

# The Free Hormone Hypothesis: A Physiologically Based Mathematical Model\*

CARL M. MENDEL

*The Cardiovascular Research Institute, Liver Center, and Department of Medicine, University of California, San Francisco, California 94143-0130*

## Summary

The free hormone hypothesis states that the biological activity of a given hormone is affected by its unbound (free) rather than protein-bound concentration in the plasma. The fundamental mathematical and physiological principles relating to this hypothesis are reviewed, along with experimental data that shed light on its validity. It is shown that whether or not this hypothesis is likely to be valid for any given hormone will depend largely on which step in the tissue uptake process (plasma flow, dissociation from plasma binding proteins, influx, or intracellular elimination) is rate-limiting to the net tissue uptake of that hormone. It is further shown that the free hormone hypothesis could hold even if tissue uptake of hormone occurred by a mechanism that acted directly on one or more circulating protein-bound pools of hormone. Indeed, many of the data previously interpreted as being inconsistent with the free hormone hypothesis are in fact readily consistent with it when its predictions are fully understood. Nevertheless, the free hormone hypothesis is not likely to be valid for all hormones with respect to all tissues. It is likely to be valid with respect to all tissues for the thyroid hormones, for cortisol, and for the hydroxylated metabolites of vitamin D. For many of the other steroid hormones, however, it is likely to be valid with respect to some tissues, but not with respect to others (in particular, the liver). And for some of the steroid hormones (in particular, progesterone) it may not hold at all.

- I. Introduction
- II. The Free Hormone Transport Hypothesis
- III. The Free Hormone Transport Hypothesis: Specific Parameter Values
- IV. The Free Hormone Hypothesis: History and Empiric Testing
- V. The Free Hormone Hypothesis: A Physiologically Based Mathematical Model
  - A. Introductory statements
  - B. When intracellular elimination is rate-limiting to the net uptake of hormone by tissues
  - C. When other steps are rate-limiting to the net uptake of hormone by tissues
  - D. General statements
- VI. The Free Hormone Hypothesis: Application to Particular Hormones

- A. Thyroid hormones
    - 1. General statements
    - 2. Changes in the elimination rate constant
    - 3. Changes in the ratio of the influx and efflux rate constants
    - 4. Changes in plasma protein binding
    - 5. Changes in intracellular binding
  - B. Cortisol
  - C. Testosterone
  - D. Estradiol
  - E. Progesterone
  - F. Aldosterone
  - G. Vitamin D
  - H. Summary
- VII. Is There Active Transport of Hormones?
- A. Thyroid hormones
  - B. Steroid hormones
- VIII. Incompatibilities with the Free Hormone Hypothesis and Traditional Models of Hormone Transport
- IX. Alternative Hypotheses
- X. Functions of Plasma Hormone-Binding Proteins
- XI. Conclusions and Areas for Future Investigation

## I. Introduction

THIS REVIEW is occasioned by the recent debate surrounding the free hormone hypothesis and by recent developments that have transformed this hypothesis from an empirical maxim into a formal mathematical and physiological model. In this review, the term "free hormone hypothesis" will refer to the hypothesis that the biological activity of a hormone is affected by the unbound (free) rather than protein-bound concentration of hormone in the plasma (as measured at equilibrium *in vitro*). Because the hormones to which this hypothesis has been applied exert their principal biological effects intracellularly, an equivalent statement is that the con-

Address requests for reprints to: Carl M. Mendel, M.D., Cardiovascular Research Institute, University of California, San Francisco, California 94143-0130.

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centration of hormone within tissues is affected by the free rather than protein-bound concentration of hormone in the plasma. As will be discussed in detail, this hypothesis does not state that intracellular hormone concentrations and biological activity are determined only by the free hormone concentration in the plasma. Nor does it make any definite predictions about the absolute magnitude of hormone that can be taken up by tissues, either unidirectionally or in a net fashion. In addition, as will be shown, this hypothesis does not require that hormones enter tissues exclusively via the free pool, and not via the protein-bound pool.

It is hoped that this review will provide an understanding of the mathematical and physiological principles governing the free hormone hypothesis, the elucidation of which followed rather than preceded the empirical demonstrations of its general validity. Although the conclusion that this hypothesis is at present a sound model of hormone transport and distribution will be firm, detailed analysis of this hypothesis will also reveal areas of exception, weakness, and lack of data.

## II. The Free Hormone Transport Hypothesis

The postulate that the thyroid and steroid hormones enter tissues exclusively via the pool of free hormone after the spontaneous dissociation of hormone-protein complexes within the tissue vasculature will be termed "the free hormone transport hypothesis," in distinction to the free hormone hypothesis defined above. Although these two hypotheses have frequently been confused, they are in fact distinct and not interdependent. That is, the free hormone transport hypothesis can be correct when the free hormone hypothesis is incorrect and vice versa (see below, *Section V*). Although this review will concentrate on the free hormone hypothesis, it is nevertheless important to consider the free hormone transport hypothesis in detail.

To facilitate discussion of this subject, I will focus on two simplified equations: the Kety-Renkin-Crone equation (1-3)

$$E = 1 - e^{-kt} \quad (I)$$

and the "modified Kety-Renkin-Crone equation" (4-7)

$$E = 1 - e^{-kt} \quad (II)$$

where  $E$  is the fractional unidirectional uptake of hormone by a given tissue,  $k$  is the uptake (influx) rate constant for free hormone,<sup>1</sup>  $t$  is the capillary or sinusoidal transit time (derived from the flow rate and the volume

of the capillaries or sinusoids within the tissue), and  $f$  is the free fraction (at equilibrium) of hormone in the plasma or other test solution. Equation I is used to describe the tissue uptake of hormone in the absence of hormone-binding proteins, and Eq II is used to describe the tissue uptake of hormone via the pool of free hormone in the presence of hormone-binding proteins. Both of these equations assume that capillary or sinusoidal transit times are uniform throughout the tissue and that capillaries and sinusoids are well-mixed along their radial axes, such that diffusional gradients do not form at the cell surface. In addition, Eq II assumes that bound and free radiolabeled hormone remain at equilibrium during transit through the tissue (*i.e.* it assumes that the influx rate constant is much smaller than the product of the on-rate constant and the free binding protein concentration). Although not all of these assumptions are likely to be valid under many of the circumstances that prevail *in vivo*, the current dialogue over the validity of the free hormone transport hypothesis concerns experimental discrepancies of orders of magnitude rather than of small numbers. Thus, these equations provide an appropriate conceptual framework for discussing relevant issues in this area (they will not be used for model development in later sections of this manuscript, however). More complicated equations which take into account more of these factors have been published elsewhere (4, 9-13).

An optimal approach to the question of whether hormone uptake from plasma by tissues occurs exclusively via the free pool or also via one or more protein-bound pools is to measure all of the various rate constants involved in the uptake process and then analyze the data with an appropriate model (*e.g.* under some circumstances, Eq II) to determine whether all of the observed uptake can be accounted for by the pool of free hormone. If the single-pass tissue uptake of hormone greatly exceeds the free fraction of hormone in the plasma or other test solution, then at least two conditions must hold for that uptake to be accounted for entirely by the pool of free hormone. The tissue influx rate constant must be high enough to cause the free pool to turn over many times during a capillary or sinusoidal transit, and the rate of dissociation of hormone from its binding proteins must be rapid enough to continuously replenish the free pool during that transit. When these conditions can be demonstrated, there usually remains little reason to postulate direct tissue uptake of protein-bound hormone.

Several techniques have been used successfully to measure rates of dissociation of hormones from binding proteins. These include stopped-flow fluorometry (14, 15), rapid precipitation of protein-bound hormone (16), and rapid separation from bulk solution of acceptor particles that bind free but not protein-bound hormone (17-21). Thus, information exists on the rates of spon-

<sup>1</sup> This equation was derived to describe the steady-state uptake of hormone by tissues. Therefore,  $k$  really equals  $k_1 k_3 / (k_2 + k_3)$  in the notation of Fig. 9.  $k$  represents the influx rate constant ( $k_1$ ) under initial extraction conditions, however, when efflux (and therefore  $k_2$ ) is effectively 0 (8).

TABLE 1. Distribution (% of total hormone in serum), equilibrium association constants ( $K_A$ ) ( $M^{-1}$ ), and half-times of spontaneous dissociation ( $t_{1/2}$ ) (sec) of hormone-protein complexes in normal human serum at 37°C

Hormone	Albumin			Prealbumin			Globulin			% Free	References
	$K_A$	% bound	$t_{1/2}$	$K_A$	% bound	$t_{1/2}$	$K_A$	% bound	$t_{1/2}$		
T <sub>4</sub>	$7 \times 10^6$ <sup>a</sup>	20	<1.5	$7 \times 10^7$ <sup>a</sup>	10	8	$1 \times 10^{10}$	70	39	(TBG)	0.03
T <sub>3</sub>	$1 \times 10^5$ <sup>d</sup>	35	<1	$1.4 \times 10^7$ <sup>a</sup>	25	<1.5	$4.6 \times 10^8$	40	4		0.4
Testosterone	$4 \times 10^4$	50 (30)	<sup>b</sup>				$2 \times 10^9$	44 (66)	22 <sup>c</sup>		2 (1)
Dihydrotestosterone	$4 \times 10^4$	39 (21)	<sup>b</sup>				$6 \times 10^9$	60 (78)	105	(SHBG)	0.9 (0.5)
Estradiol	$6 \times 10^4$	78 (61)	<sup>b</sup>				$7 \times 10^8$	20 (37)	<sup>d</sup>		2
Progesterone	$6 \times 10^4$	80	<sup>b</sup>				$2 \times 10^7$	17	0.2		2
Cortisol	$3 \times 10^3$	7	<sup>b</sup>				$8 \times 10^7$	90	0.8	(CBG)	4
Corticosterone	$1 \times 10^4$	19	<sup>b</sup>				$8 \times 10^7$	78	<sup>e</sup>		3
Aldosterone	$2 \times 10^3$	42	<sup>b</sup>				$2 \times 10^6$	21	---		37
	$5 \times 10^4$	15	<sup>b</sup>				$4 \times 10^7$	85	---	(DBP)	0.4
1,25-(OH) <sub>2</sub> D <sub>3</sub>											24

For testosterone, dihydrotestosterone, and estradiol, values outside of parentheses are for men and values within parentheses are for women. None of the other values shown are markedly different in men and women. Corresponding values for the hormone-binding proteins in sera from other animals are not necessarily the same. For example, rats have little or no circulating SHBG (25-27), and the  $K_A$  values of testosterone, dihydrotestosterone, and estradiol for rabbit SHBG are 1-2 orders of magnitude lower than for human SHBG (28, 29) (the  $t_{1/2}$  values of these complexes are expected to be correspondingly shorter). Off-rate constants ( $k_{off}$ ) can be calculated as  $\ln 2/t_{1/2}$ , or  $0.693/t_{1/2}$ . Spontaneous fractional dissociation in a given time ( $t$ ) can be calculated as  $1 - e^{-k_{off}t}$ . At least some of the information provided in this Table is likely to require modification on the basis of future investigation. 1,25-(OH)<sub>2</sub>D<sub>3</sub>, 1,25-dihydroxyvitamin D<sub>3</sub>. Dashed line indicates no data available.

<sup>a</sup> More than one binding site; the  $K_A$  of the highest-affinity binding site is shown.

<sup>b</sup> Off rates of steroids from albumin are generally assumed to be extremely rapid. Using methods previously described (20, 21), the  $t_{1/2}$ s of the cortisol-albumin complex and the testosterone-albumin complex have both been shown to be less than 1.5 sec (Mendel, C. M., unpublished observations).

<sup>c</sup> A more recent reference suggests that this  $t_{1/2}$  may be an overestimate of the true value (30).

<sup>d</sup> Estradiol dissociates from SHBG 4 times as rapidly as testosterone at 0°C (16).

<sup>e</sup> Corticosterone dissociates from CBG 1.5 times as rapidly as cortisol at 20°C (15).

TABLE 2. Estimated mean capillary transit times in various organs at physiological flow rates

Organ	Capillary length (l) <sup>a</sup> (mm · mm <sup>-3</sup> tissue)	Capillary diameter (D) (μm)	Capillary surface area (A) (cm <sup>2</sup> · cm <sup>-3</sup> tissue)	Capillary volume (V) (cm <sup>3</sup> · cm <sup>-3</sup> tissue)	Organ blood flow (F) (ml · min <sup>-1</sup> · g <sup>-1</sup> tissue)	Mean capillary transit time (t) (sec)
Rat liver				0.15 <sup>b</sup> (31)	1 (32, 33)	9
Human liver				<sup>c</sup>	1 (32, 33)	9
Mouse liver	4200	6	800	0.12 (34)	1 (32)	7
Mouse brain (cerebral pia mater)						0.7 <sup>d</sup> (35)
Rat cerebral cortex	1180	2.7	96	0.007 (34)	0.6 <sup>e</sup> (36)	0.7
Mouse renal cortex	4500	6	850	0.13 (34)	5.6 <sup>f</sup> (37)	1.4
Cat red muscle	760	4.6	110	0.013 (34)	0.2 <sup>g</sup> (38)	1 <sup>h</sup>
Cat white muscle	380	4.4	52	0.006 (34)	0.09 <sup>h</sup> (38)	1 <sup>h</sup>

The following equations for the lateral surface area and volume of a cylinder were used:  $A = 2\pi(D/2)^2l$ ;  $V = \pi(D/2)^2l$ .  $t$  was calculated as  $V/F$ ; tissue density was assumed to be 1 g/cm<sup>3</sup>. Average values for  $F$  are given; in most organs  $F$  (and therefore  $t$ ) can vary significantly *in vivo*, and when organs are perfused *in vitro*  $F$  is set by the investigator. Transit times (with catheter dead space subtracted) measured by indicator dilution studies (not shown here) represent maximal estimates of capillary transit times because they include transit through both capillaries and larger vessels. At least some of the information provided in this Table is likely to require modification on the basis of future investigation. Numbers in parentheses are references.

<sup>a</sup> Individual capillaries are heterogeneous in length. Capillary volumes and blood flow may vary markedly in different regions of organs.

<sup>b</sup> Sinusoids plus space of Disse; measured directly (stereologically).

<sup>c</sup> Assumed to be the same as in rat liver.

<sup>d</sup> Measured directly as fluorescein transit time.  $t$  was shown to vary directly with blood viscosity and inversely with blood pressure.

<sup>e</sup> Whole brain blood flow.

<sup>f</sup> Rat renal cortical blood flow.

<sup>g</sup> At rest; blood flow increased 6-fold with maximal exercise.

<sup>h</sup> At rest; calculation assumes that only 25% of the capillaries are perfused at rest.

TABLE 3. Single pass unidirectional uptake of hormone by tissues at physiological flow rates and temperature

Organ	Test solution	Hormone	Maximal predicted uptake (%) <sup>a</sup>	Observed uptake (%)	Reference
Human liver <sup>b</sup>	Human plasma	T <sub>4</sub>	35	6	39
	Human plasma	T <sub>4</sub>	35	4 <sup>c</sup>	40
	Human plasma	T <sub>4</sub>	35	6	41
	Human plasma	T <sub>4</sub>	35	3 <sup>c</sup>	42
	Human plasma	T <sub>3</sub>	90	29	43
Rat liver	Human serum	T <sub>4</sub>	35	11	19
	Human serum	T <sub>3</sub>	90	32	20
	Human TBPA <sup>d</sup>	T <sub>4</sub>	55	30	44
	Human serum (67%)	T <sub>4</sub>	35	18	45
	Human serum (67%)	T <sub>3</sub>	90	59	45
	Human serum (67%)	Dihydrotestosterone	43 (26) <sup>e</sup>	44 (34) <sup>e</sup>	46
	Human serum (67%)	Testosterone	63 (48)	49 (34)	46
	Human serum (67%)	Estradiol	>85 (>72)	69 (67)	46
	Human serum (67%)	Cortisol	100	50 (42)	46
	Human serum	Cortisol	100	59	21
Rat brain	Male human serum (67%)	T <sub>3</sub>	42	5	47
	Male human serum (67%)	Testosterone	33	26	48
	Male human serum (67%)	Estradiol	>50	45	48
	Male human serum (67%)	Progesterone	67	68	48
	Male human serum (67%)	Corticosterone	>50	6	48
	Male human serum (67%)	Aldosterone	>63	3	48
	Human serum (10%)	1,25-(OH) <sub>2</sub> vitamin D <sub>3</sub>	>9	7	49

This table is a representative rather than exhaustive list of such studies performed. The liver perfusion studies of Hillier (Refs. 50 and 51; see text) are not included in this table because most were performed at 22°C.

<sup>a</sup> Predictions based on the fraction of hormone released spontaneously from binding proteins during capillary or sinusoidal transit. Calculations were performed using the data in Tables 1 and 2; if t<sub>1/2</sub> was too rapid to measure or assumed to be very rapid (Table 1), it was arbitrarily assigned a value of 0.5 sec. The capillary transit time in rat brain was assumed to be the same as that in mouse pia mater and rat cerebral cortex (Table 2).

<sup>b</sup> In vivo kinetics studies; hepatic plasma flow was assumed to be 800 ml/min [from Table 2, an assumed liver weight of 1400 g (33), and an assumed hematocrit of 45%]. Three independent methods of obtaining and analyzing the data are represented in the studies shown.

<sup>c</sup> The liver was assumed to constitute 80% of the rapid pool (40).

<sup>d</sup> Thyroid hormone-binding prealbumin, 0.3 mg/ml.

<sup>e</sup> Values outside of parentheses are for men and values within parentheses are for women.

taneous dissociation of many protein-hormone complexes. The free hormone transport hypothesis predicts that for all protein-hormone complexes the rate of tissue uptake of hormone will never exceed the rate of spontaneous dissociation of the protein-hormone complex within the tissue vasculature. This is a very robust prediction in that a great deal of experimental data relating to it have been collected, and a single contrary finding would be enough to disprove the hypothesis. Yet, for all hormones studied, in combination with all binding proteins studied and in all tissues studied, spontaneous dissociation of the protein-hormone complex has always been found to be rapid enough to account for the observed tissue uptake of hormone entirely via the pool of free hormone (Tables 1-3). I am not aware of a single exception to this finding. These observations alone provide strong evidence that tissue uptake mechanisms for hormones act on the pool of free hormone after spontaneous dissociation of the protein-hormone complex. In addition, Hillier (50, 51) recognized that under certain con-

ditions, when the concentration of binding protein is low enough that the rate of rebinding of released hormone is very much slower than the rate of tissue uptake of hormone, the free hormone transport hypothesis predicts that the rate of tissue uptake of hormone will in fact equal the rate of spontaneous dissociation of the protein-hormone complex. Hillier (50, 51) tested this prediction with measurements of T<sub>4</sub> uptake by rat liver and found that it holds true. Based on all of these data, rejection of the free hormone transport hypothesis would seem difficult to justify at this point in time.

In contrast to measurements of rates of dissociation of hormones from their binding proteins, measurements of tissue influx rate constants for free hormones have been more difficult. Recently, two groups of investigators have attempted to directly measure influx rate constants for free hormones, Pardridge *et al.* (5, 6) and ourselves (19-21). Pardridge and his colleagues have used the Oldendorf injection technique (52) to measure rates of unidirectional tissue uptake of radiolabeled hormones in

various test solutions. The Oldendorf injection technique involves a rapid bolus injection of test solution into the vessel supplying blood to the tissue in the live, anesthetized animal (usually rat). Tissue is then sampled 15–18 sec after injection, and uptake of hormone is compared to uptake of a “freely diffusible reference” such as butanol. The bolus injection method is assumed to result in “no significant” mixing of the test solution with the plasma of the animal being studied, and hormone taken up by tissues is assumed not to return to plasma during the 15- to 18-sec period before tissue sampling (5). Uptake of free hormone is analyzed according to Eq I for calculation of the influx rate constant. Uptake of hormone in test solution containing binding proteins is then analyzed according to Eq II to determine whether uptake can be accounted for entirely via the pool of free hormone after spontaneous dissociation of the hormone-protein complex within the tissue vasculature (44). Almost invariably, Pardridge *et al.* have concluded that it cannot. This conclusion, however, is inconsistent with the majority of their data and, where it is consistent with their data, is likely to be due to experiment artifacts.

In general, the arguments of Pardridge *et al.* that tissue influx rate constants for free hormone are too low to account for observed rates of hormone uptake from serum in accordance with the free hormone transport hypothesis use  $T_4$  and  $T_3$  as examples. Thus, Pardridge *et al.* (44) pointed out that in Eq II the influx rate constant ( $k$ ) for free  $T_4$  must be “extraordinarily high” to account for uptake of  $T_4$  from serum exclusively via the free pool. That is, assuming a free fraction of  $T_4$  in human serum of 0.0003 (Table 1), a fractional unidirectional hepatic (rat) uptake of  $T_4$  from human serum of 0.15 (Table 3), and a 9-sec sinusoidal transit time (Table 2), the influx rate constant for free  $T_4$  must equal 60  $\text{sec}^{-1}$  according to Eq II to satisfy the free hormone transport hypothesis. Based on their measured fractional unidirectional hepatic uptake of free  $T_4$  of 43%, Pardridge *et al.* (44) calculated (using Eq I) the influx rate constant for free  $T_4$  to instead be only 0.11  $\text{sec}^{-1}$ , and therefore concluded that the free hormone transport hypothesis was not valid for  $T_4$ . Leaving aside for the moment the question of the validity of these data, it should first be noted that the extrapolation of this argument to other hormones (6) is difficult to justify. In the case of cortisol, for example, where the free fraction in serum is more than 100-fold greater than that of  $T_4$ , the difference between similarly predicted and measured influx rate constants is not nearly so great. Likewise, in the case of many of the hormones that Pardridge *et al.* have studied (including testosterone, dihydrotestosterone, estradiol, and progesterone), tissue uptake in the

absence of binding proteins is “nearly complete”<sup>2</sup> (46, 48, 53–55), indicating immeasurably high tissue influx rate constants. Thus, even if the data and arguments of Pardridge *et al.* are valid for  $T_4$  and  $T_3$ , their extrapolation to other hormones cannot be considered appropriate.

