Increased Thyroxine Turnover and Thyroidal Function after Stimulation of Hepatocellular Binding of Thyroxine by Phenobarbital

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A B S T R A C T Administration of phenobarbital to rats in a dosage schedule previously demonstrated to increase hepatocellular binding of thyroxine results in increased hormonal turnover, due both to increased deiodination and to fecal disposition of thyroxine iodine. The rate of biliary excretion of thyroxine iodine is roughly proportional to the hepatic content of exchangeable thyroxine. The enhanced peripheral disposition of thyroxine appears to lead to increased thyroidal function, as measured by isotopic iodine studies, and the maintenance of a normal nonradioactive serum PBI. On the other hand, thyroidectomized animals maintained on a constant replacement dose of L-thyroxine and treated with phenobarbital exhibit a marked fall in serum PBI. These findings suggest that increased thyroxine flux in phenobarbital-treated animals is secondary to primary stimulation of hepatocellular binding. Exchangeable intracellular thyroxine may thus be an important determinant of hormone turnover and, possibly, of hormonal action.

INTRODUCTION

A number of studies have now established that the liver contains substantial quantities of thyroxine in rapid equilibrium with plasma proteins (1–5). A similar exchange occurs between thyroxine bound to plasma proteins and cellular thyroxine in the kidney (5), and it appears likely that other tis-

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sue pools are also in equilibrium with plasma thyroxine. The cellular mechanism responsible for retaining thyroxine in competition with plasma proteins has not been defined but may be regarded from a kinetic standpoint as cellular binding.

Previous studies have indicated that injection of phenobarbital and chlordane stimulates hepatocellular binding of thyroxine (6). In these experiments, phenobarbital and chlordane were administered in a dosage regimen known to induce the formation of a variety of drug-metabolizing enzymes (7). Augmented liver binding resulted both from an increase in total hepatic mass and from increased binding per gram of liver. This was demonstrated by a shift of thyroxine-^{125}I (T_4-^{125}I) from plasma to liver in the absence of demonstrable alterations in plasma binding of thyroxine. Despite the redistribution of isotopic thyroxine the concentration of nonradioactive PBI in plasma remained unchanged.

The present study was undertaken in order to elucidate the effect of augmented hepatocellular binding on the peripheral disposition of thyroxine. It was felt that the results of this investigation might provide a broader understanding of the factors governing the net thyroxine flux in the whole organism.

METHODS

Male rats of the CD strain (Charles River, Boston) weighing between 150 and 250 g were used. In order to facilitate cannulation of the bile ducts larger male rats of the same strain (300–420 g) were used in determination of biliary excretion. In testing the effect of pheno-

barbital on the serum PBI in thyroidectomized animals, female CD rats (150–200 g) were used because the supplier could not provide male thyroidectomized animals at the time of these studies. Control studies had indicated that female rats and older rats were equally suitable for demonstrating the effect of phenobarbital on hepatocellular binding. Animals were housed in animal quarters under constant temperature conditions and fed a Wayne Laboratory diet containing approximately 1 μg of I per g of diet.

Plasma disappearance studies were performed in a manner similar to that used by Gregerman (8). Animals were injected under light ether anesthesia through the tail vein with 0.5 μc of T_4-^{125}I (less than 0.04 μg), dissolved in 50% propylene glycol (Abbott Laboratories, North Chicago, Ill.). All animals received intraperitoneal injections of 1 mg NaI twice a day in order to prevent thyroidal accumulation of ^{125}I. Plasma samples were obtained from freely flowing blood from the cut tail 6, 24, 30, and 48 hr after injection. Plasma samples were processed and assayed for radioactivity as previously described (5). 6 hr after injection of the tracer the plasma disappearance curve approached a straight line on a semilogarithmic plot (Fig. 1). Fractional removal rates (k) were determined by the method of least squares. The thyroxine distribution space (TDS) was calculated from the reciprocal of the zero-time extrapolation. The metabolic clearance was calculated as the product of k and TDS.

