Nonlinear (Amplified) Relationship between Nuclear Occupancy by Triiodothyronine and the Appearance Rate of Hepatic α-Glycerophosphate Dehydrogenase and Malic Enzyme in the Rat

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ABSTRACT Three separate approaches were applied to examine the general relationship between R, the rate of induction of specific enzymes (mitochondrial α-glycerophosphate dehydrogenase and cytosolic malic enzyme) and q, the fractional nuclear occupancy by triiodothyronine in male Sprague-Dawley rats. Daily 200-μg injections of triiodothyronine per 100 g body wt for 7 days resulted in saturation of the hepatic nuclear sites and the achievement of an apparent new steady state of enzyme levels. The increase achieved over base-line hypothyroid levels was then compared with the increment over hypothyroid base line characteristic of intact euthyroid animals with 47% of nuclear sites occupied. The maximal theoretical rate of steady-state enzyme induction could be projected on the basis of the observed maximal increase in enzyme activity observed 1 day after the injection of graded doses of hormone and λ, the known fractional rate of enzyme dissipation. The 24-h dose-response studies were used to generate R as a continuous function of q, both in hypothyroid as well as in euthyroid animals. This approach involved the numerical solution of an ordinary differential equation describing the rate of change of enzyme as a function of R, which was assumed to be uniquely related to q. Results of these analyses indicated that the ratio of the maximal rate of induction of enzyme at full occupancy to the rate of induction under euthyroid conditions assumes a value between 9.0 and 19.5, depending on the precise analytic and experimental approach applied. This value is far in excess of the theoretical ratio 2.13 which one would anticipate if R were linearly related to q and 47% of the nuclear sites occupied under physiological conditions. Thus, the signal for enzyme induction appears to undergo progressive amplification with increasing nuclear occupancy. Moreover, the curve describing the relationship between R and q appears highly nonlinear throughout (concave upwards). Although the molecular mechanism responsible for amplification is unknown, recognition of this phenomenon may be helpful in understanding tissue effects of thyroid hormone excess. Moreover, the analytic technique for determining R as a function of q may be of general applicability in studying hormonal response systems under nonsteady-state conditions.

INTRODUCTION

The relationship between receptor occupancy and biological response is important both in developing an understanding of the initiating mechanism and in developing a comprehensive model which will facilitate the prediction of tissue response as a function of time for any given level of plasma hormone. We have previously reviewed the evidence which supports the concept that nuclear binding sites are the points of initiation of thyroid hormone action (1). More recent supporting data have been provided by the laboratories of Samuels (2, 3), DeGroot (4), Feigelson (5), Refetoff (6), and Baxter (7). A number of investigators have proposed, primarily on the basis of in vitro data, that thyroid

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hormones may also exert some action through nonnuclear mechanisms (8–10).

We have recently presented the results of studies in which the induction of two hepatic enzymes in response to the administration of triiodothyronine (T_3)[1] was measured (11). The activity of mitochondrial α-glycerophosphate dehydrogenase (α-GPD) and cytosolic malic enzyme (ME) were evaluated primarily under circumstances in which the nuclear receptor sites were saturated. Our studies also confirmed previous reports indicating that the enhanced ME activity induced by thyroid hormone reflects increased enzyme mass rather than enzyme activation. The increase in the rates of new enzyme appearance appeared to be maximal as long as the nuclear sites remained effectively saturated. The level of enzyme appeared to be uninfluenced by the fractional rate of removal since this appeared to be remarkably independent of the thyroidal status of the animal. Necessary for such an analysis was the assumption that the number of nuclear binding sites did not change with the thyroidal status of the animal. Such an assumption appeared justified on the basis of nuclear binding analyses carried out both under in vivo (12) and under in vitro (13, 14) conditions. The relative stability in the effective number of sites measured in the liver of animals injected in vivo with T_3 or rendered hyperthyroid contrasts with the depletion in pituitary nuclear receptor sites observed by Samuels et al. (3) in tissue culture after the in vitro addition of T_3. This discrepancy may simply be a reflection of the difference in the systems studied. In our previous report (11), we did not consider the relationship between nuclear occupancy and response when the presumed receptor sites were less than fully saturated. To examine this problem, we performed additional experiments in which nuclear sites were saturated by the daily injection of large doses of T_3 for a period of 7 days and levels of hepatic mitochondrial α-GPD and ME were determined as a function of the duration of saturation. These results suggested that the relationship between nuclear occupancy and response is highly nonlinear. We, therefore, analyzed our previously published dose-response data obtained under nonsteady-state conditions (11) in an effort to calculate in greater detail the relationship between nuclear occupancy and the responsivity of the enzyme induction system. We believe that the analytic approach developed may represent a novel method for assessing endocrine data obtained under nonsteady-state conditions.

