Vol. 89, No. 3, 1979 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
August 13, 1979 Pages 1006-1017

MEMBRANE TRANSPORT OF L-TRIIODTHYRONINE BY HUMAN RED CELL GHOSTS

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Received June 22,1979

SUMMARY

L-T_3 transport has been investigated in human red cell ghosts. Determination of initial T_3 uptake revealed two separate saturable uptake systems, one with a K_m of I.6 x $IO^{-8}M$, the other with a K_m of 3.3 x $IO^{-6}M$. Binding experiments resulted in two dissociation constants, I.4 x $IO^{-7}M$ and 2.6 x $IO^{-6}M$. Uptake was dependent on the ghost volume, indicating an intravesicular location of T_3. The T_3 was concentrated 6 times by the ghosts. Ouabain reduced the uptake by the low K_m system, but was without effect on the high K_m system. Thus evidence is provided both of binding of T_3 to the ghost membrane and of its uphill transport across the membrane.

The discovery that thyroid hormones bind to a number of intracellular components, such as cytosol proteins (I), nuclei (2,3) and mitochondria (4), inevitably raises the question anew as to how the hormones enter the cells.

Early publications, referring to indirect evidence, suggested that the thyroid hormones enter by simple diffusion (5-7), a hypothesis which was subsequently supported by the observation that they bind to phospholipids (8). At present, however, the opinion is turning in favour of a carrier mediated transport (9-I2). Specific T_3 binding sites have been observed in rat liver plasma membranes and suggested as regulator of the T_3 entry into target cells (I3). The recent study by Krenning et al. using rat liver cells does in fact demonstrate a membrane linked process, which is thought to represent an active T_3 transport (I4).

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0006-291X/79/151006-12$01.00/0

For the present study on T_3 transport an easily available human cell, suitable for preparation of vesicles, was found in the red blood cell. Thanks to its lack of intracellular organelles this cell lends itself to preparation of membranes uncontaminated by foreign elements. The use of membrane vesicles in transport studies is advantageous in that it eliminates the possibility of any intracellular binding reaction interfering with the membrane process.

MATERIALS AND METHODS

[^{3}H] H_2O (5 mCi/ml) and [^{125}I]-L-triiodothyonine (I200 µCi/µg) were purchased from the Radiochemical Center, Amersham. The iodide content of the hormone preparation, occasionally determined by paper chromatography in a butanol : acetic acid : water (8 : 2 : 2) system, did not exceed 4 %. [^{131}I] -labeled albumin (0.5 µCi/mg) was kindly prepared by Dr B. Lambert. Unlabeled L-triiodothyronine (T_3) and ouabain were obtained from Sigma Chemical Company and anion exchange resin AG I-X2, chloride form, from Bio-Rad Laboratories.

Red cell ghosts were prepared by osmotic lysis using the method of Steck and Kant (I5) with slight modifications. Fresh blood from healthy donors was collected in heparinized tubes and centrifuged at 4°. After removal of the plasma and the buffy coat the red cells were washed three times in four volumes of ice-cold phosphate-buffered saline (PBS) pH 7.4. Hemolysis was initiated by pipetting one volume of pelleted cells into 40 volumes of ice-cold, well stirred 5 mM sodium phosphate pH 8.0 (5P8). Mixing was continued for I0 min. The ghosts were then pelleted by centrifugation at 22.500 g for I0 min. at 4°. They were washed in 5P8 three to five times, i.e. until creamy white. To insure impermeability to T_3 the ghosts were resealed in an isotonic phosphate-buffer (5 mM) pH 7.4 containing NaCl I30 mM, KCl 20 mM and $MgCl_2$ 4 mM. Unless otherwise indicated the experiments were performed with sealed ghosts in the same medium as that used for resealing.

Total ghost protein was estimated by dissolving the ghosts in I % sodium dodecyl-sulfate and determining the light absorbance in a Zeiss spectrophotometer at 280 nm. Bovine serum albumin was used as standard. Protein measurements allowed standardisation of results obtained in separate experiments. The hemoglobin retained by the ghosts was determined at 398 nm with bovine hemoglobin as standard. It was estimated at I-2 % of the red cell content.
The actual volume occupied by the ghosts in a ghost pellet was determined as described by Blostein and Benderoff (I6). The total pellet volume was measured with [^3H_2O] and the extravesicular volume with [^{131}I]-albumin, the difference giving the intravesicular volume.