Complete collections of urine and feces were obtained in metabolic cages. Collections were made at 24-hr intervals, at the end of which the cage and collecting syphon were carefully washed to remove residual radioactivity. Washes were pooled with the urine. Fecal collections were homogenized and digested in concentrated KOH. Aliquots of urine and fecal digests were counted in an Auto-gamma spectrometer well. Urinary and fecal clearances were calculated as the products of the metabolic clearance and the fraction of total radioactivity excreted via the urinary or fecal route. At the end of each study the
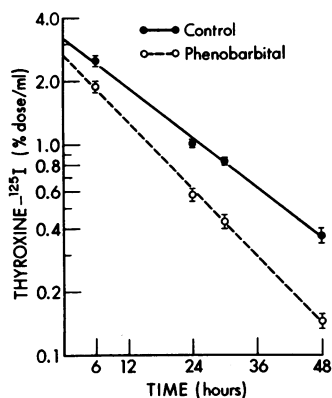
liver was rapidly removed and weighed. A tissue sample of known weight was digested in concentrated KOH and assayed for radioactivity. In order to assess the amount of radioactivity associated with trapped plasma a correction factor was introduced. On the basis of the ratio of the simultaneous albumin-^{131}I counting rates in liver and plasma in a series of animals it was estimated that each gram of liver contained approximately 0.1 ml of plasma. The amount of T_4-^{125}I contained in this volume of plasma was, therefore, subtracted from the total ^{125}I counting rate per gram of liver. Approximately 15–20% of T_4-^{125}I in tissue was found to be associated with trapped plasma.

Bile ducts were cannulated under ether anesthesia 24 hr after the intravenous injection of the dose. Biliary collections were obtained over a 30 min interval. Plasma samples were obtained just before and at the conclusion of the collection period. The biliary clearance was calculated from the quotients of the excretory rate and the mean plasma concentration of radioactivity.

For the sake of simplicity, the assumption was made in the calculations that all radioactivity in plasma and tissue during the first 48 hr was in the form of T_4-^{125}I. Chromatographic analysis (tertiary amyl alcohol:2 N NH_4OH:H_2O [4:1:5]) of plasma and tissue radioactivity after butanol extraction showed that more than 85% of extractable radioactivity was in the form of thyroxine with the remainder largely in the form of iodide. All urinary radioactivity was in the form of iodide as determined by paper electrophoresis and chromatography. Thus, disposition of radioactivity via the urinary route could be attributed to deiodinative processes.

Animals were pretreated with phenobarbital and chlordane for 5 days before the thyroid function and thyroxine turnover studies. An aqueous solution of sodium phenobarbital (The Vitarine Co., Inc., New York.) was injected daily by the intraperitoneal route (100 mg/kg of body weight). Chlordane (City Chemical Corp., New York) was dissolved in corn oil and also injected intraperitoneally (75 mg/kg). Only thyroid function tests were performed in chlordane-treated animals. Daily injections of phenobarbital were continued throughout the 48 hr turnover study. Control studies had indicated no change in liver weight, liver:plasma concentration ratios, or serum PBI during the additional 2 days of phenobarbital injections. In all studies experimental controls were injected with the appropriate diluent of the test compound.

The effect of phenobarbital treatment on serum PBI (BioScience, Van Nuys, Calif.) in a group of thyroidectomized animals maintained on a constant dose of L-thyroxine was assessed. 10 thyroidectomized animals supplied by Charles River were injected subcutaneously daily with 2 μg of thyroxine per 100 g of body weight and were maintained on 1% calcium gluconate in their drinking water. Starting on the 5th day, five animals were injected additionally with phenobarbital (100 mg/kg). The remaining five animals received daily thyroxine injections and saline alone. On the 10th day a combined dose of albumin-^{131}I, and T_4-^{125}I was injected intravenously as previously described (5). 35 min afterwards the animals were sacrificed. Liver and plasma radioactivity were measured,

FIGURE 1 Mean plasma disappearance curves of 11 control animals and 13 animals treated with phenobarbital. Vertical bars indicate ± SE.

	Animal wt		Liver wt		TDS		k		MC		UC		FC		Liver space	
	C	P	C	P	C	P	C	P	C	P	C	P	C	P	C	P
	g		*g*		*ml/100 g*		*day⁻¹*		*ml/100 g per day*		*ml/100 g per day*		*ml/100 g per day*		*ml/100 g*	
Mean	205	211	8.71	11.51	15.6	18.6	1.10	1.47	16.8	27.1	8.45	11.8	8.40	15.5	3.38	6.83
SE	5.6	7.8	0.30	0.65	0.69	0.81	0.04	0.03	0.86	1.16	0.64	0.52	0.49	0.82	0.16	0.34
P	NS		<0.001		<0.02		<0.001		<0.001		<0.001		<0.001		<0.001	

In control (C) group, 11 animals were studied; in phenobarbital (P) group there were 13 animals. TDS, total thyroxine distribution space; k, fractional turnover by plasma; MC, metabolic clearance; UC, urinary deiodinative clearance; FC, fecal clearance; liver space, total hepatic tissue radioactivity: plasma concentration of thyroxine-¹²⁵I 48 hr after injection. TDS, MC, UC, FC, and liver space are expressed in terms of 100 g of body weight.

and calculations of tissue uptake of thyroxine were performed as previously indicated (5).

