Thyroxine uptake by perfused rat liver. No evidence for facilitation by five different thyroxine-binding proteins.

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Research Article

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Thyroxine Uptake by Perfused Rat Liver

No Evidence for Facilitation by Five Different Thyroxine-binding Proteins

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Abstract

Rates of hepatic uptake of thyroxine (T_4) from dilute solutions of five different plasma T_4-binding proteins were measured in the isolated perfused rat liver using an indicator dilution method. For each protein, this rate was compared with the rate of spontaneous dissociation of the T_4–protein complex measured in vitro. Proteins studied were human T_4-binding globulin (TBG), human T_4-binding prealbumin (TBPA), human albumin, rat TBPA, and human albumin isolated from subjects with familial dysalbuminemic hyperthyroxinemia. For each of the five protein–hormone complexes studied, the rate of hepatic uptake of T_4 (measured under conditions expected to result in dissociation-limited uptake) closely approximated the rate of spontaneous dissociation of the protein–hormone complex within the hepatic sinusoids. These findings indicate an absence of special cellular mechanisms that facilitate the hepatic uptake of T_4 from its plasma binding proteins, and support the view that uptake occurs from the free T_4 pool after spontaneous dissociation of T_4 from its binding proteins. (*J. Clin. Invest.* 1990. 86:1840–1847.) Key words: computer modeling • diffusion barriers • kinetics • rates of dissociation • transport

Introduction

In human plasma, thyroxine (T_4) is extensively bound to plasma proteins that include T_4-binding globulin (TBG),[1] T_4-binding prealbumin (TBPA), and albumin. The liver plays an important role in T_4 metabolism by either activating it to 3,5,3′-triiodothyronine (T_3) or deactivating it to 3,3′,5′-triiodothyronine (reverse T_3) or T_4 conjugates, according to the metabolic state of the body. Despite the binding of > 99.9% of the circulating T_4 by proteins, the liver is able to remove T_4 efficiently from the plasma.

The mechanism of hepatic T_4 uptake remains controversial. For many years, it was assumed that protein-bound hormone was not directly available for uptake. Instead, the bound form was believed to serve primarily as a reservoir to stabilize the unbound (free) concentration of T_4 by spontaneously releasing hormone into the unbound pool during uptake and by absorbing excess T_4 during hormone secretion. In recent years, however, this view has been challenged by a number of studies showing that uptake rates in experimental transport models do not correlate with the unbound concentration measured under equilibrium conditions. In particular, when uptake rates measured in the presence and absence of binding proteins are compared, addition of binding proteins does not reduce the uptake rate as much as predicted by conventional models from the fall in the free T_4 concentration. These data have recently been reviewed (1), and will not be reiterated here.

Based on these and similar results, a number of investigators have concluded that uptake may be facilitated by interaction of the T_4–protein complex with the liver cell surface, either through collision of the complexes with the cell membrane (2), or via specific receptors for the complexes that catalyze the transfer of bound hormone from the protein to the cell (3). Other investigators have concluded that special mechanisms exist within the hepatic vasculature that cause enhanced dissociation of thyroid and steroid hormones from their plasma binding proteins and thereby facilitate uptake (4).

A common feature of these "facilitation" mechanisms is that the rate of transfer of hormone from the binding protein to the liver cells is more rapid than can be explained by simple dissociation of hormone from the protein followed by diffusion of unbound hormone to the cell surface.

In an earlier study (5), we approached this problem by asking two related questions. First, is the rate of spontaneous dissociation of T_4 from its binding proteins within the hepatic sinusoids fast enough to account for observed rates of uptake from plasma? Clearly, an uptake rate that exceeded the limit imposed by spontaneous dissociation would prove the existence of catalyzed dissociation (i.e., facilitation). For T_4 (and all other hormones studied), however, uptake rates from plasma did not exceed the dissociation limit (1, 5). It could still be argued that facilitation was present, but that it was not detected because the very high binding protein concentration in plasma reduced the uptake rate below the dissociation limit. Thus, it would be useful to repeat these studies using lower protein concentrations for which uptake rates are more rapid.

The second question we asked was whether the uptake rate for free T_4 in the absence of binding proteins is rapid enough to account for the observed rate of uptake in the presence of such proteins. Here, in contrast, the answer appeared to be no (1, 5). The absolute rate of T_4 uptake by the perfused rat liver from buffer alone was much lower than needed to account for the observed rate of uptake from plasma according to conventional rate theory (5). This result is compatible with the presence of a facilitation mechanism. However, it could also reflect experimental limitations in the methods used to measure uptake from protein-free buffers (5) or more efficient diffusion of T_4 to the cell surface in the presence of binding proteins (see Discussion).

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1. *Abbreviations used in this paper:* FDH, familial dysalbuminemic hyperthyroxinemia; TBG, T_4-binding globulin; TBPA, T_4-binding prealbumin.

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In the current study, we approached the question of facilitation more directly. Although several different mechanisms have been proposed for facilitating cellular uptake from the protein-bound pool, all involve an increase in the rate of transfer of hormone from the binding protein to the cell. We therefore used the perfused rat liver model to test the hypothesis that the rate of transfer of T_4 to liver cells is more rapid than the comparable rate of transfer to an inert acceptor, dextran-coated charcoal. Dextran-coated charcoal was chosen because the dextran coating prevents large molecules such as binding proteins from interacting with the charcoal, while still permitting binding of free T_4. To increase the probability of detecting facilitation, we studied five different T_4-binding proteins.