Furthermore, the experimental data obtained for  $T_4$  and  $T_3$  by Pardridge *et al.* are unlikely to be valid, as discussed previously (56, 57). Pardridge *et al.* (58, 59) have estimated that, due to mixing on injection, their test solutions contain approximately 5% endogenous rat plasma as they traverse the tissue capillaries or sinusoids.<sup>3</sup> Pardridge (57) has argued that this degree of contamination is not significant, using for his calculations the expected binding of steroid hormones to albumin in 5% plasma. This argument, however, is not sustainable for two reasons. First, even if only a small fraction of hormone is protein-bound, an influx rate constant calculated on the basis of a measured (for example) 75% uptake is enormously different from that calculated from a greater than 99% uptake (in which case only a minimum estimate can be obtained; see below). Second, ignoring the higher affinity binding proteins in rat plasma in this argument is difficult to justify [actually, in the case of  $T_4$ , even the binding to albumin is of high affinity compared to the binding of steroids to albumin (Table 1)]. In the case of  $T_4$ , for example, assuming a concentration of thyroid hormone-binding prealbumin (TBPA) in rat plasma of 4.3  $\mu\text{M}$  and an equilibrium dissociation constant ( $K_D$ ) of  $T_4$  for rat TBPA of  $3 \times 10^{-9}$  M (60), it can be calculated from the mass law equation that more than 98% of injected  $T_4$  (2 nM) will be bound if the test solution is contaminated with 5% rat plasma. Clearly,

<sup>2</sup> Pardridge *et al.* (53–55) consider unidirectional tissue uptakes in excess of 80% “nearly complete;” measured uptakes of 80% are thus regarded as indistinguishable from uptakes of 100% of their system. This is appropriate given the inherent experimental uncertainty in their methodology, which has at least two sources: 1) The Oldendorf injection method requires comparing the amount of hormone taken up by tissues with the amount of hormone injected. When the fractional uptake approaches 100%, this comparison requires calculating a small difference between two large numbers. Therefore, as fractional uptakes approach 100%, small errors in the measured values can result in large errors in the calculated fractional uptakes. (Techniques in which the actual measurements involve the fraction of hormone *not* taken up by tissues, such as ours (19–21), are thus expected to be more accurate when fractional uptakes are high, although less accurate when fractional uptakes are low.) 2) In addition, in the Oldendorf injection technique, the fractional uptake of hormone must be calculated using the initial uptake of a “freely diffusible” isotope as a reference. The initial uptake of the reference isotope cannot be measured, however, because significant efflux of the isotope from the tissue occurs over the time course (15–18 sec) of the experiments. The initial uptake must therefore be obtained by extrapolation (54). Statistical analyses of the fractional tissue uptakes of hormones provided by Pardridge *et al.* have never adequately taken into account the uncertainty in this latter measurement.

<sup>3</sup> Estimation of this degree of mixing involved the assumption that the tissue influx rate constant for the free ligand used was low (58). If this assumption was incorrect, the data in fact indicate a much higher degree of mixing (58).

uptake of  $T_4$  from such a solution cannot be considered equivalent to uptake of  $T_4$  in a solution lacking binding protein. It should also be noted that the "essentially protein-free" (61) lactated Ringer's solution that Pardridge *et al.* use as an injection vehicle contains 0.1% bovine albumin. Since this concentration of albumin is high enough to bind the majority of injected  $T_4$  and  $T_3$ , such an injection vehicle cannot be considered *a priori* as being appropriate for assessment of tissue influx rate constants for free hormone. Bound fractions of various hormones in 0.1% albumin and 5% rat plasma are shown in Table 4.

Pardridge *et al.* (44, 45) also contend on experimental grounds that mixing of their serum-free injection solutions with rat plasma does not account for the relatively low fractional hepatic uptakes of  $T_4$  (43%) and  $T_3$  (74%) that they observe. The basis of this contention is their observation that these uptakes are unaffected by the addition to the injection solutions of unlabeled  $T_4$  or  $T_3$  in concentrations (50  $\mu\text{M}$ ) expected to saturate the high-affinity binding proteins in 5% rat plasma. However, the

TABLE 4. Binding of hormones by 0.1% bovine albumin or 5% rat plasma in the Oldendorf injection technique

Hormone	% Bound (0.1% albumin)	% Bound (5% rat plasma)
$T_4$ (2 nM)	93	99
$T_4$ (50 $\mu\text{M}$ )	77	92
$T_3$ (2 nM)	66	88
$T_3$ (50 $\mu\text{M}$ )	45	67
Testosterone (15 nM)	37	54
Dihydrotestosterone (10 nM)	37	54
Estradiol (15 nM)	46	63
Progesterone (20 nM)	46	78
Cortisol (25 nM)	4	86
Corticosterone (20 nM)	13	66
Aldosterone (25 nM)	3	19
1,25-(OH) <sub>2</sub> vitamin D <sub>3</sub> (200 nM)	42	82

The affinities of the different hormones for bovine and rat albumin were assumed to be the same as those for human albumin and were taken from Table 1. The concentrations of rat albumin, rat TBPA, rat CBG, rat DBP, and rat SHBG were assumed to be 0.58 mM (62), 4.3  $\mu\text{M}$  (60), 1.9  $\mu\text{M}$  (63), 3.7  $\mu\text{M}$  (64), and 0 (25–27), respectively. The  $K_A$  of  $T_4$  for rat TBPA was assumed to be  $3.5 \times 10^8 \text{ M}^{-1}$  (60) and that of corticosterone for rat CBG was assumed to be  $2 \times 10^7 \text{ M}^{-1}$  (63). The affinities of other hormones for rat plasma hormone-binding proteins [ $T_3$  for TBPA, cortisol, progesterone, and aldosterone for CBG, and 1,25-(OH)<sub>2</sub>vitamin D<sub>3</sub> for DBP] were assumed to be the same as those for the human hormone-binding proteins and were taken from Table 1. Endogenous hormone concentrations in rat plasma were ignored because, for the most part, they are low (in 5% plasma) when compared to the injected hormone concentrations (shown in parentheses and taken from Refs. 45, 46, 48, and 49). Concentrations of free and bound ligand and binding protein for each of m ligands and n binding proteins were obtained by solving simultaneously by the Gauss-Newton method all equilibrium equations (based on the law of mass action) and conservation equations for given total concentrations of m ligands and n binding proteins and for given  $K_A$  values describing each ligand-protein interaction.

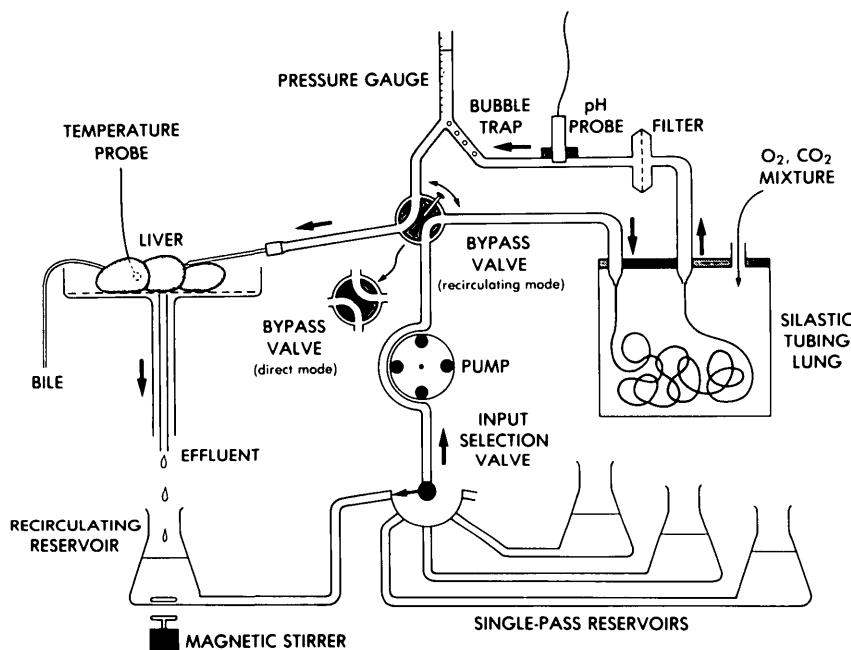
data in Table 4 show that the fraction of  $T_4$  or  $T_3$  bound by 5% rat plasma (or 0.1% bovine albumin) is relatively unaffected by the addition of 50  $\mu\text{M}$   $T_4$  or  $T_3$  to the serum-free injection solutions. Therefore, this is not a valid argument.

The fractional unidirectional hepatic uptake of free cortisol (73%) measured by Pardridge *et al.* (46) is also likely to be an underestimate of the true value, but for a different reason. Pardridge's methodology requires the assumption that once radiolabeled hormone is taken up by the liver, no efflux back to the plasma occurs within 18 sec (5, 46). When this assumption is not valid, fractional unidirectional uptakes will be underestimated. Our data with respect to cortisol uptake by the perfused rat liver indicate that this assumption is not likely to be valid for cortisol (21).<sup>4</sup> Thus, experimental artifacts are likely to account for Pardridge's data indicating that tissue influx rate constants for free  $T_4$ ,  $T_3$ , and cortisol are not high, and Pardridge's own data indicate that tissue influx rate constants for free testosterone, dihydrotestosterone, estradiol, and progesterone are indeed very high.

Our own measured values for the hepatic influx rate constants for  $T_4$ ,  $T_3$ , and cortisol are considerably higher ( $>1 \text{ sec}^{-1}$ ) than those of Pardridge *et al.* and have been obtained by two independent methods. Using an *in vitro* rat liver perfusion system (Fig. 1), we (19–21) measured rates of unidirectional hepatic uptake from protein-free solution for  $T_4$ ,  $T_3$ , and cortisol (all fractional uptakes were  $>99\%$  even at very rapid flow rates) and calculated influx rate constants according to Eq I. In addition, we calculated influx rate constants from quantification of the lobular concentration profiles of hormone taken up by the liver from protein-free solution (see Figs. 2 and 3 as examples). In all cases, our calculated influx rate constants ranged from  $1 \text{ sec}^{-1}$  to  $3 \text{ sec}^{-1}$ . In the case of cortisol, this value was high enough to fully account (according to Eq II) for the observed hepatic uptake of cortisol from serum in accordance with the free hormone transport hypothesis (21). In the cases of  $T_4$  and  $T_3$ , these values could only account for a small fraction of the observed hepatic uptakes of hormone from serum in accordance with the free hormone transport hypothesis. Nevertheless, these values of hepatic influx rate constants for free  $T_4$  and  $T_3$  stand in marked contrast to the much lower values reported by Pardridge *et al.* (44, 45). Furthermore, they are appropriately viewed as only minimum estimates of the true values. This is so for three reasons.

Single-pass uptake of free hormone cannot be used to

<sup>4</sup> We have recently confirmed this finding using classical indicator dilution methodology (9). Significant efflux of cortisol from the liver occurs within 3 sec of injection (Mendel, C. M. and R. A. Weisiger, unpublished observations).



Liver perfusion apparatus. The perfusate enters the liver through the portal vein and exits through the inferior vena cava (via the central vein). Single pass extraction is calculated as  $(C_{in} - C_{out})/C_{in}$ , where  $C_{in}$  is the concentration of labeled hormone in the perfusate entering the liver and  $C_{out}$  is its concentration in the effluent. [Reproduced with permission from P. Brissot *et al.*: *J Clin Invest* 76:1463, 1985 (273).]

detect more rapid influx rate constants because, when the fractional uptake approaches 100%, even small amounts of radiolabeled impurities or flow heterogeneity (*i.e.* shunt) within the tissue will cause major underestimation of the rate constant. For example, for a transit time of 9 sec and an influx rate constant ( $k$ ) of  $0.5 \text{ sec}^{-1}$ , the fractional uptake predicted from Eq I is 99%. For  $k = 1 \text{ sec}^{-1}$ , the fractional uptake would be 99.99%. There are not many experimental systems in which these values are distinguishable. The use of supraphysiological flow rates can increase the sensitivity of these types of measurements. Thus, for a transit time of 3 sec, a 99% uptake corresponds to an influx rate constant of  $1.5 \text{ sec}^{-1}$ , and for a transit time of 1 sec, a 99% uptake corresponds to an influx rate constant of  $4.6 \text{ sec}^{-1}$ . Nevertheless, the closer the measured fractional uptake is to 100%, the less distinguishable the derived influx rate constant is from 1, 10, 100, or even  $1000 \text{ sec}^{-1}$ .

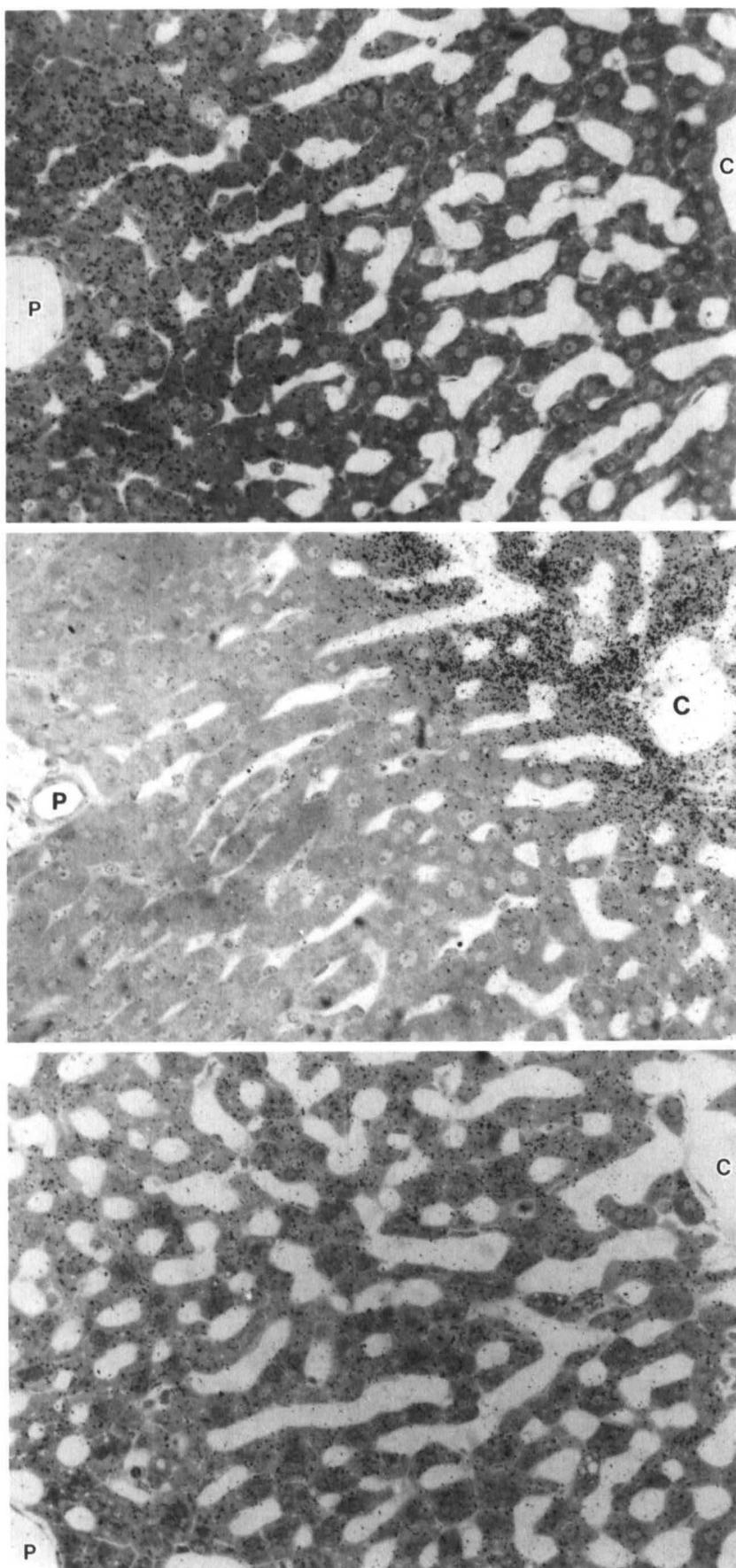
Our technique of deriving the influx rate constant from analysis of the lobular concentration profile of hormone taken up by the liver increases the sensitivity of these determinations (19, 20, 66) but is still limited in its ability to measure influx rate constants that are greater than 1 to  $3 \text{ sec}^{-1}$ . This is because axial mixing of hormone within the sinusoids or redistribution of hormone within the hepatic lobules during tissue processing would be expected to reduce the gradient of its lobular concentration profile and therefore the calculated influx rate constant. Some evidence that both of these processes may in fact occur has been presented (19).

Finally, diffusional barriers at the cell surface provide another possible explanation for why higher tissue influx rate constants for free hormones do not appear to be

measurable. Diffusional concentration gradients may form across such barriers when the influx rate constant is high enough. Such gradients reduce the hormone concentration at the cell surface below the concentration found in the bulk sinusoid or capillary bed, thereby resulting in a lower uptake rate and a lower apparent influx rate constant (12, 13). Binding proteins can then significantly enhance the apparent influx rate constant for free hormone because bound hormone at the cell surface provides an additional source of free hormone that augments the diffusional flux of free hormone across the diffusional barrier (12, 13).

Thus, there are both experimental and theoretical limitations to the direct measurement of high tissue influx rate constants for free hormone. The highest value for an influx rate constant that can be determined accurately must be carefully evaluated in each system. Values obtained in this range must then be considered only as minimum estimates of the true values. As discussed above, systems that allow for even a small amount of contamination of free hormone with binding proteins cannot be accepted *a priori* as providing valid means of determining tissue influx rate constants for free hormone.

In summary, we have recently shown that the hepatic uptakes of  $T_4$  (19),  $T_3$  (20), and cortisol (21) from protein-free solution are all described by influx rate constants that are at the upper bounds of values that can be accurately determined by presently available methods. In the case of cortisol, this rate constant is high enough to fully account for the observed hepatic uptake of cortisol from serum in accord with the free hormone transport hypothesis (21). In addition, Pardridge *et al.* (53-



**FIG. 2.** Autoradiographs of rat liver after single pass perfusion (flow rate = 1 ml·min<sup>-1</sup>·g liver<sup>-1</sup>) of [<sup>125</sup>I]T<sub>4</sub> in various tests solutions. P, Portal venule; C, central venule.

*Top,* Perfusion through the portal vein of [<sup>125</sup>I]T<sub>4</sub> in protein-free buffer. An apparent influx rate constant for free T<sub>4</sub> can be calculated from the concentration profile of [<sup>125</sup>I]T<sub>4</sub> shown in this photograph (see Fig. 3). [Reproduced with permission from C. M. Mendel *et al.*: *Endocrinology* 120:1742, 1987 (65).]

*Middle,* Retrograde perfusion (66) through the central vein of [<sup>125</sup>I]T<sub>4</sub> in protein-free buffer. This study indicates that observed gradients in the concentration profile of [<sup>125</sup>I]T<sub>4</sub> taken up by the liver reflect corresponding gradients in the concentration of [<sup>125</sup>I]T<sub>4</sub> within the sinusoids rather than differences in intrinsic uptake capacity for T<sub>4</sub> between periportal and centrilobular cells. [Reproduced with permission from C. M. Mendel *et al.*: *Am J Physiol* 255:E110, 1988 (19).]

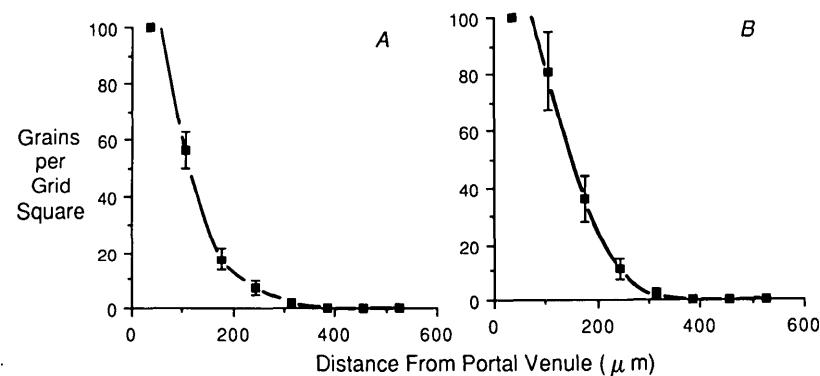
*Bottom,* Perfusion through the portal vein of [<sup>125</sup>I]T<sub>4</sub> in human serum. The uniformity of uptake [documented quantitatively (65)] can be attributed to the decreased fractional extraction of T<sub>4</sub> in serum compared with that in buffer (11% vs. > 99%). This study also indicates that all cells within the lobule have approximately equal intrinsic capacities to take up T<sub>4</sub>. [Reproduced with permission from C. M. Mendel *et al.*: *Endocrinology* 120:1742, 1987 (65).]

**FIG. 3.** Quantitation of portal-to-central concentration profiles of [<sup>125</sup>I]T<sub>4</sub> in rat liver after its single pass uptake from protein-free solution. Perfusion was through the portal vein at a flow rate of  $1 \text{ ml} \cdot \text{min}^{-1} \cdot \text{g liver}^{-1}$  (A) or  $3 \text{ ml} \cdot \text{min}^{-1} \cdot \text{g liver}^{-1}$  (B). Quantitations were performed using a microscope eyepiece grid placed over microscopic fields similar to those shown in Fig. 2. All of the grains in each grid square were assigned a distance from the portal venule equal to that of the middle of the grid square. The number of grains in each grid square was normalized to the grain density of the grid square closest to the portal venule (assigned a value of 100). Each point shown is the mean  $\pm$  SE of countings in 15 lobules from 3 livers (A) or 7 lobules from 2 livers (B). Each of these sets of data can be used to derive an apparent influx rate constant ( $k$ ) for free T<sub>4</sub> from the equation  $C/C_0 = e^{-kt}$ , where  $C$  is the number of grains in each grid square,  $C_0$  is the number of grains in the grid square closest to the portal venule, and  $t$  is time, derived from the known dimensions of each grid square and the known linear flow rate. The apparent influx rate constant derived from panel A was  $1.1 \pm 0.2 \text{ sec}^{-1}$ , and that from panel B was  $2.8 \pm 0.3 \text{ sec}^{-1}$ . [Reproduced with permission from C. M. Mendel *et al.*: Am J Physiol 255:E110, 1988 (19).]

55) have obtained similar findings for the hepatic, brain, and uterine uptakes of testosterone, dihydrotestosterone, estradiol, and progesterone. Considering these data in light of the fact that, for all cases studied to date, rates of tissue uptake of hormone have never been found to exceed rates of spontaneous dissociation of protein-hormone complexes within the tissue vasculature, the free hormone transport hypothesis must be considered to rest on firm ground at this point in time.

### III. The Free Hormone Transport Hypothesis: Specific Parameter Values

If it is assumed that thyroid hormone uptake by the liver occurs via the free pool, then approximate hepatic influx rate constants for free T<sub>4</sub> and free T<sub>3</sub> in the human can be calculated using Eq II (provided the assumptions on which this equation is based hold in this situation; see below) from data listed in Tables 1 to 3. Assuming a free T<sub>4</sub> fraction in plasma of 0.0003, an average sinusoidal transit time of 9 sec, and a fractional unidirectional hepatic uptake of 0.048, then the influx rate constant for free T<sub>4</sub> by the human liver is  $18 \text{ sec}^{-1}$  according to Eq II. Similarly, assuming a free T<sub>3</sub> fraction in plasma of 0.004 and a fractional unidirectional hepatic uptake of 0.29,



then the influx rate constant for free T<sub>3</sub> by the human liver is  $10 \text{ sec}^{-1}$ . Similar calculations performed previously for cortisol with regard to rat liver yielded an estimated influx rate constant for free cortisol by the perfused rat liver of  $2 \text{ sec}^{-1}$  (21).

The use of Eq II in these calculations requires the assumptions (as discussed above, *Section II*) that the hepatic sinusoids are well mixed along their radial axes (such that diffusional gradients do not form at the cell surface) and that bound and free radiolabeled hormone remain at equilibrium during transit through the sinusoids. The less valid these assumptions, the more likely the influx rate constants calculated above will be underestimates of the true values. The first of these assumptions is difficult to test. Although the concept of an unstirred water layer immediately adjacent to the cell surface is well accepted, the permeability of this unstirred layer is usually already taken into account in the derived influx rate constant. Thus, the question remaining is, How well mixed along the entire radial axis is the sinusoid (or capillary)? This question has not been answered either quantitatively or qualitatively (its answer may be very different in the presence or absence of red blood cells). If the assumption of complete radial mixing is not made, however, then predictions based on a more com-

plicated model can be made only in the setting of assumed quantitative values for the degree of mixing. Thus, in the complete absence of data on this point, it is difficult to incorporate deviations from this assumption into any model. It can be concluded, nevertheless, that if this consideration is ever important, it is less likely to be important in the setting of a low fractional uptake (12).