Uptake of T_3 was measured by incubating at 25° 50-200 µg ghost protein per ml with 0.I µCi of labeled T_3 and increasing concentrations of unlabeled T_3 in a final volume of 2 ml. Incubation was terminated by fixation of the free hormone to an anion exchange resin. This was achieved by pipetting 0.5 ml aliquots of the ghost suspension into tubes containing 0.I ml of resin and 0.5 ml of PBS. Agita-

tion of the tubes was started immediately and continued for 2 min, upon which they were centrifuged at 100 g for 15 sec. Aliquots of the supernatant, containing the T_3-binding ghosts, were counted in a gamma counter. Control experiments performed in the absence of ghosts showed that after 10 sec the resin had fixed 90 % of the T_3, also at the highest T_3 concentrations used. After 2 min. the resin had fixed 98.6 %. Uptake values were corrected for the non fixed T_3.

In experiments performed with ouabain, 1 mM was present in the medium during the resealing process. The ghost suspension was then used directly for incubation with T_3.

RESULTS

Resealing of the ghosts resulted in an increase in equilibrium uptake of T_3. From Fig. 1 it is evident that this increased uptake by the sealed ghosts is attributable to their greater non-saturable uptake. The saturable uptake is the same in the two preparations. The lower non-saturable uptake in the unsealed ghosts was eventually found to be due to a smaller volume rather than a permeability to T_3. In

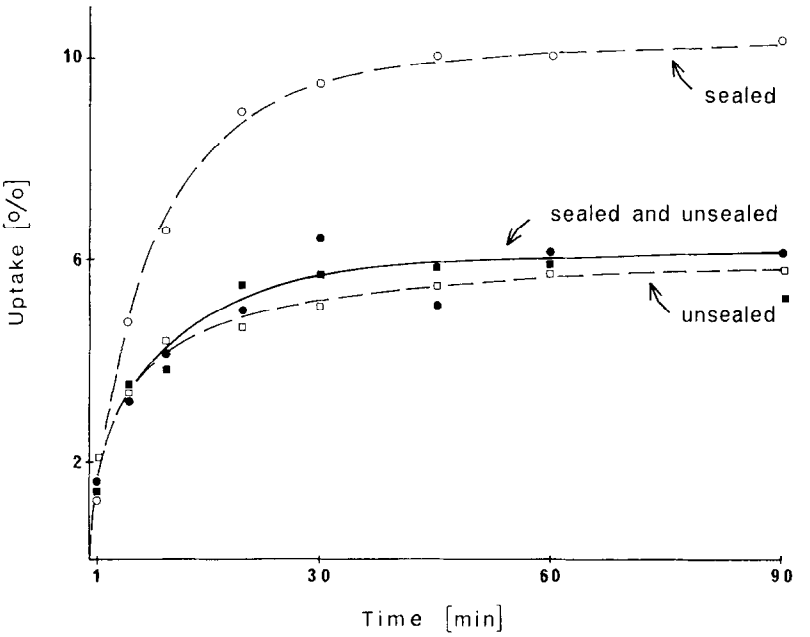

Fig. 1. Time courses of saturable (——) and non-saturable (– – –) uptake of T_3 by sealed and unsealed ghosts. Non-saturable uptake is obtained with a tracer dose of labeled T_3 and an unlabeled T_3 concentration of 6 x 10^{-5} M. Saturable uptake is obtained by incubation with the tracer alone and subsequent subtraction of the non-saturable uptake. The values represent the uptake by 50 µg of protein.