The thyroid gland was assayed both for ¹³¹I to measure thyroidal accumulation of ¹³¹I, and for ¹²⁵I to measure the thyroid: serum iodide concentration ratio (T/S ratio). 24 hr before sacrifice, 10 μc of carrier-free Na¹³¹I was administered intraperitoneally. 90 min before sacrifice, 15 mg of propylthiouracil was injected subcutaneously. 45 min later 20 μc of ¹²⁵I was injected. The animal was sacrificed by exsanguination. The thyroid gland was carefully dissected, weighed, and assayed for radioactivity. The concentration of ¹²⁵I in serum was measured, and the concentration of PB¹³¹I was determined after precipitation of serum proteins with 20% trichloracetic acid.

RESULTS

Turnover studies (Table I and Fig. 1). In agreement with previous findings (6) administration of phenobarbital resulted in an increased hepatic mass with a proportionately greater increase in hepatic thyroxine distribution volume. At the same time the metabolic clearance of thyroxine increased 62% above levels in control animals. The increased metabolic clearance was due both to a 33% increase in fractional removal rate and a 19% increase in total thyroxine distribution vol-

ume. Since the concentration of nonradioactive PBI does not change significantly under these conditions (6), these results imply an increased absolute turnover of thyroxine.

Thyroxine iodine is excreted via both fecal and urinary routes. In control animals 49.8% of excreted radioactivity was found in the urine and 50.2% in the stool. Administration of phenobarbital resulted in a significant ($P < 0.05$) change in the partition of excreted radioactivity between fecal and urinary routes. An average of 43.5% of excreted radioactivity was found in the urine and 56.5% in the feces of phenobarbital-treated animals. Recovery of radioactivity was similar in experimental and control animals (control, 84.9%; phenobarbital, 85.2%).[1] The fecal clearance in-

[1] Recovery was calculated as the percentage of administered radioactivity excreted in urine and feces plus estimated residual body radioactivity (TDS × terminal thyroxine-¹²⁵I concentration in plasma). In calculating the fecal and urinary clearance the assumption was made that the losses of radioactivity were partitioned in the same proportion between urinary and fecal routes as the recovered excretion of radioactivity.

TABLE II
Effect of Phenobarbital on Biliary Excretion of Thyroxine

	Animal wt		Liver wt		Liver space		Bile flow		Bile:plasma concn. ratio		Biliary clearance	
	C	P	C	P	C	P	C	P	C	P	C	P
	g		*g*		*ml*		*ml/hr*				*ml/hr*	
Mean	360	369	13.2	19.4	10.6	26.7	0.79	1.14	0.98	1.54	0.74	1.75
SE	11	11	1.0	1.3	0.47	1.76	0.08	0.06	0.08	0.11	0.05	0.15
P	NS		<0.005		<0.001		<0.001		<0.005		<0.001	

Biliary clearances determined 24 hr after injection of T_4-¹²⁵I in seven control (C) and six phenobarbital-treated (P) animals.

		Animal wt		Liver wt		24 hr uptake		PB^{125}I		T/S		Thyroid wt	
		C	X	C	X	C	X	C	X	C	X	C	X
		g		*g*		*% dose*		*% dose/ml*				*mg*	
Phenobarbital	Mean	248	238	9.8	10.8	6.8	12.3	0.19	0.26	19.6	29.7	6.56	7.19
	SE	2.5	2.5	0.2	0.3	0.6	1.0	0.02	0.04	1.7	3.3	0.24	0.18
	P	NS		<0.025		<0.001		<0.025		<0.02		<0.005	
Chlordane	Mean	214	208	8.2	9.2	5.8	8.8	0.19	0.25	20.8	28.8	6.62	7.59
	SE	6.7	11.1	0.4	0.6	0.7	0.8	0.01	0.01	1.4	2.7	0.41	0.49
	P	NS		NS		<0.001		<0.02		<0.05		NS	

Six animals were studied in each group. C, control groups; X, experimental groups; T/S, thyroid: serum iodide concentration ratio.

creased 88%, and urinary degradative (deiodinative) clearance increased 40% as a result of phenobarital treatment.