The validity (or lack thereof) of the second of these assumptions, that bound and free radiolabeled hormone remain at equilibrium during transit through the sinusoids, can be reasonably predicted for any given set of conditions using appropriate calculations (5, 21), and can be demonstrated by comparing predictions made using Eq II with predictions made using more complicated equations that do not depend on this assumption. The probable lack of validity of this second assumption for many hormones under many different sets of conditions in fact limits the widespread use of Eq II when rates of unidirectional uptake of hormone are considered. Nevertheless, in a recent study, such a more complicated set of equations was used to predict a fractional hepatic  $T_4$  uptake from plasma of 13.3% under the conditions considered (influx rate constant =  $110 \text{ sec}^{-1}$ ) (67), and reanalysis of that same situation (see Table 2 in Ref. 67) using Eq II yields a predicted hepatic  $T_4$  uptake of 16.1%. The similarity of these two predictions suggests that with respect to  $T_4$  in human plasma, this second assumption is not likely to be too inaccurate. Therefore, at least with regard to  $T_4$ , the influx rate constant derived using Eq II may be a fair approximation of the true value.

If both of the assumptions discussed above represent good approximations of the true conditions, and Eq II can therefore be used validly, then, in the setting of relatively low fractional uptakes, the fractional tissue uptake of hormone should be proportional to the free hormone fraction in the plasma when the free hormone fraction is varied (see Eq II). Conversely, if either of these assumptions is incorrect, such a proportionality would not be expected. Data collected by Cavalieri and Searle (39) more than two decades ago, in which the hepatic uptake of  $T_4$  was measured using an external  $\gamma$ -detector, do in fact suggest that the fractional unidirectional hepatic uptake of  $T_4$  in humans *in vivo* is proportional to the free  $T_4$  fraction in plasma (Fig. 4). Similar data obtained more recently by us (68), using a different method of data analysis [that of Oppenheimer *et al.* (40)], are also consistent with this conclusion. However, a recent study by Kaptein *et al.* (42), in which a three-pool model was used to analyze the *in vivo* kinetics data, did not find as clear a relationship between the fractional unidirectional hepatic  $T_4$  uptake and the free  $T_4$  fraction in plasma in subjects with primary abnormalities of circulating  $T_4$ -binding proteins. (Despite the different

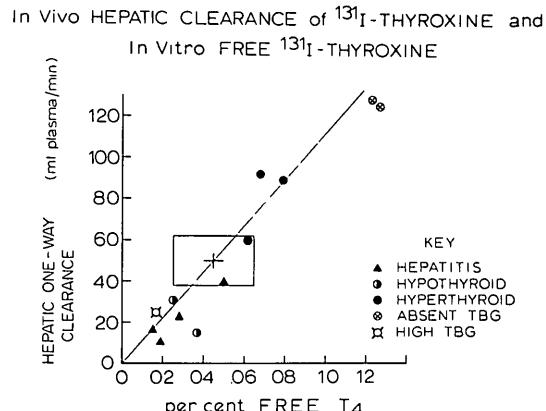


FIG. 4. Hepatic one-way clearance of  $T_4$  vs. per cent free  $T_4$  in plasma in humans. Hepatic one-way clearance was measured using an external  $\gamma$ -detector (39). Per cent free  $T_4$  was estimated using a  $T_4$  Sephadex uptake assay (39). Data were compiled by Dr. Cavalieri more than 20 yr ago from published (39, 91) and unpublished studies. Rectangle indicates normal range. The subjects with hepatitis had increased serum concentrations of TBG (91).

methods employed in each of these studies, each yielded a similar value for the unidirectional hepatic (rapid pool) uptake rate constant for  $T_4$  in normals (39, 42, 68). Therefore, the reason for their discrepant findings is not clear, although it may be related to the small number of subjects in each study.) Consequently, whether or not the hepatic influx rate constant for free  $T_4$  calculated using Eq II is a significant underestimate of the true value must be considered an unsettled issue at this point in time.

Although the mechanisms of, and rules governing, the unidirectional uptake of hormones by tissues are of considerable interest, physiological conclusions should never be based on measurements of unidirectional uptake alone. In the case of  $T_4$ , for example, more than 98% (see below, Section VI.A.1) of the hormone taken up unidirectionally by the liver *in vivo* returns to the plasma unmetabolized, indicating that measurements of unidirectional uptake bear little relevance to the steady state. Thus, some uncertainty over whether or not the unidirectional tissue uptake of  $T_4$  is proportional to the plasma free  $T_4$  concentration, and some uncertainty over the true values of tissue influx rate constants for free hormones, should not be considered a major impediment to understanding the relationships between extra- and intracellular hormone concentrations *in vivo*.

#### IV. The Free Hormone Hypothesis: History and Empiric Testing

The free hormone hypothesis was first proposed by Recant and Riggs (69) in 1952, the same year in which plasma thyroid hormone-binding proteins were discovered (70–72). Recant and Riggs noted that their patients

with nephrotic syndrome were euthyroid despite having low serum concentrations of thyroid hormone. They postulated that a then undetectable unbound fraction of thyroid hormone in plasma, rather than the protein-bound fraction, affected biological activity, and cited as precedent the previously recognized importance of ionized (*vs.* total) calcium in plasma (73). This hypothesis was subsequently formalized by Robbins and Rall (74), who stated in the summary of their 1957 review: "It appears likely that thyroid hormone action is a function of the concentration of free hormone in the blood, and . . . the amount of thyroxine metabolized may also be a function of the level of free thyroxine in serum." Ingbar and Freinkel (75) attempted to describe this hypothesis in terms of a mathematical and physiological model, but not enough was then known to complete such a formulation.

Empirical testing of the free hormone hypothesis has come from two types of observations. Thus, when subjects with primary abnormalities in the concentrations of plasma hormone-binding proteins are identified, it can be determined whether their free or protein-bound hormone concentrations in plasma are conserved (*i.e.* are in the normal range). For the thyroid hormones (22), testosterone (76), and 1,25-dihydroxyvitamin D (77, 78), it is generally accepted that the free hormone concentrations in plasma are conserved in such cases. The free hormone hypothesis can also be tested empirically using data from *in vivo* kinetics studies. For hormones whose disposal occurs intracellularly, the disposal rate can reasonably be assumed to be proportional to the intracellular hormone concentration (this requires the assumptions that the disposal rate constant is not altered in the condition being studied and that the mechanism for disposal is far from saturated under physiological conditions). Thus, when the free hormone hypothesis is correct, under most circumstances the disposal rate of hormone (which can be determined from *in vivo* kinetics studies) will be proportional to the concentration of free hormone in the plasma. This was originally pointed out in 1957 by Robbins and Rall (74), who noted that the then available *in vivo* kinetics data for T<sub>4</sub> supported the free hormone hypothesis. Data accumulated over the past 30 yr for T<sub>4</sub> and T<sub>3</sub> continue to support this hypothesis as applied to these hormones (Figs. 5 and 6). It is important to note, however, that this type of data could be misleading if the free hormone hypothesis were valid with respect to the organ responsible for the metabolism of the hormone, but not valid with respect to other organs of interest (or vice versa).

Thus, there have been a number of empirical demonstrations that the free hormone hypothesis can be appropriately applied to several hormones. However, a model based entirely on empiricism has certain weaknesses,

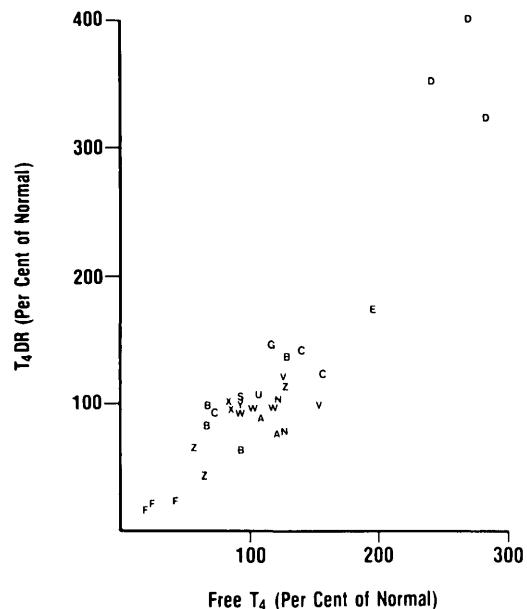


FIG. 5. Correlation between the disposal rate (DR) of T<sub>4</sub> obtained from *in vivo* kinetics studies, and the plasma free T<sub>4</sub> concentration in humans (Spearman rank correlation coefficient = 0.80,  $P < 0.001$ ). Data on both DR and free T<sub>4</sub> are expressed as fractions of the midnormal value; therefore, data on normals are not shown. These data were collected from published studies. Each data point represents the mean value in each group of subjects studied; individual studies often contained more than one group. For a study to be included in this figure, it had to fulfill the following criteria: Measurements or estimates of the plasma free T<sub>4</sub> concentration had to have been made for both the study subjects and the normals at the same time that the kinetics studies were performed; if the free T<sub>4</sub> concentration was estimated rather than measured directly, the method could not employ a T<sub>3</sub> resin uptake test in cases of nonthyroid illness or FDH; for a subject group to be included, more than one case per group had to have been studied.

No published study or subject group that met these criteria was intentionally excluded, except for studies of drug effects. Key to letter symbols and references: A, Elevated TBG (79, 80); B, decreased TBG (39, 42, 80); C, FDH (42, 68, 79); D, thyrotoxicosis of Graves' disease (80, 81); E, toxic nodular goiter (66); F, hypothyroidism (80, 81); G, treated Graves' disease (81); XYZ, nonthyroid illness (mild, moderate and severe) (42, 82–84); W, chronic renal failure (42, 85, 86); V, cirrhosis (67, 84); U, hepatitis (67); S, ethanol abuse (42); N, caloric deprivation (87, 88). In our published study of FDH (68), plasma free T<sub>4</sub> concentrations in the normals were not given, but data obtained from our records were used for this figure. [Reproduced with permission from C. M. Mendel and R. R. Cavalieri: *Thyroid Today* 11:3, 1988.]

perhaps the most important of which is that it cannot readily be extrapolated to new situations. Therefore, we (19–21) recently began to explore the fundamental mathematical and physiological principles relating to the free hormone hypothesis.

## V. The Free Hormone Hypothesis: A Physiologically Based Mathematical Model

### A. Introductory Statements

Despite the probable validity of the free hormone transport hypothesis for most or all hormones (or at least

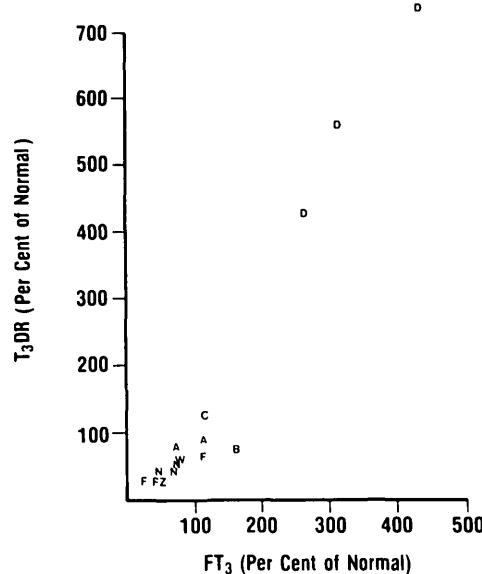


FIG. 6. Correlation between the disposal rate (DR) of  $T_3$  obtained from *in vivo* kinetics studies, and the plasma free  $T_3$  concentration ( $FT_3$ ) in humans (Spearman rank correlation coefficient = 0.93,  $P < 0.001$ ). These data were obtained from published studies (80, 82, 85, 87–90). The data presentation, letter symbols, and criteria for inclusion in the figure are the same as described for Fig. 5. [Reproduced with permission from *Thyroid Today* (see Fig. 5 for citation).]

the lack of a compelling reason to reject this hypothesis at this time), it is important to realize that this finding does not bear directly on whether or not the free hormone hypothesis is correct. Early assumptions to the contrary aside, the free hormone concentration inside of cells could under some circumstances be proportional to the free hormone concentration in the plasma even if hormone uptake were to occur directly via the protein-bound pool, and it can under some circumstances be proportional to the bound hormone concentration in the plasma even when uptake occurs exclusively via the free pool (see below).

A number of general models of ligand transport *in vivo* have been developed. If all of the rates and rate constants in the tissue uptake process of any given hormone were known, then one of these models could be used to predict intracellular hormone concentrations, and it could be determined whether or not intracellular hormone concentrations would be expected to change proportionately as the free hormone concentration in the plasma changed. Unfortunately, no hormone exists for which all of these rates and rate constants are known with any degree of certainty. Nevertheless, it will be shown that whenever the rate-limiting step in the net tissue uptake of hormone is known, useful statements regarding the validity (or lack of validity) of the free hormone hypothesis can be made based on generally available data. In this section, only hypothetical situations will be considered. In the following section (Section VI), the applica-

bility of these hypothetical situations to particular hormones will be considered.

When one step in the tissue-uptake process is very much slower than the other steps, that step can be considered rate-limiting to tissue uptake. [A formal mathematical delineation of the conditions under which a single step can be considered rate-limiting to uptake has been presented previously (11).] Possible rate-limiting steps in the net tissue uptake of hormone are 1) plasma flow, 2) dissociation of hormone from plasma binding proteins, 3) influx of hormone to tissues, and 4) intracellular elimination of hormone. Let us first consider the situation in which intracellular elimination is rate-limiting to the net uptake of hormone by tissues. For initial consideration of this situation, plasma flow will be ignored, because uptake is independent of plasma flow (see below, Section V.C).

#### B. When intracellular elimination is rate-limiting to the net uptake of hormone by tissues

Consider the two models shown in Fig. 7. For uptake via the pool of free hormone (Fig. 7A), the rate of formation of  $L_I$  (symbols are defined in the legend to Fig. 7) can be described by the differential equation:

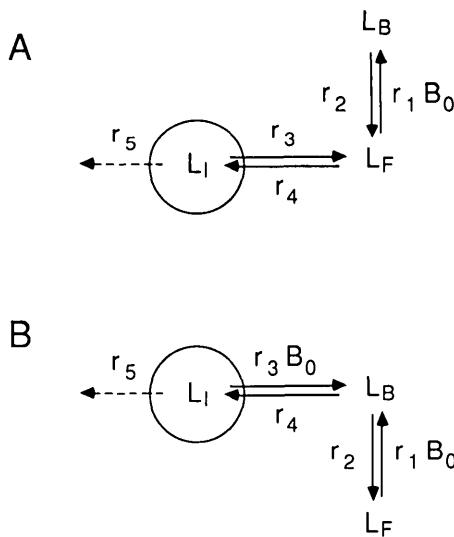


FIG. 7. Schematic of cellular uptake of hormone from plasma when uptake occurs via the free pool (A) or the protein-bound pool (B).  $L_I$ , Concentration of intracellular hormone;  $L_F$ , concentration of free hormone in plasma;  $L_B$ , concentration of protein-bound hormone in plasma;  $B_0$ , concentration of free binding protein in plasma; and  $r_1$  to  $r_5$ , rate constants describing the processes depicted. The model as presented assumes that plasma hormone-binding proteins do not enter the cell, that cellular uptake and elimination of hormone are unsaturable processes, that intracellular hormone can be treated as a single pool, and that the plasma is well stirred. Plasma flow is not considered, and only one hormone-binding protein in plasma is considered. The equations in the text relating to this model assume that intracellular and local plasma volumes are equal. [Reproduced with permission from C. M. Mendel et al.: *Endocrinology* 123:1817, 1988 (20).]

$$\dot{L}_I = L_F r_4 - L_I r_3 - L_I r_5 \quad (\text{III})$$

For consideration of the steady state,  $\dot{L}_I$  is set at 0. Thus, the relationship between  $L_I$  and  $L_F$  under steady-state conditions can be described as

$$L_I = L_F r_4 / (r_3 + r_5) \quad (\text{IV})$$

Since  $r_5 \ll r_3$  when the rate of metabolism (or other means of intracellular elimination) is much slower than the rates of efflux and influx (*i.e.* when metabolism is rate-limiting to the net tissue uptake of hormone), this equation reduces to

$$L_I = L_F r_4 / r_3 \quad (\text{V})$$

where  $r_4/r_3$  is the equilibrium partition coefficient for free ligand across the cell-surface membrane. Thus, it can be seen that under these conditions the hormone will be in approximate equilibrium between the plasma and tissues, and the free hormone hypothesis will hold.

For uptake via the pool of bound hormone (Fig. 7B), the processes depicted can be described by the following differential equations:

$$\dot{L}_I = L_B r_4 - L_I B_0 r_3 - L_I r_5 \quad (\text{VI})$$

$$\dot{L}_B = L_I B_0 r_3 + L_F B_0 r_1 - L_B (r_4 + r_2) \quad (\text{VII})$$

$$\dot{L}_F = L_B r_2 - L_F B_0 r_1 \quad (\text{VIII})$$

Under elimination-limited conditions,  $r_5$  can be set at 0 as an approximation.<sup>5</sup> For consideration of the steady state,  $L_I$ ,  $L_B$ , and  $L_F$  are set at 0. Thus, from Eq VIII:

$$B_0 = L_B r_2 / L_F r_1 \quad (\text{IX})$$

Substituting this value for  $B_0$  in Eq VII, we then derive the following equation in which the term  $L_B$  is cancelled out and  $L_I$  is found to be proportional to  $L_F$ :

$$L_I = L_F \frac{r_1 r_4}{r_2 r_3} \quad (\text{X})$$

[this relationship between  $L_I$  and  $L_F$  can be demonstrated numerically as well (19)]. Thus, it can be seen that a model in which the tissue uptake of hormone occurs via the protein-bound pool can still be compatible with the free hormone hypothesis.

The model shown in Fig. 8, which allows for cellular uptake of hormone via both bound and free pools, is more difficult to analyze. Unlike the two previous models examined, elimination-limited conditions (*i.e.*  $r_7$  set at 0) do not force this model into equilibrium at steady state, because three interconnected pathways still exist. Nevertheless, based on the above steady-state analyses, several conclusions can be drawn. If influx and efflux occur almost exclusively via one route (*i.e.* if both occur almost exclusively via either the bound or the free pool), then

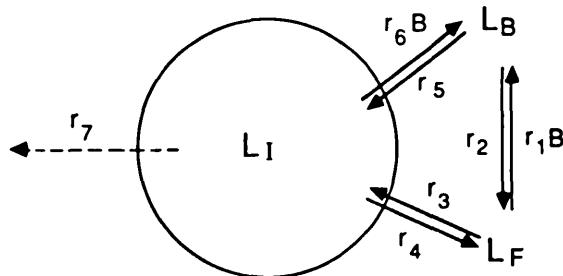


FIG. 8. Schematic of cellular uptake of hormone from plasma when uptake occurs via the protein-bound pool as well as the free pool.  $L_I$ , Concentration of intracellular hormone;  $L_F$ , concentration of free hormone in plasma;  $L_B$ , concentration of protein-bound hormone in plasma;  $B$ , concentration of free binding protein in plasma; and  $r_1$  to  $r_7$ , rate constants describing the processes depicted. Assumptions in the model depicted are the same as those for Fig. 7.

(for  $r_7$  set at 0) this model will reduce to one of the above models (Fig. 7A or Fig. 7B) and at steady state  $L_I$  will be proportional to  $L_F$  for given rate constants. Similarly, if for each route influx approximately equals efflux (*i.e.* if  $L_I r_6 B \approx L_B r_5$  and  $L_I r_4 \approx L_F r_3$ ), then equilibrium conditions will in fact be approximated and  $L_I$  will be proportional to  $L_F$  ( $L_I \approx \frac{r_3}{r_4} L_F$ ). Thus, under either of these sets of conditions, at steady state  $L_I$  will be proportional to  $L_F$  for given values of the rate constants, and the free hormone hypothesis will hold. Conversely, it can be shown that (for  $r_7$  set at 0) if influx occurs exclusively via the pool of protein-bound hormone and efflux occurs exclusively via the pool of free hormone, or if influx via the protein-bound pool ( $L_B r_5$ ) equals efflux via the free pool ( $L_I r_4$ ), then intracellular hormone concentrations will be proportional to the concentration of protein-bound hormone in the plasma ( $L_I = \frac{r_5}{r_4} L_B$ ).

For other sets of conditions, the relationships between  $L_I$ ,  $L_F$ ,  $L_B$ , and  $B$  in the model shown in Fig. 8 must be approached by numerical techniques. The limitation of this approach is that conclusions can be drawn only for the particular sets of conditions tested and cannot readily be extrapolated to other sets of conditions. Thus, in the absence of data indicating the validity of this model to describe a given situation and providing particular values for the relevant parameters, further general analyses of this model are of only limited value.

In summary, it has been shown in this section that under elimination-limited conditions, the free hormone hypothesis will hold when tissue uptake occurs exclusively via the pool of free hormone, and may still hold even if uptake were to occur via the protein-bound pool as well. It is important to recognize that the conclusions drawn from the above analyses would be unaffected by consideration of additional plasma hormone-binding proteins, of intracellular hormone divided into bound

<sup>5</sup> The following derivation is in fact valid only for  $r_5 = 0$ , in which case equilibrium obtains.

and free pools [the intracellular free pool would then bear the same relationship to the plasma hormone concentration as the total intracellular pool did in the above analyses (67)], of active transport of hormone across the cell-surface membrane (only the assigned values for the influx and efflux rate constants would be changed), or of saturability of the transmembrane transport or intracellular elimination mechanisms (additional terms would be introduced into the equations that would cause these processes to display saturation kinetics).

### C. When other steps are rate-limiting to the net uptake of hormone by tissues

For rate-limiting steps other than elimination, analyses such as those described above are more difficult. Nevertheless, from numerical solutions to a general model of ligand (or hormone) transport *in vivo* (11), important conclusions about the expected validity of the free hormone hypothesis under different sets of conditions can be reached. This model (Fig. 9) simultaneously considers the effects of plasma flow, the plasma concentration of hormone and of hormone-binding protein, and the rate constants for hormone dissociation from its binding protein, rebinding to its binding protein, influx into the cell, efflux from the cell, and intracellular elimination on the rate of uptake of hormone by tissues. It assumes that tissue uptake of hormone occurs exclusively via the pool of free hormone, but can be modified to instead consider uptake via the pool of protein-bound hormone (19, 21). It considers only a single binding protein in plasma and does not take into account diffu-

sional gradients within capillaries or sinusoids (12, 13) or nonuniformity of the capillaries or sinusoids (9, 10). For each of the extreme cases where one step is rate-limiting to uptake (*i.e.* where one step in the uptake process—plasma flow, dissociation of hormone from plasma binding proteins, influx of hormone to tissues, or intracellular elimination of hormone—is very much slower than the other steps), the net rate of uptake has been obtained numerically (11). [The conditions under which a single step can be considered rate-limiting to uptake have been described (11), although it should be emphasized that these considerations are necessarily approximations and that all steps always contribute at least minimally to limiting uptake.] Because the rate of intracellular elimination of hormone ( $L'k_3$ ) must equal the net rate of uptake of hormone ( $U$ ) at steady state, the intracellular hormone concentration ( $L'$ ) at steady state can be calculated as  $L' = U/k_3$ . Table 5 gives rates of net uptake and intracellular hormone concentrations as a function of the rate-limiting step and the mechanism of cellular uptake of hormone.

