothyronine (sp. radioactivity 3.7–5.0 MBq/ μ g) was from New England Nuclear Corp. (Dreieichenhain, Germany), and tritiated water and 14 C-labelled inulin were from Amersham-Buchler, Braunschweig, Germany. Unlabelled thyroid hormones were gifts from Dr. E. Scheiffele and Dr. H. Rokos (Henning-Berlin, Berlin, Germany).

Methods

The methods for the isolation of rat liver cells, preparation of plasma-membrane vesicles and the composition of buffers have been described previously (Eckel *et al.*, 1979). Protein was measured by the method of Lowry *et al.* (1951), with bovine serum albumin as standard. The purity of the plasma-membrane fraction was assessed by the assay of marker enzymes and by electron microscopy. Details have been reported previously (Eckel *et al.*, 1979).

Cellular cyclic AMP was determined by the method of Jakobs *et al.* (1976) and Steiner *et al.* (1972), with the test kit from New England Nuclear Corp.

Intracellular and intravesicular volumes were calculated from the difference between the total $^3\text{H}_2\text{O}$ -permeable space and [14 C]inulin-impermeable space (Hunter & Brierly, 1969).

In the present study 'uptake' is defined as the association of L-tri-[125 I]iodothyronine with intact viable cells or with plasma-membrane vesicles and is considered as 'total' uptake. Distinction between specific and non-specific uptake was not made. Uptake by cells was measured under initial-uptake (-velocity) conditions; wherever mentioned, uptake was also measured at equilibrium. The values given are means $\pm$ s.d. The absolute decay rate of L-

tri-[125 I]iodothyronine was determined by the method of Horrocks (1975), and the actual L-tri-[125 I]iodothyronine concentrations were calculated accordingly. Experimental details of the measurement of uptake by the 'multiple-time-point' method and calculation of the rates of uptake have been described previously (Eckel *et al.*, 1979). Vesicles stored at -40°C were kept overnight at -20°C , thawed for 3 h on ice, stirred for 10 min and equilibrated for 10 min at 23°C before use. During our investigations with L-tri-[125 I]iodothyronine it was invariably observed that at low concentrations of the hormone (3–50 pM), as used in the present studies, considerable losses of radioactivity occurred. These losses were traced back to adsorption of radioactivity on plastic test tubes, Microfuge tubes and plastic tips of the automatic pipettes. This problem was circumvented by diluting the stock solution and layering it on to the oil phase of the Microfuge tubes; eight to ten of these tubes were kept overnight at $2-4^\circ\text{C}$. On the next day the tubes were taken out and kept for 4–5 h at room temperature. To half of the tubes 20 μ l of the liver-cell suspension and to the other half 20 μ l of buffer were added, and after 21 s the radioactivities of portions (0.1 ml) from these tubes were counted along with their respective pipette tips. Both sets of tubes gave the same radioactivity values, indicating that under the conditions described radioactivity adsorbed on the interior of the Microfuge tubes did not dissociate into the medium when liver cells were added. The actual concentration of L-tri-[125 I]iodothyronine that was incubated with cells was calculated from the measured radioactivity and the specific radioactivity of L-tri-[125 I]iodothyronine.

For exit measurements L-tri-[125 I]iodothyronine was added to 150 μ l of cell suspension, yielding the hormone concentrations given in the legend to Fig. 3; the protein concentration was 100 μ g/20 μ l. After 60 s 20 μ l of the cell suspension was transferred to the buffer phase in Microfuge tubes. At the time intervals shown in Fig. 3, the tubes were rapidly centrifuged to stop the exit of the hormone. The value for cell-associated radioactivity at zero time was obtained by transferring 20 μ l of the equilibrated cells into Microfuge tubes that did not contain buffer, followed by centrifugation. Plasma-membrane vesicles were equilibrated with L-tri-[125 I]iodothyronine in a manner similar to that for cells (Fig. 4); 20 μ l of the suspension corresponded to 30 μ g of protein.

To prepare the homogenate and the cytosol fraction, the liver was perfused *in situ* with 200–300 ml of Hepes buffer I, pH 7.4 (Eckel *et al.*, 1979), to free it from blood. It was then removed and homogenized with Hepes buffer III, pH 7.4 (Eckel *et al.*, 1979) (1:10, w/v), in a cooled Potter-Elvehjem Teflon/glass homogenizer, followed by

filtration through a multi-pore filtration device. The cytosol fraction was obtained as described previously (Eckel *et al.*, 1979).

