Evidence that the uptake of tri-iodo-L-thyronine by human erythrocytes is carrier-mediated but not energy-dependent

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We investigated 3,3',5-tri-iodo-L-thyronine transport by human erythrocytes and by 'ghosts' prepared from these cells. Uptake of tri-iodothyronine by erythrocytes at 37°C was time-dependent with a maximum reached after 60 min. Tracer analysis after incubation for 1 min revealed only one saturable binding site, with K_m 128 ± 19 nM (mean $\pm$ S.E.M.; $n = 7$) and V_{max} 4.6 ± 0.7 pmol of tri-iodothyronine/min per 6×10^7 cells. After 10 min incubation K_m 100 ± 16 nM ($n = 10$) was found with V_{max} 7.7 ± 1.2 pmol of tri-iodothyronine/10 min per 6×10^7 cells. At 0°C the uptake system is still active, with K_m 132 ± 26 nM and V_{max} 1.8 ± 0.3 pmol of tri-iodothyronine/10 min per 6×10^7 cells. The V_{max} with intact cells is 5-fold greater than the V_{max} with membranes derived from the same amount of cells when uptake studies are performed in media with similar free tri-iodothyronine concentrations. This indicates that at least 80% of tri-iodothyronine taken up by the intact erythrocytes enters the cell. This saturable uptake system can be inhibited by X-ray-contrast agents in a dose-dependent fashion. ($\pm$)-Propranolol, but not atenolol, has the same effect, indicating that the membrane-stabilizing properties of ($\pm$)-propranolol are involved. Furthermore, there is no inhibition by ouabain or vanadate, which indicates that tri-iodothyronine uptake is not dependent on the activity of $Na^+ + K^+$ -dependent adenosine triphosphatase. We have prepared erythrocyte 'ghosts', resealed after 2.5 min with 0 mM-, 2 mM- or 4 mM-ATP inside. Inclusion of ATP and integrity of the membrane of the erythrocyte 'ghosts' were verified on the basis of an ATP-concentration-dependent functioning of the Ca^{2+} pump. No difference was found in the uptake of tri-iodothyronine by erythrocyte 'ghosts' with or without ATP included, indicating that uptake of tri-iodothyronine is not ATP-dependent. The following conclusions are drawn. (1) Tri-iodothyronine enters human erythrocytes. (2) There is only one saturable uptake system present for tri-iodothyronine, which is neither energy (i.e. ATP)-dependent nor influenced by the absence of an Na^+ gradient across the plasma membrane. This mode of uptake of tri-iodothyronine by human erythrocytes is in sharp contrast with that of rat hepatocytes, which uptake system is energy-dependent and ouabain-sensitive [Krenning, Docter, Bernard, Visser & Hennemann (1978) *FEBS Lett.* **91**, 113–116; Krenning, Docter, Bernard, Visser & Hennemann (1980) *FEBS Lett.* **119**, 279–282]. (3) X-ray-contrast agents inhibit tri-iodothyronine uptake by erythrocytes in a similar fashion to that by which they inhibit the uptake of tri-iodothyronine by rat hepatocytes [Krenning, Docter, Bernard, Visser & Hennemann (1982) *FEBS Lett.* **140**, 229–233].

The biological action of 3,3',5-tri-iodo-L-thyronine (referred to below simply as tri-iodothyronine) is initiated by the binding of the hormone to receptors in the target cell (Samuels, 1978). To reach the cellular compartment the hormone has to be translocated through the plasma

membrane. We have shown with transport studies with rat hepatocytes in primary culture (Krenning *et al.*, 1978) that this uptake of tri-iodothyronine is carrier-mediated and energy-dependent and can be inhibited by KCN, 2,6-dinitrophenol or oligomycin. Further investigations have shown that this active

transport of tri-iodothyronine is regulated by the ATP content of the hepatocytes (Krenning *et al.*, 1980). It was found that a highly significant positive correlation existed between the intracellular ATP concentration and the transport of tri-iodothyronine. The energy-dependency of tri-iodothyronine uptake is in harmony with the finding by Cheng *et al.* (1980) that the mechanism of tri-iodothyronine uptake is by the so-called 'receptor-mediated endocytosis'.