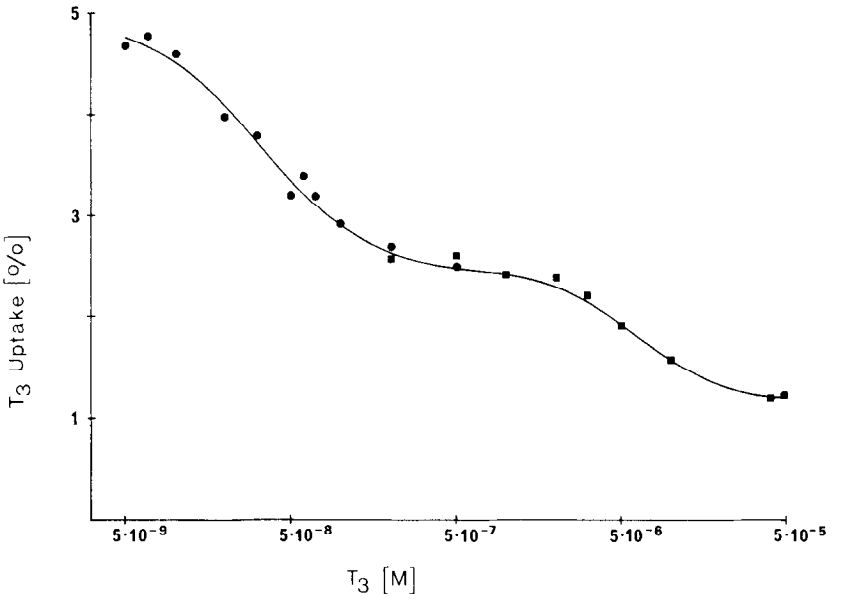

Fig. 2. I min. uptake of T_3 by sealed ghosts. The values represent the uptake by 100 µg of protein. The low (●) and the high (■) range of T_3 concentration were studied on different occasions. Each point represents the mean of triplicate determinations from two separate experiments.

pellets containing the same amount of protein the unsealed ghosts occupied 25 % of the total volume and the sealed ghosts 50 %.

At I min., the period necessary to obtain enough counts, the non-saturable uptake by the sealed ghosts was I/IO of the uptake at equilibrium and efflux should still be negligible. Hence I min. was the time chosen for initial uptake experiments.

T_3 uptake was linearly related to ghost protein concentration at least up to 220 µg/ml.

The effect of increasing concentrations of T_3 on initial uptake is shown in Fig. 2. The percent of T_3 taken up decreases as the T_3 concentration increases from 5×10^{-9} to 5×10^{-5}M. The curve presents two slopes and two plateaus, suggesting two saturable uptake systems. The first slope, in the nanomolar concentration range, reaches a plateau at about 5×10^{-7} M of T_3. This plateau can be regarded as representing non-saturable uptake relative to this first system.