Binding of L-tri-[125 I]iodothyronine to liver homogenate was determined by the combination of the rapid centrifugation technique with the charcoal-adsorption method and by the equilibrium-dialysis method. The concentrations of L-tri-[125 I]iodothyronine chosen were 400–500 pM, since these concentrations are reached in the cells when they are incubated with about 5 pM-L-tri-[125 I]iodothyronine for 60 s. In the charcoal-adsorption method, 50 μ l of the homogenate, equivalent to 300 μ g of protein, was incubated at 23°C with 100 μ l of L-tri-[125 I]iodothyronine for different lengths of time. The amount of L-tri-[125 I]iodothyronine bound to the particulate fraction was determined by transferring 100 μ l of the incubation mixture to Microfuge tubes containing 20 μ l of 1 M-HClO₄, 30 μ l of silicone oil and 100 μ l of Hepes buffer III (Eckel *et al.*, 1979). The tubes were then centrifuged immediately for 20 s at 20 000 g, and the radioactivity associated with the sediment was determined in an LKB Gamma counter. In the second set of experiments the homogenate was incubated with L-tri-[125 I]iodothyronine for different lengths of time as described above, and then transferred to Microfuge tubes containing 100 μ l of Norit A charcoal (200 mg in 10 ml of Hepes buffer III) in the buffer phase. After centrifugation, portions of the supernatant were used for determination of radioactivity. The amount of L-tri-[125 I]iodothyronine bound to the homogenate is the sum of the radioactivities in the sediment (the particulate fraction in the absence of charcoal) and in the supernatant (the particulate-free fraction in the presence of charcoal). In separate experiments the values of binding of L-tri-[125 I]iodothyronine to the cytosol fraction were found to agree well with those obtained with the supernatant in the presence of charcoal. Furthermore, identical values of binding were obtained by the equilibrium-dialysis method. All values of binding were corrected for dissociation.

In the experiments on countertransport cells or vesicles were equilibrated in the absence and in the presence of unlabelled L-tri-iodothyronine (100 nM) for 5 min at 23°C. Samples were then transferred to Microfuge tubes containing buffer and L-tri-[125 I]iodothyronine (7.5 pM); after 7, 9, 11, 14, 20 and 60 s the tubes were centrifuged to stop the fluxes. Influx was calculated from the linear portion of the time curve (i.e. up to 14 s), six to eight incubations being used per time point.

The purity of the L-tri-[125 I]iodothyronine was checked weekly by t.l.c. as described previously (Eckel *et al.*, 1979). Decomposition of L-tri-[125 I]iodothyronine during chromatography was prevented by the addition of 10 μ g of unlabelled

L-tri-iodothyronine. Only L-tri-[125 I]iodothyronine that was more than 97% pure was used. The metabolism of the hormone was investigated by incubating freshly isolated cells (100 μ g of cell protein) and plasma-membrane vesicles (30 μ g of membrane protein) with 50 pM-L-tri-[125 I]iodothyronine.

In order to investigate the influence of blood proteins on uptake, rat plasma or 3.5% (w/w) bovine serum albumin in Hepes buffer III (0.18 ml) was incubated with increasing concentrations of L-tri-[125 I]iodothyronine (20 μ l) for 20 h at 2–4°C and subsequently for 60 min at 23°C; 20 μ l of the cell suspension (100 μ g of protein) was added to 0.2 ml of these mixtures, and the rates of uptake of L-tri-[125 I]iodothyronine were measured.

The effects of vinblastine, colchicine, phenylbutazone, 6-n-propyl-2-thiouracil, methimazole and corticosterone on the uptake of L-tri-[125 I]iodothyronine were studied by incubating cells and plasma-membrane-vesicle suspensions with the different reagents at 23°C for 30 min. Cells equivalent to 100 μ g of protein were then incubated with the hormone (50 pM) in quadruplicate for 7, 14 and 21 s, and the rates of uptake were calculated as mentioned above. Uptake of L-tri-[125 I]iodothyronine (50 pM) by plasma-membrane vesicles (equivalent to 30 μ g of protein) was determined after 60 s of incubation. Uptake in the absence of reagents was taken as the control. Binding of L-tri-[125 I]iodothyronine (20 pM) to the cytosol fraction, in the absence and in the presence of the different reagents, was studied as described previously (Eckel *et al.*, 1979).

