Slow Fractional Removal of Nonextractable Iodine from Rat Tissue after Injection of Labeled L-Thyroxine and 3,5,3′-Triiodo-L-Thyronine

A POSSIBLE CLUE TO THE MECHANISM OF INITIATION AND PERSISTENCE OF HORMONAL ACTION

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ABSTRACT Previous studies have shown that a small but significant proportion of radioiodine from labeled L-thyroxine (T_4) and 3,5,3′-triiodo-L-thyronine (T_3) is incorporated into plasma and tissue proteins and is not, therefore, extractable with ethanol or other organic solvents. Other studies have shown that the complex consists, at least in part, of the iodothyronine in apparent covalent linkage with protein. In the present series of experiments the disappearance rate of nonextractable iodine (NEI) was determined in plasma, liver, and kidney after the injection of rats with a single dose of T_4 and T_3 labeled with radioiodine in the phenolic ring. The $t_{\frac{1}{2}}$ of NEI decay was substantially longer than the $t_{\frac{1}{2}}$ of the initial metabolic removal of T_4 (16 hr) and T_3 (4–6 hr). Thus, between days 3 and 11 the average $t_{\frac{1}{2}}$ of plasma NEI derived from T_4 was 2.2 days, from T_3, 1.9 days; kidney NEI from T_4, 7.4 days, from T_3, 6.1 days; hepatic NEI from T_4, 4.3 days, from T_3, 5.2 days.

The slow disappearance of liver NEI was of special interest in connection with an analysis of previously published data by Tata and associates dealing with the sequential tissue effects after the injection of a single dose of T_3 into thyroidectomized rats. The $t_{\frac{1}{2}}$ of decay of the various biological effects measured, primarily in the liver, appeared similar to each other, averaging between 4 and 6 days. These findings are compatible with the existence of a single long-lived intermediate governing the tissue expression of thyroid hormone. The $t_{\frac{1}{2}}$ of hepatic NEI in similarly prepared animals (thyroidectomized and injected with 25 μg of T_3) was found to be 4.5 days. The coincidence in the slow fractional disappearance rates of hepatic NEI and the dissipation of hormonal tissue effects raises the distinct possibility that T_3 interacts with specific cellular receptor sites to form covalent complexes which are slowly removed and serve both to initiate and to perpetuate hormonal action. A mathematical analysis of hormonal reaction mechanisms, based on the assumption of a linearly responsive system, a $t_{\frac{1}{2}}$ of T_3 of 4 hr, and a $t_{\frac{1}{2}}$ of 4.5 days for the postulated long-lived "messenger" suggests that maximal expression of hormonal activity cannot be attained before 20 hr after the injection of a hormone pulse. This value is broadly consonant with the observed data accumulated by Tata and associates. The existence of a long-lived messenger, possibly a species of NEI, would therefore explain not only the slow dissipation of hormonal effects but also the well-recognized "lag-time" in the expression of hormonal action.

Efforts were also made to define the relationship between extractable and nonextractable radioactivity in the plasma and tissue samples analyzed. The ratio of extractable radioactivity in plasma to extractable radioactivity in tissue became constant shortly after the injection of T_3 and T_4. The fractional disappearance of extractable radioactivity showed a progressive slowing over the course of the first 10 days after the injection. The distribution of extractable radioactivity between plasma and tissues was compatible with the known partition of exchangeable T_4 and T_3. In the case of T_3, di-

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rect chromatographic studies confirmed the presence of T_3 in plasma throughout the experiment and showed a progressive slowing of the fractional removal rate of this hormone. These data were interpreted to suggest that after the initial removal of exchangeable hormone, small amounts of plasma T_3 and T_4 are derived from the degradation of slowly turning over NEI. These considerations provide additional evidence that a significant proportion of NEI is composed of a complex between the injected iodothyronine and protein.

INTRODUCTION

After injection of L-thyroxine (T_4)[1] and 3,5,3′-triiodo-L-thyronine (T_3) labeled with iodine in the phenolic ring, a small but significant portion of radioactivity remains with plasma and tissue proteins after exhaustive extraction with ethanol or other organic solvents (1, 2). This moiety has been designated "nonextractable iodine" (NEI) and appears to consist, at least in part, of a covalent complex between iodothyronine molecules and protein (3, 4).

The mechanism by which NEI is formed and its biological significance remain obscure. It has been known for many years that in a variety of in vitro systems the deiodination of T_4 and T_3 is accompanied by the formation of iodoprotein (5–12). Recent in vitro studies with microsomes (13) and with plasma proteins (14) have indicated that complexes between iodothyronine and proteins can be formed under a variety of oxidizing conditions. These findings have prompted the speculation that similar mechanisms may underlie the formation of iodoprotein under in vivo conditions as well.

In the present series of studies we have analyzed in the rat the disappearance curve of liver, kidney, and plasma NEI formed after a pulse injection of T_3 and T_4 labeled with iodine in the phenolic ring. The fractional rate of disappearance of tissue NEI was found to be extremely slow in comparison with the initial metabolic disposal rate of exchangeable hormones. Specific attention was drawn to the relationship between nonextractable and extractable iodine in the later phases of these studies. Kinetic and chromatographic studies were performed which suggest that as NEI disappears from tissues and plasma, small quantities of labeled hormones derived from the iodoprotein re-enter the exchangeable hormone pools. Moreover, a striking correspondence was noted between the slow disappearance rate of liver NEI and the equally slow dissipation of a variety of hormonal effects in this tissue after pulse injection of hormone. On the basis of published data, theoretical formulations are advanced to suggest that hormonal action is mediated by a long-lived rate-limiting metabolic intermediate. The possibility is suggested that the postulated intermediate might consist of a covalent complex between the hormone and a specific cellular receptor site.

[1] *Abbreviations used in this paper:* NEI, nonextractable iodine; T_3, 3,5,3′-triiodo-L-thyronine; T_4, L-thyroxine.

METHODS

Male Sprague-Dawley rats (150–250 g) were obtained from Carworth Div., Becton Dickinson & Co., (New York) and were maintained on a Wayne Laboratory Diet and allowed water ad lib. Thyroidectomized animals which were used in the experiment illustrated in Fig. 9 were rendered completely hypothyroid after the administration of 100 μCi ^{131}I after 1 wk on a low iodine diet. Hypothyroidism became manifest 3–4 wk after ^{131}I administration by cessation of weight gain.

Both ^{131}I- and ^{125}I-labeled T_3 and T_4 were obtained from Abbott Laboratories (North Chicago, Ill.) (30–50 μCi/μg). Doses (5–60 μCi) mixed with rat serum were injected within 1 hr after preparation. In a number of experiments both ^{131}I- and ^{125}I-labeled compounds were injected simultaneously. 1 mg of NaI was administered subcutaneously daily to prevent thyroidal reutilization of radioiodine. At the indicated time periods after injection of the labeled hormones, groups of five animals were killed by exsanguination. The tissue and plasma concentrations of nonextractable iodine were determined in 1 g or 1-ml samples as previously described (3). Serial ethanolic extractions for removing iodothyronine were shown to be 95–98% efficient. For those time points in which the predominant proportion of radioactivity was in the extractable form, an iodothyronine with another radioactive label (^{131}I or ^{125}I) was added to serve as an internal extraction control. Concentrations were expressed as per cent of the injected dose per milliliter or gram, corrected to an ideal body weight of 200 g.