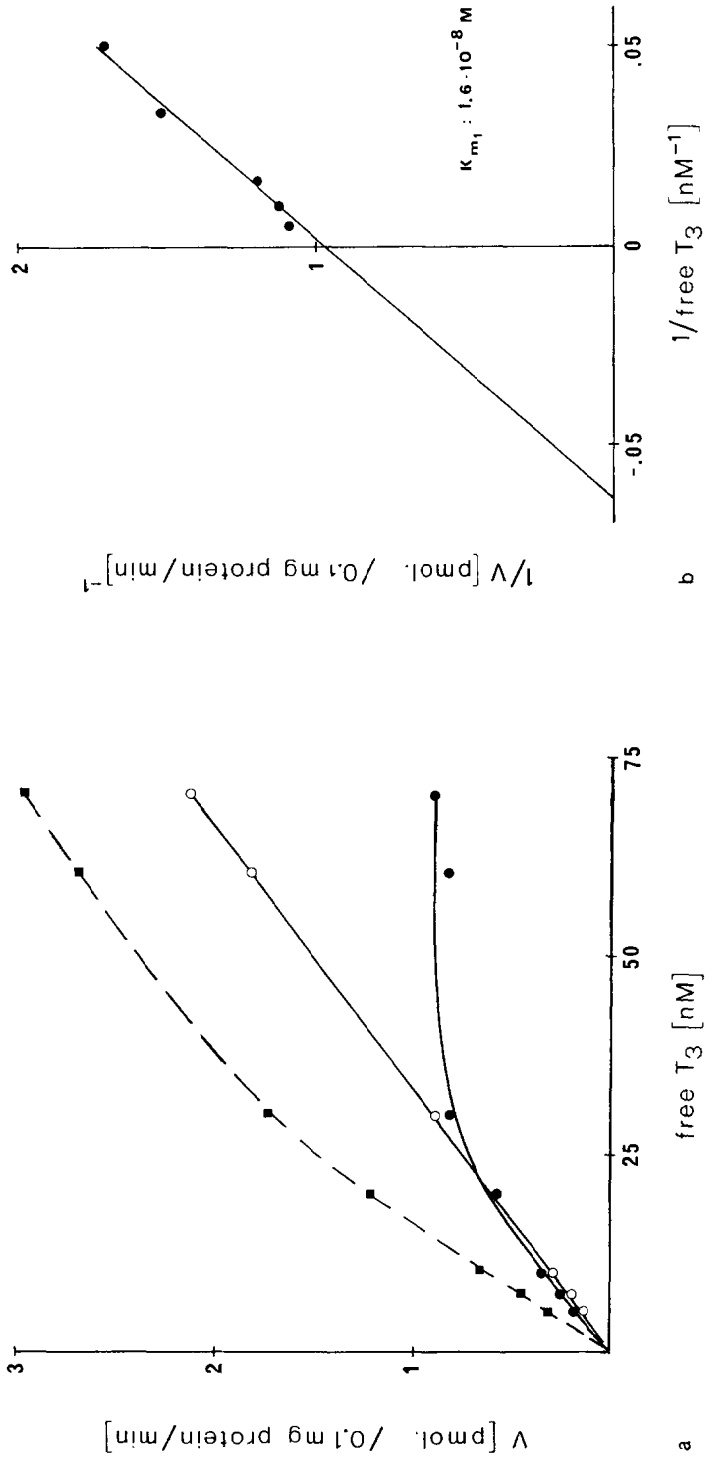

Fig. 3. a) Initial rate of T_3 uptake (■) is the product of percent of uptake in Fig. 2. and corresponding T_3 concentration. The linear component above 30 nM, extrapolated to zero concentration, gives the non-saturable uptake (O). Subtraction of this non-saturable uptake from the curve of total uptake gives the saturable component (●).
b) Lineweaver-Burk plot of the saturable component in Fig. 3a.

Fig. 3a illustrates the rate of initial uptake at the nanomolar concentrations of T_3. The non-linear relationship between total T_3 uptake and T_3 concentration below 30 nM is an evidence of saturability. Above 30 nM the rate increases linearly. This apparently non-saturable process comprises a high K_m uptake system (Fig. 2 and below), which at these lower T_3 concentrations behaves like simple diffusion. It may therefore be subtracted as a non-saturable component, thereby permitting analysis of the low K_m system (I7). Subtraction of this apparently non-saturable uptake results in a hyperbolic curve, whose corresponding Lineweaver-Burk plot gives a straight line (Fig. 3b). The calculated K_m is I.6 x IO^{-8}M and the V_{max} I pmol/O.I mg protein/ min.

The second uptake system appears as a slope in the micromolar range in Fig. 2. The initial rate is shown in Fig. 4a. Here the non-saturable component suggests diffusion. From a Lineweaver-Burk plot of the saturable component the K_m is calculated to 3.3 x IO^{-6} M and the V_{max} to 80 pmol/O.I mg protein/min. (Fig. 4b). The presence of a low K_m process with a considerable lower V_{max}, as in the present case, does not significantly interfere with the analysis of a high K_m process (I7).

To assay binding, with exclusion of transport, uptake was investigated under equilibrium conditions. From the timecourses it can be concluded that saturability is a feature also of equilibrium uptake, indicating the presence of binding sites. (Intact red cells showed no saturability at equilibrium ; not shown). To characterize the sites of this binding, the ghosts were permitted to equilibrate with T_3 in concentrations between 5 x IO^{-9} and 6 x IO^{-5}M. Preliminary experiments had given a ten times lower K_d in phosphate-buffer 5 mM than in phosphate-buffered saline I54 mM. Therefore the hypo-osmolar medium was used for the current experiments. That ghosts placed in this