Biliary clearance (Table II). The increased fecal clearance observed as a result of phenobarbital treatment could have resulted either from increased biliary excretion of thyroxine, diminished reabsorption from the gut, or both. Direct measurements of biliary clearance indicated a 137% increase above control levels. The increase in hepatic distribution space in these animals was 151%. The increase in biliary excretion was due both to an increase in the biliary flow and to an increase in the bile: plasma concentration ratio.

For technical reasons measurements of biliary excretion were made in larger animals than those employed in the turnover studies. Moreover, biliary measurements were performed shortly after surgical trauma and under ether anesthesia. Therefore, absolute biliary clearance cannot be easily compared to the fecal measurement performed in the smaller and unanesthetized animals. Nevertheless, the marked relative increase in biliary clearance induced by phenobarbital would appear to adequately account for the observed increase in fecal disposition of thyroxine.

Thyroid function (Table III). The finding of increased peripheral thyroxine disposal under steady-state conditions implies increased thyroidal release of hormone. Results of thyroid function tests supported the concept of increased thyroidal activity in animals treated with phenobarbital. Although turnover studies were not performed in chlordane-treated rats it seems reasonable to suppose that the increased thyroidal activity in these animals was secondary to previously demonstrated hepatic effects (6). Percentage increases over control animals in the phenobarbital-treated group were: 24 hr uptake, 80; PB^{131}I, 37; and T/S ratio, 51. Corresponding percentage increases in the chlordane-treated group were: 24 hr uptake, 54; PB^{131}I, 28; and T/S ratio, 38. Only slight increases in the thyroid weight were noted, 9.5% in the case of phenobarbital and 14.5% in the case of chlordane. The increase in weight was statistically significant ($P < 0.005$) in the case of phenobarbital but not so in the case of chlordane.

These results suggested that increased thyroidal secretion of thyroxine was responsible for maintaining a normal level of serum PBI in phenobarbital-treated anmials (6). Additional evidence favoring this conclusion is the result of experiments in which thyroidectomized animals were maintained on constant doses of L-thyroxine (2 μg/100 g of body weight) (Table IV). Phenobarbital administration resulted in an increase in the hepatic distribution volume, but, in contradis-

TABLE IV

Effect of Phenobarbital on Serum PBI of Thyroidectomized Rats Maintained on L-Thyroxine, 2 μg/100 g Body Weight

	Animal wt		Liver wt		Liver space		Serum PBI	
	C	P	C	P	C	P	C	P
	g		*g*		*ml*		*μg/100 ml*	
Mean	174	176	6.28	9.11	7.4	11.4	4.96	2.60
SE	5.1	4.9	0.30	0.26	0.82	0.71	0.31	0.08
P	NS		<0.001		<0.02		<0.001	

Five control (C), five phenobarbital-treated (P) animals.

tinction to the results in intact animals, the concentration of serum PBI fell. These findings also indicate that augmented hepatocellular binding is not dependent on increased thyroidal secretion.

DISCUSSION

The results of these studies clearly indicate that increased deiodination and biliary clearance of thyroxine accompany the enhanced hepatocellular binding of thyroxine induced by phenobarbital. It is tempting to suggest that these phenomena are causally related, i.e., that the hepatocellular content of exchangeable thyroxine governs the rate of hepatic deiodination and biliary transport.

This hypothesis is most readily tested by measurements of biliary clearance rather than total deiodinative clearance. The liver is the only organ directly involved in biliary transport of thyroxine, whereas a number of tissues contribute to the over-all deiodination of the thyroid hormones. The similarity in the relative increase in hepatic thyroxine space (2.5-fold) and the relative increase in clearance (2.4-fold) induced by phenobarbital supports the concept of intracellular exchangeable thyroxine as an important determinant of the rate of cellular metabolism. The enhanced biliary excretion of thyroxine also adequately accounts for the increased disposition of thyroxine via the fecal route.