In the present paper we describe studies concerning tri-iodothyronine uptake by human erythrocytes. It has been reported that erythrocyte membranes bind tri-iodothyronine and thyroxine competitively (Singh *et al.*, 1976). Holm & Jacquemin (1979) have shown that human erythrocyte membranes contain two separate saturable uptake systems for tri-iodothyronine, with K_m values almost identical with those found with rat liver cells (Krenning *et al.* 1978).

We decided to investigate further the characteristics of human erythrocytes with regard to transmembranal transport of tri-iodothyronine. Our reasoning was that, if we could confirm that transport of tri-iodothyronine into erythrocytes is based on similar principles to those that apply for hepatocytes, erythrocytes could possibly be used as a model system for tissue uptake of tri-iodothyronine (and possibly other iodothyronines) in studies performed *in vivo*.

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Materials and methods

Materials

3,3',5-Tri-iodo-L-thyronine labelled at the 3'-position ($[^{125}\text{I}]$ tri-iodothyronine, $1200\mu\text{Ci}/\mu\text{g}$) was purchased from The Radiochemical Centre (Amersham, Bucks., U.K.). The iodide content of this material, as determined by high-pressure liquid chromatography [C_{18} column; methanol/aq. $7.35\text{ mM-KH}_2\text{PO}_4$ (57:43, v/v) as solvent] did not exceed 3%. Unlabelled tri-iodothyronine, X-ray-contrast agents, inhibitors of the cytoskeleton, ouabain and oligomycin were purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.). ($\pm$)-Propranolol and atenolol were a generous gift from I.C.I. (Rotterdam, The Netherlands). All other chemicals were of reagent grade and were obtained from BDH Chemicals (Poole, Dorset, U.K.). Polypropylene reaction vessels from Eppendorf (Marpurg, West Germany) were used throughout.

Preparation of the erythrocytes

Blood from healthy volunteers was collected in heparinized tubes and centrifuged at room tem-

perature. The plasma and the buffy coat were removed and the erythrocytes were washed three times with 3 vol. of ice-cold phosphate-buffered saline (150 mM-NaCl/5 mM-sodium phosphate buffer, pH 7.4). Finally, the erythrocytes were suspended in phosphate-buffered saline and diluted to obtain a relative packed-cell volume of 0.25, as estimated with a haematocrit centrifuge. This suspension will contain 3×10^5 cells/ $10\mu\text{l}$ (Dacie & Lewis, 1968). Erythrocytes were kept at 4°C until use.

Preparation of erythrocyte lysate

Erythrocytes were mixed with 5 mM-sodium phosphate buffer, pH 7.4, containing 4 mM- MgCl_2 . After 10 min membranes were separated by centrifugation at $9500g$ for 1 min. Then $10\mu\text{l}$ of 2 M-KCl and $65\mu\text{l}$ of 2 M-NaCl per ml were added to the supernatant to obtain iso-osmoticity (130 mM-NaCl and 20 mM-KCl).

Uptake studies with erythrocytes

Incubation mixtures (0.8 ml) were made in Eppendorf reaction vessels containing 6×10^7 cells in phosphate buffer (130 mM-NaCl/20 mM-KCl/4 mM- MgCl_2 /5 mM-sodium phosphate buffer, pH 7.4). After temperature equilibration at 37°C during 15 min, the reaction was started by the addition of $[^{125}\text{I}]$ tri-iodothyronine with various concentrations of unlabelled tri-iodothyronine in 0.2 ml of phosphate buffer at 37°C . The reaction vessels were closed and the contents were mixed and incubated for 1 min or 10 min. The cells were then separated from the medium by centrifugation at $9500g$ for 1 min. Cells were pelleted within the first 10 s of centrifugation. The supernatant was discarded and the radioactivity in the pellet was counted after an additional wash with ice-cold phosphate buffer. Blanks for all hormone concentrations used were prepared in the same way, but the centrifugation was omitted, to correct for non-specific binding to the reaction vessels.

Binding studies with erythrocyte membranes were performed similarly, except that NaCl and KCl were omitted from the phosphate buffer, which caused lysis of the cells. To correct for non-specific binding to the membrane and/or diffusion the fractional uptake at the highest tri-iodothyronine concentration used ($7.7\mu\text{M}$) was subtracted from the fractional uptake values obtained at lower concentrations of tri-iodothyronine. Then a double-reciprocal plot was constructed to assess the kinetic parameters.