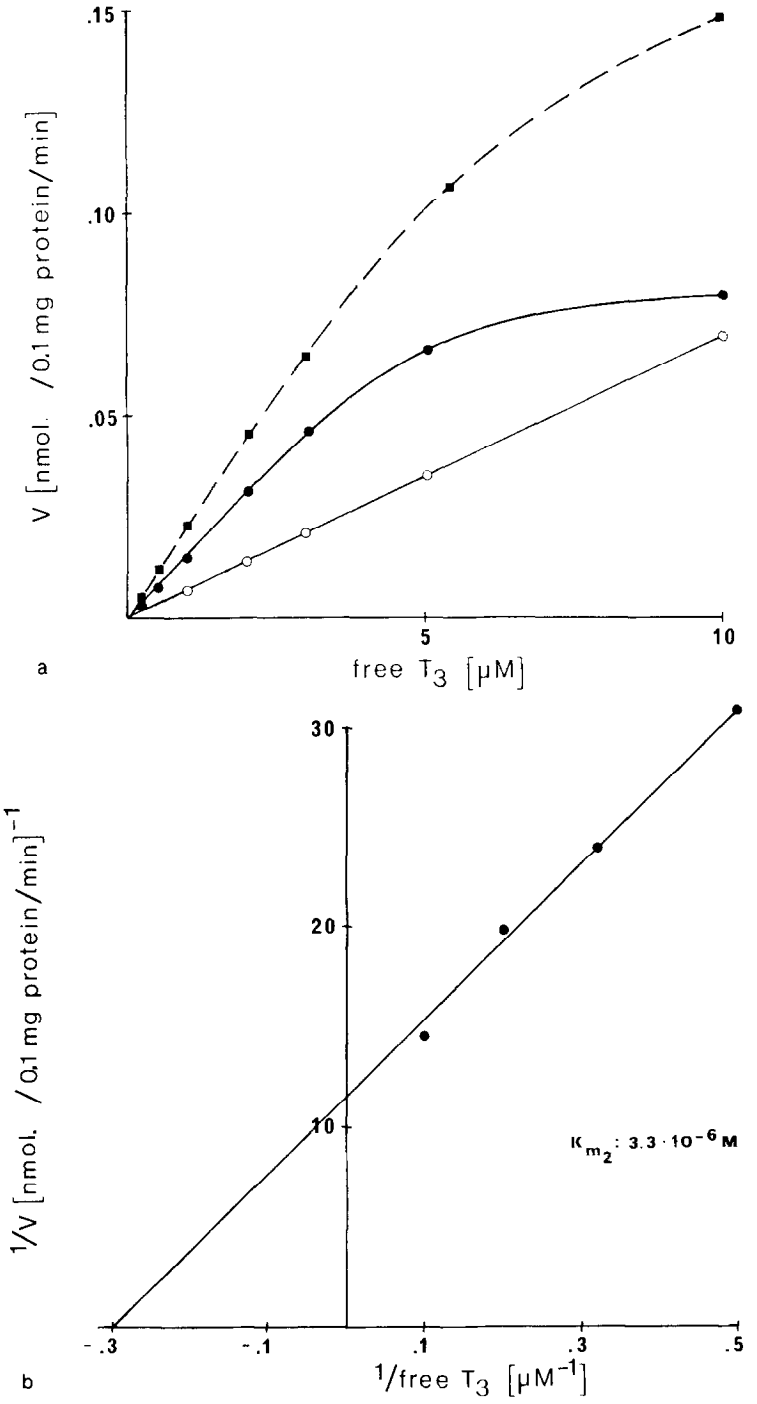

Fig. 4. a) Initial rate of T_3 uptake by the high K_m system. The curves are constructed as described under Fig. 4a. b) Lineweaver-Burk plot of the saturable component in Fig. 4a.

medium, i.e. permeable ghosts (see below), maintained a vesicular form was verified by phase microscopy. The percent of uptake decreased with increasing T_3 concentrations (not shown). Two plateau values were obtained and each one was treated as non-saturable uptake relative to its respective uptake system. Fig. 5 shows the data of saturable binding plotted according to Scatchard. The high affinity system gives a K_d of I.4 x IO^{-7} M and the low affinity system a K_d of 2.6 x IO^{-6} M.

In view of the presence of these membrane binding sites, the initial uptake might represent membrane-bound T_3 just as well as transported T_3. To resolve this problem the location of T_3 had to be determined. To this end, the non-saturable uptake at equilibrium was compared in different types of ghosts. As seen in Table I, the sealed ghosts having a bigger volume than the unsealed ghosts show a greater non-saturable uptake, II,O % versus 7,3 %. Further, ghosts incubated in a hypo-osmolar medium, in which they were likely to be permeable to T_3, do not take up more than 2,8 %. With a knowledge of the ghost volume it could be calculated that the ratio of intravesicular to extravesicular T_3 concentration was 4.8 for the sealed ghosts and 6.2 for the unsealed. For the ghosts incubated in the hypo-osmolar medium this ratio was I,O, indicating that these ghosts were in fact permeable to T_3. It should be noted that the

Table I. Effect of ghost volume and impermeability on T_3 uptake.

	non-saturable (%)	saturable (%)	ghost volume (ul)
Sealed ghosts	II.00 ± I.79	6.20 ± 0.98	6.25
Unsealed ghosts	7.30 ± I.73	5.93 ± 0.74	3.I2
Permeable ghosts	2.80 ± 0.59	6.20 ± 0.90	7.75

Sealed and unsealed ghosts were incubated in the iso-osmolar standard medium and the permeable ghosts in a hypo-osmolar phosphate buffer 5 mM. Incubation was for 60 min. with tracer ± unlabeled T_3, 6 x IO^{-5} M. The percentages represent the uptake by 50 µg of protein, which correspond to the indicated volumes.