If a facilitation mechanism for T_4 were active in the liver, we would expect that the uptake rate, measured under conditions for which the rate-limiting step is transfer of T_4 from the protein to the cell, would be significantly more rapid than the corresponding rate of transfer to the inert acceptor for at least one binding protein. In contrast, conventional theory predicts that these rates should be identical in the two systems for all five binding proteins. This approach thus provides a simple and direct method of detecting facilitation of the cellular uptake of T_4 by T_4-binding proteins.

Methods

Materials

^{125}I-T_4 (1,100–1,300 μCi/μg) was purchased from New England Nuclear (Boston, MA). The T_4 was purified immediately before each use by reverse-phase chromatography using Sep-Pak C18 cartridges (Waters Associates, Milford, MA) as previously described (6), and was kept shielded from light until used (6). ^{131}I (20 mCi/μg) was purchased from Amersham Corp., Arlington Heights, IL; TBG isolated from pooled normal human sera was obtained from Protos Laboratories, San Francisco, CA; human TBPA was from Calbiochem-Behring Corp., La Jolla, CA; fatty acid-free human serum albumin (product number A3782) and ovalbumin (grade V) were from Sigma Chemical Co., St. Louis, MO; Affi-Gel blue was from Bio-Rad Laboratories, Richmond, CA; CNBr-activated Sepharose 4B was from Pharmacia, Inc., Piscataway, NJ; rabbit anti-rat albumin (IgG fraction) was from Cooper Biomedicals, Malvern, PA; and goat antiserum to human albumin was from International Immunology Corp., Murietta, CA.

Affinity chromotography

Albumin was removed from pooled normal rat serum by chromatography with Affi-Gel blue, as described previously (6). 95% of the albumin was removed from the serum by this method, as assessed by immunodiffusion (6). Repeat passage of the albumin-depleted serum over the column did not result in further removal of albumin. The resulting albumin-depleted serum is called "rat TBPA" below, in reference to its functional role in this study.

Selected-affinity immunoaffinity chromatography (7, 8) was used to isolate albumin from the serum of two unrelated human subjects with familial dysalbuminemic hyperthyroxinemia (FDH). These subjects were the index cases described in a previous report (9). Details of this method have been published elsewhere (8). Briefly, human serum albumin (HSA) covalently coupled to CNBr-activated Sepharose 4B was used to isolate goat antibodies directed against HSA. Goat antiserum to human albumin was washed through the column with 0.9% NaCl, 15 mM Tris (pH 7.4), and 0.05% sodium azide (wash buffer) at 5°C. A subset of the bound antibodies was selectively eluted with 0.9% NaCl, 0.2 M acetic acid, pH 3.0 (elution buffer), at 5°C and then covalently coupled to CNBr-activated Sepharose 4B. The resulting immunoaffinity column was used to isolate albumin from human serum at 5°C by passing human serum through the column with wash buffer and then eluting the bound albumin with elution buffer. The eluted albumin solution (column capacity, 40 mg of albumin) was immediately neutralized with 2 M Tris. The albumin thus isolated from the two subjects with FDH (FDH-albumin) was pooled; it migrated as a single band on SDS-PAGE.

Solutions eluted from both the Affi-Gel blue and immunoaffinity columns were concentrated back to their original volumes under 40 psi pressure in an ultrafiltration cell fitted with a YM 10 membrane (Amicon Corp., Danvers, MA), diluted 20-fold into wash buffer and reconcentrated twice, and stored at −70°C until used.

In vitro dissociation rates

Rates of dissociation of T_4 from serum binding proteins were determined from the rate of transfer of T_4 to acceptor particles (dextran-coated charcoal) under appropriate conditions. The theoretical basis and details of this method have been described in detail previously (5, 10). Briefly, in each experiment, 5 μl of a physiological concentration of serum T_4-binding protein (TBG, 0.2 μM; human TBPA, 0.33 mg/ml; albumin and FDH-albumin, 40 mg/ml; albumin-depleted rat serum ["rat TBPA"], 30 mg/ml) that had been preincubated with 0.1–0.2 pmol of radiolabeled T_4 for 10 min at 37°C in Krebs-tricine buffer (10) or in wash buffer was rapidly injected into 20 ml of a vigorously stirred slurry of dextran-coated charcoal (0.2% wt/vol) in Krebs-tricine buffer, pH 7.4 (37°C), containing 1 mg/ml ovalbumin to prevent nonspecific binding of the T_4-protein complexes. The rate of transfer of hormone to the charcoal was then determined by periodic (5-s intervals) rapid filtration (GF/C glass fiber filters, Whatman, Inc., Clifton, NJ) of 1-ml aliquots of the slurry. Radioactivity trapped on each filter was measured in an automated γ counter and compared with the radioactivity in 1 ml of the unfiltered slurry. Dissociation rate constants were estimated by fitting the sum of one or more exponential functions to the data by computerized nonlinear least-squares analysis. Data for replicate experiments were analyzed separately, and the resulting parameter values were averaged to determine the mean and SD values presented in the text.