All experiments were repeated three to five times with cells and plasma-membrane vesicles from different animals.

Results

Viability and intracellular space of freshly isolated liver parenchymal cells

The viability of isolated rat liver parenchymal cells, when examined by exclusion of Trypan Blue, was $88.3 \pm 3.5\%$ ($n = 35$). Intracellular water space was found to be $4.35 \pm 0.60 \mu$ l/mg of cell protein ($n = 6$) under the conditions of incubation in the presence of L-tri-[125 I]iodothyronine. The amount of L-tri-[125 I]iodothyronine associated with the extracellular water space after incubation and centrifugation was 1.3%.

Nature of plasma-membrane vesicles

As judged by the increase in the relative specific activity of phosphodiesterase I (EC 3.1.4.1), the plasma-membrane fraction was purified 28–31-fold compared with the homogenate. Contamination by the endoplasmic reticulum and mitochondria was

6% and 4% respectively. The intravesicular water space was estimated to be $3.18 \pm 0.40 \mu\text{l}/\text{mg}$ of membrane protein ($n=6$). After incubation of vesicles with L-tri- ^{125}I iodothyronine the residual amount of L-tri- ^{125}I iodothyronine associated with the extravesicular water space amounted to an average of 0.4%.

Influence of adrenaline, glucagon, insulin and L-tri-iodothyronine on the cyclic AMP content of isolated liver cells

When cells were exposed for 30 s at 37°C to adrenaline ($10 \mu\text{M}$) and glucagon ($1 \mu\text{M}$) the content of cyclic AMP increased by $170 \pm 15\%$ and $420 \pm 30\%$ (mean $\pm$ S.E.M., $n=5$) respectively as compared with the amount found in untreated cells ($100 \pm 9\%$). The values then gradually decreased and reached those of the untreated cells after 1800 s. Insulin ($1 \mu\text{M}$) lowered the cyclic AMP content by $34 \pm 5\%$.

A dose-response relationship with L-tri-iodothyronine showed that after 10 s of exposure of cells to 10 pM -hormone the content of cyclic AMP increased by $300 \pm 115\%$. After 1800 s the values returned to those of the untreated cells. The changes in concentrations of cyclic AMP in response to hormones show that the isolated liver cells possessed intact membrane receptors, active adenylate cyclase and an adequate amount of ATP.

Uptake of L-tri- ^{125}I iodothyronine at low concentrations

In the present investigation the kinetics of the uptake system were examined at low concentrations of L-tri- ^{125}I iodothyronine, which included the range

of the free concentration of the hormone in rat plasma ($3\text{--}5 \text{ pM}$). This concentration was arrived at by assuming that, as in the human, 0.3% of the total amount of L-tri-iodothyronine, about 1 nM (Fukuda *et al.*, 1975, and from our own estimation), in the rat is in the free form. The rates of uptake of L-tri- ^{125}I iodothyronine by isolated cells increased and attained saturation as the concentrations in the incubation medium increased from 3 to 55 pM (Fig. 1a). The Eadie-Hofstee plot of the data, $v/[\text{L-tri-}^{125}\text{I}]\text{iodothyronine}]$ versus v (v is the rate of uptake), did not yield a straight line (Figure not shown). When the data were analysed by the method of least squares (non-linear regression), an exponent $n=2.42$ was obtained. A likelihood ratio test of the hypothesis $n=2$ gave χ^2 value of 0.84^- (the minus denotes no significance), whereas the hypothesis of $n=1$ gave a χ^2 value of 9.18^{++} (the plus signs denote significance at the 1% level). Thus an excellent fit was obtained for $n=2$, whereas $n=1$ must be rejected (error probability $\alpha < 0.01$). In support of this finding, when the concentrations of L-tri- ^{125}I iodothyronine were squared a straight line was obtained (Fig. 1b). The apparent concentration of L-tri- ^{125}I iodothyronine required for half-maximal uptake, K_{t} , of $8.6 \pm 1.6 \text{ pM}$ was calculated by taking the square root of $1/\text{slope}$ of the regression line; the maximal uptake velocity was found to be $12.1 \pm 0.6 \text{ fmol}/\text{min}$ per mg of protein. From the Hill plot (Fig. 1b inset) h was calculated to be 1.6, suggestive of positive co-operativity.