It can thus be seen from Table 5 that, for given rate constants, when plasma flow is rate-limiting to the net uptake of hormone by tissues, intracellular hormone concentrations at steady state will be proportional to the concentration of total hormone in the plasma. When

TABLE 5. Net rate of hormone uptake ( $U$ ) and intracellular hormone concentrations ( $L'$ ) at steady state under different conditions

Rate-limiting step	Uptake via which pool	$U =$	$L' =$	Free hormone hypothesis valid?
Flow	Free or bound	$QL_{t,in}$	$\frac{QL_{t,in}}{k_3}$	No
Dissociation	Free	$r_2 C_{in}$	$\frac{r_2 C_{in}}{k_3}$	No
Influx	Free	$k_1 L_e$	$\frac{k_1 L_e}{k_3}$	Yes
Influx	Bound	$k_1 C_{in}$	$\frac{k_1 C_{in}}{k_3}$	No
Elimination	Free	$\frac{k_1 k_3 L_e}{k_2}$	$\frac{k_1 L_e}{k_2}$	Yes
Elimination	Bound	$K k_3 L_e$	$K L_e$	Yes

$L_{t,in}$  is the total ligand concentration entering each sinusoidal or capillary element.  $C_{in}$  is the equilibrium-bound ligand concentration entering each element, and  $K$  is an equilibrium partition coefficient ( $= r_1 r_4 / r_2 r_3$  in the notation of Fig. 7B). Other symbols are defined in Fig. 9. For uptake via the bound pool,  $k_1$  acts on  $C$ . Values of  $U$  for the model shown in Fig. 9 were obtained numerically for uptake via the free pool (11) or by inference from these numerical solutions for uptake via the bound pool (19, 21). By definition, at steady state the net rate of uptake ( $U$ ) must equal the rate of intracellular elimination ( $L'k_3$ ). Therefore, intracellular hormone concentrations ( $L'$ ) were calculated from  $L' = U/k_3$ . Note that for elimination-limited uptake, the values for  $L'$  obtained here are the same as those given by Eq V and Eq X.

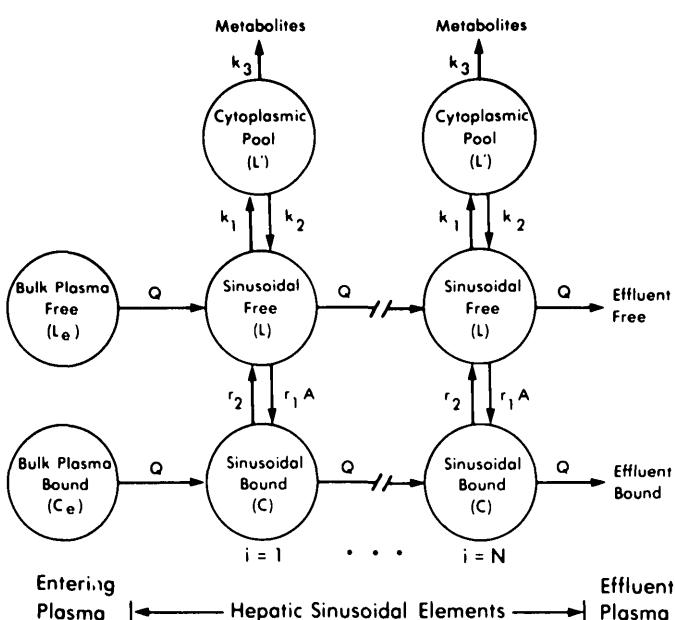


FIG. 9. Model of hormone uptake by the liver. This model can be applied to any organ. [Reproduced with permission from R. A. Weisiger: *Proc Natl Acad Sci USA* 82:1563, 1985 (11).]

dissociation from binding proteins is rate-limiting, intracellular hormone concentrations will be proportional to the concentration of protein-bound hormone in the plasma. When influx is rate-limiting, intracellular hormone concentrations will be proportional to the concentration of free hormone in the plasma if influx occurs via the pool of free hormone, but will be proportional to the concentration of protein-bound hormone in the plasma if influx occurs via the pool of protein-bound hormone. When elimination is rate-limiting to the net uptake of hormone by tissues, intracellular hormone concentrations at steady state will be proportional to the concentration of free hormone in the plasma regardless of whether influx occurs via the pool of free hormone or via the pool of bound hormone. It can also be seen from Table 5 that intracellular hormone concentrations will differ among cells at different axial locations along capillaries or sinusoids if uptake is flow- or dissociation-limited, but not if uptake is influx- or elimination-limited.<sup>6</sup> Under the latter conditions intracellular hormone concentrations will be independent of plasma flow. Finally, it can be seen from examination of Table 5 that the free hormone hypothesis can be correct even if the free hormone transport hypothesis is incorrect, and that the free hormone transport hypothesis can be correct even when the free hormone hypothesis is not.

It is important to note that the presence of more than one plasma hormone-binding protein in this model would lead to a considerably more complex situation, and that this situation has not been explicitly considered in the above analyses. Nevertheless, certain inferences about it can be reached based on those analyses. For example, if a hormone were bound to two plasma binding proteins, one with a very slow rate of dissociation and one with a very rapid rate of dissociation compared to the rate of tissue blood flow, then, in the setting of high enough influx and elimination rate constants, cellular uptake of hormone and intracellular hormone concentrations would be primarily dependent on the plasma concentration of the rapidly dissociating hormone-protein complex.

It is also important to note that when one step is not clearly rate-limiting to the net uptake of hormone by tissues, information about more of the relevant parameters in the tissue uptake process is needed to draw these types of conclusions. Furthermore, the factors not taken into account in this model (such as diffusional gradients within capillaries or sinusoids, and heterogeneity of capillaries or sinusoids) are likely to become more important. Thus, the above analysis is not meant to be considered

<sup>6</sup> If uptake is influx limited and occurs via the bound pool, then the concentration of hormone within cells will also differ among cells at different axial locations along the capillaries or sinusoids, and will not be independent of plasma flow.

complete. I do consider it useful, however (see below, *Section VI*). It should also be noted that the nonsteady state has not been considered at all in this analysis. However, it can be calculated that most hormones with binding proteins *in vivo*, even those with relatively short half-lives such as cortisol, are usually at or close to steady state most of the time (92). This is presumably because changes in the rates of secretion of hormone are slow compared with the processes (other than elimination) depicted in Fig. 9.

#### D. General statements

From the above analyses, it should be apparent that the free hormone hypothesis is not expected to be valid under all possible sets of conditions. It should also be apparent that intracellular hormone concentrations and disposal rates can be affected by factors other than circulating hormone concentrations. Thus, the free hormone hypothesis predicts that free rather than protein-bound hormone concentrations in the plasma affect intracellular hormone concentrations, but it does not state that intracellular hormone concentrations are affected *only* by the plasma free hormone concentration.

Several other general observations, not all of which are intuitively obvious, can be made from the above analyses. 1) Changes in the rate constant for intracellular elimination will greatly affect intracellular hormone concentrations at steady state under all conditions *except* when elimination is rate-limiting to uptake (Table 5). That is, intracellular hormone concentrations will be relatively independent of changes in intracellular concentrations of metabolizing enzymes when uptake is elimination-limited, but not when other steps limit uptake (Table 5). In contrast, elimination (disposal) rates will be greatly dependent on the intracellular elimination rate constant *only* when elimination is rate-limiting to uptake (Table 5). 2) If a hormone exerts intracellular effects in a tissue but is not metabolized in that tissue, then the steady-state uptake of that hormone by that tissue must by definition be elimination-limited. In such a case, however, the measured *in vivo* disposal rate would not reflect the intracellular hormone concentration in that tissue. Thus, for a given hormone, the free hormone hypothesis may be valid with respect to the tissues of interest, but its validity may not be apparent from total body *in vivo* kinetics studies. 3) Similarly, it should be apparent that, for a given hormone, the free hormone hypothesis may be valid with respect to some tissues but not with respect to others, if different steps are rate-limiting to uptake in different tissues. Thus, if a hormone were cleared largely by the liver in the setting of dissociation-limited uptake, and if the net uptake of that hormone by other tissues was elimination-limited, then

an increase in the plasma concentration of protein-bound hormone (with an unchanged plasma free hormone concentration) would result in increased delivery of that hormone to the liver and increased intracellular hormone concentrations within the liver, but intracellular hormone concentrations in other tissues would be unchanged. 4) It should also be apparent that even if the free hormone hypothesis is valid for a given hormone with respect to a number of tissues, intracellular (free) hormone concentrations in those different tissues may still differ, if, for example—taking the case of elimination-limited uptake—the ratio of the influx and efflux rate constants differs in the different tissues. 5) Finally, it should be apparent that the highest intracellular free hormone concentration possible is that which would exist in the absence of intracellular elimination and, in the absence of active transport, is equal (at steady state) to the free hormone concentration in the plasma. Thus, although uptake for a given hormone may be flow-limited and dependent on the total hormone concentration in the plasma, in the absence of active transport the intracellular free hormone concentration at steady state will nevertheless be less than the free hormone concentration in the plasma. Because of this consideration, it could be argued on teleological grounds that, *in vivo*, hormone uptake by tissues in which that hormone exerts significant physiological effects might generally be anticipated to be—assuming optimal economy—elimination-limited, at least when the physiological effects of that hormone are not related to its metabolism.

It can thus be seen that, although the situation may not always be simple, a reasonable prediction as to whether or not the free hormone hypothesis is likely to be valid for any given hormone with respect to any given tissue can usually be made if a single step is effectively rate-limiting to the net uptake of that hormone by that tissue. Ekins (12) has previously suggested that different (and conflicting) models of hormone delivery evolved because hormones with different rates of net tissue uptake were considered in each case.<sup>7</sup> Thus, the free hormone hypothesis as advocated by Robbins and Rall (74) was correct when applied to a hormone ( $T_4$ ) whose net

<sup>7</sup> Despite the considerable insights of Ekins on this subject, his own discussions relating to the free hormone hypothesis (4, 12, 95) have centered on descriptions of the unidirectional uptake of hormone by tissues. Although he has acknowledged the importance of considering net uptake rather than unidirectional uptake in this context, his descriptions of intracapillary events nevertheless ignore hormone efflux from tissues (4, 12, 95) and are therefore difficult to justify in this context. This is particularly true when these descriptions are applied to the thyroid hormones, for which very rapid efflux of hormone from tissues occurs (96). Thus, *in vivo* the free  $T_4$  concentration within hepatic sinusoids is maintained at equilibrium in the face of rapid tissue uptake not only by rapid dissociation of  $T_4$  from its plasma binding proteins (4), but also by rapid efflux of  $T_4$  from the liver (see below, Section VI.A.).

tissue uptake was slow and limited by the rate of intracellular elimination. In contrast, Tait and Burstein (93) and Baird *et al.* (94) considered the hepatic uptake of cortisol, whose net uptake was much faster. If their assumption that the rate of dissociation of cortisol from corticosteroid-binding globulin (CBG) was very slow had been correct,<sup>8</sup> then their conclusion that the delivery of cortisol to the liver was a function of the free plus albumin-bound fractions of hormone in plasma rather than just the free fraction would have been correct (see below, Section VI.B).

In the section that follows, predictions will be made about the validity (or lack thereof) of the free hormone hypothesis with respect to particular hormones and particular tissues. These predictions will be based on the concepts developed in this section but will also depend on data that is often incomplete. Therefore, they should be considered subject to modification as more complete data become available.

## VI. The Free Hormone Hypothesis: Application to Particular Hormones

### A. Thyroid hormones

1. *General statements.* It has been calculated that even if all of the metabolism of  $T_4$  and  $T_3$  in humans occurred in the liver, more than 99% of the  $T_4$  and  $T_3$  taken up unidirectionally by the liver would still return to the plasma unmetabolized (96).<sup>9</sup> Thus, intracellular elimination is more than 100-fold slower than the hepatic influx and efflux of these hormones, and elimination can be considered almost exclusively rate-limiting to the net uptake of thyroid hormones by the liver. Therefore, the free hormone hypothesis is expected to be valid for  $T_4$  and  $T_3$  with respect to the liver. Given that rates of metabolism are almost certainly slower in other organs (and, presumably, nonexistent in some), and that thyroid hormones display a high avidity for lipid membranes in general (97), it seems unlikely that a different step is rate-limiting to uptake in other organs. Nevertheless, because of a lack of data on rates of influx and metabolism in particular organs, this cannot be concluded with certainty. What can be concluded more certainly, however, is that neither plasma flow nor dissociation from plasma binding proteins is likely to be rate-limiting to the net uptake of the thyroid hormones by any tissue.

<sup>8</sup> It was not until 1978 that the rapid rate of dissociation of cortisol from CBG was documented (15).

<sup>9</sup> Calculations using the data from Table 3, assumed disposal rates for  $T_4$  and  $T_3$  of 80  $\mu\text{g}/\text{day}$  and 30  $\mu\text{g}/\text{day}$ , respectively, and assumed plasma concentrations for  $T_4$  and  $T_3$  of 8  $\mu\text{g}/\text{dl}$  and 150 ng/dl, respectively, indicate that more than 98% of the  $T_4$  and more than 94% of the  $T_3$  taken up unidirectionally by the liver in humans would return to the plasma unmetabolized even if the entire disposal of these hormones occurred in the liver.

This is because, for a given hypothetical organ (in the human), even assuming the shortest capillary transit time shown in Table 2 (0.7 sec), an organ blood flow rate 1/50 that of the liver (28 ml/min), and an organ rate of metabolism such that 25% of the total body metabolism of the thyroid hormones occurred in that organ, it can be calculated that less than 1% of the  $T_4$  and less than 15% of the  $T_3$  released from plasma binding proteins during a capillary transit would be metabolized.<sup>10</sup> Thus, if a step other than elimination is rate-limiting to the net uptake of the thyroid hormones by any tissue, it is likely to be influx. Under influx-limited conditions, the free hormone hypothesis would still be expected to hold, provided that uptake occurred via the pool of free hormone (Table 5). Therefore, even for organs for which no specific data are available, it is exceedingly likely that intracellular concentrations of  $T_4$  and  $T_3$  are dependent on the plasma free rather than protein-bound hormone concentration. This conclusion is supported by more than 30 yr of clinical experience and numerous *in vivo* kinetics studies (Figs. 5 and 6).

The consequences of changes in the plasma concentrations of free (and protein-bound) thyroid hormones have been documented extensively during the past 30 yr (e.g. Figs. 5 and 6). In addition, some of the factors other than the plasma free hormone concentration that can affect intracellular hormone concentrations and disposal rates have been documented to vary *in vivo* for the thyroid hormones. Although the changes in these factors that have been observed are in general proportionately smaller than observed changes in the plasma free hormone concentration (*cf.* Figs. 5 and 6), they are nevertheless of importance. When considering the consequences of such changes, it will be important to keep in mind the homeostatic and feedback mechanisms that operate *in vivo*.

**2. Changes in the elimination rate constant.**  $T_4$  is secreted by the thyroid gland and converted to the metabolically more active  $T_3$  by 5'-monodeiodination in peripheral tissues such as the liver and kidneys. Elimination of the thyroid hormones occurs mainly by deiodinative metabolism, although nondeiodinative metabolism and biliary excretion also occur. Hyperthyroidism can increase the fractional turnover of the thyroid hormones independently of changes in plasma hormone binding. This was demonstrated most lucidly more than 20 yr ago by Schusssler and Vance (98), who measured the fractional turnover of  $T_4$  in subjects before and after administration of  $T_3$  and found that it increased after  $T_3$  administration despite the fact that the serum free  $T_4$  fraction did not

change. The hyperthyroid-induced increase in the fractional turnover of  $T_4$  and  $T_3$  is also apparent on inspection of Figs. 5 and 6. An increased fractional turnover of  $T_4$  in the setting of an unchanged plasma free  $T_4$  fraction could theoretically result from changes at either one of two steps in the tissue uptake process. Assuming elimination-limited uptake, an increase in either the ratio of the influx and efflux rate constants or in the elimination rate constant could account for these data (Eq V and Table 5). Based on *in vitro* work with rat liver homogenates demonstrating that the tissue content of 5'-deiodinase is dependent on thyroid-metabolic status (99, 100), it seems reasonable to attribute the hyperthyroid-induced increase in the fractional turnover of  $T_4$  and  $T_3$  to an increase in the elimination rate constant. It should be noted in this context that changes in the elimination rate constant are expected to affect rates of intracellular elimination (and thereby total body disposal) only under conditions of elimination-limited uptake (Table 5; also see above, *Section V.D*). These observations therefore provide support for the conclusion that thyroid hormone uptake by tissues *in vivo* is elimination-limited.

Several drugs also apparently affect the elimination rate constant for  $T_4$ . Phenobarbital, which exerts minimal or no effects on the serum free  $T_4$  fraction, causes an increase in the fractional turnover of  $T_4$  in both rat and man (101, 102). Serum  $T_4$  and free  $T_4$  concentrations are maintained in normals, presumably by increased secretion of  $T_4$  from the thyroid gland (these concentrations decrease in hypothyroid subjects on a fixed replacement dose of  $T_4$ ). This indicates that the increase in the fractional turnover of  $T_4$  caused by phenobarbital does not result in increased biological activity of  $T_4$ , as pointed out by Oppenheimer *et al.* (103). It also suggests that the mechanism underlying the increased fractional turnover of  $T_4$  is an increase in the elimination rate constant. If this increased fractional turnover were due to an increase in the ratio of the influx and efflux rate constants, intracellular (free) hormone concentrations would initially increase, resulting in increased biological activity for a given plasma free  $T_4$  concentration. Homeostatic mechanisms would then be expected to come into play, causing a decrease in the plasma free  $T_4$  concentration to maintain euthyroid status. In contrast, in the case of elimination-limited uptake changes in the elimination rate constant are not expected to affect intracellular (free) hormone concentrations (Eq V and Table 5; also see above, *Section V.D*). The known enzyme-induction effects of phenobarbital (104) and the fact that, in the rat at least, hepatic mass is increased during phenobarbital administration (104) (see also below, *Section VI.A.3*) are also consistent with this conclusion. Although the observed increase in the hepatic accumulation of  $T_4$  after phenobarbital administration (102, 104) might

<sup>10</sup> These calculations were performed using the data in Table 1 (as described for Table 3) and assume the same disposal rates and plasma concentrations for  $T_4$  and  $T_3$  as in footnote 9.

be interpreted as evidence against this conclusion, it more likely is solely the result of increased intrahepatic binding of T<sub>4</sub> (see below, Section VI.A.5). Effects of phenobarbital on T<sub>3</sub> metabolism are similar but less marked (105, 106).

Diphenylhydantoin also may affect the elimination rate constant for T<sub>4</sub>, although these data are more difficult to interpret than those for phenobarbital. In contrast to the actions of phenobarbital, diphenylhydantoin causes a decrease in the concentration of T<sub>4</sub> in serum (107). This observation was initially attributed to a dilantin-induced displacement of T<sub>4</sub> from T<sub>4</sub>-binding globulin (TBG), since such displacement could be documented *in vitro* (108, 109), but Chin and Schussler (110) subsequently demonstrated that the serum concentration of free T<sub>4</sub> was also decreased in patients receiving diphenylhydantoin. Because patients receiving diphenylhydantoin are clinically euthyroid and have normal serum TSH concentrations (106), they presumably exhibit increased biological activity for a given plasma free T<sub>4</sub> concentration. Larsen *et al.* (111) demonstrated that diphenylhydantoin causes an increase in the fractional turnover rate of T<sub>4</sub> *in vivo*, thus maintaining a normal absolute T<sub>4</sub> turnover rate despite low serum free T<sub>4</sub> concentrations. Although the diphenylhydantoin-induced increase in the fractional turnover of T<sub>4</sub> could be accounted for by an increase in the ratio of the influx and efflux rate constants (and this explanation would be consistent with the apparent increase in biological activity of T<sub>4</sub> for a given plasma free T<sub>4</sub> concentration), the other known intrahepatic effects of diphenylhydantoin make an increase in the elimination rate constant more likely. The decrease in serum free T<sub>4</sub> concentrations in patients treated with diphenylhydantoin would then need to be explained by an increase in the biological activity of a given intracellular (free) T<sub>4</sub> concentration, which would presumably be due to an increase in the fractional rate of conversion of T<sub>4</sub> to T<sub>3</sub>. Consistent with this explanation is the fact that serum T<sub>3</sub> concentrations are normal in patients receiving diphenylhydantoin (106, 112-115). A recent case report describing increased T<sub>4</sub> replacement requirements in a hypothyroid patient receiving diphenylhydantoin (116) is not consistent with either possibility [or with the *in vivo* kinetics studies of Larsen *et al.* (111)], however, and the situation is complicated further by the fact that diphenylhydantoin suppresses TSH secretion (117, 118). Carbamazepine apparently produces effects on thyroid hormone metabolism that are similar to those of diphenylhydantoin (106).

Propranolol, glucocorticoids, radiographic contrast agents, and amiodarone all inhibit the peripheral conversion of T<sub>4</sub> to T<sub>3</sub> (106) and are expected, thereby, to decrease the elimination rate constant for T<sub>4</sub>.

Drugs and thyroid hormone-metabolic status are not

the only things documented to result in altered elimination rate constants for the thyroid hormones. A syndrome of generalized 5'-deiodinase deficiency, which presumably results in decreases in the elimination rate constants for T<sub>4</sub> and T<sub>3</sub>, has been described recently (119, 120). The near-normal serum T<sub>3</sub> concentrations in this syndrome are apparently maintained by elevated serum T<sub>4</sub> and free T<sub>4</sub> concentrations. In addition, the low serum T<sub>3</sub> concentrations in nonthyroid illness and calorie deprivation have generally been viewed as the result of a primary decrease in the rate of conversion of T<sub>4</sub> to T<sub>3</sub> (121). *In vivo* kinetics data in these subjects are consistent with this explanation. Although the disposal rates of T<sub>4</sub> in such patients remain roughly proportional to their free T<sub>4</sub> concentrations (Fig. 5), Kaptein (122) has pointed out that free T<sub>4</sub> clearances (*i.e.* T<sub>4</sub> clearances corrected for the serum free T<sub>4</sub> concentration) are in fact reduced compared to normal. A change in the ratio of the influx and efflux rate constants could account for these data (see below, Section VI.A.3), but a decrease in the elimination rate constant is a more likely explanation, since the fractional rate of T<sub>4</sub> to T<sub>3</sub> conversion is decreased in liver homogenates from fasted rats (99) (however, see also Section VI.A.3).