The in vitro dissociation assay was validated for each T_4–protein complex by the following studies: (*a*) When the amount of T_4-protein complex added to the slurry was doubled, the apparent dissociation rate constant was unchanged. This indicates that the T_4-binding proteins added to the slurry did not significantly compete with the charcoal for the binding of free T_4. (*b*) When T_4 was added to the charcoal slurry in the absence of binding proteins, the rate constant describing the binding of T_4 to the charcoal was $> 0.5\ s^{-1}$ (data not shown). Because binding of free T_4 to the charcoal is rapid compared with the transfer rates observed for the complexes (see Results), the observed rates of transfer of T_4 to the charcoal were not significantly limited by the rate of binding of free T_4 to the charcoal. When both conditions *a* and *b* hold, the observed rate of transfer of T_4 to the charcoal should equal the rate of dissociation of T_4 from its binding protein (10). Control studies indicated that neither ^{125}I-albumin (human) nor ^{125}I-TBPA (human) bound to the charcoal under the experimental conditions employed (< 5%).

Liver perfusion

Solution preparation. Solutions for bolus injection containing T_4-binding proteins were prepared in modified Krebs-Henseleit bicarbonate (Krebs) buffer (11). Approximately 0.4 μCi of ^{125}I-T_4 and 0.2 μCi of a single ^{131}I-labeled binding protein were used for each injection. Indicator proteins were ^{131}I-labeled human TBPA for studies of human and rat TBPA, ^{131}I-labeled human albumin for studies of human albumin and FDH-albumin, and ^{131}I-TBG for studies of human TBG. In the latter case, the amount of ^{125}I-T_4 used was reduced to 0.1 μCi to maintain the T_4/TBG molar ratio < 0.5. Total binding protein concentrations used (TBG 4 nM, TBPA 7 μg/ml, albumin and FDH-albumin 1 mg/ml, albumin-free rat serum ["rat TBPA"] 0.6 mg/ml) were ~ 2% of normal serum values. These concentrations were selected to be high enough to bind nearly all of the T_4 (> 90% in all cases, as assessed by equilibrium dialysis), yet to be low enough to minimize the rate of rebinding of T_4 released from the binding proteins. Rate theory indicates that slow rebinding favors dissociation-limited uptake (12). Specifically, when the tissue influx rate constant (or the binding rate constant in the case of the charcoal studies) is much greater than the product of the rate constant for rebinding to the protein and the available binding protein concentration, the steady-state rate of uptake of hormone from the bound hormone pool approaches the rate of dissociation of the binding protein–hormone complex within the hepatic sinusoids. As described below, this makes it possible to experimentally measure the rate of dissociation of the complex within the hepatic sinusoids.

Perfusion. Animal surgery and liver perfusion were performed as previously described (11). Briefly, livers were removed from anesthetized 55- to 65-d-old male Sprague-Dawley rats and perfused via the portal vein with recirculating fluorocarbon emulsion at 2–2.5 $ml \cdot min^{-1} \cdot g^{-1}$ liver for 30 min (Fig. 1). The fluorocarbon was then washed from the liver by single-pass perfusion with Krebs buffer for 4 min, and the flow rate was adjusted to between 1.5 and 3.0 $ml \cdot min^{-1} \cdot g^{-1}$liver as specified in Results. Flow rates were chosen to maintain T_4 extractions within the most readily measured range. Ovalbumin (0.1% wt/vol) was added to all single-pass solutions to minimize nonspecific adsorption of binding proteins to the liver and tubing. Effluent samples were collected directly from the hepatic vein outflow catheter into 0.4-ml polypropylene tubes (Fisher Scientific Co., Pittsburgh, PA) at 0.5-s intervals for a period of 10 s using a high-speed rotary fraction collector constructed specifically for this purpose. Bolus injections of ^{125}I-T_4 and the specified ^{131}I-labeled binding proteins (total volume 0.05 ml) were delivered directly into the inflow catheter above the portal vein using a 1-ml syringe and a 23-gauge needle (Fig. 1).

Sample processing and data analysis. Tubes containing effluent samples were placed inside carrier tubes and the total radioactivity in each tube was determined using an automated multichannel gamma counter. Correction was made for spillover of ^{131}I into the ^{125}I channel. Results were expressed as the ratio (T_4 cpm)/(protein cpm) (a unitless concentration ratio). Because T_4 is removed from the perfusate by the liver while the indicator in theory is not, this ratio declines during passage of the perfusate through the liver.

Uptake by the liver is often modeled by the "parallel tube" or "sinusoidal perfusion" model. According to this model, uptake for a linear (nonsaturating) transport system at steady state may be expressed by the simple relationship first proposed by Kety (13), Renkin (14), and Crone (15):

$$C_{out} = C_{in}e^{-kt}, \tag{1}$$

where C_{in} and C_{out} are the ligand concentrations entering and exiting the liver, respectively, k is the rate constant for removal of ligand from the perfusate within the liver, and t is the sinusoidal transit time. Because we are concerned with transients rather than the steady state, we can correct for dilution of the ligand in the bolus by replacing the concentration terms C_{in} and C_{out} with the corresponding concentration ratios of ligand (T_4) to nontransported indicator (binding protein), R_{in} and R_{out}. Thus, we have

$$R_{out} = R_{in}e^{-kt}. \tag{2}$$

Dividing both sides of Eq. 2 by R_{in} and taking the natural logarithm, we obtain

$$\ln(R) = -kt, \tag{3}$$

where R is the normalized ratio R_{out}/R_{in}. For each aliquot, R is related to the single-pass extraction of T_4 (E) according to the simple relationship:

$$E = 1 - R. \tag{4}$$

The determination of k takes advantage of the fact that the transit time t is not uniform, but displays a distribution of values due to variation in length and flow rate among different sinusoids (16, 17). Thus, indicator molecules exiting the liver at different times after administration of a bolus reflect different values of t. In consequence, Eq. 3 predicts that a plot of ln(R) as a function of t will be linear with a slope of $-k$ at early time points.