The time course of uptake reached a steady state at 60 s. When the uptake of L-tri- ^{125}I iodothyronine by cells was measured after 60 s of incubation it was found to increase linearly with increasing con-

Fig. 1. (a) Rate of uptake of L-tri- ^{125}I iodothyronine by isolated rat liver cells as a function of the concentration of the hormone in the medium and (b) Eadie-Hofstee plot of the data in (a)

(a) Cells equivalent to $100 \mu\text{g}$ of protein were incubated at 23°C for 7, 14 and 21 s in quadruplicate. Each value was obtained from the slope of the regression line of the plot of time versus uptake. The vertical bars in this and other Figures represent 1 S.D. (b) v denotes the rate of uptake, and the concentration of L-tri- ^{125}I iodothyronine is raised to the power of 2. The inset shows the Hill plot of the data. In this and the following Figures L-tri-iodothyronine represents L-tri- ^{125}I iodothyronine.

centrations, suggesting that other processes in the cell play a decisive role when the initial uptake condition is not maintained.

Uptake of L-tri-[125 I]iodothyronine by plasma-membrane vesicles was linear up to $160\mu\text{g}$ of membrane protein/ml, and increased with time up to 60s, after which a plateau was reached. When uptake was measured after 60s an apparent saturation occurred as the concentration increased from 2 to 55pM (Fig. 2a). The Scatchard plot of the data was non-linear, suggesting positive co-operativity. However, a straight line was obtained when the concentrations of L-tri-[125 I]iodothyronine were raised to the power of 2 (Fig. 2b). The resulting apparent K_d value was calculated to be $6.1 \pm 1.3\text{pM}$, and the maximal uptake capacity $65.5 \pm 5.8\text{fmol/mg}$ of protein. Cells or plasma-membrane vesicles did not metabolize L-tri-[125 I]iodothyronine up to 2min of incubation.

When cells were incubated with the hormone (4.75pM) for 7s the total concentration of L-tri-[125 I]iodothyronine inside the cells was 181pM, which is 38-fold higher than the extracellular concentration (data from Fig. 1a). When vesicles were incubated with 5.0pM-L-tri-[125 I]iodothyronine for 7s, the intravesicular concentration was calculated to be 1834pM; this leads to an apparent gradient (defined as the ratio of the concentration of L-tri-[125 I]iodothyronine inside the lumen of the vesicle to the concentration of L-tri-[125 I]iodothyronine in the external medium) of 366:1. It is possible to calculate the gradient in the case of vesicles since they contain buffer in their lumen. That uptake of L-tri-[125 I]iodothyronine by vesicles was consistent with transport into the lumen of the

vesicles and not adsorption on the vesicle exterior was demonstrated in an experiment in which the uptake responded to changes in the osmolarity of the external medium, in that an inverse relationship was observed between uptake and osmolarity (Eckel *et al.*, 1979).

The vesicles were contaminated with very small amounts (0–3%) of cytoplasmic soluble binding components, as judged by the assay of the cytoplasmic marker enzyme glucose 6-phosphate dehydrogenase (EC 1.1.1.49). The contribution of binding proteins of the cytosol fraction to the uptake by vesicles was studied by using conditions for uptake measurements, for 60s at 23°C at a concentration of 7.5pM-L-tri-[125 I]iodothyronine, in the linear range of protein concentration. The cytosol fraction bound 0.65fmol of L-tri-[125 I]iodothyronine, whereas the vesicles took up 3.05fmol of L-tri-[125 I]iodothyronine. Therefore uptake due to the 3% contamination by cytosol proteins amounted to 0.02fmol of L-tri-[125 I]iodothyronine/mg of protein. Thus the contamination of the vesicles by cytosol binding proteins contributes to less than 0.7% to the uptake by vesicles.