3. *Changes in the ratio of the influx and efflux rate constants.* Although many of the effects attributed above to changes in the elimination rate constant for the thyroid hormones could also result from changes in the ratio of the influx and efflux rate constants, there is yet no documentation that changes in this ratio occur *in vivo*. A number of investigators have suggested that changes in thyroid hormone influx may affect intracellular thyroid hormone concentrations, but, as has been shown (Eq V and Table 5), proper assessment of the physiological effects of a change in the influx rate constant in the case of elimination-limited uptake requires a simultaneous assessment of any possible change in the efflux rate constant. Such an assessment seems unobtainable at present. This is because rate constants calculated from *in vivo* kinetics studies have certain limitations in a physiological sense. Among these is the fact that they act on pool sizes (mass or volume) rather than on concentrations. They are therefore not equivalent to physiologically relevant rate constants. Since the concentration of thyroid hormones in plasma is known, physiologically relevant influx rate constants (that act on concentration) can be derived from the influx rate constants calculated in such studies, assuming that the tissue surface area available for exchange with plasma is known. But because the concentration of (free) thyroid hormones within tissues is unknown (and cannot be assumed to remain constant during manipulations), physiologically relevant efflux rate constants cannot be

derived. In addition, it should be noted that because the rate constants calculated from such studies relate to fractional transfers of entire pools, changes in the mass of organs during experimental manipulations would be expected to result in calculated changes in influx, efflux, and elimination rate constants (and in intracellular pool sizes) when in fact no such changes occur per unit mass of tissue.

Because transmembrane transport of thyroid hormones is a bidirectional event, in the absence of active transport at the plasma membrane at least (which may or may not occur; see below, *Section VII*), one might expect *a priori* that anything that changes the rate constant for influx would also cause a proportionate change in the rate constant for efflux. Thus, in the absence of assessments of the physiologically relevant efflux rate constants, the possible physiological implications of demonstrations that the influx of thyroid hormones to tissues can be decreased independently of changes in the plasma free thyroid hormone concentration should be considered with caution. In addition, other factors complicate many of the studies indicating that tissue influx of the thyroid hormones can be decreased independently of the plasma free hormone concentration. For example, Jennings *et al.* (123) observed that  $T_4$  uptakes were decreased in perfused livers from fasted rats compared to those from fed rats, whereas fractional rates of  $T_4$  to  $T_3$  conversion (per unit mass of tissue) were unchanged. Those investigators therefore concluded that the decreased rate of hepatic  $T_4$  to  $T_3$  conversion that occurs with fasting is due to decreased hepatic influx of  $T_4$ , resulting in decreased intracellular (free)  $T_4$  concentrations. However, reanalysis of the data in that study yields a different conclusion. Such analysis is made difficult because the measured uptakes in that study were equivalent to neither unidirectional uptakes nor the steady-state uptakes that exist *in vivo*. Nevertheless, it is important to note that the liver weights of the fasted rats in that study were almost 50% lower than those of the control rats [a finding consistent with the observations of others (124)], and that hepatic  $T_4$  uptake on a per unit mass basis in fact did not change. In the absence of data on hepatic structural changes during fasting, it seems most reasonable to conclude therefore that the physiologically relevant hepatic influx rate constant for  $T_4$  did not change with fasting. Since the fractional rates of  $T_4$  to  $T_3$  conversion on a per unit mass basis also did not change in that study, the observed decrease in the hepatic production of  $T_3$  from  $T_4$  during fasting in those experiments was likely due solely to a decrease in the mass of the liver. No change in the ratio of the influx and efflux rate constants, or in the intracellular (free)  $T_4$  concentration, needs to be postulated. Whether or not the decrease in the mass of the liver (with its apparent

accompanying decrease in 5'-deiodinase mass but not concentration) should be considered a decrease in the elimination rate constant for  $T_4$  is a question of semantics.

Similarly, decreased hepatic influx of  $T_4$  independent of changes in the plasma free  $T_4$  concentration has been documented in humans by *in vivo* kinetics studies in calorically deprived obese subjects (125) and in subjects with nonthyroid illness (42). van der Heyden *et al.* (125) concluded from their study that fasting-induced decreases in the serum  $T_3$  concentration are caused by decreased influx of  $T_4$  to the liver which, in turn, results in decreased intracellular (free)  $T_4$  concentrations within the liver and a decreased rate of conversion of  $T_4$  to  $T_3$ . As discussed above, however, this conclusion is not clearly warranted in the absence of assessment of the physiologically relevant hepatic efflux rate constant. The possibility that these data could be at least partially accounted for by a change in liver weight must also be considered carefully. During fasting, the liver appears to be autophagic; in the mouse and rat it loses 25–40% of its mass, protein, and RNA (but not DNA), presumably to sustain the needs of the rest of the body (124). There is no reason to think that the human liver does not act similarly, although the changes may be of lesser magnitude. Thus, as discussed above, the decreased hepatic influx observed by van der Heyden *et al.* (125) and Kaptein *et al.* (42) could be at least partially due to a decrease in hepatic mass, resulting in a decrease in total hepatic  $T_4$  influx but not in a decrease in influx per unit of tissue. The physiologically relevant influx rate constant, let alone the ratio of the influx and efflux rate constants, therefore, may not have been altered. [The increase in the calculated influx rate constant of  $T_4$  to the liver in patients with chronic ethanol abuse studied by Kaptein *et al.* (42) could similarly be partially explained by an increased hepatic mass in these patients.] The decrease in the apparent influx rate constant of  $T_4$  to the more slowly equilibrating tissues in both of these studies is not readily explained by this consideration, however. It should also be kept in mind that it is not absolutely certain that influx of  $T_4$  to tissues is expected to be wholly independent of the circulating protein-bound pools of  $T_4$  (see above, *Section III*).

Decreased hepatic influx of the thyroid hormones was also observed in a study performed by Cavalieri *et al.* (126). Those investigators used an external  $\gamma$ -detector to measure hepatic influx of  $T_3$  in controls, in subjects with untreated Graves' disease, and in subjects treated for Graves' disease 6 months to 15 yr previously. They found that hepatic influx of  $T_3$  was increased in subjects with active or treated Graves' disease beyond what could be accounted for by changes in plasma hormone concentra-

tions and binding. The physiological significance of this observation remains to be elucidated.

**4. Changes in plasma protein binding.** Changes in plasma protein binding are not expected to exert any effects on the metabolism of thyroid hormones at steady state (see above, *Section VI.A.1*). Thus, the recent suggestion that patients with familial dysalbuminemic hyperthyroxinemia who develop thyroid dysfunction may need to be treated differently than other subjects with thyroid dysfunction (127) is unfortunate. It is also inconsistent with the documented normal  $T_4$  and  $T_3$  disposal rates in this syndrome (68, 128). In the nonsteady state, however, such as may occur after drug administration, changes in  $T_4$ -plasma protein binding can lead to changes in thyroid hormone metabolism. Thus, the displacement of thyroid hormones from their circulating binding proteins by aspirin (129) or furosemide (130) can result in acutely increased clearance and biological activity until a new steady state is reached.

Because tissue uptake of the thyroid hormones is elimination-limited, these hormones exist in approximate equilibrium [“pre-equilibrium” (131)] between the plasma and tissues (Eq V), and intracellular thyroid hormone concentrations reflect the plasma free (at equilibrium) thyroid hormone concentration. Under equilibrium conditions, the free fraction of hormone in the plasma will be determined mainly by the stronger binding components present and will be less affected by weaker binding components. Therefore, the findings that a small fraction of the thyroid hormones in plasma may bind to lipoproteins (132), red blood cells (133), or other blood components should not be assigned foremost physiological significance, unless it can be demonstrated that such binding selectively directs these hormones to particular metabolic pathways.

**5. Changes in intracellular binding.** In the models developed above (*Section V*), intracellular hormone was treated as a single pool. It can readily be shown, however, that when intracellular hormone is divided into rapidly exchanging bound and free pools, the intracellular free pool will bear the same relationship to the plasma hormone concentration as the total intracellular pool did in the above models (67). In order to understand the possible significance of changes in intracellular hormone binding, it is important to recognize that these binding sites fall into one or more of at least three distinct categories: 1) sites that perform no known metabolic function, 2) sites that result in degradation of hormone, and 3) sites that effect the biological action of hormone. In the case of  $T_4$ , some of the category 2 sites (*i.e.* 5'-deiodinase) are also category 3 sites (*i.e.* they convert  $T_4$  to the more biologically active  $T_3$ ). In general, a change in the concentration of category 1 sites would be analogous

to a change in the concentration of plasma hormone-binding proteins. The concentration of total intracellular hormone would change, but the concentration of intracellular free hormone would not. A change in the concentration of category 2 sites (which is equivalent to a change in the elimination rate constant) would be expected, for elimination-limited uptake, to result in an altered rate of hormone disposal, but, provided that the rate of hormone secretion changed concomitantly to maintain metabolic status, plasma and intracellular free hormone concentrations would be unchanged (Table 5; also see above, *Section V.D*). In contrast, a decrease (for example) in the concentration of category 3 sites would be expected, through feedback mechanisms, to result in proportionate increases in intracellular free and plasma free hormone concentrations (if biological activity was to be maintained). Examples *in vivo* of changes in all of these types of sites have been documented for the thyroid hormones.

As discussed above (*Section VI.A.2*), phenobarbital increases hepatic mass and the hepatic content of  $T_4$  (104). Because fractional  $T_4$  turnover is also increased by phenobarbital (and in roughly the same proportion as the increase in hepatic content), Oppenheimer *et al.* (134) were led to postulate that  $T_4$  turnover was determined by “the total exchangeable cellular  $T_4$  pool.” This postulate is consistent with the above analyses only to the extent that metabolizing enzymes constitute some of the intracellular binding sites for  $T_4$ . The proportionate increase in hepatic binding of  $T_4$  and  $T_4$  disposal (which is generally assumed to occur largely in the liver) observed by Oppenheimer *et al.* in phenobarbital-treated rats suggests that category 1 and category 2 sites are increased in roughly equal proportions by phenobarbital (it seems unlikely that the majority of hepatic  $T_4$  binding is to category 2 sites). Radiographic contrast agents, in contrast, acutely decrease the hepatic binding of  $T_4$  (135). Since these agents also inhibit the conversion of  $T_4$  to  $T_3$  (106), they presumably interact with both category 1 and category 2 sites as well. The previously reported inhibition of intracellular  $T_4$  binding by heparin (136, 137) may have been the result of an *in vitro* artifact (138).

The syndrome of thyroid hormone resistance (139, 140) illustrates the effects of changes in category 3 sites. In this syndrome the diminished binding of  $T_3$  to nuclear receptors results in compensatory elevations of serum total and free thyroid hormone concentrations. Intrahepatic  $T_4$  is increased roughly proportionately to the increase in the serum free  $T_4$  concentration (as is influx of  $T_4$  to the liver) (139).  $T_4$  turnover is also increased in proportion to the serum free  $T_4$  concentration (139). (Also see *Section VI.A.2* above in this context.)

### B. Cortisol

In humans, the liver removes (net) approximately 15–20% of the cortisol presented to it in the plasma (94). Because virtually 100% of the cortisol in plasma can dissociate from its binding proteins during hepatic sinusoidal transit (Table 3), net hepatic uptake is unlikely to be dissociation-limited. The unidirectional fractional uptake of cortisol from human serum by the perfused rat liver is approximately 60% (Table 3). Therefore, assuming the same unidirectional fractional uptake by human liver, it seems likely that the net hepatic uptake of cortisol *in vivo* is predominantly elimination-limited, although it may be partially influx-limited as well. Because evidence indicates that hepatic influx of cortisol occurs via the free pool (21), the concentration of intracellular cortisol in the liver is predicted to be proportional to the concentration of free cortisol in the plasma regardless of whether or not there is a component of influx-limitation to its net uptake (Table 5). Thus, the free hormone hypothesis is likely to be valid for cortisol with respect to the liver. It is also likely to be valid with respect to most other tissues, since the majority of cortisol metabolism occurs in the liver (141), and net uptake by most other tissues can therefore reasonably be assumed to be elimination-limited (see the above corresponding discussion for the thyroid hormones, Section VI.A.1).

Consistent with this conclusion are the descriptions of familial decreases (142) and increases (143) in the plasma concentration of CBG. In these syndromes, the concentration of total cortisol in plasma is affected by the concentration of CBG, but the plasma free cortisol concentration and the urinary excretion of cortisol and its metabolites are normal. In women who are pregnant or receiving estrogens, the plasma concentrations of CBG and cortisol are markedly increased. Interestingly, plasma free cortisol concentrations in these women may also be increased (144), thus suggesting refractoriness of their tissues to the effects of cortisol. A syndrome of familial cortisol resistance, apparently analogous to the syndrome of thyroid hormone resistance (139, 140), has also been described (145).

Recently, it has been recognized that a fraction of the cortisol in blood (roughly equivalent to the unbound fraction plus the fraction bound by albumin) is bound to erythrocytes (146). The half-time of dissociation of cortisol from erythrocytes was found to be rapid ( $t_{1/2} < 2.3$  sec), indicating that erythrocyte-associated cortisol may readily exchange with cortisol in plasma and tissues *in vivo*. Nevertheless, the possible physiological significance of this observation must be considered with caution. Since the rate of dissociation of cortisol from CBG is also extremely rapid ( $t_{1/2} < 1$  sec; Table 1), the cortisol

in plasma (or, now, whole blood) should, under most circumstances, be viewed as a single pool. The physiological significance of a small increase in this pool size attributable to the binding of cortisol by erythrocytes, without a change in the free cortisol concentration, is not readily apparent.

### C. Testosterone

*In vivo*, the human liver removes (net) 40–50% of the testosterone presented to it in the plasma (94). This fraction is close to the measured fractional unidirectional uptake of testosterone by rat liver and to the maximal possible fractional unidirectional uptake (for both rat and human liver) predicted on the basis of the rates of dissociation of testosterone from its plasma hormone-binding proteins (Table 3). Thus, dissociation—and, to a lesser extent, perhaps also influx—appears to play the major role in limiting the net rate of uptake of testosterone by the liver. The free hormone hypothesis, therefore, is unlikely to be valid for testosterone with respect to the liver. Intracellular testosterone concentrations in the liver are likely to be a function of the amount of hormone that can dissociate from its plasma binding proteins during sinusoidal transit, as well as of the elimination rate constant (Table 5).

In normal women there is very little nonhepatic metabolism of testosterone (94). Consequently, it seems likely that nonhepatic tissue uptake *in vivo* is generally elimination-limited and that, therefore, the free hormone hypothesis will hold for most other tissues. In men and androgenized women, more of the metabolism of testosterone occurs in nonhepatic tissues [although the majority still occurs in the liver (94)], so that such a statement must carry somewhat less certainty in the absence of data on rates of influx and metabolism in specific organs.

Because the net uptake of testosterone by the liver appears to be dissociation-limited and, according to available data, only 25% of the testosterone-sex hormone-binding globulin (SHBG) complex dissociates spontaneously during hepatic sinusoidal transit (Tables 1 and 2), the metabolic clearance rate (MCR) of testosterone is expected to be related inversely to the concentration of SHBG in the plasma. Thus, women, who have higher plasma concentrations of SHBG than men, have lower MCRs of testosterone than men (147), and treatment of men with estrogens, which increases their plasma concentrations of SHBG, results in decreased MCRs of testosterone (148). Because the biological activity of testosterone in nonhepatic tissues is likely to be a function of the plasma free hormone concentration [a prediction that is consistent with clinical observations as well (76)], it also—for different reasons—is expected

to be inversely related to the concentration of SHBG in the plasma.

#### D. Estradiol

Estradiol is cleared almost entirely by the liver, and the MCR is similar to the rate of hepatic plasma flow (94, 149). Hence, its net hepatic uptake appears to be primarily flow-limited, although dissociation or influx may play some role (Table 3). Thus, the free hormone hypothesis is not likely to hold for estradiol with respect to the liver. Because there is little metabolism of estradiol outside of the liver (149), however, its uptake is likely to be elimination-limited in most nonhepatic tissues, and the free hormone hypothesis is therefore likely to hold for those tissues.

#### E. Progesterone

The liver removes (net) more than 80% of the progesterone presented to it in the plasma (94). Because virtually 100% of the protein-bound progesterone can dissociate during a 9-sec transit through the liver (Table 1), uptake is unlikely to be dissociation-limited. Plasma flow almost certainly plays a role in limiting the rate of net uptake of progesterone by the liver, but the relative contribution of influx or elimination may be more important. Regardless, the free hormone hypothesis is unlikely to hold in pure form.

Almost half of the (very rapid) metabolism of progesterone *in vivo* occurs in nonhepatic organs (94). Thus, it is difficult to generally assume elimination-limited uptake. In the absence of specific data relating to particular organs, speculation about whether or not the free hormone hypothesis is likely to hold for nonhepatic organs is not feasible.

#### F. Aldosterone

Unlike the other hormones discussed here, a very large fraction of the aldosterone in plasma is free (almost 40%; Table 1); only 20% is bound to CBG. No data are available on the rate of dissociation of aldosterone from CBG, but because of the low affinity of the aldosterone-CBG interaction (23) this rate would generally be assumed to be very rapid. Aldosterone is primarily metabolized in the liver; net fractional hepatic uptake approaches 100% (94), suggesting predominantly flow-limited uptake. As expected for the case of flow-limited uptake, cardiac dysfunction, when it results in decreased hepatic plasma flow, appears to decrease the MCR of aldosterone (150). The study demonstrating this, however, was complicated by the fact that not only hepatic blood flow but also the hepatic extraction of aldosterone was decreased (from more than 90% to 71%) in patients

with marked cardiac dysfunction, indicating that factors other than plasma flow also affect hepatic removal of aldosterone.

Less than 20% of the circulating aldosterone is metabolized in nonhepatic tissues (the kidney is apparently the other organ primarily responsible for its metabolism) (94, 151). Thus, its uptake may be elimination-limited in many tissues. Evidence that the nonhepatic clearance of aldosterone is dependent on the plasma free hormone concentration has in fact been presented. Zager *et al.* (151) reported that the MCR of aldosterone increased markedly during manipulations that resulted in increased free aldosterone concentrations in plasma. Because the liver normally clears almost 100% of the aldosterone presented to it, and because hepatic plasma flow was not increased by these manipulations, the increased MCR observed in this study was presumably caused by increased metabolism in nonhepatic organs.

#### G. Vitamin D

Of all the steroid hormones, vitamin D and its metabolites appear to most closely resemble the thyroid hormones in their transport and distribution. Their free fractions in plasma are very small, and their half-lives in plasma are very long compared to those of other steroid hormones. Vitamin D formed in the skin diffuses into the blood, where it is bound primarily by vitamin D-binding protein (DBP) (152). It circulates with a half-life of approximately 12 h (153) before being taken up by the liver, where it is converted to 25-hydroxyvitamin D. 25-Hydroxyvitamin D circulates with a half-life in plasma of approximately 20 days (153). It is converted to the metabolically more active 1,25-dihydroxyvitamin D in the kidney or to 24,25-dihydroxyvitamin D in the kidney and other tissues (154). 1,25-Dihydroxyvitamin D circulates in plasma with a half-life of approximately 14 h (155); the half-life of 24,25-dihydroxyvitamin D in plasma is probably somewhat shorter. The liver converts all of these vitamin D metabolites to inactive compounds (152). Because of their long plasma half-lives (and because the lipid solubilities of the steroid hormones in general suggest that they should cross membranes rapidly), it seems likely that the uptake of all of these metabolites by most or all organs is elimination-limited and that the free hormone hypothesis therefore generally applies.

Vitamin D absorbed from the diet, on the other hand, appears to enter the circulation bound to chylomicrons and is cleared rapidly. Although evidence indicates that some of the vitamin D bound to chylomicrons transfers to DBP before hepatic uptake occurs (156), the fact that hepatic removal of dietary vitamin D appears to be much more rapid than that of vitamin D derived from cuta-

neous sources (152, 156) is hard to reconcile with a large fraction of the removal of dietary vitamin D occurring via DBP. Fraser (152) has proposed that vitamin D taken up by the liver on chylomicron remnants may be preferentially shunted to metabolically inactivating pathways, thus accounting for the inefficiency of oral vitamin D as compared to vitamin D derived from the skin in maintaining vitamin D status.

In this respect, a recent study by Haddad *et al.* (157) deserves detailed comment. Those investigators perfused (single pass) rat livers with radiolabeled vitamin D in solutions of DBP, albumin, chylomicrons, chylomicron remnants, low density lipoproteins (LDL), or high density lipoproteins. They noted that hepatic uptake of vitamin D was greatest when it was perfused bound to chylomicron remnants or LDL, and least when it was perfused bound to DBP. Based on these data, they concluded that the predominant mechanism of hepatic uptake of vitamin D from cutaneous as well as dietary sources *in vivo* may involve lipoproteins, which "facilitate" this uptake. Extrapolation of this study to the *in vivo* situation is difficult, however. Assuming (in humans) a plasma half-life of 12 h for vitamin D (153), hepatic plasma flow of 800 ml/min (Table 3), and total body plasma volume of 3 liters, and assuming that all of the vitamin D metabolism *in vivo* occurs in the liver, it can be calculated that the liver removes less than 0.3% of the vitamin D in the plasma during each hepatic transit. Yet, in the study by Haddad *et al.* the fractional hepatic uptake of vitamin D perfused bound to DBP was observed to be 2%. Thus, assuming similar fractional uptakes by human and rat liver, no facilitation of the uptake of DBP-bound vitamin D is necessary to account for the *in vivo* situation. Furthermore, the suggestion that lipoproteins (in particular, LDL) could perform such facilitation *in vivo* is difficult to accept. Although chylomicrons do deliver dietary vitamin D to the liver via lipoprotein receptors, the assumption in that study that the observed hepatic uptake of vitamin D bound to (human) LDL was mediated by LDL receptors is difficult to justify. The plasma half-life of human LDL in the rat is approximately 8 h, the majority of the hepatic uptake of LDL proceeds via LDL receptors, and LDL taken up through the LDL receptor pathway is degraded (158). Therefore, the single-pass uptake of human LDL by the perfused rat liver is expected to be negligible, and LDL receptors cannot be expected to account for the more than 50% single-pass uptake of LDL-bound vitamin D that was observed in that study.

Thus, the more likely mechanism of the hepatic uptake of LDL-bound vitamin D observed in that study involves spontaneous dissociation of the vitamin D-LDL complex. In plasma, vitamin D circulates bound primarily to DBP, but also to weaker binding sites that include, presumably,

albumin (Table 1) and, apparently, lipoproteins (157) (a situation analogous to that of the thyroid hormones). In such a setting, the free vitamin D fraction will be influenced primarily by the concentration of DBP. Thus, if tissue uptake occurs via the free pool and is dependent on the free vitamin D concentration (as appears likely), the weak binders in plasma will affect uptake only minimally. If Haddad *et al.* had perfused livers with vitamin D in the presence of both LDL and DBP, they presumably would have found almost the same fractional uptake as when vitamin D was perfused in the presence of DBP alone. Certainly when the *in vivo* steady state is considered, this situation seems likely to hold.

#### H. Summary

The above discussions are summarized in Table 6. Most of the specific conclusions in this section should be considered provisory, and may need to be modified as more complete data are obtained.