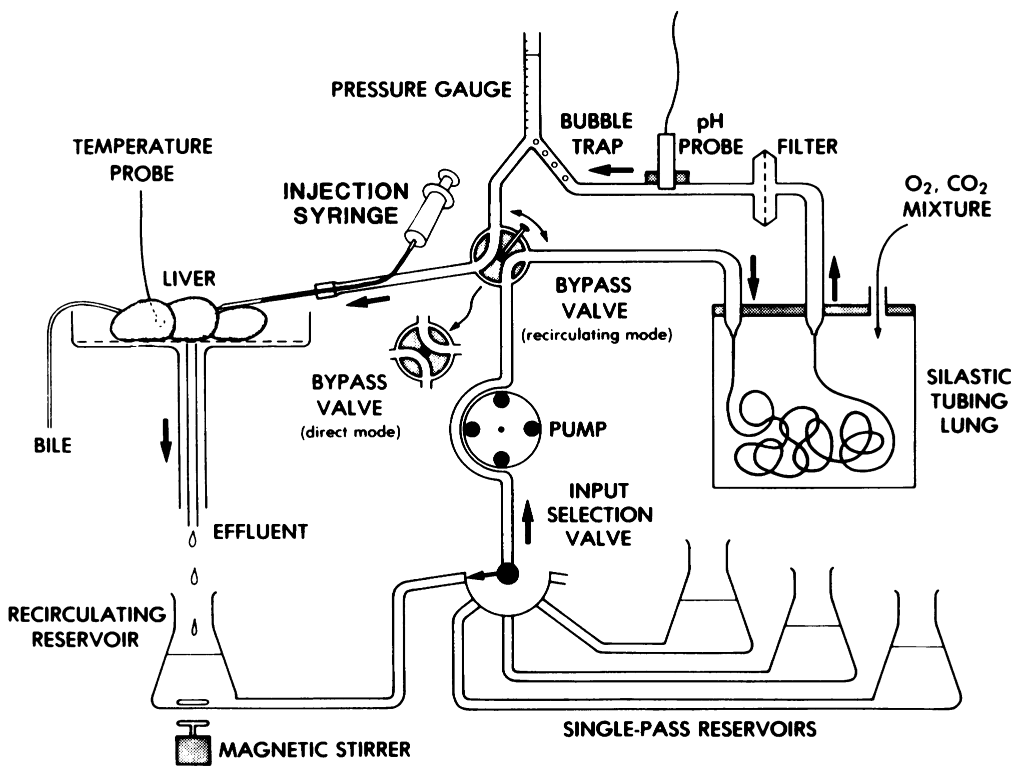

Figure 1. Diagram of liver perfusion apparatus. In the single-pass mode, a small bolus of ^{125}I-T_4 and ^{131}I-labeled binding protein was injected directly into the portal vein and effluent samples were collected at 0.5-s intervals using an automatic fraction collector. Conditions were selected so that uptake was dissociation-limited (i.e., every T_4 molecule released entered the liver before it could rebind to its binding protein). From the $^{125}I/^{131}I$ ratio, the fractional extraction was calculated for each effluent sample. Expressing extraction as a function of retention time in the liver permits calculation of the dissociation rate constant for the T_4–protein complexes within the liver.

This approach is a highly simplified version of the indicator dilution method developed for the liver by Goresky et al. (16) for determination of influx, efflux, and elimination rate constants. The simplification is made possible by the fact that we limit our study to very early time points before efflux and metabolism become significant. Goresky (18) has shown that under these conditions the initial slope of $\ln(R)$ vs. t is a measure of the influx rate constant k. This simplification has been used previously (18).

Miscellaneous

Radioiodination of TBG, TBPA, and albumin (to a level of not more than one iodine per molecule of protein) was performed by the chloramine-T method (19). Equilibrium dialysis was performed as previously described (20), except that the buffer used was Krebs-Tricine, pH 7.4, and the samples in the dialysand were not diluted. Dextran-coated charcoal was prepared as described previously (21).

Results

In vitro dissociation. After addition of T_4–protein complexes to the suspension of dextran-coated charcoal, the amount of radiolabeled T_4 remaining in the protein-bound (filterable) pool declined with time due to transfer of the T_4 to the charcoal. For TBG, rat TBPA, human TBPA, and FDH-albumin, this decay was linear when plotted on logarithmic coordinates (Fig. 2). Results are not shown for normal human albumin, because transfer to the charcoal was too rapid to measure by our assay (> 95% within 5 s; rate constant > 0.5 s^{-1}). The apparent dissociation rate constant for each T_4–protein complex at 37°C is given in Table I.