Additional evidence to support that uptake of L-tri-[125 I]iodothyronine by plasma-membrane vesicles represented transport into their lumen and not adsorption was obtained from experiments on countertransport. Vesicles equilibrated with unlabelled L-tri-iodothyronine (100nM) exhibited a transient increase in the rate of uptake of L-tri-[125 I]iodothyronine, which amounted to 71.6%. When the data were analysed by the general linear model (computer program developed by Quednau, (1979), the increase was found to be highly

Fig. 2. (a) Uptake of L-tri-[125 I]iodothyronine by purified plasma-membrane vesicles at equilibrium as a function of the concentration of the hormone in the external medium and (b) modified Scatchard plot of the data in (a)

(a) Plasma-membrane vesicles equivalent to $30\mu\text{g}$ of protein were incubated at 23°C for 60s. Each point is the mean of quadruplicate incubations. (b) The concentration of L-tri-[125 I]iodothyronine is raised to the power of 2.

significant, with $P < 0.001$. In a similar experiment when cells were equilibrated with unlabelled L-tri-iodothyronine the rate of uptake of L-tri-[125 I]-iodothyronine increased by 64%. If accumulation of L-tri-iodothyronine were solely due to adsorption, then one would expect to find no increase in the rates of uptake of L-tri-[125 I]-iodothyronine.

Effects of inhibitors of the microtubule assembly, drugs and steroids on the uptake of L-tri-[125 I]-iodothyronine by cells, plasma-membrane vesicles and binding to the cytosol fraction

In a previous study (Eckel *et al.*, 1979) it was shown that thiol-group-blocking agents and inhibitors of metabolic energy decreased the uptake of L-tri-[125 I]-iodothyronine by cells. The object of the present experiment was to extend these findings by studying the effects of cytoskeleton-perturbing agents, drugs and steroids on uptake by cells and vesicles and binding to the cytosol fraction (see Table 1). Vinblastine and colchicine, both inhibitors of the assembly of microtubules, decreased the uptake of L-tri-[125 I]-iodothyronine by intact cells, but had no effect on the uptake of L-tri-[125 I]-iodothyronine by plasma-membrane vesicles or on the binding of the hormone to the cytosol fraction. Phenylbutazone, an antipyretic and analgesic drug (Smith, 1958), inhibited the uptake of L-tri-[125 I]-iodothyronine by cells and plasma-membrane vesicles and binding to the cytosol fraction. 6-n-Propyl-2-thiouracil, an inhibitor of L-thyroxine 5'-deiodinase, an enzyme that converts L-thyroxine into L-tri-iodothyronine (DeGroot & Stanbury, 1975), did not inhibit the uptake of L-tri-[125 I]-iodothyronine by liver cells and their plasma-membrane vesicles, but decreased the binding of the

hormone to the cytosol fraction. The anti-thyroid drug methimazole (DeGroot & Stanbury, 1975) inhibited the uptake of L-tri-[125 I]-iodothyronine by cells but not that by plasma-membrane vesicles; binding of the hormone to the cytosol fraction was not affected. Corticosterone is taken up by liver cells (Rao *et al.*, 1977) and plasma-membrane vesicles (Alléra *et al.*, 1979) by a membrane-mediated process; in its presence the uptake of L-tri-[125 I]-iodothyronine was inhibited by more than 50%. Binding of L-tri-[125 I]-iodothyronine to the cytosol fraction was not affected by the glucocorticoid. Oestradiol-17 β and testosterone did not affect uptake or binding.

Influence of rat plasma and bovine serum albumin on the uptake of L-tri-[125 I]-iodothyronine by cells

In the presence of plasma from normal rats or 2.8% (w/w) bovine serum albumin, uptake of L-tri-[125 I]-iodothyronine (400–1200 pM in the external medium) by cells was decreased by 80–95%.

Studies on the exit of L-tri-[125 I]-iodothyronine

In the intact liver cell a certain amount of L-tri-iodothyronine is derived from the deiodination of L-thyroxine. The question is: does L-tri-iodothyronine flow out of the cell and to what extent? Moreover, since the fraction of the hormone that is not bound will flow out, it was thought that this amount would represent the free form. Cells were equilibrated for 60 s with different concentrations of L-tri-[125 I]-iodothyronine (5–123 pM), after which outflow was determined (Fig. 3a). The cell-associated radioactivity decreased linearly with time up to 21 s, after which a plateau was reached. The cells that were equilibrated with 5 pM-L-tri-[125 I]-iodothy-

Table 1. *Influence of microtubule-assembly-perturbing agents, drugs and steroid on the uptake of L-tri-[125 I]-iodothyronine by cells and plasma-membrane vesicles and on binding to the cytosol fraction*

Cells and plasma-membrane vesicles were incubated with the different reagents at 23°C for 30 min; portions were then used to study the uptake of L-tri-[125 I]-iodothyronine (50 pM). The rate of uptake was determined with cells, whereas plasma-membrane vesicles were incubated for 60 s; the values are expressed as percentages of the control. Binding of L-tri-[125 I]-iodothyronine to the cytosol fraction was determined as described previously (Eckel *et al.*, 1979). Results are expressed as means $\pm$ S.D. for three to five experiments.