### VII. Is There Active Transport of Hormones?

#### A. Thyroid hormones

Evidence that active transport of T<sub>3</sub> occurs *in vivo* comes primarily from studies of the fractional occupancy of nuclear receptors isolated after the injection of [<sup>125</sup>I] T<sub>3</sub> into the plasma of the live animal. Free T<sub>3</sub> normally circulates in plasma at concentrations of 5–10 pM, yet the nuclear T<sub>3</sub> receptor, which has an equilibrium dissociation (K<sub>D</sub>) for T<sub>3</sub> of approximately 1 nM (159), appears to be half-saturated *in vivo* (159, 160). Thus, the free T<sub>3</sub> concentration in the nucleus must be approximately 1 nM, or more than 100-fold greater than the plasma free T<sub>3</sub> concentration. Hence, active transport must be postulated [although some of this gradient could be accounted for by intracellular T<sub>3</sub> synthesis (see below, Section VIII and Appendix)]. Until recently, it had been assumed that the active transport must occur at the

TABLE 6. Predicted validity of the free hormone hypothesis (FHH) for particular hormones in humans, based on available data

Hormone	FHH valid for liver?	FHH valid for other tissues?
T <sub>4</sub>	Yes	Yes
T <sub>3</sub>	Yes	Yes
Cortisol	Yes	Yes
Testosterone	No	Yes
Estradiol	No	Yes
Progesterone	No	?
Aldosterone	No	Yes
Vitamin D [1,25-(OH) <sub>2</sub> and 25-(OH)] <sup>a</sup>	Yes	Yes

<sup>a</sup> Vitamin D (unhydroxylated) derived from the skin is probably delivered to the liver in accordance with the principles of the free hormone hypothesis, but vitamin D derived from diet probably is not.

plasma membrane, and a number of *in vitro* studies appeared to be consistent with this. Thus, specific cell-surface binding sites for T<sub>3</sub> were identified in a variety of cell types (161–164). Affinity labeling demonstrated T<sub>3</sub> binding to a 55-kilodalton (kDa) membrane protein (163), and cellular uptake of T<sub>3</sub> was observed to occur by receptor-mediated endocytosis (161, 162, 165). The cellular uptake of T<sub>3</sub> was shown to be saturable, stereospecific, and sodium- and energy-dependent in a variety of cell types and studies (166–172). And, T<sub>3</sub> uptake by rat hepatocytes was inhibited by a monoclonal antibody to a specific 52-kDa membrane protein (168). (Findings for T<sub>4</sub> have been similar, but more variable.<sup>11</sup>)

Extrapolation of these studies to the *in vivo* situation, however, requires caution. The Michaelis-Menten constants (K<sub>m</sub>) and K<sub>D</sub>s described in all of these reports are many orders of magnitude higher than the circulating free hormone concentrations. While this is not sufficient reason to reject the physiological relevance of these studies [and it is not inconsistent with perfused organ studies showing the relative unsaturability of uptake (19, 20, 45, 51)], it is a reason to remain cautious. More specifically, in most of the studies demonstrating active transport at the plasma membrane, only a minority of the total observed transport could be accounted for by active transport. The quantitative significance of this fact can perhaps best be appreciated by a detailed analysis of a recent study by Hennemann *et al.* (167), who measured T<sub>4</sub> and T<sub>3</sub> uptake under equilibrium conditions by cultured rat hepatocytes in the presence or absence of ouabain or an antibody to the plasma membrane. They found that the cellular uptakes of both T<sub>4</sub> and T<sub>3</sub> were inhibited by approximately 50% by the antibody or ouabain. They also found that deiodination of the T<sub>4</sub> and T<sub>3</sub> in their system was decreased by approximately 50% by the antibody or ouabain, and therefore assigned physiological relevance to their observations. However, when considering the *in vivo* situation, the opposite conclusion should have been reached. Given that the K<sub>m</sub> for rat liver 5'-deiodinase is in the micromolar range (100), and that the experiments of Hennemann *et al.* were conducted with picomolar concentrations of T<sub>4</sub> and T<sub>3</sub>, the observed 50% reduction in deiodination is consistent with a 50% reduction in intracellular (free) thyroid hormone concentrations. Thus, the active transport process at the plasma membrane described by Hennemann *et al.* accounts for

only a doubling of (free) T<sub>4</sub> and T<sub>3</sub> concentrations. But the considerations that led to the postulate of active transport (nuclear T<sub>3</sub> receptor occupancy *vs.* circulating free T<sub>3</sub> concentration) require that the active transport account for more than a 100-fold gradient of free T<sub>3</sub>. Thus, these (and other similar) *in vitro* studies must so far be considered to have explained very little about how thyroid hormones enter cells and reach the nucleus *in vivo*.

A possible solution to this puzzle has recently come from an imaginative study conducted by Oppenheimer and Schwartz (159), whose observations in rats suggest active transport of T<sub>3</sub> across the nuclear membrane in a magnitude sufficient to account for the receptor occupancy of T<sub>3</sub>. The assumptions involved in the data analysis and the complexity of the experimental methodology employed in this study necessitate that its findings still be considered preliminary. Furthermore, the fact that only a much lesser degree of active transport of T<sub>3</sub> across the nuclear membrane can be demonstrated using cells in tissue culture (175) suggests that obtaining additional information about this process will be difficult. Nevertheless, these observations must be considered of particular importance to investigators interested in solving this puzzle.

#### B. Steroid hormones

There is presently little evidence for active transport of steroid hormones. For some of the steroid hormones, the normal free concentrations in plasma are similar to the K<sub>D</sub> values of the receptors. Thus, the plasma free cortisol concentration is approximately 15 nM (23), while the K<sub>D</sub> of cortisol for the glucocorticoid receptor appears to be approximately 10 nM (176). Similarly, the plasma free aldosterone concentration is approximately 0.1 nM (23), while the K<sub>D</sub> of aldosterone for its receptor in human kidney appears to be approximately 0.5 nM (177, 178). For some of the other steroid hormones, however, there appears to be a discrepancy between the normal plasma free hormone concentration and the receptor K<sub>D</sub>, just as is the case for the thyroid hormones. In the case of estradiol, for example, the normal plasma free hormone concentration in women ranges between 5 and 15 pM, whereas the K<sub>D</sub> for the receptor in the uterus appears to be approximately 1 nM (176). There is little direct evidence that the steroid receptors exist *in vivo* near half-saturation, however, so it is not certain that active transport must occur. Nevertheless, in the case of hormones such as estradiol, it would seem reasonable to search for such transport. Since steroid receptors appear to be localized in the nucleus *in vivo* (179), such transport could occur at the nuclear membrane.

It should also be noted in this context that, unlike the

<sup>11</sup> Considerations of fractional receptor occupancy for T<sub>4</sub> also suggest that active transport of T<sub>4</sub> occurs *in vivo*. The fraction of nuclear receptors occupied by T<sub>4</sub> *in vivo* (~5%) appears to be much less than that occupied by T<sub>3</sub> (160, 173), but the K<sub>D</sub> of the nuclear receptor for T<sub>4</sub> (20 nM) is approximately 20-fold higher than that for T<sub>3</sub> (174). Since the circulating concentration of free T<sub>4</sub> (20–30 pM) is only approximately 4-fold greater than that of free T<sub>3</sub>, the predicted plasma to nuclear gradient of free T<sub>4</sub> is less than that of free T<sub>3</sub>, but by less than 1 order of magnitude.

case of the thyroid hormones, where uptake appears to be almost purely elimination-limited, the rate-limiting step in the net uptake of steroid hormones by tissues is not always expected to be elimination, particularly when the liver is considered. Hence, in the absence of active transport, intracellular and plasma free steroid hormone concentrations would not necessarily be expected to be identical. In situations other than elimination-limited uptake, the intracellular free hormone concentration at steady state will be lower than the plasma free hormone concentration (see above, *Section V.D*). This fact needs to be taken into account when deciding whether or not active transport must be invoked to account for receptor occupancy. The role played by intracellular metabolism in determining intracellular steroid hormone concentrations under certain conditions is well illustrated in a recent study by Funder *et al.* (180). Those investigators demonstrated that in mineralocorticoid-responsive tissues steroid hormone receptors were selective for mineralocorticoids rather than glucocorticoids not because of their higher affinity for mineralocorticoids, but because the rate of intracellular metabolism of glucocorticoids was higher than that of mineralocorticoids.

### VIII. Incompatibilities with the Free Hormone Hypothesis and Traditional Models of Hormone Transport

Studies purporting to disprove the free hormone hypothesis by demonstrating that the unidirectional tissue uptake of hormone from plasma exceeds the free fraction of hormone in that plasma (6, 53) should not be assigned undue physiological significance and will be discussed separately below (*Section IX*). Most of the other data that have been interpreted as contradicting the tenets of the free hormone hypothesis or other traditional models of hormone transport can in fact be shown to be consistent with these models (see below, this section). Nevertheless, there remain at least two observations that are inconsistent with the traditional models of hormone transport described in this review. Both are related to thyroid hormone metabolism.

The models of hormone transport described in this review make definite predictions about the relationship between intracellular hormone concentrations and plasma hormone concentrations. More certain than for any other hormone, the predictions of these models for the thyroid hormones are that, given constancy of the ratio of the influx and efflux rate constants, intracellular free hormone concentrations and therefore biological activity will be proportional to the plasma free hormone concentration (*Section VI.A*). In cases of primary changes in the concentration or affinity of plasma thyroid hormone-binding proteins, the concentrations of

free thyroid hormones in plasma should therefore be normal. Although most data are consistent with this prediction, not all are. A number of subjects with primary abnormalities of plasma thyroid hormone-binding proteins have been described who appear to have elevated or decreased concentrations of free thyroid hormones in plasma (42, 137, 181–188). Since abnormalities of thyroid hormone metabolism other than hyper- and hypothyroidism (e.g., thyroid hormone resistance) are rare, it seems unlikely that all of these observations can be accounted for by the coexistence of two unrelated conditions affecting thyroid hormone metabolism in individual subjects.

I believe that the most likely explanation for these observations is that, although measurement of free thyroid hormone concentrations in plasma by equilibrium dialysis is considered a "gold standard" technique and so routine that methods are usually no longer detailed in research articles, these measurements are nevertheless difficult to perform and subject to more error than is generally appreciated. In reaching this conclusion, I rely mainly on personal experience with these measurements, but also note that the published midnormal value for the free fraction of  $T_4$  in serum as measured by equilibrium dialysis varies more than 3-fold (42, 137, 189–191). We should not forget, after all, that the free fraction being measured is only 1/10,000 of the total. The alternative possibility, that the models of hormone transport described above are wholly or partially incorrect, must also be considered, however.

The other piece of evidence inconsistent with the above models of hormone transport and distribution is the demonstration that the majority of nuclear  $T_3$  in the anterior pituitary comes from local conversion of  $T_4$  to  $T_3$  rather than from the  $T_3$  in the plasma (192–194). Local conversion of  $T_4$  to  $T_3$  appears to be an important source of nuclear  $T_3$  in the central nervous system and in brown adipose tissue as well (194, 195). This is not readily consistent with the rapid *in vivo* equilibration between plasma and tissues envisioned for the thyroid hormones in the above models. One possible explanation of these data is that the anterior pituitary, central nervous system, and brown adipose tissue do not share in this rapid equilibration. However, there is no reason to think that this is the case.  $T_3$  uptake by rat brain from human serum is extremely rapid (47). Furthermore, in each of the studies demonstrating the importance of local synthesis of  $T_3$  to nuclear receptor occupancy, the substrate for the locally synthesized  $T_3$  was the injected  $T_4$  that was taken up rapidly by the tissue. Another possible explanation would be a very rapid rate of conversion of  $T_4$  to  $T_3$  in these tissues (196). If the rate of intracellular synthesis of  $T_3$  were greater than the rate of influx of  $T_3$  from the plasma, then even in the absence of active

transport the concentration of free  $T_3$  within the cells would be markedly increased above that in the plasma, and the majority of intracellular  $T_3$  would in fact be derived from intracellular synthesis (see *Appendix*). Given the rapid rates of influx of  $T_3$  to tissues (Table 3), however, this explanation alone may be difficult to reconcile with the long half-life of circulating  $T_4$ .<sup>12</sup> Yet another possible explanation would be some sort of intracellular compartmentalization in these tissues with preferential shunting of newly synthesized  $T_3$  to the nucleus. In any event, until a firm explanation of these findings is forthcoming, the above models of hormone transport must be considered at least incomplete. The apparent physiological importance of the local conversion of testosterone to dihydrotestosterone in some tissues may present an analogous difficulty with these models.

Most other studies that could be or have been interpreted as contradicting the free hormone hypothesis or other traditional models of hormone transport can in fact be readily reconciled with these models. Thus, Bianchi *et al.* (79) concluded that  $T_4$  bound to the albumin of subjects with familial dysalbuminemic hyperthyroxinemia (FDH) was "more available to tissues" than the  $T_4$  bound to TBG based on their *in vivo* kinetics studies showing that disposal rates of  $T_4$  in subjects with FDH were 43% higher than those of normal controls and of patients with elevated serum concentrations of TBG. However, what those investigators did not note was that their subjects with FDH had serum free  $T_4$  concentrations that were on average 40% higher than those of their controls and of their patients with elevated serum concentrations of TBG. Thus, their data are fully accounted for by the traditional tenets of the free hormone hypothesis.

An early study by Hennemann *et al.* (183) examined the relationship between the concentration of free  $T_4$  in serum and the  $T_4$  disposal rate determined from *in vivo* kinetics studies in normal subjects and in subjects with increased and decreased serum concentrations of TBG. They found no such relationship and appropriately concluded that factors other than the plasma free  $T_4$  concentration influenced  $T_4$  disposal [for elimination-limited uptake, these factors are the ratio of the influx and efflux rate constants and the elimination rate constant;

(see Table 5)]. Unfortunately, they also concluded that the plasma concentration of free  $T_4$  "may not be a clear determinant of  $T_4$  turnover" in euthyroid subjects, and this article is therefore often cited as providing evidence against the free hormone hypothesis. However, this conclusion is not likely to be valid. The entire body of data in that study are most appropriately viewed as being scattered about a single point, that of a normal serum free  $T_4$  concentration and a normal  $T_4$  disposal rate. Although experimental error no doubt accounts for some of this scatter, differences among individuals in the ratio of the influx and efflux rate constants or in the elimination rate constant for  $T_4$  would also be expected to result in slightly different disposal rates for a given concentration of free  $T_4$  in plasma.

The concept that the biologically active fraction of hormone in plasma is the free plus albumin-bound fraction, rather than the free fraction, based solely on consideration of rates of hormone dissociation from binding proteins and unidirectional uptake by tissues, has perhaps been most pervasive in the recent literature dealing with testosterone metabolism (197-202). The models of hormone transport described above predict that the free hormone hypothesis is likely to be valid for testosterone with respect to nonhepatic tissues (*Section VI.C*). Cumming and Wall (197), using assumed values for the equilibrium binding constants, recently calculated serum free and free-plus-albumin-bound testosterone concentrations in sera from normal women and from women with hirsutism (with or without oligoamenorrhea) from measurements of serum concentrations of testosterone, SHBG, and albumin. They found that serum concentrations of both free and free-plus-albumin-bound testosterone accurately predicted androgen-metabolic status, but that the serum concentration of free-plus-albumin-bound testosterone was a marginally better predictor. They interpreted these findings as supporting the concept that the "biologically available testosterone" was the free-plus-albumin-bound fraction in plasma. However, a better test of which measurement more accurately predicts androgen-metabolic status would have been one in which the two measurements yielded divergent rather than convergent results, such as the evaluation of androgen-metabolic status in patients with nephrotic syndrome or analbuminemia. Such a study performed recently in analbuminemic rats supported the validity of the free hormone hypothesis with respect to testosterone (203). This is not to say, however, that the only way to determine androgen-metabolic status is to measure the serum free testosterone concentration. Indices of the serum free hormone concentration often yield reliable information.

Recently, Siiteri *et al.* (176) pointed out that the MCRs of steroid hormones that circulate bound to cortisol-

<sup>12</sup> Assuming a plasma flow to the brain in humans of 400 ml/min (272), assuming a plasma  $T_3$  concentration of 2 nM, and assuming that 5% of the plasma  $T_3$  enters (unidirectionally) the brain during each transit (47), it can be calculated that the one-way flux of  $T_3$  from the plasma to the brain is approximately 60 nmol/day. If 50% of the intracellular  $T_3$  in the brain is postulated to be derived from local synthesis, then the amount of  $T_3$  synthesized locally in the brain would have to be approximately 60 nmol/day (*Appendix*). For this to be the case, more than half of the total *in vivo* disposal of  $T_4$  [100 nmol/day (39, 42, 80-82)] would have to be devoted to  $T_3$  production by the brain.

binding globulin or to SHBG correlate with both the serum free hormone concentrations and with serum non-globulin-bound hormone concentrations, but correlate better with the latter. Indeed, the latter correlation is quite remarkable, with only 3 of 12 points lying measurably off the line. Siiteri *et al.* interpreted this observation as indicating that steroid hormone clearances are more dependent on the circulating non-globulin-bound fraction of hormone than on the free fraction. The models of hormone transport described above do not suggest rejection of this interpretation (see Section VI) but do suggest that the reason for the observed correlation may be somewhat more complex. First, it is important to point out that whenever different hormones that are metabolized by different pathways or that have different affinities for the same metabolizing enzymes are compared, there is no *a priori* reason to expect any correlation between measurements in plasma and MCRs. Factors other than plasma hormone concentrations and binding that affect metabolic clearance (influx, efflux, and elimination rate constants) cannot be assumed to be the same for different hormones. Thus, the findings that the MCRs of different steroid hormones do not always correlate with the non-globulin-bound fractions of hormone in serum (204, 205) should not be considered surprising. The correlation between MCRs and non-globulin-bound fractions of steroid in serum observed by Siiteri *et al.* would be expected if, for all of the hormones considered, elimination were very rapid, dissociation from albumin were very rapid, and dissociation from globulin were very slow compared to the rate of hepatic blood flow (assuming that most metabolism occurs in the liver). In such a setting, virtually all of the free and albumin-bound hormone in the plasma would be metabolized during a single pass through the liver, whereas virtually none of the globulin-bound hormone would. However, such a situation exists at most for only a minority of the hormones examined by Siiteri *et al.* (Section VI). Therefore, at the present time, the reason for the very close correlation that they observed must be considered incompletely understood.

Siiteri *et al.* (176, 206) have also recently questioned the traditional tenets of the free hormone hypothesis based on studies of New World monkeys. These monkeys have serum cortisol concentrations that far exceed the binding capacities of CBG in serum and thus have very high circulating free cortisol concentrations. The serum concentrations of other steroid hormones in these monkeys may also be affected similarly (206). Although not all of the findings in these monkeys are readily explained, there presently seems little reason to reject the traditional explanation that their high circulating free cortisol concentrations are due to resistance at the level of the glucocorticoid receptor (207-209) or postreceptor events.

Some, although not all, *in vitro* studies show a marked decrease in the affinity of dexamethasone for the glucocorticoid receptor in these monkeys (207). Indeed, the close homology between the glucocorticoid receptor and other steroid hormone receptors (210) would then suggest a possible mechanism for the more generalized steroid hormone resistance observed in some of these monkeys. Furthermore, it should be emphasized that comparative studies in different species must be interpreted cautiously. For example, glucocorticoid metabolism and action may differ in New World monkeys and humans for reasons unrelated to receptors or plasma hormone binding, and this would not be inconsistent with traditional models of hormone transport and distribution. For this reason, studies of the effects of manipulations within species on hormone metabolism and action may be more telling. In this light, the recent study of Coe *et al.* (211), in which New World monkeys were treated with estrogens, is of particular interest. Those investigators found that when male New World monkeys that had very high serum total and free cortisol concentrations were treated with estrogens, their serum concentrations of both cortisol and CBG rose. Although those investigators interpreted their findings as indicating a primary effect of estrogens on adrenal cortisol secretion, in the absence of data on serum free cortisol concentrations and on cortisol production rates after estrogen administration, those data are just as consistent with a primary effect of estrogens on CBG production and a compensatory increase in serum cortisol concentrations to maintain normal or near normal (for those monkeys) plasma and intracellular free hormone concentrations and disposal rates. Such a situation would be predicted by traditional models of hormone transport and distribution if the net tissue uptake of cortisol in New World monkeys is elimination-limited in both metabolizing and nonmetabolizing organs, as appears to be the case in humans (Section VI.B), and if estrogens exert no primary effects on cortisol production or on the (intracellular) biological activity of cortisol. The decreased MCRs of cortisol in those monkeys (208) as compared to Old World monkeys, despite increased free fractions of cortisol in serum (206), would then be explained by a relatively decreased elimination rate constant in those monkeys due either to intrinsic differences in metabolism between the two types of monkeys or to near-saturation of the metabolizing enzymes in the New World monkeys (caused by the very high plasma free hormone concentrations).

Immunocytochemical localizations of plasma steroid hormone-binding proteins within cells have also been used to argue against the traditional tenets of the free hormone hypothesis (reviewed in Ref. 212). Leaving aside the problem that the techniques employed cannot be used to demonstrate a quantitative importance of the

findings, such findings must still be considered with caution in light of apparent structural similarities between intracellular and plasma steroid hormone-binding proteins. For example, in a recent study four separate monoclonal antibodies to the intracellular androgen-binding protein were found to cross-react with SHBG (213). Similarly, demonstrations that there are "specific" binding sites for plasma steroid hormone-binding globulins on cells (214, 215) should not result in the automatic assignment of biological significance to these sites. Because "specific" binding sites for biological ligands can probably be found anywhere they are sought (including inert materials such as glass), if searched for hard enough (216), physiological relevance of such binding cannot be assumed until it is demonstrated. Nevertheless, the possibility that circulating steroid hormone-binding globulin-hormone complexes are internalized by cells and exert biological effects cannot be excluded. Such a finding, however, would not necessarily be incompatible with traditional models of hormone transport and distribution, since it could represent an additional (rather than substitute) mechanism of hormone action.

The study perhaps most often cited as being contradictory to the traditional tenets of the free hormone hypothesis is that of Keller *et al.* (217). This was an elegant and carefully performed series of experiments, in which the biological effects of corticosterone in rats were compared at different circulating concentrations of CBG. However, what seems not to be widely appreciated about this study is that the experimental design was extremely complex, and there was a great deal of uncertainty—openly discussed and appropriately emphasized by the authors—in the interpretation of the results. Now, 20 yr later, with little additional corroboration, it would seem appropriate to no longer assign undue weight to it. In a different study, Rosner and Hochberg (218) examined the biological effects in adrenalectomized rats of a single iv injection of free cortisol or of cortisol bound to CBG and found no difference between the two types of injection. They therefore concluded that CBG did not appear to influence the biological activity of cortisol. However, it seems likely that under the non-steady-state conditions of that experiment, all of the cortisol in both types of injection was cleared very rapidly from the plasma [in the presence of CBG, the single-pass extraction (net) of cortisol by the human liver is 15–20% (94)] and therefore exerted similar (summed) biological effects (which were measured 2–5 h later). Consequently, the conclusions reached in that study should not be extrapolated to the normal *in vivo* situation. An earlier, similar study by Slaunwhite *et al.* (219) had reached the opposite conclusion utilizing an experimental design that can be more appropriately extrapolated to the normal *in vivo* situation (219).