These values might underestimate the true dissociation rate constant for two reasons. First, some of the newly dissociated T_4 may rebind to the protein before it can bind to the charcoal. This possibility was minimized by the very low concentrations of binding protein present in the charcoal suspension. Moreover, in each case, the apparent rate constant was unaffected by doubling the amount of binding protein, indicating that rebinding was negligible. Secondly, reversible binding of T_4 to the charcoal might interfere. However, in each case the lines in Fig. 2 remained linear for at least three half-times (90% dissociation), indicating no measurable release of T_4 from the charcoal at late time periods when it would be most readily detected. This high degree of linearity also suggests that the T_4 was present in a single protein-bound pool. From these results, we conclude that the rate of transfer was limited by dissociation in each case, and that the slope of each line in Fig. 2 is an accurate measure of the dissociation rate constant.

In vivo dissociation. After injection of a bolus of dual-labeled T_4–protein complex into the portal vein of a perfused rat liver, the concentration of the protein in the hepatic effluent was distributed over time (Figs. 3–7), reflecting a corresponding distribution of vascular transit times. After correction for delays due to the volumes of the inflow and outflow catheters (< 0.2 s), the weighted mean of these transit times was used to determine the apparent vascular volume of the perfused liver (Table II). For each protein, preliminary studies indicated that > 95% of the radiolabeled protein was recovered within 10 s after injection, indicating no measurable protein uptake. This fact allowed the protein to be used as an indicator of the amount of T_4 that would have been present in the same sample had no uptake occurred (normalized ratio $R = 1$).

The degree to which R declines during passage of the perfusate through the liver is determined by the uptake rate constant (k) and the transit time (t) according to Eq. 3 (10). In our analysis, k is assumed to be similar for sinusoids with short and long transit times. If conditions are selected so that the rate of uptake is dissociation-limited (12), then k is a measure of the rate of dissociation of T_4 from its binding protein within the hepatic sinusoids (10). The value of k is determined in an analogous fashion to the charcoal studies, from the slope of a plot of the logarithm of R as a function of time.

For each T_4–protein complex, R declined with time of retention within the liver (square symbols in Figs. 3–7), while the extraction rose (triangles). The line in each case is the best fit of Eq. 3 to the data, determined by nonlinear regression. Because of systematic deviation at later time points, k for normal serum albumin was determined from the data for 0–2 s rather than the full data set. This deviation was probably caused by instability of the ^{125}I-T_4-albumin solutions, resulting in release of some nontransported ^{125}I (see Discussion). With this exception there was no measurable deviation of R to higher than expected values at later time points, indicating the absence of measurable efflux of T_4 from the liver. From the slopes of the $\ln(R)$ vs. time curve, the uptake rate constant (fraction of total ligand taken up per second) was readily determined (Table I). This method has an advantage over steady-state methods employed earlier for similar studies of fatty acid uptake (10) in that the vascular volume of the liver does not need to be known to calculate the uptake rate constant.

As a further test of our methods, we determined the net extraction of T_4 by the liver (fraction of the T_4 in the injected bolus not recovered within 20 s, assuming 100% recovery of

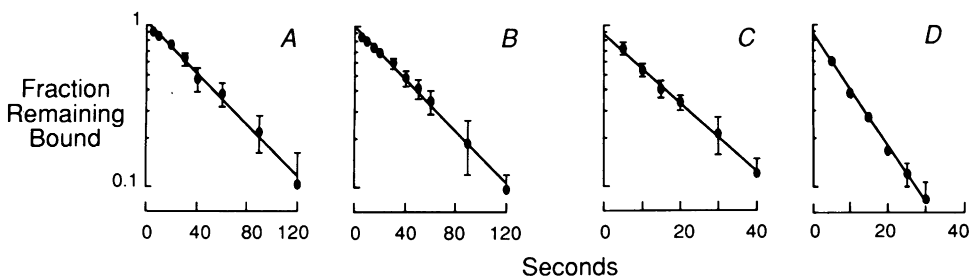

Figure 2. Rates of spontaneous dissociation. A small amount of ^{125}I-T_4 complexed to a binding protein was rapidly mixed with a suspension of dextran-coated charcoal, and the charcoal was separated by rapid filtration after varying incubation times. Conditions were chosen so that virtually every T_4 molecule released bound to the charcoal before it could rebind to the binding protein, thus making the rate of transfer dissociation-limited. The ordinate is the fraction of T_4 remaining in the protein-bound (filterable) form expressed on a logarithmic scale while the abscissa is incubation time. Data for three to four experiments are shown ±SD for (*A*) TBG, (*B*) rat TBPA, (*C*) FDH-albumin, and (*D*) human TBPA. Error bars are not shown where they are smaller than the plotting symbol used. In each case, the amount of complex remaining undissociated declined exponentially as a function of time as expected for a first-order process, allowing the dissociation rate constant to be determined from the slope of the plot. Note that the *x*-axis is different in *C* and *D* than in *A* and *B*.