Measurement of the inhibition of tri-iodothyronine uptake by various compounds

Incubation mixtures (0.8 ml) were made in phosphate buffer containing 6×10^7 cells, with or without

the compound to be tested (added as 0.08 ml of a 10-fold concentrated solution in phosphate buffer). After 30 min preincubation at 37°C, the reactions were started by the addition of tri-iodothyronine and tracer tri-iodothyronine in 0.2 ml of phosphate buffer. Incubation for 10 min and separation of cells and medium were performed as described above. Specific uptake of tri-iodothyronine was calculated as the difference in percentage uptake between a low dose of hormone (1.5 nM-tri-iodothyronine), far below the K_m of the transport process, and a very high concentration of tri-iodothyronine (7.7 μ M). Percentage inhibition was calculated as the percentage fall in specific uptake in erythrocytes in the presence of the compound tested versus control incubations of mixtures not containing the inhibitor.

Preparation of pink erythrocyte 'ghosts'

Erythrocyte 'ghosts' were prepared as previously described (Larsen *et al.*, 1978), with some modifications. Erythrocytes were haemolysed by mixing 1 vol. of packed cells with 10 vol. of hypo-osmotic buffer containing 10 mM-Tris/HCl buffer, pH 7.4, 4 mM-MgCl₂, 1 mM-CaCl₂ and various concentrations of ATP (0–4 mM). After 2.5 min the erythrocyte 'ghosts' were resealed by addition of 2 M-KCl and 2 M-NaCl solutions to adjust the concentration of KCl to 20 mM and that of NaCl to 130 mM. The whole procedure was performed at 0–4°C, and the erythrocyte 'ghosts' were kept on ice until use. When the erythrocyte 'ghosts' were used to study the function of Ca²⁺-stimulated ATPase, ⁴⁵Ca²⁺ was included in the hypo-osmotic buffer (final specific radioactivity 10⁵ d.p.m./ μ mol of Ca²⁺) and the sealed erythrocyte 'ghosts' were washed twice with Tris buffer (4 mM-MgCl₂ / 1 mM-CaCl₂ / 130 mM-NaCl / 20 mM-KCl / 10 mM-Tris/HCl buffer, pH 7.4) to remove radioactive Ca²⁺ not incorporated into the erythrocyte 'ghosts'.

Preparation of white erythrocyte 'ghosts'

For comparison purposes erythrocyte 'ghosts' were also prepared as described by Holm & Jacquemin (1979). Erythrocytes were haemolysed by mixing 1 vol. of packed cells with 40 vol. of 5 mM-sodium phosphate buffer, pH 8.0. After 10 min the membranes were collected by centrifugation at 22 000 g for 10 min at 4°C. They were washed five times with the same buffer to remove all haemoglobin. Then the membranes were suspended in the hypo-osmotic buffer used to prepare pink erythrocyte 'ghosts' and treated further as described in the preceding paragraph.

Measurement of the Ca²⁺ pump in erythrocyte 'ghosts'

Transport experiments were started by warming

the ⁴⁵Ca²⁺- and ATP-loaded erythrocyte 'ghosts' suspended in Tris buffer at 37°C. Packed 'ghosts' were diluted 4-fold. After various time intervals, 0.5 ml samples were chilled on ice and centrifuged at 9500 g for 1 min. The packed 'ghosts' were washed once with ice-cold Tris buffer, treated with 6% (w/v) HClO₄ and centrifuged, and the radioactivity of samples of supernatant were counted.

Statistical analysis

Statistical analysis of the data was performed by using Student's *t* test for unpaired groups (Snedecor & Cochran, 1967).

Results

Uptake of tri-iodothyronine at 37°C by human erythrocytes is time-dependent (Fig. 1). Binding of the hormone is maximal and constant after 60 min of incubation; 60% of the maximal value is obtained within 10 min. There is a linear relationship between the number of erythrocytes in the incubation mixture and the uptake of tri-iodothyronine by the cells, both at a low concentration of hormone and at a much higher concentration (Fig. 2). From the fact that the fraction of the hormone bound by the cells is lower at high concentrations of tri-iodothyronine, it can be concluded that the process is saturable.