Reagent	Uptake by cells (%)	Uptake by plasma-membrane vesicles (%)	Binding to the cytosol fraction (%)
None (control)	100.0 $\pm$ 14.0	100.0 $\pm$ 10.8	100.0 $\pm$ 9.5
Vinblastine (50 μ M)	44.3 $\pm$ 10.6	103.0 $\pm$ 2.9*	91.5 $\pm$ 19.1*
Colchicine (50 μ M)	41.2 $\pm$ 11.2	104.0 $\pm$ 11.8*	112.8 $\pm$ 11.5*
Phenylbutazone (1 mM)	34.6 $\pm$ 5.0	57.3 $\pm$ 6.9	48.4 $\pm$ 13.4
6-n-Propyl-2-thiouracil (1 mM)	121.0 $\pm$ 13.0*	91.9 $\pm$ 9.1*	65.0 $\pm$ 9.2
Methimazole (1 mM)	57.3 $\pm$ 11.0	96.0 $\pm$ 4.5*	105.4 $\pm$ 12.9*
Corticosterone (1 μ M)	53.5 $\pm$ 16.2	61.1 $\pm$ 8.9	99.8 $\pm$ 10.7*

* Values not significantly different from control, $P > 0.05$.

Fig. 3. (a) Time-dependence of exit of L-tri-[125 I]iodothyronine from isolated rat liver cells equilibrated with different concentrations of the hormone and (b) efflux of the hormone from cells in dependence of the intracellular concentration of L-tri-[125 I]iodothyronine

Cells equivalent to $100\mu\text{g}$ of protein/ $20\mu\text{l}$ were exposed for 60 s at 23°C to the following concentrations of L-tri-[125 I]iodothyronine: $\square$, 5.2 pM; $\blacksquare$, 15.8 pM; $\triangle$, 35.3 pM; $\blacktriangle$, 47.4 pM; $\circ$, 71.8 pM; $\bullet$, 123.7 pM. Details of measurement of exit are mentioned in the Experimental section. The points are the means of quadruplicate incubations; the coefficients of variation ranged between 2 and 5%. (b) The intracellular concentration was obtained after taking into account the cell-associated radioactivity at zero time of efflux and the intracellular volume. The approximate values of efflux were obtained from the slopes of the regression line from the linear part of the time curve between 0 and 21 s. The vertical and horizontal bars represent 1 S.D.

ronine released an average of $17.7 \pm 3.8\%$ of L-tri-[125 I]iodothyronine into the medium. From the slopes of the linear part of the curves, from 0 to 21 s from Fig. 3(a), efflux values were calculated and plotted versus the total intracellular concentration of L-tri-[125 I]iodothyronine at zero time (Fig. 3b). Efflux appears to be a saturable process. The apparent concentration for half-maximal efflux was found to be in the nanomolar range. When the logarithms of the values on the ordinate of Fig. 3(a) were plotted against time, a linear relationship was obtained from 0 to 60 s (Figure not shown). From this plot the $t_{1/2}$ for exit (the time required for exit of 50% of cellular or vesicle-associated L-tri-[125 I]iodothyronine at 23°C) from cells was calculated to be 97 ± 23 s ($n = 5$). After a 60 s period of equilibrating the vesicles with different concentrations of L-tri-[125 I]iodothyronine (556–1226 pM) the apparent gradient ranged between 165 and 117. Exit proceeded in two phases (Fig. 4); after 21 s $66.8 \pm 5.8\%$ ($n = 5$) of the hormone had flowed out of the vesicles. The $t_{1/2}$ from 7 to 60 s, for vesicles was calculated to be 84 ± 48 s. These results suggest that L-tri-[125 I]iodothyronine taken up by liver cells and plasma-membrane vesicles can be released into the medium when the external concentration is considerably lowered.