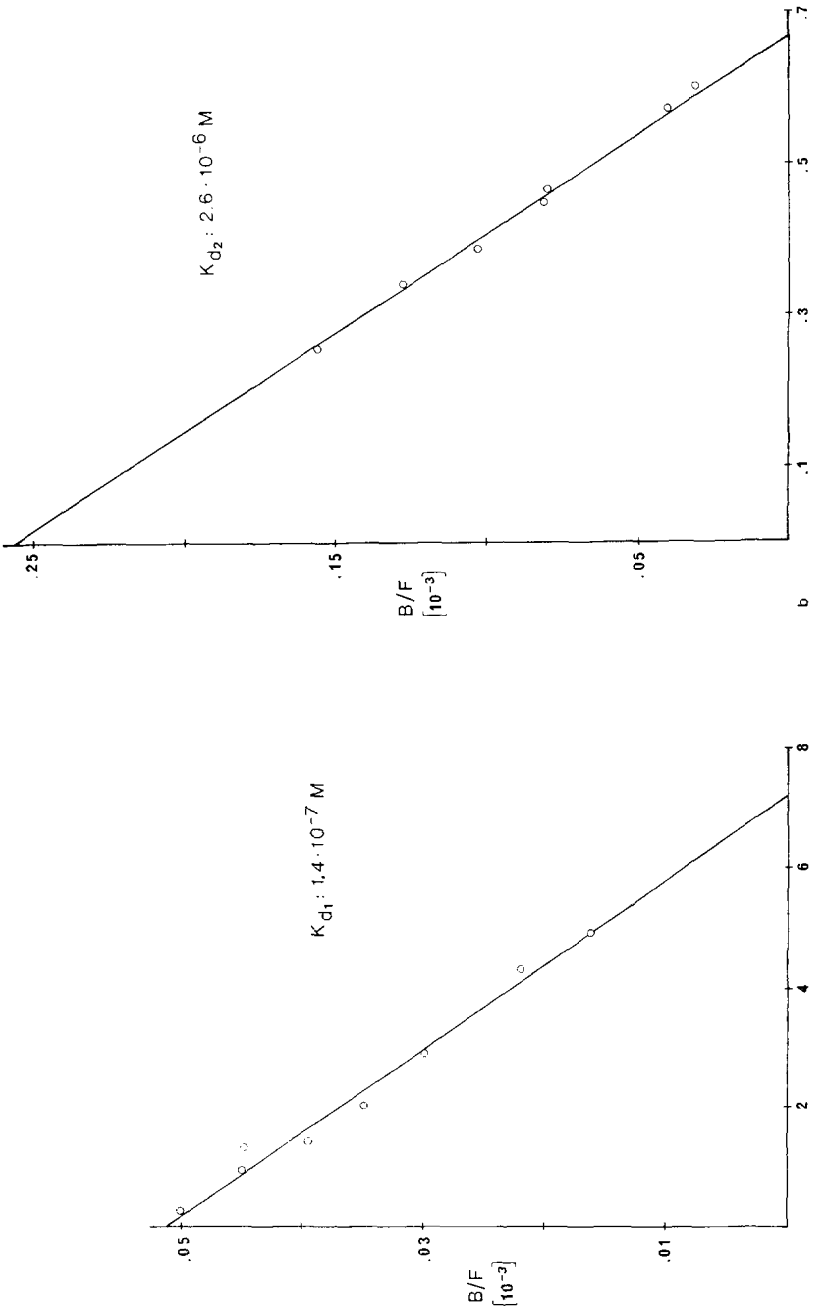

Fig. 5. Equilibrium T_3 uptake by ghosts incubated for 60 min. in a 5 mM phosphate buffer. Saturable uptake was obtained by subtraction of non-saturable from total uptake. Incubations were performed with T_3 concentrations of
a) 5 x IO-9 to I x IO-6 M and
b) I x IO-6 to 6 x IO-5 M.