To extend this finding further, cells were incubated with various concentrations of tri-iodothyronine, and the fraction associated with the cells was plotted against the logarithm of the con-

Fig. 1. Tri-iodothyronine uptake by erythrocytes plotted versus time

Erythrocytes (6×10^7) were incubated with 15 pmol of tri-iodothyronine in 1 ml of phosphate buffer for various time intervals. For full experimental details see the text. Time in minutes is plotted on the abscissa. On the ordinate the amount of tri-iodothyronine associated with the erythrocytes is shown as percentage of total tri-iodothyronine added. Bars indicate s.d. Each data point is the mean for five different experiments performed in duplicate.

Fig. 2. Tri-iodothyronine uptake (1 min) by erythrocytes plotted versus the number of cells

Various amounts of erythrocytes were incubated with 15 pmol (●) or 3 nmol (▲) of tri-iodothyronine in 1 ml of phosphate buffer. For full experimental details see the text. Number of erythrocytes is plotted on the abscissa, and the amount of tri-iodothyronine bound to the erythrocytes after 1 min of incubation is given on the ordinate. Each data point is the mean of two experiments, performed in duplicate.

centration of the hormone (Fig. 3). A sigmoidal pattern, typical for a saturable process, is observed, both after 1 min and after 10 min incubation.

Extension of the concentration range down to 0.5 pM-tri-iodothyronine did not reveal a second saturable binding site; extension to higher concentrations was not possible owing to the limited solubility of the hormone. From these findings it can be concluded that only one saturable uptake system for tri-iodothyronine exists in erythrocytes. Plotting these data in a double-reciprocal plot (Fig. 4) revealed that both curves have the same intercept with the abscissa. This indicates that the K_m of the uptake process is not dependent on incubation time. The intercepts with the ordinate are different, showing a higher maximal uptake after 10 min incubation than after 1 min. A summary of the uptake parameters is listed in Table 1.

If maximal uptake velocities are calculated it appears that uptake during the first minute is 6-fold greater than the mean uptake during the first 10 min. This finding is in accordance with the curved relationship between uptake of hormone and time (Fig. 1). At 0°C the uptake system is still active, although the maximal velocity is significantly lower than at 37°C (Table 1).

Fig. 3. Tri-iodothyronine uptake by 6×10^7 erythrocytes plotted versus the concentration of tri-iodothyronine. For full experimental details see the text. On the ordinate the fractional uptake of hormone is plotted, and on the abscissa the logarithm of the concentration of tri-iodothyronine in the incubation mixtures. Data points are means $\pm$ S.D. for ten (■; 10 min incubation time) or seven (▲; 1 min incubation time) experiments. Each experiment was performed in triplicate and the data points were corrected for non-saturable binding by subtraction of the percentage uptake of hormone at a tri-iodothyronine concentration of $7.7 \mu\text{M}$ before further calculations were performed. Non-saturable binding was the same at $1.5 \mu\text{M}$ - and $7.7 \mu\text{M}$ -tri-iodothyronine added to the cells and amounted to $2.5 \pm 0.8\%$ (mean $\pm$ S.D., $n = 7$) after 1 min incubation and $6.5 \pm 2.1\%$ (mean $\pm$ S.D., $n = 10$) after 10 min incubation.

In order to answer the question whether tri-iodothyronine enters human erythrocytes, we measured the binding of tri-iodothyronine to erythrocyte membranes, prepared by lysis of cells, and compared the results obtained with the uptake parameters for intact cells. Because the incubation mixture of the membranes still contained the cell cytosol liberated during lysis, we have incorporated a similar amount of cytosol in the incubation mixtures of the intact erythrocytes. Although it is known that erythrocyte cytosol contains tri-iodothyronine-binding proteins (Yoshida & Davis, 1977; Davis *et al.*, 1980), it has also been shown that association of tri-iodothyronine with these proteins proceeds only slowly, about 5% of the added tri-iodothyronine being bound during the first 30 min. Because we started the incubations by the addition of hormone, it is therefore assumed, for the calculations of the kinetic parameters, that all tri-iodothyronine in the incubation mixtures was unbound.