Fig. 4. Time-dependence of exit of L-tri-[125 I]iodothyronine from purified plasma-membrane vesicles. Vesicles equivalent to $30\mu\text{g}$ of protein/ $20\mu\text{l}$ were exposed for 60 s at 23°C to the following concentrations of L-tri-[125 I]iodothyronine: $\triangle$, 556 pM; $\square$, 647 pM; $\circ$, 825 pM; $\bullet$, 1226 pM. Other details are similar to those mentioned in the legend to Fig. 3. The coefficients of variation ranged between 5 and 15%.

Determination of binding of L-tri-[¹²⁵I]iodothyronine to the liver homogenate

In order to estimate the amount of free L-tri-[¹²⁵I]iodothyronine in cells, the binding of L-tri-[¹²⁵I]iodothyronine to the liver homogenate was studied, since it was reasoned that inside the cell L-tri-iodothyronine should exist bound to different intracellular components and in the free form at equilibrium. The amount of the latter should then be the fraction that is able to leave the cell during the initial phase of outflow. At equilibrium of binding (after 60 s at 23°C) the amount of L-tri-[¹²⁵I]iodothyronine present in the free form estimated by the charcoal-adsorption method was $22.0 \pm 3.3\%$; the amount of free hormone determined by equilibrium dialysis (after 5 h at 23°C) was $17.5 \pm 0.5\%$. At the end of the equilibrium dialysis more than 95% of the radioactivity corresponded to L-tri-[¹²⁵I]iodothyronine.

Discussion

Under initial-velocity conditions and with low concentrations of L-tri-[¹²⁵I]iodothyronine, including the range of the hormone that occurs in the free form *in vivo*, uptake by rat liver cells was saturable. In previous reports (Rao *et al.*, 1976; Eckel *et al.*, 1979) several different uptake systems were found; however, this may have been due to the fact that the concentrations of the hormone in the medium ranged from 50 pM to 2500 nM. The important uptake system is the one that is operating at or near the physiological concentration. In the present study this system was found to have an apparent K_t value of 8.6 ± 1.6 pM.

After 7 s of exposure of cells to 4.74 pM L-tri-[¹²⁵I]iodothyronine, the intracellular total concentration was 38-fold higher than the concentration of the hormone in the medium. The ratio of free hormone inside the cell to that in the medium was obtained by two different experimental approaches: (1) by estimation of the efflux of L-tri-[¹²⁵I]iodothyronine from cells equilibrated with the hormone and (2) by measurement of binding of L-tri-[¹²⁵I]iodothyronine to the liver homogenate. When cells were equilibrated with L-tri-[¹²⁵I]iodothyronine for 60 s, the average intracellular total concentration was 74 ± 12 -fold higher than that in the incubation medium. From exit experiments it was found that from cells equilibrated with 5 pM-hormone 17.7% of the accumulated L-tri-[¹²⁵I]iodothyronine flowed out, indicating that this amount might represent the free form. But it is essential to remember that L-tri-[¹²⁵I]iodothyronine that dissociates from the bound pool will also contribute to the exit of L-tri-[¹²⁵I]iodothyronine. However, the amount of L-tri-[¹²⁵I]iodothyronine

that was in the unbound form in the homogenate (as determined by the charcoal-adsorption method) was found to be 17.5%. With this method the detection of rapidly dissociating components (in the range of milliseconds) is not feasible; therefore binding studies were done with the equilibrium-dialysis method. The amount of free L-tri-[¹²⁵I]iodothyronine was estimated to be 22.6%. On the basis of these findings it appears that L-tri-iodothyronine is accumulated by the isolated liver cell against a concentration gradient; the actual calculated gradient is approx. 13:1. The concentration of free L-tri-[¹²⁵I]iodothyronine in the cell at physiological concentration of the hormone in the external medium may thus amount to about 65 pM. This concentration is arrived at from studies made *in vitro*; whether it does occur in the liver cell *in vivo* is not known.