The results of our studies were interpreted to indicate that with progressive nuclear occupancy there is a major amplification of the signal for the appearance of new enzyme. Such amplification may govern the expression of a number of thyroid hormone effects at the tissue level and may be important in interpreting the clinical symptomatology of hyperthyroidism. Moreover, the model of thyroid hormone action on which our calculations were based appears to have at least limited value in predicting tissue response to thyroid hormone administration.

METHODS

Male Sprague-Dawley rats (150–225 g) were obtained from Charles River Breeding Laboratories, Wilmington, Mass. Tap water and food (Wayne Lab-Blox, Allied Mills, Inc., Chicago, Ill.) containing 1 μg iodine/g were freely available. Surgically thyroidectomized rats were obtained from the breeder at a weight of 100–125 g. Upon arrival, each rat was placed on a low iodine diet (<0.05 μg iodine/g) for 7 days and then injected intraperitoneally with 100 μCi of ^{131}I-Na (Mallinckrodt, Inc., St. Louis, Mo.). The animals were weighed bi-weekly and used experimentally when their weights had stabilized, generally 4–5 wk after radioiodine administration. The weight of the rats at that time ranged between 180 and 210 g. Mitochondrial α-GPD activity was measured by the method of Lee and Lardy (15) with the synthetic electron acceptors, phenozine methosulfate and *p*-iodonitrotetrazolium violet. Hepatic ME activity was measured by the method of Ochoa (16) as modified by Hsu and Lardy (17).

To relate the plasma concentration to the dose of T_3 administered, 1, 50, and 1,000 μg T_3/100 g body wt were injected into groups of hypothyroid and euthyroid animals. Animals were killed at the intervals indicated in Fig. 1. Plasma T_3 concentrations were determined by the method of Surks et al. (18) and expressed as percent of the injected dose per milliliter of plasma. Although with increasing doses of T_3, the percent of the dose per milliliter appeared to fall, the shift was not large. In calculating the plasma concentration at any time after the injection of a given dose, the percent of the dose per milliliter for the intermediate 50-μg dose was used both for euthyroid and hypothyroid animals.

To saturate the nuclear sites for a 7-day period animals were given a daily intraperitoneal dose of 200 μg of T_3/100 g body wt. Control experiments demonstrated relatively rapid absorption of administered T_3. Calculations based on the plasma T_3 concentration indicated that >95% of the nuclear sites were occupied for the 24 h after injection. Animals were killed at 1, 3, 5, and 7 days after the injection. A group of five euthyroid and five hypothyroid animals were killed at the same time to assess the increment in enzyme activity above euthyroid and hypothyroid base-line levels.

COMPUTATIONS

A method was developed to analyze the results of previously published dose-response studies in which the level of α-GPD and ME activity were determined 24 h after the intravenous injection of increasing weight adjusted doses of T_3. For this purpose, the primary dose-response data were just fitted by applying the equations proposed by Rodbard (19) (Fig. 2).

On the basis of our previous studies (11) we now propose that the rate of increase of enzyme activity with respect to time can be represented by the following ordinary dif-

[1] *Abbreviations used in this paper*: α-GPD, α-glycerophosphate dehydrogenase; ME, malic enzyme; *q*, the fractional nuclear occupancy by triiodothyronine; *R*, the rate of induction, of specific enzymes; T_3, triiodothyronine.

ferential equation:

$$\frac{dE}{dt} = R(q) - \lambda E, \quad (1)$$

where E is the instantaneous enzyme activity per milligram protein; R, the rate of instantaneous new enzyme induction which is assumed to be a unique function of q, the fraction of nuclear sites occupied at any time t, and λ, the fractional rate of disappearance of enzyme which averages 0.25/day and appears to be independent of the thyroidal status of the animal (11).