The disappearance of T_3 from plasma was determined by direct serial chromatographic analysis in two animals. Each animal was injected with 100 μCi of T_3-^{125}I. 10–200 μl of plasma obtained from the cut tail was directly applied to a paper chromatogram which was developed in a tert-amyl alcohol: 2 N ammonia: hexane solvent system (15). Authentic T_3-^{131}I was applied to the origin in addition to carrier T_3. After the T_3 area was identified by staining with diazotized sulfanilic acid it was excised and assayed for radioactivity. The proportion of total ^{125}I in the T_3 area was then calculated from the corresponding proportion of applied T_3-^{131}I in the T_3 area. Those counts of ^{131}I not in the T_3 zone represented material either adherent to the origin or scattered as T_3 or degradation products in the remainder of the chromatogram. At the time of sacrifice, a venous blood was also obtained and subjected to extraction and chromatography. Analyses of radioactivity were carried out in two-channel well counters (Packard Instruments, Downers Grove, Ill. and New England Nuclear, Boston, Mass.). Appropriate corrections were made for physical decay and "spill over" of ^{131}I into the ^{125}I counting register. The counting rates were carried out for sufficient length of time to achieve a statistical accuracy of at least 5%.

RESULTS

The disappearance of NEI from plasma and tissues. The fractional rate of disappearance of NEI from plasma and tissue was extremely slow in comparison to the initial fractional metabolic removal of exchangeable T_4 and T_3. In previous studies we have reported

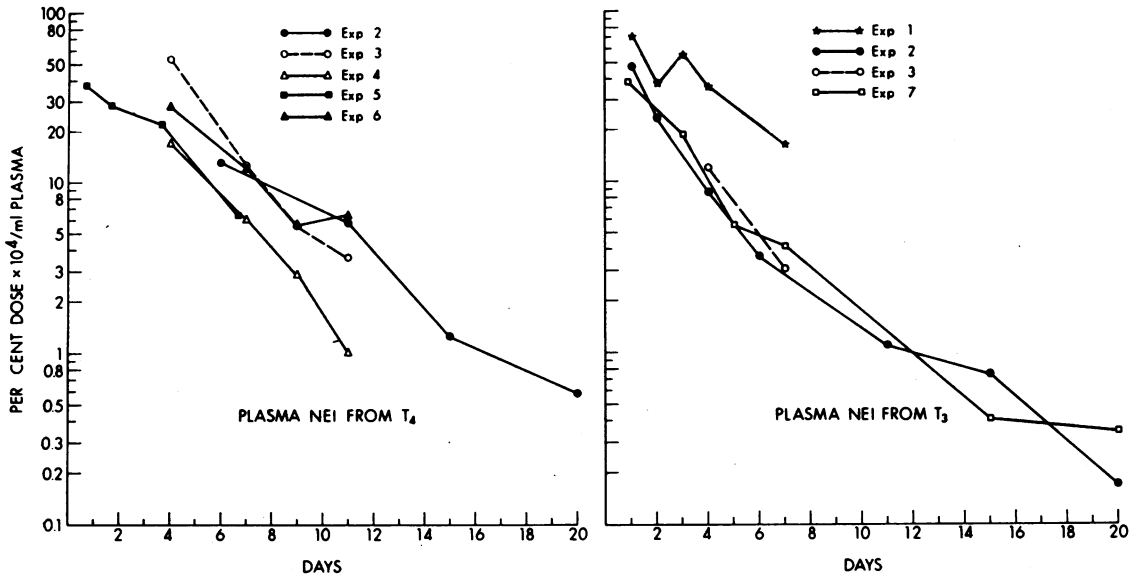

FIGURE 1 Disappearance of plasma NEI derived from a single dose of labeled T_4 (left) and T_3 (right) injected intravenously at $t = 0$. Each point represents the mean of determination in five animals. T_4-^{131}I was injected in experiments 2, 3, and 6; T_4-^{125}I, in experiments 4 and 5; T_3-^{125}I, in experiments 1, 2, 3, and 7.

that the $t_{\frac{1}{2}}$ of the irreversible disappearance of T_4 was 16 hr and for T_3 5–7 hr (16, 17). In contrast, the disappearance of plasma and tissue NEI was measured in terms of days. Results of individual experiments are illustrated in Figs. 1–3. In those experiments conducted for 20 days after the injection of labeled hormone there was a suggestion of a multiexponential curve with a gradually decreasing fractional removal rate of the NEI. The average half time of disappearance of NEI between days 3 and 11 are listed in Table I. These values were obtained from the regression equations calculated by least squares from the geometric means of individual experiments. As is apparent from the inspection of Figs. 1–3, the fractional removal rate of plasma NEI

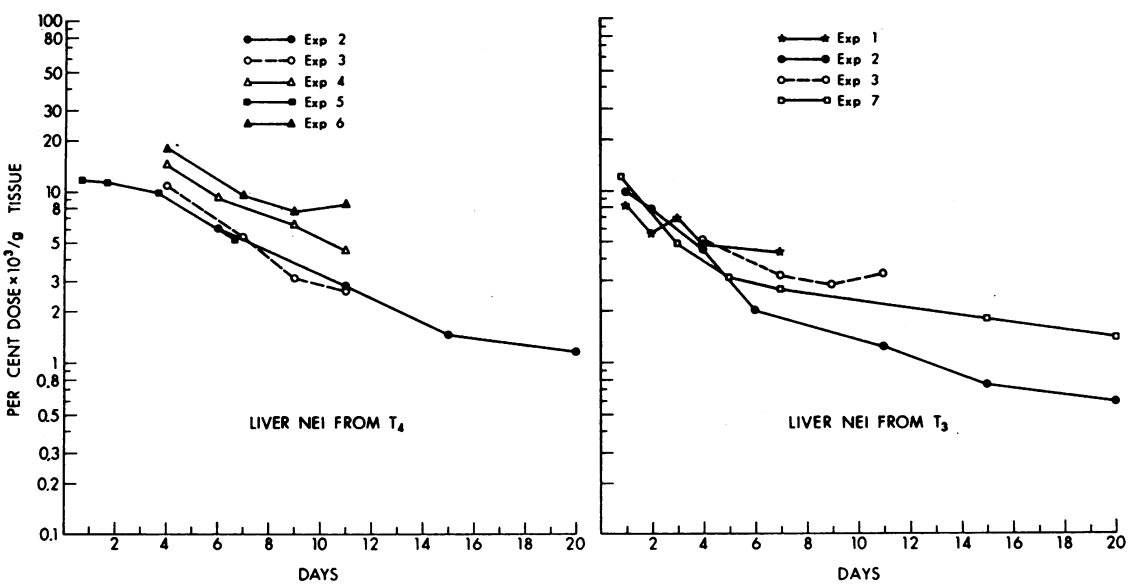

FIGURE 2 Disappearance of kidney NEI derived from T_4 (left) and from T_3 (right). Each point represents the mean of determinations in five animals. Experiments as in Fig. 1.