The increased total deiodinative clearance probably reflects enhanced metabolism of thyroxine in the stimulated liver. In previous studies (6) it was shown that 35 min after the intravenous injection of tracer, the increased total volume of distribution of T_4-^{125}I could be attributed exclusively to the increased hepatic distribution volume. Similarly, in the present experiments the differences in the total thyroxine distribution volume between the phenobarbital-treated and control animals was 7.2 ml, whereas the difference between hepatic thyroxine distribution volumes was 7.5 ml. These findings strongly suggest that the phenobarbital effect on peripheral thyroxine metabolism is confined to the liver.

If we accept the likelihood that phenobarbital acts only upon the liver, we may calculate the contribution of hepatic deiodination to total tissue deiodination. Thus, total deiodinative clearance (D_t) represents the sum of hepatic deiodinative clearance (D_h) and the deiodinative clearance by

extrahepatic tissue (D_e). If we assume that deiodination is proportional to the content of exchangeable tissue thyroxine,[2]

$$D_h = \alpha V_h \quad \text{and} \quad D_e = \beta V_e \qquad (1, 2)$$

in which V_h = hepatic thyroxine distribution volume, V_e = extrahepatic cellular thyroxine distribution volume, and α, β are proportionality constants. Thus,

$$D_t = \alpha V_h + \beta V_e. \qquad (3)$$

Since we assume that phenobarbital effects are limited to the liver,

$$(D_t)_p = \alpha(V_h)_p + \beta(V_e)_c \qquad (4)$$

$$(D_t)_c = \alpha(V_h)_c + \beta(V_e)_c \qquad (5)$$

in which the subscripts c and p refer to control and phenobarbital-treated groups. Solutions of simultaneous equations 4 and 5 from data in Table I will yield the following values:

$$(D_h)_c = \alpha(V_h)_c = 3.2 \text{ ml/100 g per day}$$
$$(D_h)_p = \alpha(V_h)_p = 6.5 \text{ ml/100 g per day}$$
$$(D_e)_c = (D_e)_p = \beta V_e = 5.2 \text{ ml/100 g per day.}$$

Thus, under control conditions the liver is responsible for approximately 38% of the thyroxine deiodinated in the animal. In the phenobarbital-treated group, the contribution of hepatic deiodination rises to 55% of the total.

In previous studies phenobarbital was found not to cause a statistically perceptible change in serum PBI despite the marked shift of isotopic T_4 between plasma and liver observed 35 min after

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[2] It appears quite likely that hepatic thyroxine as determined from the product of the hepatic thyroxine distribution volume and the plasma thyroxine concentration is largely, if not exclusively, in the exchangeable form. After initial equilibration which takes place in the first 15 min after intravenous injection of labeled thyroxine, the plasma and hepatic radioactivity decline at nearly parallel rates over the ensuing several days (9). Since hepatic radiothyroxine has been demonstrated to be entirely exchangeable in the early phase (5), and since hepatic radioactivity is largely in the form of thyroxine, it follows that at the intervals considered in this study hepatic thyroxine is also in the exchangeable form. This conclusion is further strengthened if one compares the liver: plasma thyroxine concentration ratio 35 min after injection of T_4-^{125}I, 0.6 (6) to that obtained in animals equilibrated with ^{131}I, 0.5 (10).

intravenous injection of the tracer. These findings raised the possibility that the thyroid was releasing additional amounts of hormone which served to sustain the plasma PBI at normal levels. In the present series of experiments the increased thyroidal activity was confirmed by isotopic measurements of thyroidal accumulation of iodine, the thyroid : plasma iodide gradient, and the concentration of $PB^{131}I$ in plasma. These results confirm previous reports of increased thyroidal activity in rats after barbiturate treatment (11, 12). Although it is difficult quantitatively to relate these increases to the observed augmentation of peripheral turnover, the general direction of the changes serves to support the idea that increased thyroidal secretion of thyroxine is responsible for maintaining a normal PBI. Additional data favoring this explanation were obtained in the study of thyroidectomized animals maintained on a constant dosage regimen of T_4. In this group there was a marked fall in serum PBI in the subgroup treated with phenobarbital. The fall in serum PBI was somewhat greater than one would have anticipated on the basis of the observed increase in liver space, but this discrepancy can probably be attributed to limitations in interpretation imposed by the small number of animals studied.