Eq. 1 can be solved by multiplying both sides of Eq. 1 by the factor $e^{\lambda t}$, intergrating both sides of the equation, and dividing by $e^{\lambda t}$. Under boundary conditions such that when $t = 0$, $E = 0$, the following equation results:

$$E(q) = \frac{R(q)}{\lambda}(1 - e^{-\lambda t}) \quad (2)$$

It is now possible to determine the function $R(q)$ from available data. First, consider the hypothyroid animal without endogenous T_3. Divide the interval of q from $q = 0$ (unoccupied) to $q = 1$ (fully occupied) into 10 equal segments designated $q(0.1)$, $q(0.2)$, $q(0.3)$, . . . $q(0.9)$, $q(1.0)$. For a very large dose of T_3 the sites will be completely occupied for the duration of the experiment; i.e., q will assume the value of $q(1.0)$ throughout. During this time, R can also be presumed to assume a maximal value of $R(1.0)$. Furthermore, let us designate the terminal enzyme activity for a given dose D by the terminal nuclear occupancy q. Thus, designate as $E(1)$ the response to a large dose which saturates the sites throughout the preceding 24 h and will result in a terminal nuclear occupancy $q(1)$. The function t is the time from the onset of induction. Because of the lag time τ between injection and the onset of the effect, $t = t' - \tau$, where t' is the actual time from the injection to the point of observation. The lag time τ has previously been shown to be equal to 9.0 h for α-GPD in hypothyroid animals, 13.4 h for α-GPD in euthyroid animals, and 8.2 h for ME in euthyroid animals (11).

From the above considerations, it follows that for a large dose:

$$E(1) = \frac{R(1)}{\lambda}(1 - e^{-\lambda t}). \quad (3)$$

For α-GPD response in hypothyroid animals, t is 15.0 h at the time of observation ($t' = 24$ h). Eq. 3 can now be rearranged to solve for $R(1)$.

Now, consider that dose of T_3 which is associated with a terminal nuclear occupancy of $q = 0.9$ designated $q(0.9)$. Since the value of q at $t = 0$ is nearly equal to 1.0 and since there is a monotonic decrease in q over the interval under consideration, it follows that there must be some average value R for that interval. Designate this value $R(0.9)$. As a first approximation, therefore:

$$E(0.9) = \frac{R(0.9)}{\lambda}(1 - e^{-\lambda t}). \quad (4)$$

Again, $E(0.9)$ represents the enzyme activity when the terminal $q = 0.9$.

As previously shown (11), the value of q can be estimated from the plasma concentration of T_3, p, according to the expression:

$$q = \frac{p}{p + 0.671} \quad (5)$$

Conversely, it is possible to determine the value of p for $q = 0.9$. From the data in Fig. 1A and Eq. 5, it is possible to estimate $D(0.9)$, the dose which will yield a terminal value of $q(0.9)$ and from the empirical relationships illustrated in Fig. 2C, the value $E(0.9)$, the induced enzyme level corresponding to a terminal q of (0.9).

Next, consider the situation in which the terminal $q = 0.8$. The interval from $t = 0$ to t when $q = 0.8$, $t(0.8)$ can be subdivided into two periods, $\Delta t(1.0,0.9)$ and $\Delta t(0.9,0.8)$. These intervals represent respectively the time during which q assumes values from 1.0 to 0.9 and from 0.9 to 0.8. It is apparent that:

$$E(0.8) = \frac{R(0.9)}{\lambda}[1 - e^{-\Delta t(1.0,0.9)}]e^{-\lambda[t(0.8)-t(0.9)]} + \frac{R(0.8)}{\lambda}[1 - e^{-\lambda t(0.9,0.8)}]. \quad (6)$$

Since $q(t)$ can be evaluated for $D(0.8)$ both intervals can be defined and Eq. 6 solved for $R(0.8)$.