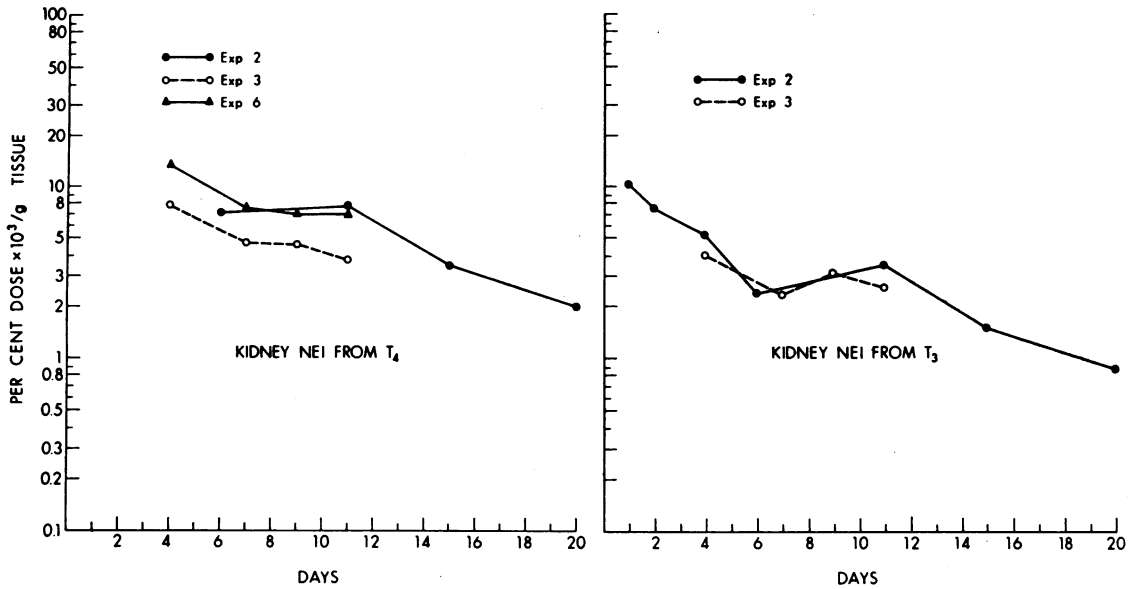

FIGURE 3 Disappearance of hepatic NEI derived from T_4 (left) and from T_3 (right). Each point represents the mean of determinations in five animals. Experiments as in Fig. 1.

($t_{\frac{1}{2}}$, 2.2 days for T_4, 1.9 days for T_3) was clearly more rapid than the disappearance of tissue NEI derived from both hormones ($t_{\frac{1}{2}}$, 4.3–7.4 days). The disappearance of tissue NEI from kidney appeared to be somewhat slower than that from liver, although the limited data in these experiments did not allow any firm conclusions. There was no gross difference in the fractional disappearance rates of NEI derived from T_3 and T_4. On the other hand, NEI derived from T_4 appeared to be present in somewhat higher concentration than NEI derived from T_3 when comparisons were made in the same tissue and at the same time after injection. This is reflected (Table I) in the concentration of NEI extrapolated to $t = 0$ from the regression equations based on the average values between days 3 and 11.

The disappearance rate of extractable radioactivity. The behavior of the extractable radioactivity during the period of observation is illustrated in the representative results of experiment 2 (Figs. 4–7). In this experiment T_3-^{125}I only was injected in animals sacrificed 1, 2, and 4 days after injection, but both T_3-^{125}I and T_4-^{131}I were injected in the animals sacrificed on days 6, 11, and 20. The extractable radioactivity was measured as the difference between the total radioactivity and NEI and was considered to include the injected iodothyronine, iodide, and other hormonal degradative products. It is apparent from inspection of Fig. 4 that the extractable radioactivity derived from T_3 in liver, kidney, and plasma appeared to be in secular equilibrium, i.e., the ratio of radioactivity in any one of these compartments to any other did not change with time. The relative stability of the values for the tissue: plasma concentration ratios of extractable radioactivity and the steadily increasing tissue: plasma concentration ratios of NEI are illustrated in Fig. 5. Thus, the liver: plasma ratio of extractable radioactivity varied in an apparently random fashion about a mean value of 2.5 throughout the 20 days of the experiment. Similarly, the mean kidney: plasma ratio showed no consistent directional shifts during this interval and averaged 3.3. In contrast, the tissue: plasma concentration ratio of nonextractable radioactivity increased progressively in the course of the experiment (liver: from 2.0 on day 1 to 32 on day 20; kidney, from 2.1 on day 1 to 49 on day 20).

Similar types of relationships were apparent in the case of extractable radioactivity derived from T_4 (Figs.

TABLE I
Least Square Analysis of Kinetic Data

	Liver	Kidney	Plasma
T_4 $t_{\frac{1}{2}}$, *days*	4.3	7.4	2.2
c_0, *% dose* × 10^3*/g or ml*	21.8	13.8	9.2
T_3 $t_{\frac{1}{2}}$, *days*	5.2	6.1	1.9
c_0, *% dose* × 10^3*/g or ml*	7.8	7.4	6.2

Least square analysis was performed on the geometric mean of all experimental points between days 3 and 11 after injection of the tracer (Figs. 1–3). The $t_{\frac{1}{2}}$ of the resulting curves and c_0, the values extrapolated to $t = 0$, are tabulated.