*Table I. Comparison of the Rates of Spontaneous Dissociation of T_4–Protein Complexes with the Rates of Hepatic Uptake of T_4 from Those Complexes under Conditions Predicted to Result in Dissociation-limited Uptake**

Protein	Dissociation rate constant	Hepatic uptake rate constant‡	Hepatic uptake rate constant§
	s^{-1}	s^{-1}	s^{-1}
TBG	0.018±0.005	0.015±0.001	0.014±0.002
Rat TBPA	0.017±0.005	0.017±0.005	0.016±0.005
FDH-albumin	0.047±0.012	0.046±0.003	0.045±0.004
Human TBPA	0.082±0.007	0.095±0.027	0.109±0.031
Albumin	>0.5	0.68±0.08	0.576±0.035

* All results are for 37°C and are given as mean±SD (n = 3–4).
‡ Calculated from slope of ln(R) vs. t plot (Eq. 3).
§ Calculated from net extraction using Eq. 5.

the indicator, Table II). We then used this value to calculate an independent estimate for the uptake rate constant as follows. Solving Eq. 4 for R, substituting in Eq. 3, and rearranging gives

$$k = \frac{-\ln(1 - E)}{t}. \quad (5)$$

The mean transit time (t) is the sinusoidal volume (cm³/g liver) divided by the flow rate (cm³/s per g liver; see Table II). The estimates for k determined from Eq. 5 are given in Table I. The agreement between k determined by this method and k determined from the ln(R) vs. time plot was very good (mean ratio 0.99±0.05). We conclude that both methods provide valid estimates of the uptake rate constant under the conditions of the current study.

In every case, the uptake rate constant in the perfused liver was indistinguishable from the dissociation rate constant determined in vitro (mean ratio = 0.99–1.00, correlation coefficient > 0.99 for both methods of determining the uptake rate constant). Had a special mechanism been present to facilitate transfer of T_4 to the liver cell for any one or more of these proteins, we would have expected hepatic uptake of T_4 from that protein to have been more rapid. Instead, the precise agreement is exactly what is predicted by the traditional uptake model under dissociation-limited conditions. We conclude that if such facilitation mechanisms exist, they are quantitatively insignificant under the conditions of the current study.

*Table II. Perfusion Parameters**

Protein	Flow rate	Vascular volume	Net extraction
	$ml \cdot s^{-1} \cdot g^{-1}$ *liver*	$cm^3 \cdot g^{-1}$ *liver*	
TBG	0.0378±0.0033	0.168±0.007	0.061±0.005
Rat TBPA	0.0287±0.0033	0.134±0.007	0.072±0.008
FDH-albumin	0.0488±0.0050	0.159±0.005	0.144±0.009
Human TBPA	0.0473±0.0050	0.146±0.010	0.371±0.045
Albumin	0.0443±0.0035	0.178±0.016	0.899±0.017

* ±SE (n = 4).

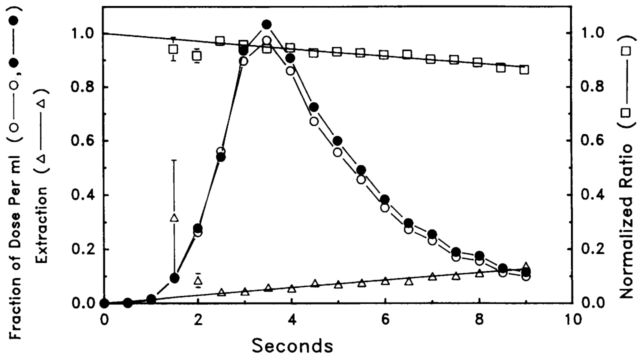

Figure 3. Hepatic uptake of T_4 from human thyroid hormone-binding globulin (TBG). After injection of a small bolus containing ^{125}I-T_4 bound to ^{131}I-labeled TBG, the quantity of T_4 (○) and binding protein (●) in each effluent sample was determined as a function of time. Results are plotted as a fraction of the total injected dose per milliliter. The $^{125}I/^{131}I$ ratio, R (□), expressed as a fraction of the starting value, declined progressively with the retention time of the complex within the liver due to hepatic uptake of T_4 but not of TBG. The extraction of T_4 (△), determined as 1-R, rose correspondingly with time. The apparent dissociation rate constant for the T_4–TBG complex within the sinusoids was determined by nonlinear least squares fitting of R to a single exponential decay function (*solid line at top*). Results are shown for four experiments±SE bars except when these were so small they were hidden by the plotting symbol.

Discussion

The present study provides the strongest evidence to date that the hepatic uptake of T_4 from plasma follows spontaneous, rather than facilitated, dissociation of T_4 from its binding proteins. When rat liver was perfused with dilute solutions of T_4 complexed to five different plasma T_4-binding proteins, the observed rate of T_4 uptake closely matched the measured rate of spontaneous dissociation of the T_4–protein complex in all cases (Table I), as predicted by conventional rate theory (10, 12) for the case of dissociation-limited uptake. There was no evidence for catalysis of T_4 uptake as would be expected for a facilitation mechanism.