From the difference in maximal uptake velocity between membranes and intact erythrocytes (Table 1) it can be concluded that after 10 min only 16% of the hormone is associated with the membranes of

Fig. 4. Double-reciprocal plot of tri-iodothyronine uptake by erythrocytes

Erythrocytes (6×10^7) were incubated with various concentrations of tri-iodothyronine for 1 min (▲) or 10 min (■). Means $\pm$ s.d. are plotted of the reciprocals of values for tri-iodothyronine uptake for seven (▲) and ten (■) experiments respectively. For further experimental detail see the text and the legend to Fig. 3.

erythrocytes, the remainder (i.e. 84%) being transported into the interior of the cell.

In order to determine whether the uptake of tri-iodothyronine by erythrocytes is dependent on ATP, we prepared pink erythrocyte 'ghosts' with-out or with 4 mM-ATP included after resealing. With these 'ghosts' we performed tri-iodothyronine-uptake studies during 10 min at 37°C. There is no significant difference between the kinetic parameters of the erythrocyte 'ghosts' with ATP included and those without ATP (Table 1). This indicates that the presence of ATP is not necessary for the uptake process.

To assess the integrity of the membranes of the erythrocyte 'ghosts' used in the tri-iodothyronine-uptake studies and to verify the inclusion of the ATP inside these 'ghosts', we performed studies with $^{45}\text{Ca}^{2+}$ to measure the activity of the Ca^{2+} pump. Normally the erythrocyte membrane maintains a steep Ca^{2+} gradient, with concentrations of Ca^{2+} higher outside than inside the cells. The gradient is maintained by a low permeability to Ca^{2+} in the inward direction and an ATPase that is stimulated by Ca^{2+} and requires Mg^{2+} . This ATPase is directly responsible for Ca^{2+} efflux (Schatzmann, 1973). The enzyme sites for ATP and Ca^{2+} are located on the inner face of the membrane.

Fig. 5 shows that the efflux of Ca^{2+} from the interior of pink erythrocyte 'ghosts' is dependent on the concentration of ATP inside. This indicates that ATP is indeed included inside these erythrocyte 'ghosts'. Since only a small amount of Ca^{2+} (about 15%) diffuses out of the erythrocyte 'ghosts' when no ATP is present inside, it can be concluded that the membranes of pink erythrocyte 'ghosts' are still impermeable for small ions.

Finally, we performed studies with various compounds to test interference with tri-iodothyronine uptake. A summary of the results is given in Table 2.

Table 1. Kinetic parameters of tri-iodothyronine uptake in erythrocytes (6×10^7), erythrocyte membranes (of 6×10^7 cells) or erythrocyte 'ghosts' (6×10^7)

For full experimental details see the text. Key to medium used: A, phosphate buffer; B, phosphate buffer with lysate of 6×10^7 cells; C, phosphate buffer without NaCl and KCl, which causes lysis of the added cells. All experiments were performed in triplicate. Kinetic parameters of each individual experiment were calculated and means $\pm$ s.e.m. are reported.

Incubation conditions			Temp. (°C)	No. of observations	K_m (nM)	V_{max} , (pmol per 6×10^7 cells)
Material	Medium	Time (min)				
Erythrocytes	A	10	37	10	100 ± 16	7.7 ± 1.2
Erythrocytes	A	1	37	7	128 ± 19	4.6 ± 0.7
Erythrocytes	A	10	0	3	132 ± 26	1.8 ± 0.3
Erythrocytes	B	10	37	6	127 ± 12	9.6 ± 1.1
Membranes	C	10	37	5	75 ± 10	1.6 ± 0.3
'Ghosts' (0 mM-ATP)	A	10	37	4	118 ± 13	3.5 ± 0.3
'Ghosts' (4 mM-ATP)	A	10	37	4	128 ± 8	3.9 ± 0.4

Fig. 5. ATP-dependent efflux of Ca^{2+} from erythrocyte 'ghosts'

Pink erythrocyte 'ghosts' were prepared with 0mM- (●), 1mM- (■) and 4mM- (▲) ATP and $^{45}\text{Ca}^{2+}$ included. For full experimental details see the text. The amount of radioactivity present in the erythrocyte 'ghosts' at the start of the incubation at 37°C was taken as 100%. Depicted are means $\pm$ S.E.M. for four experiments performed in duplicate. A similar amount of white erythrocyte 'ghosts' prepared with 1mM-ATP and $^{45}\text{Ca}^{2+}$ (□) included only 5% of the radioactivity compared with the pink erythrocyte 'ghosts', and no transport could be measured.