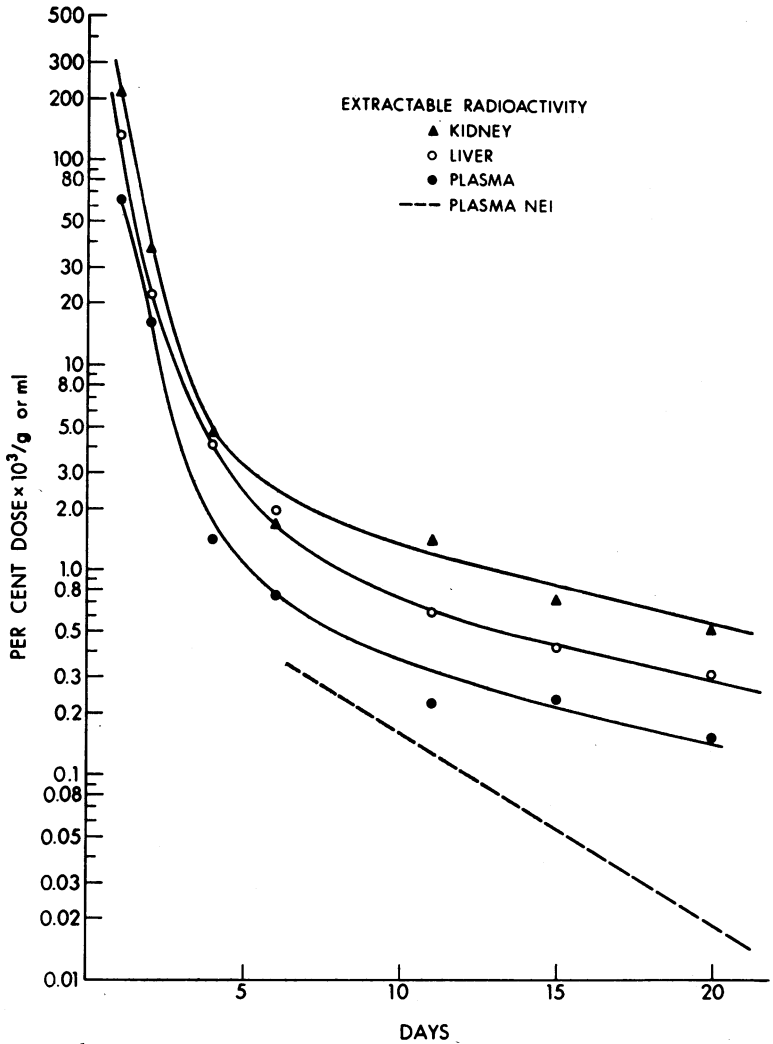

FIGURE 4 Concentration of extractable radioactivity (^{125}I) after injection of T_3-^{125}I in experiment 2 at t = 0. Note that concentrations of extractable radioactivity in kidney, liver, and plasma appear in secular equilibrium and show a progressive decrease in the fractional disappearance. Broken line indicates the disappearance of plasma NEI from T_3-^{125}I in the same experiment. Since the terminal slope of extractable radioactivity is less than the slope of plasma NEI, the plasma NEI cannot be the exclusive precursor of extractable activity.

6 and 7). As is illustrated in Fig. 7, the tissue: plasma concentration ratio of extractable radioactivity averaged 0.54 for liver and 0.65 for kidney. On the other hand, the tissue: plasma ratio of NEI rose progressively, from 4.7 on day 6 to 19.7 on day 20 in the case of liver, and from 5.4 to 34.1 in the case of kidney.

On the basis of previous experiments with rats, it has been established that when secular equilibrium is attained between T_3 and iodide after the injection of T_3 labeled in the outer ring with iodine, the ratio of T_3: iodide approximates 1:2. In the case of T_4, the slower fractional disappearance of T_4 compared to iodide establishes a T_4: iodide ratio of approximately 9:1. On the basis of these considerations we can estimate[2]

[2] It is known that iodide is essentially extracellular in its distribution. Thus, if the liver: plasma ratio of extractable radioactivity after injection of labeled T_3 is 2.5 as observed in the experiment illustrated in Fig. 5, and if one-third of the plasma radioactivity is in the form of T_3 (i.e., with a T_3: iodide ratio of 2:1), the calculated liver: plasma T_3 ratio would be 2.5/(1/3) = 7.5. Similarly, if the liver: plasma ratio of extractable radioactivity after injection of T_4 is 0.54, as in the experiment illustrated in Fig. 7, and if 0.9 of plasma radioactivity is in the form of T_4, the calculated liver: plasma ratio would be 0.54/0.9 = 0.60.

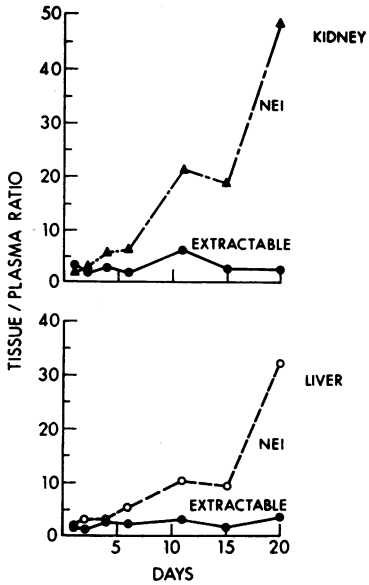

FIGURE 5 Concentration ratio of NEI and extractable radioactivity between tissue and plasma after injection of T_3-^{125}I in experiment 2. Note constancy of ratio of extractable radioactivity and progressive increase in the ratio of NEI.

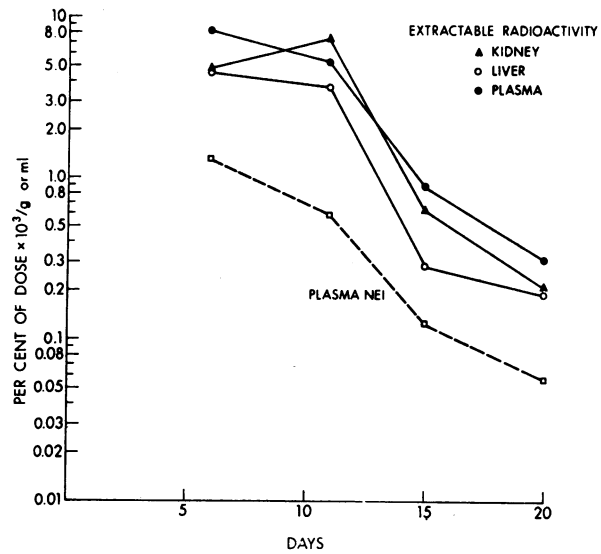

FIGURE 6 Concentration of extractable radioactivity in experiment 2 after injection of T_4-^{131}I. There also appears to be a secular equilibrium. Unlike the situation after injection of labeled T_3, however, the fractional disappearance of extractable radioactivity does not differ greatly from the decline in plasma NEI, as indicated by the broken line.

that the liver: plasma concentration ratio of extractable radioactivity, exclusive of iodide, should be 7.5 in the case of T_3 and 0.6 in the case of T_4. These calculated values are in good agreement with the previously established tissue: plasma concentration ratios for exchangeable T_3 and T_4 (17). These findings were compatible with the possibility that through the entire experiment T_3 and T_4 remain the principal noniodide constituents of the extractable moiety after injection of T_3 and T_4, respectively. This was somewhat surprising in view of the fact that at 20 days the $t_{\frac{1}{2}}$ of the disappearance rate of extractable radioactivity was 6.5 days for T_3 and 5.0 days for T_4, some 8–20 times greater than the $t_{\frac{1}{2}}$ fractional metabolic disposal of T_3 and T_4 in the early phases of the experiment.