Authors of previous studies have pointed out that the uptake rate constant for T_4 would need to be extremely large to account for uptake exclusively via the unbound T_4 pool (4, 22). The current data suggest that the rate constant for hepatic T_4 uptake is indeed quite large, because uptake could not have

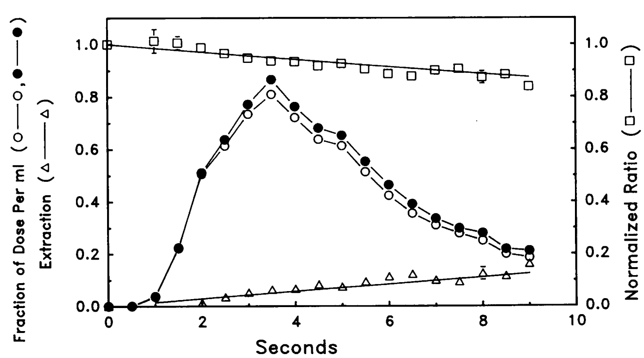

Figure 4. Hepatic uptake of T_4 from rat thyroid hormone-binding prealbumin (TBPA). Interpretation of these results is the same as for Fig. 3 (n = 4).

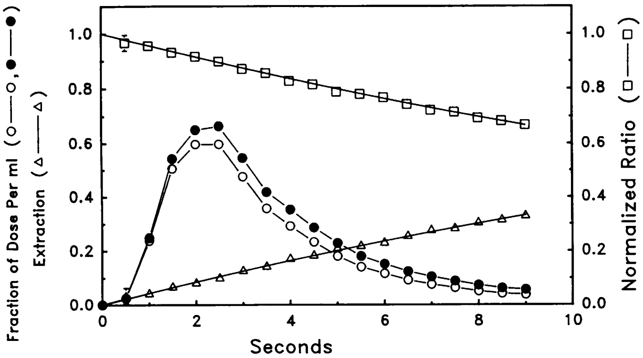

Figure 5. Hepatic uptake of T_4 from pooled albumin obtained from two patients with FDH. Interpretation of these results is the same as for Fig. 3 (n = 4).

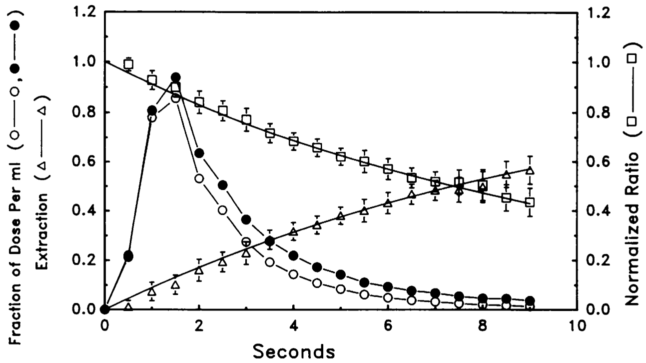

Figure 6. Hepatic uptake of T_4 from human thyroid hormone-binding prealbumin (TBPA). Interpretation of these results is the same as for Fig. 3 (n = 4).

been dissociation-limited otherwise. We have previously shown that dissociation-limited uptake requires an uptake rate constant much greater than the product of the association rate constant and the concentration of unoccupied binding sites (12). For the current study, these products may be calculated[2] as being 0.7 s^{-1} for TBG, 1.5 s^{-1} for TBPA, and 4.5 s^{-1} for FDH-albumin. Thus, the rate constant for uptake of T_4 must be much > 1–4 s^{-1}. In contrast, the hepatic uptake rate constant for inorganic chloride (Cl^-), which is considered a permeant anion, is < 0.001 s^{-1} (24). Highly efficient cellular transport mechanisms for T_4 and very low concentrations of unbound T_4 in plasma may have evolved in parallel.

It should be emphasized that no kinetic study can fully exclude alternative mechanisms of uptake. However, the fact that our data are fully accounted for by the simple traditional model without postulating facilitation mechanisms suggests that if such mechanisms do exist, their quantitative importance must be small.

Several methodological aspects of this study deserve comment. Theoretically, it is possible that an interaction of the T_4-binding proteins with the charcoal in our dissociation assay facilitated dissociation of the T_4–plasma protein complexes in vitro. If so, this would compromise our conclusions regarding the lack of such facilitation within the hepatic sinusoids. However, facilitation by the charcoal seems unlikely for three reasons. First, the close agreement between the rates of dissociation and the rates of uptake for five different T_4–plasma protein complexes argues against this possibility. It seems highly unlikely that charcoal and the liver would have facilitated dissociation to the same degree in all five cases. Secondly, the presently determined values for the dissociation rate constants of the TBG–T_4 and human TBPA–T_4 complexes agree closely with our previously reported values obtained using different acceptor particles (5) and with the previously reported values of Hillier (25), who also used different acceptor particles as well as an entirely different method. To account for these data by facilitation mechanisms, an equal degree of facilitation by all acceptors in each experimental system would need to be postulated. Finally, the dextran coating on the charcoal should have prevented direct interaction of protein with the charcoal.

Values for the dissociation rate constants of the T_4-FDH albumin and the T_4–rat TBPA complexes have not been reported previously. The present value for the dissociation rate constant of the T_4–FDH albumin complex is intermediate between that of the T_4–TBG complex and the T_4–human TBPA complex. Also of interest, since rats do not normally have circulating TBG, is the fact that the rate we observed for dissociation of the T_4–rat TBPA complex is very similar to that of the T_4–TBG complex in humans (Table I). Thus, slow dissociation of the major T_4–protein complex in plasma may serve some as yet undefined physiologic function.