By extension, we can deal in a similar fashion for q terminating in a value 0.7. Thus:

$$E(0.7) = \frac{R(0.9)}{\lambda}[1 - e^{-\lambda\Delta t(1.0,0.9)}]e^{-\lambda[t(0.7)-t(0.9)]} + \frac{R(0.8)}{\lambda}[1 - e^{-\lambda\Delta t(0.9,0.8)}]e^{-\lambda[t(0.7)-t(0.8)]} + \frac{R(0.7)}{\lambda}[1 - e^{-\lambda\Delta t(0.8,0.7)}]. \quad (7)$$

Again, Eq. 7 can be solved for $R(0.7)$. This process can be generalized to yield the expression:

$$E(j + z) = \sum_{q=j}^{q=j+z} \frac{R(q)}{\lambda}[1 - e^{-\lambda[t(q)-t(q-1)]}]e^{-\lambda[t(j+z)-t(q)]}. \quad (8)$$

where at $t = 0$, $q = j$ and the terminal value of $q = q(j + z)$, there being z equal intervals of q designated j, $j + 1$, $j + 2$, ... $j + i$ . . . . $j + z$. It is furthermore assumed in this analysis that $q(j)$ is maximal for the range of values of q studied, that $q(t)$ declines in a monotonic fashion, and lastly, that there is no endogenous T_3 in the system.

The process of successive solution by iteration enumerated above can be carried out by computer methods. A program was written in Fortran IV (version MNF) and calculated by the use of a Control Data Computer CYBER 74 (Computer Center, University of Minnesota). The computer program will be available upon request. The intervals of q used were in steps of 0.001 from $q = 0$ to $q = 1$, yielding 1,000 values for R per run. R was normalized to 1.0 for $R(1)$.

Modification of this analytic scheme was adapted to assess the relationship between R and q in euthyroid animals given increasing doses of intravenously injected T_3. Account was taken of the endogenous level of plasma T_3 at the beginning of the experiment as well as the endogenous level of T_3-dependent enzyme activity.

The theoretical basis for this modification was as follows. If euthyroid animals which can be presumed (20) to have a constant level of nuclear occupancy ($q = 0.47$) are given maximal doses of T_3 so that for the duration of the experiment q is fully saturated ($q = 1.0$) then from the considerations implicit in Eq. 8 it follows that E_t, the level of enzyme at any time t can be represented by the following expression.

$$E_t = E_{eu}e^{-\lambda t} + E_{max}(1 - e^{-\lambda t}), \quad (9)$$

where all enzyme levels E are expressed as increments over hypothyroid base line, including E_{eu}, the euthyroid enzyme

level ($q = 0.47$) and E_{max} the maximal level attainable with full saturation ($q = 1$) at $t = \infty$. Again, $t = t' - \tau$, the lag period between the injection of T_3 and the time that an increase in the accumulation of new enzyme is observed.

On rearranging Eq. 9 we obtain:

$$E_{max} = \frac{E_t - E_{eu}e^{-\lambda t}}{1 - e^{-\lambda t}}. \quad (10)$$

Since the enzyme concentration 24 h after the injection of T_3 is known and the level of enzyme in euthyroid and hypothyroid animals can be determined, the value of E_{max} can be assessed. Moreover, the ratio of $R(1.0)/R(0.47)$, designated as the response factor f, can also be evaluated since it is apparent that $f = E_{max}/E_{eu}$. Evaluation of the function f now makes it possible to apply the numerical techniques described above to evaluate R between $q = 0.47$ and $q = 1.0$. The plasma disappearance curves for varying doses of T_3 are illustrated in Fig. 1 and allow approximation of p, the plasma T_3 concentration at any time t, from a given dose of T_3. Analogous to the hypothyroid state described by Eq. 5, the nuclear occupancy q can then be related to the plasma concentration by the following equation:

$$q = \frac{p + 0.59}{p + 1.261}. \quad (11)$$

This expression assumes an average base-line value of 0.59 ng/ml in plasma during the period of observation. The observed increment E above the euthyroid base line 24 h after injection of varying doses of T_3 is now related to the calculated nuclear concentration of T_3 during the interval after T_3 injection and preceding killing. The analytic approach taken is analogous to that described above for hypothyroid animals with the modification that the euthyroid enzyme value is assumed as the base line. Moreover, it is clear that the value R' generated will assume a value of 0 when $q = 0.47$ and by the normalization process, 1.0 when all the sites are occupied. We can now correct for the endogenous T_3 and the background T_3-dependent enzyme value by applying the following relationship for R, the true responsivity with baseline q = 0. Thus:

$$R = R'\left(1 - \frac{1}{f}\right) + \frac{1}{f}, \quad (12)$$

where f is the response factor defined above.