An experiment was conducted in order to determine the fractional disappearance rate of chromatographically identified T_3 at various time periods after the injection of 100 μCi of T_3-^{125}I. Serial plasma samples were obtained from two animals and subjected to paper chromatography as detailed under Methods. The experiment was terminated after 6 days because of low counting rates. The results of this experiment are illustrated in Fig. 8. It can be seen that the curve describing T_3 is not, as one might anticipate, a single exponential decay function. At day 0.5 the $t_{\frac{1}{2}}$ is 4 hr. At 6 days the $t_{\frac{1}{2}}$ is 3.1 days, approximately the same as that of the extractable radioactivity determined at the same time (Fig. 4). The concentration of T_3-^{125}I in terminal plasma (vena cava) was determined by chro-

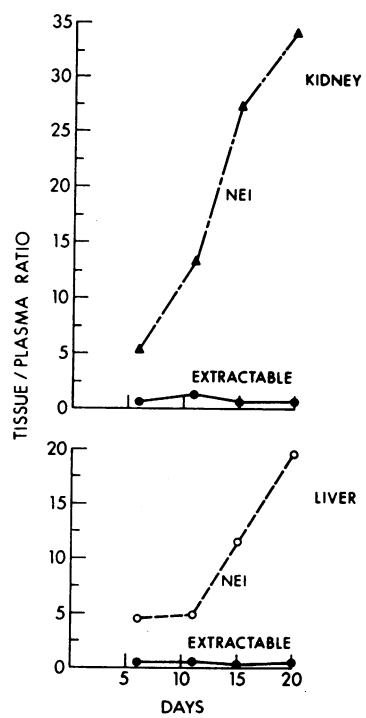

FIGURE 7 Concentration ratio of NEI and extractable radioactivity between tissues and plasma after injection of T_4-^{131}I in experiment 2. As with T_3, the ratio of extractable radioactivity remains constant, whereas the ratio of NEI progressively increases.

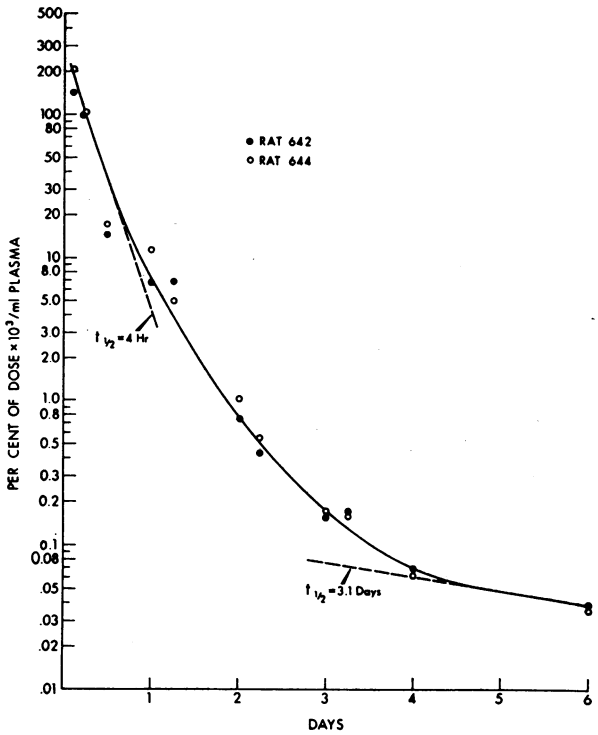

FIGURE 8 Concentration of T_3-^{125}I in plasma was determined by direct chromatography of plasma after injection of T_3-^{125}I into two rats. In confirmation of results with the crude extractable fraction (Fig. 4), there is a progressive slowing of the fractional disappearance rate. The initial and terminal $t_{\frac{1}{2}}$ values are presented only to provide a gross estimate of the magnitude of the change in the fractional removal rate of labeled T_3 from plasma.

matography with the ethanolic extracts through two paper cycles (18). A constant specific ratio of T_3-^{125}I to authentic added T_3-^{131}I was achieved. The calculated values were similar to those obtained from terminal tail-blood sample analyzed by a single chromatographic cycle.

These results support the concept that T_3 constitutes a significant proportion of the extractable radioactivity throughout the entire course of the experiment and indicate that T_3 disappears at a slow fractional rate from plasma during the later phases of the study. Since it has been established (3) that NEI consists, at least in part, of the iodothyronine injected, it seems probable that the slow disappearance rate of residual T_3 is due to the slow release of T_3 from the metabolism of NEI. Thus, the rate-limiting factor in determining the plasma disappearance of T_3 in the later phases appears to be not the metabolism of T_3 itself but the rate of delivery of T_3 from NEI. Inspection of Fig. 4 will reveal that the plasma NEI cannot be the sole precursor of the extractable plasma radioactivity since in the later phases of the study the fractional rate of disappearance of plasma NEI is considerably more rapid than the fractional disappearance rate of the extractable moiety. The probable source of the extractable radioactivity is tissue NEI, in view of the fact that it disappears at approximately the same slow fractional rate as the extractable radioactivity.

It is important, however, to exclude the possibility that the progressive slowing of the fractional removal of chromatographically identifiable T_3 simply reflects the recycling through an incompletely blocked thyroid of labeled iodide derived from the deiodination of the injected radioactive hormone. We have established in a group of four animals that the blocking dose of iodide used, 1 mg NaI daily, limits the accumulation of thyroidal radioactivity to less than 0.06% of the counts of T_3-^{125}I injected. Calculations based on the known fractional release of thyroidal iodine (20% per day), the probable percentage of T_3 in the thyroidal secretion (30%) and the known distribution and metabolism of T_3 in the rat (17) suggest that the concentration of T_3 derived from recycling would be at least one order of magnitude less than the lowest values of T_3 measured in the experiment illustrated in Fig. 8 (determined on day 6). To confirm these predictions we injected ^{131}I together with T_3-^{125}I in animals treated daily with NaI. Significant quantities of ^{131}I could not be demonstrated in the T_3 area of paper chromatograms of serum obtained 5 days after the injection. Recycling of iodide through an incompletely blocked thyroid therefore cannot account for our findings. The question can also be raised if the slowing of fractional removal is due to the conversion to T_3 of the 1–2% radioactive T_4 contaminating the injected T_3 dose. Again, quantitative considerations based on known conversion kinetics in the rat (18) indicate that such a mechanism could account for only a negligible proportion of the observed T_3 counts.

In the case of T_4, the plasma NEI and the extractable iodine disappearance curves are generally parallel and decline at a somewhat greater fractional rate than the tissue NEI. Thus, it is possible that during the period of this experiment the extractable radioactivity is in part derived from plasma NEI. Additional chromatographic studies, analogous to those performed for T_3, are required. Technical factors, however, complicate these experiments since tetraiodothyroacetic acid (tetrac), a known metabolic product of T_4 metabolism (3, 19), has a chromatographic mobility similar to that of T_4 itself. Moreover, the tissue: plasma concentration ratio of tetrac has not as yet been defined.

Decay of liver NEI after injection of T_3-^{125}I into thyroidectomized animals. The possibility was considered that the slow removal rate of NEI from tissue

might be related to the slow disappearance rate of a number of tissue effects of thyroid hormones. Since extensive data bearing on the appearance and disappearance of a variety of tissue effects after a single injection of 20–30 μg of T_3 into thyroidectomized rats have been published by Tata and associates (20–22), we determined the disappearance rate of liver NEI in a similar animal preparation. 25 μg of T_3 was injected along with 20 μCi T_3-^{125}I. The disappearance of liver NEI showed an average $t_{\frac{1}{2}}$ of 4.5 days, a value not significantly different from the $t_{\frac{1}{2}}$ of liver NEI in the intact animal. The theoretical relationship between the disappearance rate of NEI and the decay of the biological functions will be discussed in the following section.