Our observations on the albumin–T_4 complex also deserve comment. It is possible that the rate of spontaneous dissociation of this complex is actually much faster than the rate of hepatic uptake of T_4 that we observed, as no upper limit could be obtained for the dissociation rate constant (Table I). Because of this rapid dissociation, dissociation-limited uptake could not be confirmed (12). Thus, our results for albumin do not argue strongly for or against facilitation. However, there would seem to be little apparent need for facilitating dissociation from albumin because it is already very rapid.

The deviation of the uptake data for T_4 from albumin solutions from the predicted curves at later time points (Fig. 7) suggests that a nontransported ^{125}I impurity was present in the ^{125}I-T_4–^{131}I-albumin bolus injection. As no impurity was detected in the other solutions prepared by the same methods, this impurity most likely formed after preparation of the injection solution. It has previously been reported that aqueous solutions of ^{125}I-T_4 are unstable in the absence of protein binding (26, 27). Because the binding of T_4 to albumin is much weaker than its binding to the other binding proteins studied, the free T_4 concentration in the albumin solutions was presumably great enough to allow measurable breakdown of the T_4. It may also be that some breakdown of the ^{125}I-T_4 occurred in the other solutions as well, but was not detected due to the lower extractions of T_4.

The current findings support the conclusion that no special facilitation mechanism exists within the liver to separate T_4 from any of its binding proteins. Instead, the process appears to involve only spontaneous dissociation of T_4–protein com-

2. The association rate constant for each binding protein was determined as the ratio of the dissociation rate constant (current study) and the equilibrium dissociation constant (K_D). K_D values were taken from Barlow and coworkers (23) as 0.1 nM for TBG, 7 nM for TBPA, and 50 nM for FDH-albumin. One binding site was assumed for each protein molecule except for FDH-albumin, where we assumed that one-third of the total binding sites were of high affinity (23).

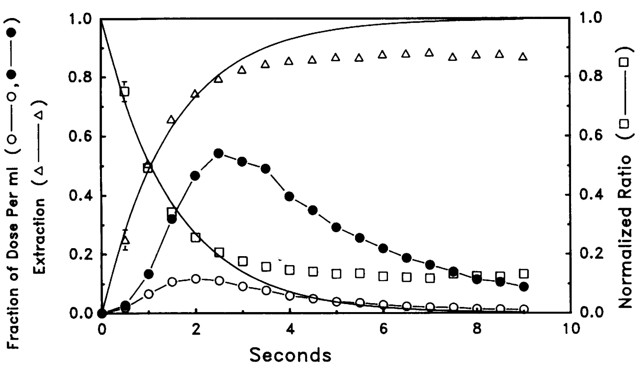

Figure 7. Hepatic uptake of T_4 from human albumin. Interpretation of these results is the same as for Fig. 3 (n = 4).

plexes within the sinusoids followed by uptake of the free T_4 by the liver cell, as has been traditionally assumed.

These results do not, however, explain why the uptake rate in the absence of binding proteins is much lower than expected based on uptake rates measured in the presence of physiologic concentrations of these proteins (1, 5). For example, conventional rate theory suggests that the value of the uptake rate constant k in Eqs. 1–3 should decrease on addition of binding protein in proportion to the fall in the free fraction produced by protein binding. When this has been studied, however, the reduction has always been less than expected (5). This result suggests that uptake is somehow more efficient in the presence of binding proteins than in their absence. We earlier speculated (5) that this apparent discrepancy could simply reflect the great difficulty in accurately measuring rapid uptake rates in the absence of binding proteins. However, a more elegant explanation has recently been proposed.

Bass and Pond (28) reported in 1988 that binding proteins may play an important role in delivering bound ligands to the cell surface across adjacent diffusion barriers such as unstirred layers. These barriers likely include the subendothelial space of Disse. In the absence of binding proteins, diffusion barriers may cause a concentration gradient to form that reduces the ligand concentration at the cell surface to a value below that present in the bulk sinusoidal fluid. As a result, the uptake rate may be much lower than would be true if no diffusion barrier existed. When binding proteins are added, however, bound ligand provides a second diffusional flux that helps overcome the diffusional resistance of the unstirred layer, thus increasing delivery of ligand to the cell surface and the apparent uptake rate constant. The net result is an increase in the uptake rate even when the free ligand concentration in the bulk perfusate remains unchanged.

The model of Bass and Pond (28) has successfully explained a 30-fold enhancement of the transport rate observed using a simplified model of transport (29), and appears able to explain enhancement of fatty acid uptake by perfused liver as well (R. A. Weisiger, S. Pond, and L. Bass, manuscript submitted for publication). This model is not needed to interpret the results of the current study because conditions for the perfused liver experiments were carefully chosen to make dissociation of the T_4–protein complex, rather than diffusion, rate-limiting to uptake.

In summary, the present studies employing five different T_4–plasma protein complexes indicate that, under conditions predicted to result in dissociation-limited uptake, rates of hepatic uptake of T_4 from T_4–plasma protein complexes closely matched the rates of spontaneous dissociation of T_4 from these complexes. These results provide no evidence for facilitation mechanisms in the hepatic uptake of T_4. In the absence of such evidence, traditional models for T_4 uptake that assume spontaneous dissociation of T_4–protein complexes remain the preferred explanation.

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