In context with the data on efflux, it was interesting to compare them with those on influx. At a physiological concentration (4 pM) of free L-tri-[¹²⁵I]iodothyronine in the external medium the total concentration of L-tri-[¹²⁵I]iodothyronine inside the cell was 320 pM after 60 s of exposure. Since about 20% represented L-tri-[¹²⁵I]iodothyronine in the free form, the concentration of the free hormone was calculated to be about 60 pM. Efflux at this concentration was 0.63 fmol/min per mg of protein. When cells were exposed to 56.6 pM free L-tri-[¹²⁵I]iodothyronine, influx was 12 fmol/min per mg of protein. Therefore a comparison of the fluxes at the same concentration of free L-tri-[¹²⁵I]iodothyronine shows that influx proceeds 19 times faster than efflux. With increasing concentrations of total L-tri-[¹²⁵I]iodothyronine in the cell, efflux reached an apparent saturation, suggesting that it may also be a mediated process. The transient increase in the uptake of L-tri-[¹²⁵I]iodothyronine by vesicles and cells, known as accelerative exchange diffusion, is suggestive of a 'mobile' carrier (Christensen, 1975) in the plasma membrane involved in the fluxes of L-tri-[¹²⁵I]iodothyronine. This phenomenon cannot be explained by adsorption.

The 'carrier'-mediated transport of L-tri-[¹²⁵I]iodothyronine is inhibited by microtubule-assembly-perturbing agents, suggesting the involvement of these structures in the transport of the hormone into cells. Vinblastine has been reported to affect the energy metabolism of Ehrlich ascites cells (Fyfe & Goldman, 1973). Previous studies (Eckel *et al.*, 1979) did suggest the involvement of cellular energy in the uptake of L-tri-[¹²⁵I]iodothyronine by intact liver cells. Colchicine has been shown to inhibit nucleoside transport in HeLa cells (Mizel & Wilson, 1972), and the inhibitory effect was attributed to an interaction with membrane component(s). The inhibition by colchicine of the transport of L-tri-[¹²⁵I]iodothyronine appears to be connected not

with plasma-membrane components but with some unknown events in the cell. The inhibition by vinblastine and colchicine of the transport of L-tri-[125 I]iodothyronine cannot be attributed to their effects on the binding of the hormone by cytoplasmic soluble components, since binding to the cytosol fraction was not affected by these agents.

L-Tri-[125 I]iodothyronine in the presence of plasma proteins, existing probably as hormone-protein complex, is not taken up by liver cells during the initial phase. It cannot be excluded, however, that at some stage dissociation of L-tri-[125 I]iodothyronine from the proteins may eventually occur, followed by uptake of the free hormone by cells.

The drugs phenylbutazone, 6-n-propyl-2-thiouracil and methimazole possess anti-thyroid activity; corticosterone in pharmacological doses decreases iodine uptake by the thyroid, clearance and turnover rates in man (Ingbar & Woeber, 1974). The present results show that these diverse compounds also affect the transport of L-tri-[125 I]iodothyronine into the liver cell of the rat and/or binding to the cytosol fraction.

It is usually accepted that steroid and thyroid hormones enter the cells by simple diffusion and that saturation of entry is due to saturation of intracellular binding proteins (for review see Rao, 1981). If binding to intracellular proteins mimics the uptake process, then it would be logical to assume that in their absence uptake should not be a saturable process. However, the uptake of L-tri-[125 I]iodothyronine by plasma-membrane vesicles does exhibit saturability with a K_d value of 6.1 ± 1.3 pM. The contribution of cytosol binding to uptake by vesicles was negligible. These results strongly suggest a mediated process of uptake for L-tri-[125 I]iodothyronine by membrane vesicles even in the absence of intracellular binding proteins. The purified plasma-membrane vesicles also accumulate L-tri-[125 I]iodothyronine against a concentration gradient. This accumulation in the absence of a known source of energy is at the moment difficult to explain. In the search for a possible explanation we considered whether the accumulation was due to the solubility of L-tri-[125 I]iodothyronine in the membrane. On the basis of a distribution coefficient of L-tri-iodothyronine of 22000 (Hillier, 1970) between the phospholipid and the buffer phase, one can imagine that L-tri-iodothyronine will be taken up instantaneously from the aqueous into the lipid phase, but it is unlikely that it would show saturability and co-operativity at the concentrations of L-tri-[125 I]iodothyronine used in the present study. Moreover, the results from experiments on the sensitivity of uptake to osmolarity, the efflux of the hormone from cells and vesicles and the phenomenon of counter-transport do not support the idea that the accumulative process is related to the solubility of L-tri-

[125 I]iodothyronine in the lipid phase of the plasma membrane.

Finally, the close correlation of the K_t and K_d values of the uptake system of cells and plasma-membrane vesicles respectively with the concentration of free L-tri-iodothyronine in rat plasma confers physiological importance to the hormone-transporting system.

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