The following sequence of events can be postulated to explain the observed effects of phenobarbital on thyroxine metabolism and thyroid function. Administration of phenobarbital causes a primary increase in hepatocellular binding, as a result of both increased liver mass and increased binding per gram liver. The ensuing shift of thyroxine from extrahepatic hormonal stores to the liver results in an increased pool of exchangeable hepatic thyroxine. This serves to stimulate both hepatic deiodinative and excretory processes. Since no major alterations in enteric absorption of thyroxine occur, both fecal and deiodinative clearances increase. The fall in plasma and extrahepatic thyroxine activates a negative feedback system which results in increased thyroidal secretion. It seems possible that depletion of the hypothalamic or pituitary content of exchangeable thyroxine serves as a stimulus to augment pituitary secretion of thyroid-stimulating hormone (TSH) and to increase thyroidal activity. A new steady state is achieved in which plasma and extrahepatic thyroxine tissue stores have returned to near

normal levels. The total body pool of exchangeable thyroxine has increased as has the net turnover of hormone. Although our previous studies have failed to demonstrate any statistically significant fall in serum PBI in the intact phenobarbital-treated rat (6),[3] it is conceivable that a small reduction in PBI might not have been detectable with relatively crude chemical methods. A small reduction might serve to explain the perpetuation of increased thyroidal activity and thyroxine flux in the new steady state.

Although considerable attention has been given to the role of plasma proteins in regulating the peripheral turnover of thyroxine, the importance of cellular factors has also been recognized (13–16). Discrepancies between the turnover of thyroxine and the serum-free thyroxine concentration have been attributed to alterations in the cellular disposal of hormone (17–21). On the basis of experiments employing tissue slices, Freinkel, Ingbar, and Dowling have previously demonstrated the existence of an equilibrium state between thyroxine bound to cellular and extracellular sites (13) and have discussed the possible role of such binding sites in thyroxine metabolism. The nature of thyroxine binding by subcellular components has also been extensively investigated by Tata (16). The precise relationship between intracellular binding and thyroxine metabolism, however, was not experimentally defined in these studies.

Our analysis of intracellular binding differs from previous approaches inasmuch as binding is defined in terms of the in vivo tissue : plasma thyroxine concentration ratio (4, 5). A much larger fraction of total exchangeable thyroxine appears to be associated with cellular binding sites than was suggested by earlier studies. Our results also allow some tentative generalizations regarding the relationship of intracellular binding and metabolism. The direct correlation between hepatocellular binding, deiodination, and biliary transport suggests that the thyroxine–cellular binding complex might participate as a unit in intracellular metabolic reactions. If it were necessary for thyroxine to dissociate from the intracellular binding

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[3] Additional studies have failed to reveal statistically significant decreases in the serum PBI after 1, 5, and 10 days of treatment with phenobarbital (100 mg/kg). Groups of five control and five experimental animals were used at each time interval.

complex into a free form before becoming available for deiodination or biliary excretion, increased hepatocellular binding, like increased plasma protein binding, might be expected to result in decreased rather than increased fractional disposition.

Our results raise the possibility that exchangeable intracellular thyroxine may play a pivotal role in determining the peripheral metabolism of thyroxine. One would anticipate that pertubation in plasma protein binding would lead to a new steady state characterized by a normal exchangeable intracellular pool and a normal hormonal turnover. The constancy of hormonal turnover with selective alterations in plasma binding is well recognized (22). Although comparatively few measurements of exchangeable intracellular thyroxine have been made, patients with an idiopathic decrease in thyroxine-binding globulin show no systematic variation in cellular hormone (2, 4). With primary alterations in intracellular binding, however, one would expect changes in steady-state thyroxine flux under all circumstances in which the operation of the normal pituitary feedback mechanism remains unimpaired. It is quite likely that simultaneous alterations in cellular and extracellular binding may occur under specific physiological and pathological settings. The possibility should also be recognized that some factors may independently influence the rate constants describing the relationship between intracellular binding and subsequent metabolic transformations.

Changes in intracellular binding after administration of phenobarbital are confined to the liver. It would be important to uncover examples of primary changes in intracellular binding involving other organs and tissues. Lastly, it seems reasonable to suppose that the metabolic action of thyroxine as well as its rate of disposal is closely geared to the intracellular exchangeable thyroxine pool. This suggestion appears amenable to experimental verification.

ACKNOWLEDGMENTS

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