Discussion

The present study demonstrates that uptake of tri-iodothyronine by human erythrocytes involves only one saturable process. Careful examination of tri-iodothyronine uptake over a wide range of hormone concentrations (from 1pM to $7.7\mu\text{M}$) did not reveal a second system. This is in contrast with the existence of two saturable uptake systems in rat hepatocytes (Krenning *et al.*, 1978, 1980; Eckel *et al.*, 1979) and with the presence of two different saturable binding sites on rat liver membranes (Pliam & Goldfine, 1977). We have shown (Krenning *et al.*, 1978, 1980) that the hepatic transport system with the highest affinity is energy (i.e. ATP)-dependent and can be inhibited by metabolic inhibitors such as KCN, 2,6-dinitrophenol and oligomycin, or by ouabain (Krenning *et al.*, 1981a), which last-mentioned finding indicates that the Na^+

Table 2. Percentage inhibition of tri-iodothyronine uptake in erythrocytes by various compounds

For full experimental details see the text. Each experiment was performed in triplicate. N.S., No significant inhibition.

Compound	Concentration (μM)	Inhibition (%)	No. of observations
Tyropanoic acid	100	22	6
	10	12	5
Iopanoic acid	100	78	6
	10	37	6
Ipodic acid	100	86	4
	10	32	5
(±)-Propranolol	100	30	6
	10	N.S.	4
Atenolol	100	N.S.	6
	10	N.S.	2
Ouabain	1000	N.S.	9
Vanadate	10	N.S.	3
Colchicine	125	16	7
	25	N.S.	7
Vinblastine	60	57	3
	12	22	3
Cytochalasin	100	N.S.	2
	20	N.S.	2
Oligomycin	110	73	2
	22	43	2

gradient over the membrane may be of importance. However, the presence or absence of ATP in erythrocyte 'ghosts' seems not to be important for the uptake of tri-iodothyronine by these 'ghosts' (Table 1), which finding argues against a role of ATP in the uptake process.

Nevertheless the possibility remains that an Na^+ gradient generated by the presence of ATP via $\text{Na}^+ + \text{K}^+$ -dependent ATPase is important for the uptake process. Because the method used for the preparation of erythrocyte 'ghosts' may partially restore the Na^+ gradient across the membrane, independent of the presence of ATP, it is therefore possible that the effect of the depletion of the erythrocyte 'ghosts' of ATP is masked. However, the fact that ouabain or vanadate does not inhibit the uptake of tri-iodothyronine by erythrocytes (Table 2) makes this hypothesis unlikely. This implies that the tri-iodothyronine-transport system present in erythrocytes is not energy-dependent. This conclusion is further substantiated by the finding that this tri-iodothyronine-transport system is still active at 0°C (Table 1), in contrast with the energy-dependent uptake system of rat hepatocytes, which is not measurable at 0°C (Krenning *et al.*, 1978). That oligomycin strongly inhibits tri-iodothyronine uptake by erythrocytes (Table 2) cannot be attributed to its inhibition of the $\text{Na}^+ + \text{K}^+$ pump (Hoffman *et al.*, 1980), because neither ouabain nor vanadate, which also block $\text{Na}^+ + \text{K}^+$ -dependent

ATPase (Hoffman *et al.*, 1980), inhibits the uptake of tri-iodothyronine by erythrocytes (Table 2). Furthermore, it is known that oligomycin blocks the production of ATP (Cantarow & Schepartz, 1967) by inhibition of the phosphorylation of ADP, but this mechanism also cannot be involved, because erythrocytes do not contain mitochondria, the usual site of action of oligomycin. The mechanism of action of this antibiotic therefore remains unclear, although it is possible that this compound has membrane-stabilizing properties too.

That membrane stabilization causes an inhibition of tri-iodothyronine uptake by erythrocytes can be concluded from the finding that ($\pm$)-propranolol in high concentrations inhibits tri-iodothyronine transport by erythrocytes, whereas atenolol does not (Table 2), although both are β -blocking agents. This discrepancy can be explained by the membrane-stabilizing properties of ($\pm$)-propranolol (Pritchard, 1978), in contrast with atenolol. The mechanism by which colchicine and vinblastine interfere with tri-iodothyronine uptake by erythrocytes is unclear. It is known that these two compounds inhibit the assembly of microtubules (Sternlicht & Ringel, 1979; Beck, 1980). However, these structures are not present in erythrocytes (Nicolson, 1976). On the other hand, erythrocytes do contain microfilaments and their associated proteins (Goldman *et al.*, 1979; Nicolson, 1976), but cytochalasin, which inhibits these structures (Weihsing, 1976), does not impair the uptake of tri-iodothyronine by erythrocytes.