DISCUSSION

Our studies clearly show that both tissue and plasma NEI formed from T_4 and T_3 are removed at a very slow fractional rate compared with the initial metabolic disposition of the exchangeable hormones. Moreover, the half time of disappearance of plasma NEI appears to be considerably faster than that of NEI formed in liver and kidney. These findings explain the earlier observations of Albert and Keating (23) who showed a progressive increase in the tissue: plasma concentration ratio of total radioactivity after the injection of T_4-^{131}I. Their findings appear to be due to the relatively greater accumulation of NEI in tissues. On the other hand, our studies indicate that the tissue: plasma concentration ratio of extractable radioactivity remains remarkably constant throughout the duration of the experiment.

We have previously pointed out that the formation of the nonextractable component in plasma is responsible for the progressive slowing of the fractional disappearance rate of total and trichloroacetic acid-precipitable radioactivity after the injection of T_3 (1). The present studies indicate that chromatographically identifiable T_3 also undergoes a multiexponential decay. Our data suggest that the curvilinear nature of the T_3-disappearance curve on semilogarithmic plot is due to the slow liberation of T_3 from tissue NEI, presumably as a result of metabolism of T_3-protein complex. This conclusion is entirely consistent with the previous demonstration in our laboratory that tissue and plasma NEI consists, at least in part, of the injected iodothyronine in probable covalent linkage with protein (3). Thus, an entirely different set of factors governs the plasma disappearance curve of T_3 in the early and in the late phase after injection. The rate-limiting step initially is the metabolism of T_3 itself; subsequently, it appears to be the release of T_3 from some tissue NEI.

In this study we have not presented any experimental data dealing specifically with the kinetics of formation of NEI. Precise measurements of the concentration of NEI shortly after the injection of labeled hormone are technically difficult because of the overwhelming preponderance of extractable hormone in plasma and tissues at this time. Preliminary data suggest that the peak concentration of NEI in plasma and tissues occurs within the first 2 days after injection of T_3. If one assumes that a constant fraction of exchangeable hormone is converted to NEI within plasma and tissues, one can calculate the precise time when maximal concentration of NEI would occur.[3] Thus, if one assumes that the $t_{\frac{1}{2}}$ of T_3 is 6 hr, the $t_{\frac{1}{2}}$ plasma NEI 2 days, and the $t_{\frac{1}{2}}$ of liver NEI is 4.5 days, maximal concentration of NEI would be achieved at 21 hr in plasma and at 26 hr in liver after the injection of the labeled hormone. If one assumes a shorter $t_{\frac{1}{2}}$ for T_3, 4 hr, as suggested in the present set of experiments, the maximal concentration of NEI in plasma would occur at 15.7 hr and in liver at 19.7 hr. The possible significance of these calculations will be discussed below.

The mechanism of formation of NEI remains a matter for speculation. Data in the present set of experiments, however, do not support the concept that tissue NEI is a precursor of plasma NEI since the $t_{\frac{1}{2}}$ of plasma NEI is considerably shorter than that of tissue NEI. On the other hand, it must be recognized that previous studies have shown that NEI is heterogeneous both with respect to subcellular localization as well as its composition. The possibility that a small proportion of tissue NEI is exported into the plasma, therefore, cannot be rigorously excluded. Nevertheless, recent studies in our laboratory (14) have shown that T_3 incubated with diluted plasma can form complexes with plasma proteins which cannot be dissociated with a variety of organic solvents, 6 M guanidine, 8 M urea, and sodium dodecyl sulfate. These findings have suggested covalent-bond formation between the iodothyronine and the proteins under in vitro conditions. Similar complexes were formed after incubation of T_4 and T_3 with hepatic microsomes (13). It seems reasonable, therefore, to suggest that the possibility that under in vivo conditions NEI is also formed locally within tissue or plasma, analogous to these in vitro reactions.[4]

[3] The time t_m when the concentration of NEI is maximal can be calculated from the following equation:

$$t_m = \frac{\left[\frac{(\lambda)_{T_3}}{(\lambda)_{NEI}}\right]}{\lambda_{T_3} - \lambda_{NEI}},$$

where the subscripts T_3 and NEI designate the fractional removal rate of T_3 and NEI respectively [$=0.693/t_{\frac{1}{2}}$.] This equation is analogous to equation 25 in the Appendix and can be derived by similar considerations to those used in its derivation.

[4] The concept that liver and plasma NEI are formed independently, however, does not afford an easy explanation

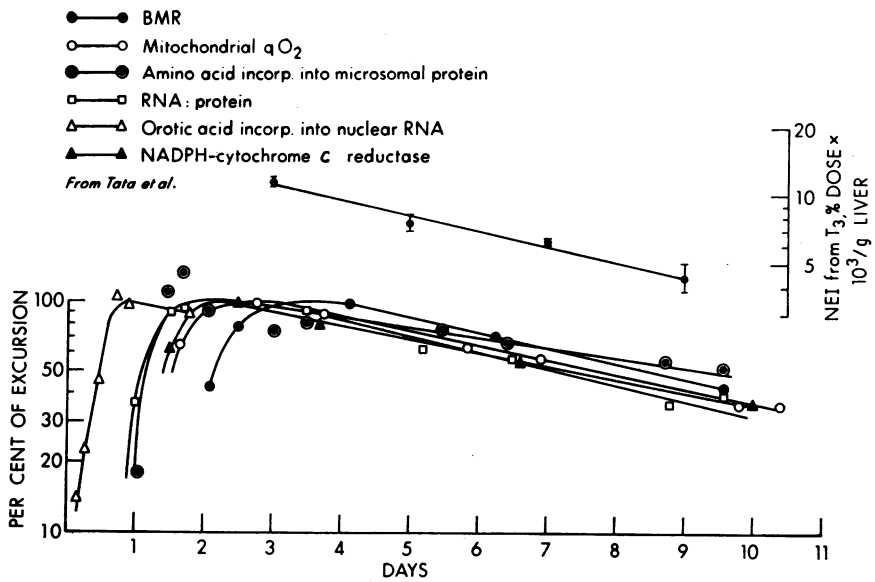

FIGURE 9 Sequential tissue response after the injection of single dose of 20–30 μg T_3 into thyroidectomized rats, replotted from the published data of Tata and associates (20–22). Results of individual determinations have been normalized by plotting the per cent of maximal increase (excursion) as a function of time. Thus, if the maximal oxygen consumption of mitochondria attained after injection is designated E_m, the base line oxygen consumption, E_b, and the oxygen consumption at any time t, E_t, then the percentage increase can be calculated from the expression

$$100\left(\frac{E_t - E_b}{E_m - E_b}\right)$$

The approximate $t_{\frac{1}{2}}$ (days) and time of maximal activity (hr) of individual parameters of hepatic tissue function are as follows: Orotic acid incorporation into rapidly labeled nuclear RNA, 5.2, 19 (20); microsomal RNA: protein ratio, 4.3,48 (21, 22); amino acid incorporation into microsomal protein, 6.6,32 (21); activity of NADPH-cytochrome *c* reductase, 5.2,60 (21); mitochrondrial oxygen consumption, 4.5,66 (21); total body oxygen consumption, 4.2,96 (21). The similarity in the final exponential decay of individual tissue functions raises the possibility that a slowly turning over "messenger" governs the expression of hormonal effect at the tissue level. The existence of such an intermediate would also explain the necessity for the lag-time required for the earliest expression of peak hormonal effect (see text and Appendix for details).