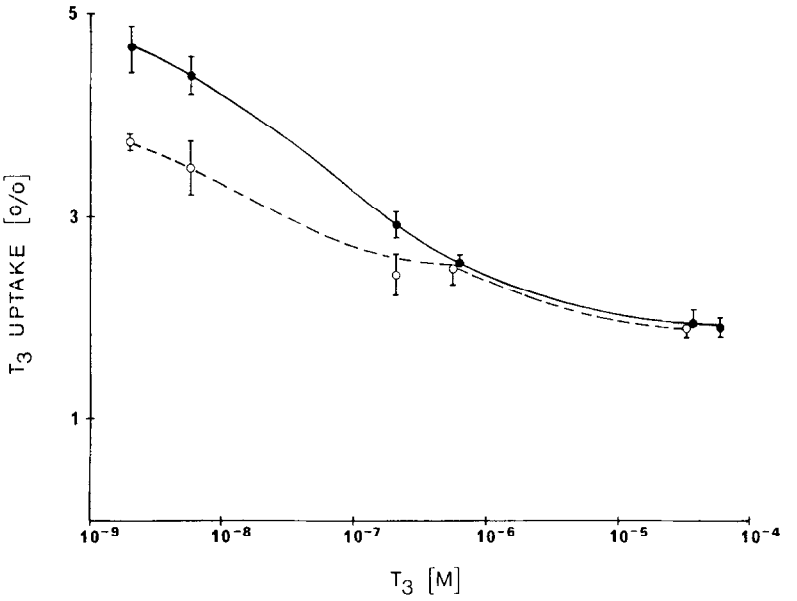

Fig. 6. Effect of ouabain on initial T_3 uptake. Ghosts were pre-incubated with I mM of ouabain for 30 min. at 37°, then incubated with T_3 for I min. at 25°. The data represent the mean of triplicate determinations ± standard deviation.

saturable uptake, representing membrane-bound T_3, is virtually the same in all types of ghosts.

The effect of I mM ouabain on initial T_3 uptake was examined over a range of T_3 concentration covering the two uptake systems. Fig. 6 shows that the high K_m system remains unaffected by ouabain, whereas the low K_m system is inhibited by 43 %.

DISCUSSION

Two saturable uptake systems for T_3 have been demonstrated in the human red cell ghost, one with a K_m of I.6 x IO^{-8} M, the other with a K_m of 3.3 x IO^{-6} M. The same values have recently been found in isolated rat liver cells (I4). The most important question to be resolved is whether the observed uptake represents binding to the membrane or transport across the membrane. The fact that the non-saturable uptake is related to the ghost volume indicates that

this uptake represents intravesicularly located T_3. Moreover, permeable ghosts take up 80 % less than a corresponding volume of impermeable ghosts. This lower uptake obviously results from a leak of T_3 from the intravesicular compartment. Consequently, T_3 does cross the membrane. The further observation that the ghosts are capable of a 6-fold concentration of T_3 leaves no doubt as to the operation of a transport system. It should be noted that there is no danger of the observed concentration being only an apparent concentration, resulting from membrane binding, since it is estimated from the non-saturable uptake.

The two K_d constants, 1.4×10^{-7} M and 2.6×10^{-6} M, most likely relate to the same uptake systems as the two K_m constants. In the case of transport K_m may or may not be identical to K_d, i.e. may or may not measure the affinity of the transport system, all depending on the relative magnitudes of the rate constants (18). A comparison of the current K_m and K_d values is not warranted however, since they were not obtained under identical conditions, the reason for which is explained under " results ". Nevertheless, the two K_d values permit the recognition of two types of binding sites in the ghost membrane.

The question now arises as to which of the two uptake systems is responsible for the uphill transport. In view of the sensitivity to ouabain, it is reasonable to assume that it is the low K_m system which carries out the active T_3 transport. As for the uptake by the high K_m system, its interpretation remains open. It might possibly represent a facilitated diffusion or binding to receptors mediating any of the described T_3 actions at the membrane level (19-22). However, the T_3 concentrations used are far from physiological and the relevance for in vivo conditions do therefore seem questionable.

ACKNOWLEDGMENTS

This study was supported by grants from the Institut National de la Santé et de la Recherche Médicale, the Ministère des Affaires Etrangères and the Fondation pour la Recherche Médicale Française.

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