Studies concluding that circulating free fatty acids (FFA) influence plasma free hormone concentrations could also be interpreted as being inconsistent with the free hormone hypothesis. Because FFA concentrations in plasma can vary from minute to minute *in vivo*, a significant influence exerted by them on plasma free hormone concentrations would be difficult to reconcile with the free hormone hypothesis, given the body's assumed interest in optimal regulation of hormone economy. Thus, despite earlier studies to the contrary (220, 221), we (138, 222) have recently shown that circulating concentrations of FFA are unlikely to affect plasma free thyroid hormone concentrations. We have also recently shown that oleic acid, when added to normal human serum in concentrations (up to 4 mM) that far exceed those circulating *in vivo*, exerts virtually no effect on the serum free cortisol concentration (Mendel, C. M., and J. T. Murai, unpublished observations). This finding appears to be inconsistent with the previous findings of Martin *et al.* (223), who observed that micromolar concentrations of FFA affected the interaction of cortisol with purified CBG. However, the findings of Martin *et al.* should be interpreted with caution. We (222) have pointed out previously that because most FFA circulate *in vivo* bound to albumin, possible physiological effects of FFA should not be studied in the absence of albumin. The experiments reported by Martin *et al.* did not employ albumin.<sup>13</sup> Thus, the concentrations of FFA free to interact with CBG in that study, although only micromolar, apparently exceeded physiological unbound FFA concentrations by more than 3 orders of magnitude.<sup>14</sup> Similar considerations force rejection of previous conclusions that FFA affect the interactions of steroids with SHBG (225) and with intracellular steroid-binding proteins (226) *in vivo*.<sup>15</sup> On the other hand, the studies of Ramsey and Westphal (228), in which an effect of FFA on the binding of progesterone to albumin was demonstrated, were performed under physiological conditions and should be considered relevant to the *in vivo* situation, particularly since the majority of progesterone in plasma circulates bound to albumin (23). It should be noted,

<sup>13</sup> In some of these studies "serum" was used also. But the serum was really an ammonium sulfate-precipitated fraction of serum (that is expected to contain albumin) that was used at a 400-fold dilution. We (222) have previously pointed out that it is the FFA/albumin molar ratio, rather than the absolute concentration of FFA, that must be considered when extrapolating results of this type to the *in vivo* situation.

<sup>14</sup> Using the data of Ashbrook *et al.* (224) for the  $K_D$  values for the interaction of oleic acid with albumin, it can be calculated (as per Table 4) that, at a concentration of 1 mM oleic acid and 0.6 mM albumin, the unbound concentration of oleic acid will be only 9 nM.

<sup>15</sup> Consideration of possible physiological effects of intracellular FFA must similarly take into account the presence of fatty acid-binding proteins within cells (227). Unbound FFA concentrations within cells are likely to be even lower than those in plasma.

however, that the free hormone hypothesis is not necessarily predicted to be valid for progesterone (Table 6).

In summary, there is presently little evidence that forces rejection of traditional models of hormone transport. One set of studies in particular, however [i.e., those of Silva *et al.* (192–194)], do serve as a reminder that these models are still incomplete.

### IX. Alternative Hypotheses

Perhaps the most vocal opposition to the free hormone hypothesis and other traditional models of hormone transport has come from investigators measuring rates of unidirectional uptake of hormone by tissues. When considering those studies, it is important to note that the theoretical basis on which such measurements have been interpreted as contradicting the free hormone hypothesis has changed over the years. In fact, during the past decade at least three different models of hormone transport and distribution have been proposed by those investigators. And, although those models have been treated as if they are similar, they are in fact mutually exclusive.

In a review in this journal in 1981, Pardridge (53) summarized his model of hormone transport and distribution as it existed at that time. In that model, the traditional view that the unidirectional tissue uptake (influx) of hormone from plasma occurred by mechanisms that acted on the pool of free hormone after spontaneous dissociation of the hormone-binding protein complex within the tissue vasculature<sup>16</sup> was accepted. Nevertheless, it was claimed that the free hormone hypothesis must necessarily be incorrect whenever the unidirectional uptake of hormone by tissues exceeds the free fraction of hormone in plasma (as measured *in vitro*). This statement was justified by the derivation of an equation

$$K_D^a = K_D e^{k_3 t} \quad (\text{XI})$$

where  $K_D^a$  is the "apparent  $K_D$ " (or the  $K_D$  that exists *in vivo* within the tissue vasculature) governing the protein-hormone interaction within the tissue vasculature,  $K_D$  is the equilibrium dissociation constant governing the protein-hormone interaction *in vitro*,  $k_3$  is the tissue influx rate constant for free hormone, and  $t$  is the capillary or sinusoidal transit time. This equation predicts that the  $K_D$  governing the protein-hormone interaction within the tissue vasculature will increase (and that the free hormone concentration within the tissue vasculature will therefore also increase) whenever tissue influx of hormone occurs. Under such circumstances, free hormone concentrations measured in plasma *in vitro* would not be

<sup>16</sup> The term "tissue vasculature" will be used in this section to refer to capillary beds or sinusoids of tissues, where exchange of hormone between the plasma and the tissue occurs.

expected to bear any relationship to intracellular hormone concentrations. Instead, intracellular hormone concentrations would be predictable only from measurements of the unidirectional uptake of hormone by tissues, and that is, in fact, what Pardridge (53, 229) concluded was the case. However, the derivation of this equation contained a critical mathematical error, as pointed out by Ekins *et al.* (4), and the conclusions based on it were thus shown to be invalid.

Although never acknowledging the role played by Ekins *et al.* and never formally retracting (and even continuing to cite) his 1981 model, Pardridge nevertheless abandoned that model in 1984 in favor of a new model that also predicted that intracellular hormone concentrations could be determined only from measurements of unidirectional uptake of hormone by tissues (5).<sup>17</sup> This new model (5, 6)—the "enhanced dissociation" hypothesis—can be described as follows. Influx rate constants for free hormones are too low to account for observed rates of tissue uptake of hormone from serum by spontaneous mechanisms acting on the pool of free hormone (cf. Eq II). Instead, this uptake is accounted for by a mechanism (i.e. that of "enhanced dissociation") operating within the tissue vasculature that somehow increases  $K_D$  values governing plasma protein-hormone interactions and, thereby, free hormone concentrations. Because of this mechanism, free hormone concentrations within the tissue vasculature are not predictable from those measured in plasma *in vitro*. Instead, these concentrations can be predicted only by measuring the rates of unidirectional tissue uptake of hormone. Other aspects of this model are similar to the more traditional models of hormone transport discussed above. Thus, tissue uptake of hormone occurs via the (now much larger) free pool, and intracellular hormone concentrations are proportional to the concentration of free hormone within the tissue vasculature.

Initial assessment of any model must proceed with at least four questions: 1) Why does it need to be proposed?—that is, What does it explain that previous models do not? 2) What assumptions does it contain? 3) What is the evidence to support these assumptions? And, 4) What testable predictions does it make?

When the enhanced dissociation hypothesis was first proposed, its stated need was that the free hormone hypothesis could no longer be considered valid because it was incorrect when it "assumed that the concentration of circulating hormone that is available for uptake by tissues *in vivo* is equal to the concentration of free hormone measured *in vitro*" (5). This assumption was

<sup>17</sup> In proposing the new model, Pardridge never admitted that his assignment of primary physiological relevance to such measurements thus preceded the theoretical basis on which he justified such assignment.

presented as being incorrect because previous studies had "shown that the apparent dissociation constant,  $K_D^a$ , of plasma protein binding of ligands *in vivo* in the brain capillary is in most cases many times greater than the *in vitro* dissociation constant,  $K_D$ " (5). The previous studies referred to here (47, 230-233), however, were studies showing only that the unidirectional tissue uptake of hormone from serum frequently exceeds the free fraction of hormone in serum (as measured *in vitro*). And such findings, of course, are not incompatible with the free hormone hypothesis or other traditional models of hormone transport (see above, *Section II*). It is only in the context of Pardridge's models that such findings can be interpreted as being incompatible with the free hormone hypothesis. In other words, the stated need for this proposed new model of hormone transport was that the free hormone hypothesis was considered to be invalid in the context of this new model. Thus, it is clear that sufficient reason for the proposal of the enhanced dissociation hypothesis was never provided.

Despite this fact, the enhanced dissociation hypothesis now stands in the literature side-by-side with the free hormone hypothesis. Therefore, the second question, What are its assumptions?, must be asked. Recent publications claim that this model contains no assumptions, only proven facts (57, 234). This can be disputed, however. There are three principal assumptions (or facts) contained in the model: 1) Influx rate constants for free hormone are low compared to tissue blood flow<sup>18</sup> (*cf.* Eq II), such that spontaneous mechanisms acting on the free pool cannot account for the observed rapid rates of uptake of hormone from plasma; 2) The mechanism operative within tissue vascular beds that results in rapid rates of hormone uptake from plasma is one that acts by, quite literally, altering the  $K_D$  governing the binding protein-hormone interaction; and 3) This altered  $K_D$  can be determined from measurement of the unidirectional tissue uptake of hormone. If *any* of these three assumptions is incorrect, the model is either wholly invalid or unusable. Assumption 1 has an experimental basis; assumptions 2 and 3 do not.

The assumption that tissue influx rate constants for free hormone are low compared to tissue blood flow is based on experimental evidence obtained using the Old-

endorf (52) injection technique. In the above section on the free hormone transport hypothesis (*Section II*), some of the limitations of this technique were pointed out. For many hormones, mixing of the injected "free" hormone with the plasma of the animal being studied, or binding of the "free" hormone by the 0.1% bovine albumin in the injection solution, would be expected to result in a large majority of the hormone being protein-bound by the time it reaches the tissue capillary beds or sinusoids (Table 4). Under such circumstances, the influx rate constant calculated from the rate of initial (unidirectional) uptake cannot be assumed to be that of free hormone. Therefore, this method cannot be expected to yield accurate estimates of tissue influx rate constants for free hormone. It was also pointed out (*Section II*) that in the majority of cases Pardridge's own data, in fact, did not support this assumption. Thus, for free testosterone, dihydrotestosterone, estradiol, and progesterone, Pardridge's measured fractional unidirectional uptakes in several different tissues were all indistinguishable from 100% (53-55), indicating immeasurably high tissue influx rate constants (Eq I). Therefore, even in the absence of data contradicting this assumption, its validity should have been questioned more vigorously. In addition, recent data do contradict this assumption. The experimental basis for this assumption was the relatively low fractional unidirectional tissue uptakes of "free" T<sub>4</sub>, T<sub>3</sub>, and cortisol [ranging from 43% to 74% in the liver (45, 46)] observed by Pardridge *et al.*, indicating relatively low tissue influx rate constants. As discussed above (*Section II*), however, our data (19-21), which were obtained using two independent methods, indicate that the hepatic influx rate constants for these hormones are really all immeasurably high (the unidirectional fractional uptakes from protein-free solution all exceeded 99%).<sup>19</sup> Explicit reasons for the

<sup>18</sup> In his reply (57) to our previous criticism (56), Pardridge claimed that the wording of this phrase misstated his assumption, and that the proper wording should have been that the influx rate constant is low "compared with either hormone dissociation from the binding protein or hormone association with the binding protein." Pardridge's wording here, however, refers to a different assumption that he also makes, but that is not germane to the discussion at hand and is not referred to here. This different assumption is required whenever Eq II is used to describe the uptake of hormone by tissues (see *Section II*). The assumption referred to here is worded correctly. This can be seen from inspection of Eq II when it is recognized that  $t$  is an inverse function of the tissue blood flow (19, 21).

discrepancies between our data and those of Pardridge *et al.* have been provided in each case (*Section II*).

The second assumption in the enhanced dissociation hypothesis is that observed rates of tissue uptake of hormone from serum are accounted for by changes in the  $K_D$  values governing the protein-hormone interactions within the tissue vasculature. Although this assumption is absolutely critical to the enhanced dissociation hypothesis, its critical nature has never been explicitly presented by proponents of this hypothesis. In their steady state modeling study, Pardridge and Landaw (235) found that, in the absence of active transport or rapid tissue metabolism, their model predicts that free hormone concentrations inside of cells are equal to those in the plasma within the tissue vasculature. But if facilitation of hormone uptake from binding proteins occurred by a receptor-mediated mechanism or by a nonspecific interaction of the binding protein with the cell membrane, rather than by a change in the  $K_D$  of the protein-hormone complex, then the free hormone concentration within the tissue vasculature would still be the same as that in bulk plasma and the predictions of this model would be the same as those of the free hormone hypothesis (*cf.* the above analysis in *Section V.B*, relating to the models shown in Figs. 7B and 8). Thus, evidence to support this assumption is essential. Such evidence, however, is wholly lacking.<sup>20</sup> In the absence of any evidence, how can it be justified to propose the existence of an unprecedented phenomenon when there are precedented phenomena that serve equally well? In the context of this model, the finding of specific binding sites for the

plasma steroid hormone-binding proteins (214, 215) and for albumin (239–241) on a variety of cell surfaces cannot be ignored.

Although Pardridge (57) has recently claimed that this second assumption has been proven correct, the only evidence cited to support this statement is that the enhanced dissociation model can be fitted to his data (5, 6, 57). This is circular reasoning. Many possible models can always be successfully fitted to any given set of data [particularly if, as in the case of Pardridge's model and data, two independent parameters are allowed to vary and only four data points are considered (5)].<sup>21</sup> Such fitting can be used only to reject a model, not to prove it correct. Proof must come from independent evidence. That Pardridge recognizes this is apparent. Ekins and Edwards (7) recently proposed a model of hormone transport that conflicted with the enhanced dissociation model, but that fitted Pardridge's data at least as well as the enhanced dissociation model did.<sup>22</sup> Yet, Pardridge and Landaw (242) rejected the model of Ekins and Edwards for reasons that had nothing to do with how well it fitted the data.

The third assumption in the enhanced dissociation hypothesis, that the altered  $K_D$  (and therefore the free hormone concentration) in the plasma within the tissue vascular bed can be accurately determined from measurements of unidirectional uptake, is important because,

<sup>20</sup> Recent studies have shown that the electron spin resonance spectrum of albumin changes in the presence of hepatocytes (236). This finding has been interpreted as indicating a conformational change in the albumin molecule during interaction with hepatocytes. Pardridge and Landaw (235) have previously claimed that such studies support their model. However, the physiological significance of such studies cannot be assumed. If the observed "conformational change" affects the hormone-albumin interaction, then this observation could be assigned meaning within the context of the enhanced dissociation model. However, this observation alone cannot be said to support this model. Similarly, the recent observations of Terasaki *et al.* (237, 238) that different hormones may bind differentially to different isoforms of the same hormone-binding globulin cannot be reasonably interpreted as supporting the enhanced dissociation model. Apart from the experimental uncertainty in these observations, the question, again, must first be, What is the demonstrated physiological significance of these findings? That a physiological significance can be attributed to them in the context of the enhanced dissociation model does not mean that they *have* a physiological significance or that they support the model. After all, the observations that they are purported to explain, namely the "differential availability" (*i.e.* the different rates of unidirectional tissue uptake) of different hormones carried on the same binding protein, already have an explanation in the free hormone transport hypothesis, namely, the different rates of dissociation of the protein-hormone complexes (among other factors) (Table 1). Furthermore, it should be noted that, in this context, the binding of different hormones to different isoforms of the same protein could even be postulated to be the reason for the different rates of dissociation of the protein-hormone complexes.

<sup>21</sup> Pardridge (5) has claimed that the enhanced dissociation model fits his data better than the free hormone transport hypothesis does. In previous criticism, we (56) pointed out that this is true only if the influx rate constant is assumed *a priori* to be very low. Pardridge disagreed (57). However, the equation that Pardridge used to fit the free hormone transport hypothesis to this data does in fact depend on this assumption (5, 7). In addition, it should be pointed out that this disagreement is at least partly only semantic. Thus, Pardridge's curve fitting included his measured values for the uptake of "free" hormone. If only 50% of the free hormone is taken up, for example, the calculated influx rate constant will be low and the free hormone transport hypothesis will not be able to account for observed rates of tissue uptake of hormone from serum. Computer-modeling studies demonstrating this fact are superfluous; if such a point is included in the curve, the free hormone transport hypothesis cannot be fitted to the data. If, on the other hand, it is acknowledged that there is debate over the experimental validity of this point, then this point should not be included in the curve fitting. When the curve fitting is performed in this manner, the two models are not distinguishable.

<sup>22</sup> Ekins and Edwards (7) pointed out that Pardridge's data could be fully explained in the absence of enhanced dissociation if a "more correct" equation [besides the "modified Kety-Renkin-Crone" equation (Eq II)] had been used in the analysis. What Ekins and Edwards did not explicitly point out, however, is that the more correct equation used by them reduces to the simpler modified Kety-Renkin-Crone equation used by Pardridge if the tissue influx rate constant for free hormone is very low. Since Pardridge and colleagues (5, 6) explicitly assume that tissue influx rate constants for free hormone are very low (and have provided experimental evidence to support this assumption), dismissal of their work (7) on the basis of their use of a "simplified" equation, in the absence of data that contradict this assumption, is not strictly justified. In this regard, our data (19–21) and arguments (see above, *Section II*) contradicting this assumption must assume a primary importance even in the criticisms of Ekins and Edwards (7).

without it, even if the model were correct, no physiologically relevant predictions could be made. The experimental uncertainties in the techniques used (discussed above in this section and in more detail in *Section II*) are reason enough to test this assumption carefully. Pardridge's (57) claim that this assumption has been proven to be correct because his measurements led him to different conclusions in different test systems is difficult to sustain. The way to test this assumption is to determine whether the measurements that are performed lead to accurate physiological predictions. And this brings us to our final question about this model, What testable predictions does it make?

If we do not allow the free hormone hypothesis to be rejected just because the unidirectional tissue uptake of hormone exceeds the free fraction of hormone in the plasma, then in many cases the predictions of the enhanced dissociation model are similar to those of the free hormone hypothesis. Indeed, this has made physiologically based experiments designed to distinguish the two models difficult. Nevertheless, there is enough cumulative data in the literature to force sound rejection of the enhanced dissociation hypothesis.

Pardridge and Landaw (235) recently used computer simulations to examine the steady state predictions of their model for intracellular  $T_3$  concentrations in the liver. They claimed that the steady state predictions of their model were reasonable. However, this claim was based on a selective analysis of their data. If they had considered more of their data, they would have uncovered unreasonable predictions, and if they had generated additional data, they would have found that their model collapses altogether. Thus, the model predicts that cytosolic and intranuclear free  $T_3$  concentrations in the liver in the normal human are approximately 0.5 nM, which is almost 200-fold higher than the plasma free  $T_3$  concentration and which is close to the presumed  $K_D$  of the nuclear  $T_3$  receptor (see above, *Section VII.A*). Because the hepatic nuclear  $T_3$  receptor is thought to be half-saturated *in vivo*, this prediction is attractive, as Pardridge and Landaw pointed out. However, this prediction is not superior to the corresponding prediction made by the free hormone hypothesis. The free hormone hypothesis simply postulates active transport of  $T_3$  to account for the half-saturation of the nuclear  $T_3$  receptor. In fact, conclusive demonstration of active transport would force rejection of the enhanced dissociation model, because a marked free hormone gradient across the plasma membrane in the setting of an already increased plasma free hormone concentration within the tissue vasculature would then result in intranuclear free  $T_3$  concentrations far exceeding those needed to saturate the  $T_3$  receptor. In addition, it should be pointed out that the enhanced dissociation model predicts that cytosolic

free  $T_3$  concentrations (0.5 nM) will be similar to the predicted intranuclear free  $T_3$  concentrations, whereas the free hormone hypothesis makes no predictions on this point (*i.e.* on whether active transport occurs at the plasma membrane or at the nuclear membrane). These considerations should be examined carefully in light of the recent findings of Oppenheimer and Schwartz (159) which suggest that active transport of  $T_3$  does occur *in vivo*, and that this transport occurs primarily at the level of the nuclear membrane.

The simulations of Pardridge and Landaw also revealed that the enhanced dissociation model predicts that subjects with high plasma concentrations of TBG will have high intracellular free  $T_3$  concentrations within the liver, and subjects with low plasma concentrations of TBG will have low intracellular free  $T_3$  concentrations within the liver (see simulations 3 and 8 of Ref. 235).<sup>23</sup> That this model makes these predictions can be understood intuitively when it is recognized that in this model TBG is seen as delivering  $T_3$  (but not  $T_4$ ) to the liver (45). Although Pardridge (57) subsequently attempted to justify one of these predictions, and although these predictions cannot be said to be absolutely disproven, they are nevertheless contrary to clinical experience and, to the extent that metabolism of  $T_3$  occurs in the liver, they are contrary to *in vivo* kinetics studies (Fig. 6).

The simulations that Pardridge and Landaw did not do are more telling. If they had performed similar simulations for  $T_4$ , they would have found that the enhanced dissociation model predicts that virtually none of the  $T_4$  in plasma can be taken up by the liver during a single pass. This prediction is incorrect (Table 3). That this model makes this incorrect prediction has been demonstrated by computer-modeling studies (67), but can be understood intuitively as well. Unlike the case of  $T_3$ , the  $K_D$  governing the  $T_4$ -TBG complex within the hepatic

<sup>23</sup> We (56) made this point in previous criticism. Inexplicably, Pardridge (57) denied that his model predicts low intracellular  $T_3$  concentrations within the liver in the case of TBG deficiency. He claimed that the simulation that we interpreted as indicating this (simulation 8 in Ref. 235) was indeed a case of TBG deficiency, but that the serum  $T_3$  was lowered disproportionately further (implying that this patient was also hypothyroid), to a value of 35 ng/dl. The basis of this claim was an article stating that serum  $T_3$  concentrations in TBG deficiency average 70 ng/dl (186), not 35 ng/dl. But this is misleading. In that article the normal serum  $T_3$  concentration was found to average 140 ng/dl. Thus, serum  $T_3$  concentrations were reduced by 50% in TBG deficiency. In the modeling study of Pardridge and Landaw (235), where assumed values for the various parameters were used, the normal serum  $T_3$  concentration was set at 70 ng/dl, not at 140 ng/dl. Thus, in their simulation 8, the serum  $T_3$  concentration of 35 ng/dl was 50% of the normal value, just as would be expected for the case of TBG deficiency without other complicating factors. Indeed, this is how the simulation was originally presented by Pardridge and Landaw (235). In fact, it should also be noted that this simulated patient was really slightly hyperthyroid (not hypothyroid) in addition to lacking TBG, as indicated by the elevated plasma free  $T_3$  concentration. In the absence of hyperthyroidism, the reduction in intracellular  $T_3$  due to TBG deficiency would have been found to be even more striking.

sinusoids is postulated, based on data interpreted within the context of this model, *not* to differ from the  $K_D$  measured *in vitro* (44). In the context of the enhanced dissociation model, with an assumed low value for the tissue influx rate constant, it would be predicted that any  $T_4$  released from the other  $T_4$ -binding proteins within the tissue vasculature (by changes in their  $K_D$  values) would bind to TBG rather than be delivered to the tissue. This is because, in the setting of a low tissue influx rate constant (here "low" refers to comparison with the rate of association of hormone with TBG; see footnote 18) the rate of association of free  $T_4$  with TBG will be much more rapid than the rate of tissue uptake of  $T_4$  (5, 67). Therefore, the low tissue influx rate constant (here "low" refers to comparison with the rate of tissue blood flow; see footnote 18) postulated in this model will be unable to account for more than a very small (<<1%) fractional unidirectional uptake of  $T_4$  by the liver (67).