The disappearance of hepatic NEI in an animal preparation similar to that used by Tata and associates (thyroidectomized, injected with 25 μg T_3) is also indicated. Each point represents the mean of results in five animals and the bars indicate ±SEM. The $t_{\frac{1}{2}}$ disappearance is 4.5 days. The similarity between the $t_{\frac{1}{2}}$ of NEI disappearance prompts the speculation that a covalent T_3-protein complex could serve as the postulated rate-determining intermediate.

An equally speculative area is the possible biological role of NEI. In this connection, it is interesting to note that the slow fractional decay rates of various hormonal tissue effects in the rat liver correspond to the slow fractional decay rate of NEI observed in these experiments. This relationship is illustrated in Fig. 9 in which we have plotted sequentially determined biochemical effects in the liver and the basal metabolic rate of whole animal as determined by Tata and associates after the injection of single 20–30 μg dose of T_3 into thyroidectomized rats. These reactions include orotic acid incorporation into rapidly labeled nuclear RNA (20), the rate of microsomal protein synthesis (21, 22), the RNA/protein ratio (21, 22), the concentration of NADPH-cytochrome *c*-reductase (21), oxygen consumption of mitochondria (21), and the basal metabolic rate of the whole animal (21). Within the limits of the experimental and biological variability in these determinations, the observed decay rates are strikingly similar to each other ($T_{\frac{1}{2}}$, 4.3–6.6 days).[5] This finding is compatible with the existence of a single rate-limiting reaction.

of our previous finding that both liver and plasma NEI are increased in phenobarbital-treated animals (2).

[5] Two exceptions to the concept of a single rate-limiting intermediate can be found in the data of Tata and associates. The apparent half time of disappearance of liver glycogen

The general problem of the relationship of a series of sequential reaction terminating in hormonal expression is more rigorously developed in the Appendix. In this analysis, the assumption is made that we are dealing with a linearly responsive system. It is readily apparent from the equations derived that if any intermediate is formed which possesses a half-life greater than those of the subsequent intermediates, then such a long-lived intermediate will be rate-determining for subsequent reactions. Since all of the observed biochemical reactions after injection of T_3 appear to decay with the $t_{\frac{1}{2}}$ approximately 4.5 days, it appears reasonable to postulate the existence of an intermediate with the similar half-life. Moreover, the earliest possible intermediate represents a direct reaction product between T_3 and the tissue receptor sites (i.e., $p_1 = 1$). If the initial $t_{\frac{1}{2}}$ of T_3 is 4 hr (Fig. 8) and the $t_{\frac{1}{2}}$ of the intermediate is 4.5 days, the maximal concentration of the intermediate can be calculated to occur at 20 hr (equation 25). Thus, if the model is correct, no hormonal effect can be *maximal* earlier than 20 hr after the injection of hormone. At the same time, it is clear that the maximal effect can occur subsequent to 20 hr after the injection of a single bolus of hormone. If biologic effects of T_3 can be shown with a maximum before 20 hr, such a finding would necessitate a revision of the basic hypothesis. It should be emphasized that these criteria are stated for the time of maximal effect and not the "earliest observable effects" since the latter index is clearly dependent on the precision of measurment and probably on the dose of hormone injected.

In connection with these considerations inspection of Fig. 9 will reveal that the earliest peak effect is attained by orotic acid incorporation into rapidly labeled nuclear RNA. The maximal value is attained at 19 hr, The discrepancy between this value and the predicted earliest time of 20 hr probably can be attributed to the inherent variability in the biologic measurements. All other biologic effects measured peak well after 20 hr. These findings are thus broadly compatible with the theoretical formulation suggesting the existence of a long-lived hormonal messenger. Moreover, since the peak of orotic acid incorporation occurs very close to the predicted earliest time of peak hormonal action, it follows that this process must be one of the earliest manifestations of T_3 activity in the liver, a conclusion which supports the earlier arguments advanced by Tata and Widnell (20). Under any circumstances, the postulated long-lived messenger would readily explain the well-recognized "lag-time" in the onset of thyroid hormone action.

It should be emphasized that the considerations formalized in the Appendix are applicable to any long-lived messenger, whether or not such a messenger is a species of NEI. Nevertheless, the similarity between the $t_{\frac{1}{2}}$ in the decay of biologic functions and the $t_{\frac{1}{2}}$ in the decay of hepatic NEI leads to the speculation that a T_3 receptor complex with the $t_{\frac{1}{2}}$ similar to that observed for total NEI is the postulated long-lived messenger in the liver. Soffer (25) has also suggested that the action of the thyroid hormone involves incorporation of iodothyronine into protein through a specific enzyme mechanism.

Reservations regarding the postulated role of NEI, however, must be considered. The similarity in the $t_{\frac{1}{2}}$ of hormonal response within the liver and the decline of NEI cannot be considered to constitute proof of a precursor-product relationship. This correlation may be entirely fortuitous. Moreover, the objection could be raised that the NEI formation does not have the requisite degree of specificity required for a hormonal messenger. Thus, NEI is formed in plasma as well as tissues, and presumably the more rapidly disappearing plasma NEI would lack functional significance. Also, we have previously shown that phenobarbital, which accelerates the hepatic metabolism of thyroid hormones (16), leads to an increase in liver NEI associated with the microsomal fraction (2). Since phenobarbital administration leads to a diminution rather than to an increase in hormonal action (17), it is apparent that NEI associated with metabolizing smooth endoplasmic reticulum cannot subserve the postulated role of the hormonal intermediate messenger. Lastly, L-T_4 and D-T_4 (26, 27) show an equally strong capacity to form NEI complexes. Since recent studies have suggested that the biologic activity of L-T_4 is largely due to its transformation to T_3 (18), we would not expect significant biologic activity from NEI composed of L-T_4, and certainly not from NEI derived from the more inert dextroisomer.