RESULTS

The results of the plasma disappearance curves are illustrated in Fig. 1 and are expressed as the percent of the injected dose per milliliter in a 100-g animal. As indicated under Methods, there appears to be a tendency to lower values with increasing doses, although the depression was not striking. The consistently higher values for a given dose in a hypothyroid as compared to a euthyroid animal is undoubtedly a reflection of the well-established increase in plasma protein binding of thyroid hormones which characterizes the hypothyroid state. Fig. 2 summarizes the basic dose-response data and the results of the best fit functions generated by application of the Rodbard Equation (19).

Fig. 3 illustrates the results of one of two experiments in which serial determinations of enzyme activity were performed in animals injected with a daily

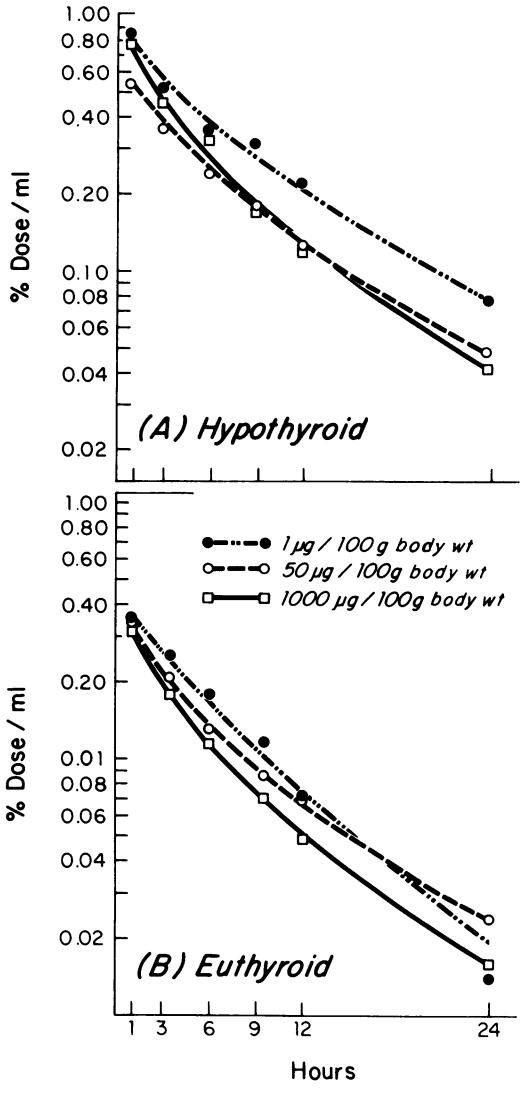

FIGURE 1 Plasma disappearance curves of intravenously injected T_3 in hypothyroid (A) and euthyroid (B) animals. The concentration of T_3 was determined by radioimmunoassay (18) and is expressed as percent of the injected dose per milliliter in an ideal animal weighing 100 g. Each point represents the average determination in three animals. Initial plasma concentrations were uniformly higher in hypothyroid animals and the fractional fall in plasma T_3 tended to be less in the hypothyroid animals. Although there appeared to be a decrease in the percent of the dose per milliliter with the larger doses of T_3, the shift was small in relationship to the 1,000-fold increase in the dose injected. The values for the 50-µg doses were used in the calculations of the nuclear occupancy-responsivity relationships described in the text.

dose of 200 µg T_3/100 g body wt, a dose designed to occupy nearly all the available nuclear binding sites and to yield a near-maximal response. In the experiment illustrated in Fig. 3 both ME and α-GPD were measured. In the other experiment, which is not illustrated,