At least two sets of physiologically based experiments explicitly designed to test important predictions of the enhanced dissociation hypothesis have been conducted recently. We (243) performed *in vivo* kinetics studies to examine  $T_4$  transport and distribution in Nagase analbuminemic rats (244, 245). Serum albumin concentrations in these rats were less than 0.1% of normal, whereas serum  $T_4$  and free  $T_4$  concentrations were normal. According to the enhanced dissociation model, both the rate of hepatic influx of  $T_4$  and the intracellular  $T_4$  concentration within the liver should have been markedly reduced in these rats (44). Instead, our studies indicated that the one-way flux of  $T_4$  from the plasma to the liver and the size of the rapidly exchangeable intracellular pool of  $T_4$  within the liver were unchanged. Thus, the enhanced dissociation model must be rejected. [Additional studies relating to testosterone metabolism in these rats similarly force rejection of this model (203).]

The second set of physiologically based experiments explicitly designed to test the enhanced dissociation model was conducted by Dubey *et al.* (246, 246a). Those investigators examined the predictions of this model for extracellular concentrations of diazepam in rat brain. Pardridge *et al.* (233) have previously applied the enhanced dissociation model to plasma protein-drug interactions as well as to plasma protein-hormone interactions, and studies of the unidirectional uptake of diazepam by rat brain had been interpreted within the context of this model as indicating that the concentration of free diazepam in brain extracellular fluid was 25-fold higher than that in bulk plasma (247). However, when these concentrations were directly measured by Dubey *et al.* (246, 246a) using microdialysis probes (248), they were found to be identical. Thus, the enhanced dissociation model must be rejected.

Other predictions made by the enhanced dissociation model that are unlikely to be correct have been enumerated elsewhere (19). However, even in the absence of demonstrable incorrect predictions, the predictive ability of this model must be considered poor. For example, this model is able to make absolutely no predictions about which protein-hormone complexes are likely to have their  $K_D$  values altered within the tissue vasculature. Furthermore, Pardridge (57) has recently emphasized that none of his experiments performed in one organ can be extrapolated to other organs, because the handling of protein-hormone complexes appears to vary from organ to organ. Therefore, if we embrace this model, we can expect to have absolutely no knowledge about intracellular hormone concentrations in organs other than those that have been directly studied by the Oldendorf injection technique. How, then, could we have any confidence that the data obtained by this technique using (primarily) rat organs have any relevance whatsoever to human organs?

The proponents of the enhanced dissociation model have clearly not abandoned it. Nevertheless, it is important to note that those investigators have interpreted certain recent findings in the context of an entirely new model. Thus, Pardridge and colleagues (249) recently observed that both rabbit SHBG and the sex steroids bound to it were significantly cleared (unidirectionally) in a single pass through rat testis and prostate. Based on these findings, they concluded that intracellular concentrations and biological activities of sex steroid hormones in the testis and prostate of the rabbit are determined by the circulating concentrations of SHBG-bound hormone. Although the model in which this conclusion was considered appropriate was not provided, it is important to note that this conclusion does not follow from interpretation of the data in the context of the enhanced dissociation model, which (like the free hormone hypothesis) predicts that intracellular hormone concentrations are proportional to the free hormone concentration within the tissue vasculature. In fact, the only obvious way that physiological relevance can be assigned to these findings as reported is if measurements of unidirectional uptake are in and of themselves assigned physiological relevance. And that, in fact, is the only way that the three different models of hormone transport proposed by Pardridge over the years can be considered similar.

## X. Functions of Plasma Hormone-Binding Proteins

When entertaining possible functions for plasma hormone-binding proteins, the first possibility that must be considered is that they have no function, at least as regards the hormones they carry, and that they bind their hormones by "accident," like other lower affinity nonspecific binding components in plasma. Investiga-

tions conducted in recent years force serious consideration of this possibility. Thus, the recently described plasma binding protein for growth hormone (GH) (250–253) appears to be the extracellular, hormone-binding domain of the plasma membrane GH receptor (254–257). In addition, circulating insulin-like growth factor II and transferrin receptors have also been described in plasma recently (258, 259). Although these receptors are all plasma membrane receptors, and therefore more accessible to the circulation than the intracellular thyroid and steroid hormone receptors, they nevertheless serve as examples of circulating binding proteins that apparently perform no essential biological function.

The fact that plasma hormone-binding proteins can be described to perform a number of functions does not detract from this consideration; that a function can be described is not sufficient reason to consider it essential or important. Thus, plasma hormone-binding proteins transport hormones and serve as reservoirs for their hormones. In addition, under appropriate sets of conditions, they can decrease the MCRs of their hormones, protect their hormones from hepatic degradation and glomerular filtration, "buffer" free hormone concentrations,<sup>24</sup> and provide an increased circulating pool of hormone that can be delivered to tissues. Certain of these actions seem important on an intuitive basis. For hormones whose hepatic influx and metabolism are very rapid, such that all of the hormone in the plasma would be cleared by the liver in a single pass, binding proteins that form slowly dissociating complexes with their hormones will decrease the disposal rates of these hormones. In addition, when hormones are tightly bound in the plasma, the free hormone concentration will be relatively unaffected by hemoconcentration or hemodilution (260). Both of these actions seem important. Nevertheless, neither seems to be essential, in that SHBG, which forms relatively slowly dissociating complexes with testosterone and dihydrotestosterone, is absent in many species (25), and CBG, which binds cortisol much more tightly in plasma than does albumin, is functionally absent in

New World monkeys [in that its concentration in plasma is far exceeded by the cortisol concentration (206)].

Other possible functions of plasma hormone-binding proteins have been proposed. Burke and Anderson (261) suggested that SHBG may serve as an estrogen amplifier, in that estrogens increase the plasma concentration of SHBG, and an increase in the plasma concentration of SHBG is expected to result in a relatively greater decrease in the free fraction of testosterone than in that of estradiol. However, the functional role played by such physicochemical events in the setting of an intact hypothalamic-pituitary-gonadal axis is unclear. Ewing *et al.* (262), who perfused rabbit testes *in vitro*, showed that the secretion of testosterone was increased 4-fold when the perfusates contained bovine albumin (3 g/dl), as compared to perfusates containing either no protein or a protein that did not bind testosterone (ovalbumin). Thus, they concluded that binding proteins facilitate the diffusion of hydrophobic steroids through capillary endothelium. Although this was an interesting and important observation, such facilitation is probably a general function of albumin, and there seems no clear need for specific hormone-binding proteins in this process. Tipping and Ketterer (263) have presented a model showing that binding proteins in general are expected to increase diffusional fluxes of lipophilic molecules that bind extensively to cell constituents.

A number of possible functions of plasma hormone-binding proteins have been proposed that would be possible only in the context of nontraditional models of hormone transport and distribution that have not been proved (see above, Sections VIII and IX). Keller and associates (217) proposed that CBG selectively delivers its hormones to tissues with "protein-permeable vascular beds," based on a number of complex findings. Siiteri and Simberg (212) proposed that cell-surface receptors for steroid hormone-binding proteins may provide for selective delivery of steroids to different tissues and, furthermore, that cellular uptake of steroid still bound to plasma proteins may result in protection of the steroid from intracellular metabolizing enzymes. Pardridge (53) proposed that plasma hormone-binding proteins cause selective delivery of steroids to tissues with long capillary transit times (see Eq XI). When he revised his model, he proposed that plasma hormone-binding proteins cause selective delivery of hormones to tissues that have unspecified mechanisms within their vascular beds to increase the  $K_D$  values of the protein-hormone complexes (6).

Selective delivery of hormones to tissues (those with rapid rates of metabolism) is also a possible function of plasma hormone-binding proteins in the context of the general models of hormone transport discussed in this review. Thus, if hormone uptake by one tissue (e.g. the

<sup>24</sup> Here, "buffer" usually refers to the ability to attenuate the biological effects of a change in the rate of hormone secretion. If hormone secretion ceases suddenly, a circulating pool of slowly cleared hormone will continue to supply hormone to tissues. If hormone secretion increases suddenly, there will be less of an increase in the plasma free hormone concentration for a given absolute (but not fractional) increase in the rate of secretion. Thus, a binding protein that is half-saturated is a poorer buffer than one that is much further from being saturated, in that both provide the same circulating pool of hormone, but the relative increase in free hormone concentration for a given relative increase in the total hormone concentration will be greater in the case of the protein closer to saturation. The buffering ability of plasma hormone-binding proteins should therefore not be considered analogous to that of chemical buffers, which are most effective at their pK values. The "buffering capacity" of plasma proteins has also been used to refer to their effects during hemoconcentration and hemodilution (see below).

liver) is flow- or dissociation-limited, and uptake by other tissues is elimination-limited, then an increase in the concentration of protein-bound hormone in the plasma, with an unchanged free hormone concentration, will result in a selective increase in the delivery of hormone to the tissue with flow- or dissociation-limited uptake (intracellular hormone concentrations in that tissue will still be lower than those in tissues with elimination-limited uptake, however) (see above, *Section V.D*). In such a setting, separate regulation of the concentration of binding protein and free hormone in the plasma would allow for separate regulation of the delivery of hormone to different tissues. Regulation of the concentrations of plasma hormone-binding proteins in response to tissue needs of hormone has not been demonstrated to occur, however.

Ekins *et al.* (4, 12, 95) have recently proposed a novel function for TBG that involves an important analysis of intracapillary phenomena that may occur when tissue influx rate constants are high enough. Those authors pointed out that, in the setting of a high enough tissue influx rate constant for free hormone, the free hormone concentration at the cell surface will be expected to fall below that in the bulk capillary. In such a setting, binding proteins can increase the apparent rate constant for influx of free hormone by providing an additional source of hormone at the cell surface that augments (through spontaneous dissociation of the protein-hormone complex) the concentration of free hormone and thereby increases the diffusional flux of hormone. Bass and Keiding (13), using a different mathematical development, independently demonstrated that such effects of hormone-binding proteins are expected. In the proper contexts, this theoretical work is of enormous significance. However, it is not at all clear that these theoretical considerations can reasonably be expected to be important with respect to the steady state transport and distribution of the thyroid hormones.

Some of the difficulties of applying these theoretical considerations to real situations have been discussed above (*Section III*). In addition, Ekins *et al.* (4, 12, 95) have correctly pointed out that such intracapillary phenomena are likely to be important at steady state only in the setting of rapid rates of net tissue uptake of hormone. [This is because, at steady state, the same equations apply, but the influx rate constant, which must be very high for these phenomena to be important, is replaced by the rate constant describing the net uptake of hormone. This rate constant equals  $k_1 k_3 / (k_2 + k_3)$  in the notation of Fig. 9, and is necessarily lower than the influx rate constant. Perhaps the best way to appreciate intuitively that such phenomena are likely to be important only in the setting of rapid rates of net tissue uptake of hormone is to recognize that, in the setting of rapid

influx but slow net uptake, the free hormone concentration at the cell surface will be replenished not only by dissociation of hormone from its binding protein, but also by rapid efflux of hormone from the tissue.] The role for TBG proposed by Ekins *et al.*, then, is that it selectively increases delivery of thyroid hormones to tissues with rapid rates of net uptake. (In our model, which does not take into account these intracapillary phenomena, this same role for plasma binding proteins is viewed as being possible in that the net uptake of hormone by tissues can be elimination-limited in one organ but flow- or dissociation-limited in another; see above, this section and *Section V.D*.) In particular, Ekins *et al.* proposed that during early pregnancy the increase in the plasma concentration of TBG may allow increased maternal  $T_4$  to be extracted by the placenta and thus serve as a source of fetal iodide. However, calculations based on reasonable assumptions indicate that net rates of tissue uptake of thyroid hormones are likely to be very slow compared with intracapillary events in all organs (see above, *Section VI.A.1*). Ekins *et al.* apparently postulate that the placenta (in early pregnancy) may be an exception, but data and calculations indicate that this is unlikely to be the case. *In vivo* kinetics studies of  $T_4$  metabolism have been conducted both in early pregnancy and in late pregnancy (264, 265), and the  $T_4$  disposal rate is normal. This confirms the clinical observation that  $T_4$  dosage in hypothyroid women who become pregnant after starting therapy does not need to be changed (266). Therefore, unless  $T_4$  disposal in other organs is markedly reduced during pregnancy, it is unlikely that net uptake of  $T_4$  by the placenta is extraordinarily high. In addition, if we assume a placental blood flow throughout pregnancy of approximately  $1.3 \text{ ml} \cdot \text{min}^{-1} \cdot \text{g tissue}^{-1}$  (267–269), and assume that the placenta takes up (net)  $T_4$  at the same rate on a per gram of tissue basis as the liver ( $80 \mu\text{g}/\text{day}$  for a 1400-g liver, or  $4 \text{ ng} \cdot \text{min}^{-1} \cdot 100 \text{ g tissue}^{-1}$ , as a maximal estimate; see above, *Section VI.A.1*), as the data of Woods *et al.* (270) might suggest, then, even assuming the shortest capillary transit time shown in Table 2 (0.7 sec), it can be calculated from the data in Table 1 that, for normal serum  $T_4$  ( $8 \mu\text{g}/\text{dl}$ ) and TBG concentrations, less than 0.2% of the  $T_4$  dissociating within the capillaries will be taken up (net) by the placenta at steady state. Similar calculations for  $T_3$  (using the same assumed values for parameters of  $T_3$  metabolism as in *Section VI.A.1*) indicate that only 1% of the  $T_3$  dissociating during capillary transit will be taken up (net) at steady state. Thus, net uptake of the thyroid hormones from plasma by the placenta, just as appears to be the case for other tissues, is likely to be very slow compared with intracapillary events, and at steady state the thyroid hormones are likely to exist in approximate equilibrium between the plasma and the placenta (as-

suming a rapid influx rate constant). Given this situation, it would seem unlikely that the intracapillary phenomena described by Ekins *et al.* are quantitatively important with respect to thyroid hormone metabolism by the placenta.

Nevertheless, for hormones in which net rates of tissue uptake are much more rapid, such considerations are more likely to be important. Whether increasing the apparent tissue influx rate constant for free hormone under such circumstances is an important function of plasma hormone-binding proteins (and it should be noted that albumin probably does this for a variety of ligands), or just a physicochemical event, must await further elucidation.

We (65) have recently proposed what we consider to be a novel and important function of plasma thyroid hormone-binding proteins. All species that rely on thyroid hormone metabolism seem to have high affinity plasma thyroid hormone-binding proteins; TBPA appears to be more ubiquitous than TBG (271). In the human, no subject with both TBG and TBPA deficiency has been described. We postulated that plasma thyroid hormone-binding proteins function principally to ensure a uniform distribution of the thyroid hormones among all cells of each tissue. We tested this postulate in a rat liver perfusion system using autoradiography to study the distribution of radiolabeled T<sub>4</sub> within hepatic lobules after its perfusion through the portal vein in solutions containing or lacking plasma thyroid hormone-binding proteins. When the rat liver was perfused single pass in the absence of plasma thyroid hormone-binding proteins, virtually all of the T<sub>4</sub> in the perfusate was taken up by the first cells it contacted and was unavailable to cells farther along the sinusoid (Fig. 2). In contrast, when the T<sub>4</sub> was perfused in solutions containing plasma thyroid hormone-binding proteins, the fractional extraction was decreased and the T<sub>4</sub> was taken up uniformly by all the cells throughout the hepatic lobule (Fig. 1).

What was novel about this study was not these unidirectional tissue uptake findings, but the fact that we were able to show that these findings were likely to have relevance to the *in vivo* situation. We did this by repeating the (1-min) perfusion of radiolabeled T<sub>4</sub> in solution lacking binding proteins, but this time we continued to perfuse the liver with protein-free solution for an additional hour before processing the tissue. When this was done, we found that virtually no redistribution of the T<sub>4</sub> initially taken up had occurred (65). We therefore concluded that in the absence of plasma thyroid hormone-binding proteins, T<sub>4</sub> circulating through tissues would be taken up by the first cells it contacted and would be slow—on a physiologically relevant time scale—to distribute to other cells within the tissue. In the absence of circulating plasma thyroid hormone-binding proteins,

then, changes in the rate of T<sub>4</sub> secretion by the thyroid gland would be expected to be communicated nonuniformly to cells in different locations within each tissue.

In our studies we found that albumin alone, in the absence of the high affinity plasma thyroid hormone-binding proteins, could perform this function just as well. However, we found that in a solution of albumin alone, unlike in serum, the free T<sub>4</sub> concentration was very sensitive to physiological concentrations of FFA (Fig. 10). Thus, albumin's function as a transporter of FFA and other organic anions appears to make it unsuitable as a sole transporter of T<sub>4</sub>. Based on all of these findings, we believe that plasma thyroid hormone-binding proteins function principally to increase—over a physiologically relevant time frame—the rate of diffusional equilibration of thyroid hormones between the plasma and tissues. In doing so, they promote a uniform distribution of thyroid hormones among all cells of each tissue. They also provide for an efficient control and feedback mechanism in that the rapid diffusional fluxes of the thyroid hormones across plasma membranes serve to maintain these hormones in approximate equilibrium between the plasma and tissues, thereby ensuring that all tissues throughout the body are exposed to the same physiologically relevant circulating thyroid hormone concentrations. The high affinity plasma thyroid hormone-binding proteins also appear to function specifically to ensure nonfluctuating concentrations of free thyroid hormones in the plasma *in vivo*.

Unfortunately, these findings may not be readily extrapolatable to the plasma steroid hormone-binding proteins. We (21) recently observed that the bidirectional

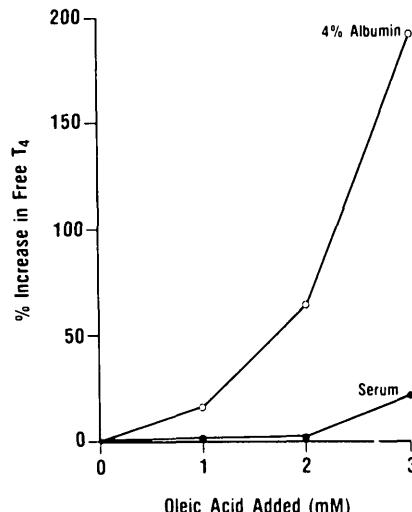


FIG. 10. The effect of oleic acid on the concentration of free T<sub>4</sub> in pooled normal human serum and in delipidated human serum albumin (4 g/dl). The concentration of endogenous FFA in the serum was 0.7 meq/liter. The concentration of FFA generally does not exceed 1.5 meq/liter in human plasma *in vivo*. [Reproduced with permission from C. M. Mendel *et al.*: *Endocrinology* 120:1742, 1987 (65).]

fluxes of cortisol across the plasma membrane in the liver are rapid even in the absence of circulating binding proteins. Thus, equilibration of cortisol between plasma and tissues might be expected to be rapid even in the absence of plasma binding proteins. (It should be noted, however, that the half-life of cortisol in plasma is very much shorter than that of the thyroid hormones. Therefore, the diffusional fluxes observed in the absence of any binding proteins might nevertheless not be rapid enough to maintain uniform tissue distribution of hormone in the face of the rapid rates of tissue removal.) Furthermore, even if plasma hormone-binding proteins were necessary to maintain rapid diffusional fluxes of other hormones as well, it would seem that albumin alone could serve, because the physical or functional absence of SHBG and CBG seem to occur commonly among other species (see above, this section). In this light, we have recently found that the concentration of free cortisol in a solution of delipidated human serum albumin (4 g/dl) is virtually unaffected by the addition of oleic acid in concentrations of up to 3 mM (Mendel, C. M., and J. T. Murai, unpublished observations).

It is possible that our observations on the function of plasma thyroid hormone-binding proteins are more relevant to vitamin D transport than to the transport of other steroid hormones. It was pointed out above (*Section VI.G*) that the transport and distribution of vitamin D and its hydroxylated metabolites seem to share a number of common features with those aspects of thyroid hormone metabolism. Regarding vitamin D, it should also be noted that the "buffering" capacity afforded by DBP in serum is enormous, since only 1–3% of the DBP binding sites are normally occupied (154). This seems likely to afford some protection against vitamin D intoxication.

## XI. Conclusions and Areas for Future Investigation

As we have seen, traditional models of hormone transport, distribution, and metabolism are consistent with the vast majority of experimental and clinical data collected over the past 30 yr. The recent realization that the particular predictions made by these models often depend on which step in the tissue uptake process is rate-limiting to the net uptake of hormone by tissues has strengthened these models and allowed reconciliation of a number of previous apparent conflicts of data obtained for different hormones. Nevertheless, these models are likely to be incomplete. Additional usable general models of hormone transport that take into account more of the possible factors affecting the tissue uptake of hormones from plasma need to be developed. More data of the type shown in Tables 1 to 3 and more data on rates of hormone

influx and metabolism in individual organs need to be accumulated. The possibility that hormones exist within the cell in more than one metabolic pool must be carefully addressed. And, the mechanisms and locations of active transport of hormones into cells require elucidation.

## Acknowledgments

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## Appendix: Intracellular Synthesis of Hormone

In the models discussed in this review, intracellular synthesis of hormone was assumed to be nonexistent or at least negligible compared with the rate of influx of hormone from the plasma. When this is not the case, intracellular hormone concentrations will be increased. Thus, in the model shown in Fig. 11, the rate of formation of  $L_I$  (symbols are defined in the legend to Fig. 11) can be described as:

$$\dot{L}_I = r_1 L_p + r_3 S - r_2 L_I - r_4 L_I \quad (\text{XII})$$

For consideration of the steady state,  $\dot{L}_I$  is set at 0 and it can be seen that

$$L_I = \frac{r_1 L_p + r_3 S}{r_2 + r_4} \quad (\text{XIII})$$

It can thus be seen that at steady state the fractional increase in  $L_I$  (over that which is due to transport from the plasma) due to intracellular synthesis of hormone will be  $r_3 S / r_1 L_p$ , and the fraction of  $L_I$  derived from intracellular synthesis of hormone will be  $r_3 S / (r_1 L_p + r_3 S)$ .

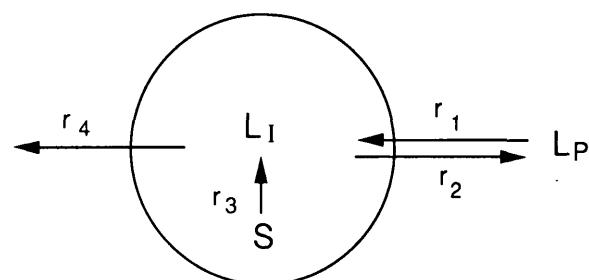


FIG. 11. Schematic of cellular hormone as a function of uptake from plasma, intracellular synthesis, and cellular elimination.  $L_I$ , Concentration of intracellular hormone;  $S$ , concentration of substrate for hormone synthesis;  $L_p$ , concentration of hormone in plasma; and  $r_1$  to  $r_4$ , rate constants describing the processes depicted. Neither plasma flow nor hormone-binding proteins in plasma are considered. Assumptions in the model depicted are the same as those for Fig. 7.

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