These considerations make it extremely unlikely that all of the tissue NEI is involved in hormonal action. In point of fact, our most recent in vitro findings would suggest that the capacity of iodothyronines to interact with proteins may be a general chemical property of this class of compounds which has hitherto attracted little attention. Nevertheless, the ability of T_3, the presumed active hormone, to form covalent linkages with specific intracellular receptor sites would be precisely what is needed to endow the hormone with the prolonged action which it exhibits after the injection of a single dose. Our hypothesis rests on the assumption that the postulated specific NEI complexes between T_3 and the recep-

was found to be approximately 20 hr (21). Glycogen depletion, however, is not believed to be a specific acute effect of thyroid hormone action but a consequence of alteration in the feeding pattern of the animals (24). A second exception, the apparently rapid fall in the Mg^{++}-activated RNA polymerase (20), is clearly a function of a single low terminal value. Additional experiments are necessary in order to determine with requisite precision the $t_{\frac{1}{2}}$ of this nuclear enzyme.

tor sites have the same over-all decay characteristics as total liver NEI. Clearly, additional studies are required to characterize the specific intracellular receptor sites and to define the decay pattern of the postulated T_3 receptor complexes. Apart from these considerations, however, the concept of a long-lived hormonal messenger deserves independent consideration since it serves to explain both the delay in onset and the slow decline of thyroid hormone activity.

The correlation between the decline in tissue NEI and hormonal function after pulse injection of hormone has been restricted largely to the liver. Only fragmentary quantitative data are available for other organs and tissues. A possible exception to the temporal dissociation between plasma hormone concentration and tissue effect is the pituitary which on the basis of limited published data appears to be rapidly responsive to changes in the plasma hormone (28). It is possible that a mechanism other than NEI formation is involved in shutting off thyroid-stimulating hormone (TSH) secretion by pituitary. On the other hand, a very rapid fractional turnover of NEI in the pituitary would still allow rapid responsivity to changing plasma hormone levels.

APPENDIX

General Kinetic Analysis of Hormonal Action

Hormonal action can be regarded as a chain of biochemical events starting with a given interaction for a specific cellular receptor and the hormone under consideration. For the sake of simplicity, let us assume a linear system in which we designate $p_0 \cdots p_i \cdots p_n$ the tissue concentration of individual rate-determining reactants; $r_0 \cdots r_i \cdots r_{n-1}$ individual receptor sites; $k_0 \cdots k_1 \cdots k_n$ the proportionality constants between the rate of a given reaction and the concentration of the reactant; $\lambda_0 \cdots \lambda_i \cdots \lambda_n$, the net fractional removal from the system of the reactants. It is apparent that p_0 is the tissue concentration of hormone. The following reactions can be written:

$$p_0 + r_0 \rightarrow p_1 \quad (1)$$

$$p_1 + r_1 \rightarrow p_2 \quad (2)$$

$$\vdots$$

$$p_{i-1} + r_{i-1} \rightarrow p_i \quad (3)$$

$$\vdots$$

$$p_{n-1} + r_{n-1} \rightarrow p_n \quad (4)$$

We can also write the following differential equations following injection of a hormone pulse:

$$\dot{p}_0 = -\lambda_0 p_0 \quad (7)$$

$$\dot{p}_1 = k_1 p_0 - \lambda_1 p_1 \quad (8)$$

$$\dot{p}_2 = k_2 p_1 - \lambda_2 p_2 \quad (9)$$

$$\vdots$$

$$\dot{p}_i = k_i p_{i-1} - \lambda_i p_i \quad (10)$$

$$\vdots$$

$$\dot{p}_n = k_n p_{n-1} - \lambda_n p_n \quad (11)$$

Equations 7–11 can be solved by standard techniques to yield the following general solutions:

$$p_0 = A_{0,0} e^{-\lambda_0 t} \quad (12)$$

$$p_1 = A_{1,0} e^{-\lambda_0 t} + A_{1,1} e^{-\lambda_1 t} \quad (13)$$

$$p_2 = A_{2,0} e^{-\lambda_0 t} + A_{2,1} e^{-\lambda_1 t} + A_{2,2} e^{-\lambda_2 t} \quad (14)$$

$$\vdots$$

$$p_i = A_{i,0} e^{-\lambda_0 t} + A_{i,1} e^{-\lambda_1 t} + \cdots A_{i,i} e^{-\lambda_i t} \quad (15)$$

$$\vdots$$

$$p_n = A_{n,0} e^{-\lambda_0 t} + A_{n,1} e^{-\lambda_1 t} + \cdots A_{n,i} e^{-\lambda_i t} + \cdots A_{n,n} e^{-\lambda_n t} \quad (16)$$

and clearly,

$$\dot{p}_0 = -\lambda_0 A_{0,0} e^{-\lambda_0 t} \quad (17)$$

$$\dot{p}_1 = -\lambda_0 A_{1,0} e^{-\lambda_0 t} - \lambda_1 A_{1,1} e^{-\lambda_1 t} \quad (18)$$

$$\vdots$$

$$\dot{p}_i = -\lambda_0 A_{i,0} e^{-\lambda_0 t} - \lambda_1 A_{i,1} e^{-\lambda_1 t} \cdots - \lambda_i A_{i,i} e^{-\lambda_i t} \quad (19)$$

$$\vdots$$

$$\dot{p}_n = -\lambda_0 A_{n,0} e^{-\lambda_0 t} - \lambda_1 A_{n,1} e^{-\lambda_1 t} - \cdots - \lambda_i A_{n,i} e^{-\lambda_i t} \cdots - \lambda_n A_{n,n} e^{-\lambda_n t} \quad (20)$$

The coefficient A_{ji} is a constant which may assume either a positive or negative value. In a pulse injection of a hormone into a hypothyroid animal it is clear that if at $t = 0$, $p_0 = 1$, then $p_1 = 0$, and $p_n = 0$.

Inspection of equations 16 and 20 will reveal that the terminal slope of p_n and $\dot{p}_n$ will be determined by the smallest value of λ in the series. Designate this value λ_f. It is clear that the terminal slopes of p_f through p_n and $\dot{p}_f$ through $\dot{p}_n$ will have the same terminal slope. This will be compatible with the postulate that the formation of p_f is the rate-limiting step. If $f = 1$, then all sequential hormonal products and rates of reactions will have a terminal slope of λ_f, and $p_1 = p_f$ can be considered a "hormonal messenger." If this is the case, then the earliest theoretical time for maximal hormonal effect after a pulse injection, t_m, can be calculated since this will be the time when p_1 is maximal. If we assume that at $t = 0$, $p_0 = 1$ (i.e. $A_{0,0} = 1$) then from equation 12, $p_0 = e^{-\lambda_0 t}$.

Substituting into equation 8

$$\dot{p}_1 = k_1 e^{-\lambda_0 t} - \lambda_1 p_1 \quad (22)$$

Solving equation 22 we obtain

$$p_1 = \frac{k_1}{\lambda_1 - \lambda_0} (e^{-\lambda_0 t} - e^{-\lambda_1 t}) \quad (23)$$

and

$$\dot{p}_1 = -\frac{\lambda_0 k_1}{\lambda_1 - \lambda_2} e^{-\lambda_0 t} + \frac{\lambda_1 k_1}{\lambda_1 - \lambda_0} e^{-\lambda_1 t} \quad (24)$$

and when $\dot{p}_1 = 0$

$$t_m = \frac{\ln \frac{\lambda_0}{\lambda_1}}{\lambda_0 - \lambda_1} \quad (25)$$

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