More than 80% of the tri-iodothyronine associated with erythrocytes after 10 min exposure to the hormone is transported into the interior of the cell. This can be concluded from the difference between the maximal uptake velocity of intact erythrocytes and the maximal binding of the hormone by a membrane preparation derived from the same amount of erythrocytes (Table 1). It is rather likely that the affinity of the carrier in the membrane will be different when binding is measured in the membrane preparation as compared with intact cells, because in the former case both sides of this carrier are exposed to the uptake buffer, whereas this is not the case when intact erythrocytes are used. Therefore a difference in K_m can be expected.

We prepared white (i.e. haemoglobin-free) erythrocyte 'ghosts' by washing the lysed erythrocytes extensively before resealing them. In our hands it was not possible to show any saturable uptake process in this type of 'ghosts', in contrast with pink erythrocyte 'ghosts', which are prepared by opening the erythrocytes for only a short time.

The absence of a saturable uptake process in white erythrocyte 'ghosts' may be due to damage of the membranes. Electron microscopy revealed that the membranes of this type of erythrocyte 'ghosts'

show many blebs, representing small protrusions at the edge of the 'ghosts' (Ting-Beall *et al.*, 1981). Furthermore, it appears not possible to confine $^{45}\text{Ca}^{2+}$ inside the white erythrocyte 'ghosts', which indicates that these 'ghosts' are leaky for small ions, and no active transport of Ca^{2+} could be measured (Fig. 5). These findings shed some doubt on the structural integrity of these white erythrocyte 'ghosts'.

Our results are in sharp contrast with a previous report (Holm & Jacquemin, 1979) in which the existence is described of two saturable tri-iodothyronine-uptake processes in white erythrocyte 'ghosts'. The uptake process with the highest affinity could be inhibited by ouabain (Holm & Jacquemin, 1979). In our study only one saturable transport system for tri-iodothyronine in intact erythrocytes or pink erythrocyte 'ghosts' (Table 1) could be demonstrated. This system could not be inhibited by ouabain (Table 2). The reason for this discrepancy is not clear, although Holm & Jacquemin (1979) did not describe any test to assess the structural integrity of the erythrocyte 'ghost' preparations that they used.

From the data in Table 2 it can be concluded that X-ray-contrast agents such as ipodic acid, tyropanoic acid and iopanoic acid are strong inhibitors of tri-iodothyronine uptake by erythrocytes. A similar influence was found on the uptake of tri-iodothyronine and thyroxine by the high-affinity system of rat hepatocytes (Krenning *et al.*, 1981b) and *in vivo* on the uptake of thyroxine by human liver (Felicetta *et al.*, 1980). It is also known that these compounds strongly inhibit the conversion of thyroxine (3,3',5,5'-tetraiodo-L-thyronine) into tri-iodothyronine (Suzuki *et al.*, 1979) *in vivo* as well as *in vitro* (Kaplan *et al.*, 1979). However, it is possible that at least part of the described inhibition of conversion *in vivo* is due to the inhibition of the transport of the thyroid hormones across the cell membrane.

The K_m of the tri-iodothyronine-transport system in erythrocytes (100/130 nM) that we describe in the present paper compares well with the K_m reported for the tri-iodothyronine-transport system present in lymphocytes (100 nM) (Holm *et al.*, 1980). This uptake system appeared to be energy-dependent. It is possible that the uptake systems of erythrocytes and lymphocytes are similar, because both cells come from the same stem cell (Quesenberry & Levitt, 1979a,b,c), but that the erythrocyte system lost its energy-dependency at the time that its nucleus disappeared, a target of the tri-iodothyronine inside the cell.

Finally, we can conclude on the basis of the results presented above that the human erythrocyte cannot be used as a model system for tissue uptake of tri-iodothyronine in studies performed *in vivo*.

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