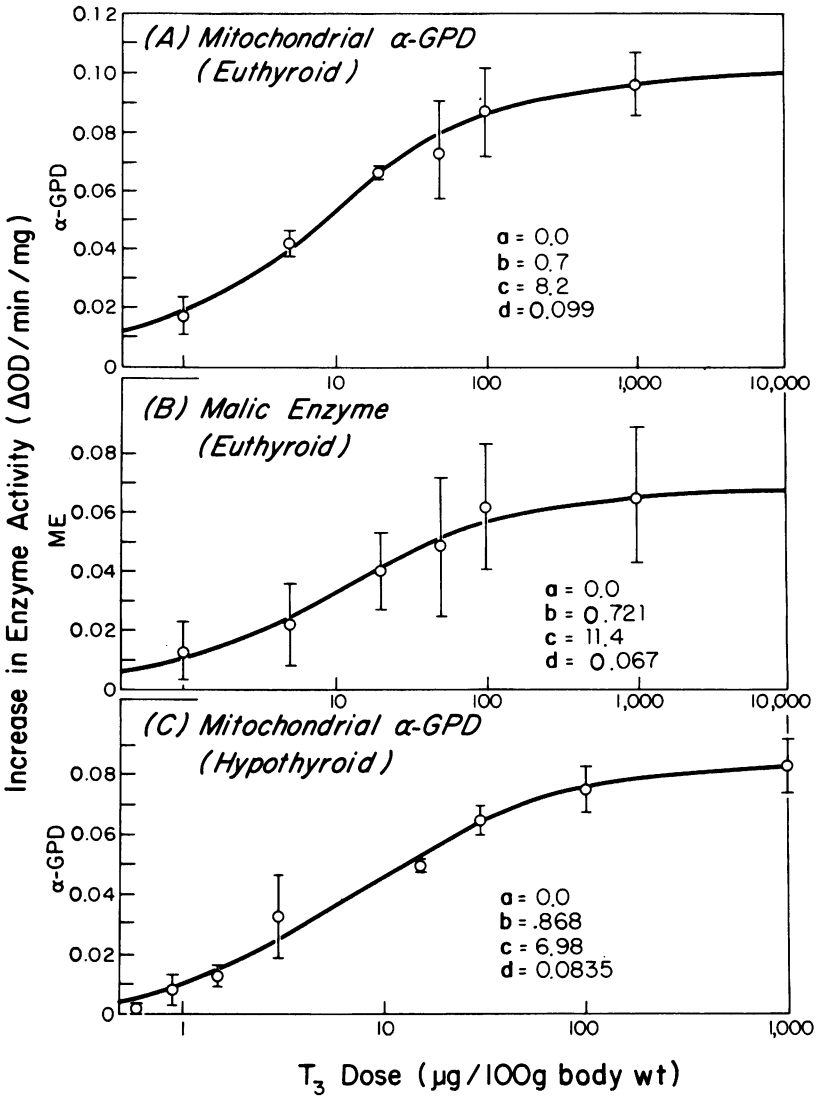

FIGURE 2 Dose-response relationship for mitochondrial α-GPD and ME in initially euthyroid animals (A, B) and initially hypothyroid animals (C) injected with increasing intravenous doses of T_3. Results in A and B are average values of four sets of experiments, each set consisting of four animals per point. Indicated in panels A and B are ±SD of the mean value of the four individual experiments. Results illustrated in panel C represent only one experiment consisting of four animals per point with ±SD indicated by bars. The primary data were used in a previous study (11) but the best fit was obtained in the present analysis by the application of the equation proposed by Rodbard (19): $y = [(a - d)/(1 + (x/c)^b] + d$, where y is the observed response; x, the dose administered, c, the dose producing half-maximal values, a, the initial value of y, and d, the linear value of y as x approaches ∞. The term b is a constant identified with the Hill-coefficient.

only α-GPD was determined. Hepatic enzyme levels were also measured concomitantly in groups of untreated euthyroid and thyroidectomized animals. The results were then expressed as the relative increase over euthyroid increments according to the formula

$$\frac{E_t - E_{hypo}}{E_{eu} - E_{hypo}},$$

where E_t is the enzyme level achieved in T_3-treated animals at time t; E_{eu}, the enzyme level in untreated euthyroid animals; and E_{hypo}, the level of enzyme in hypothyroid animals. In both experiments, the results show a progressive rise in the enzyme increment with the achievement of an apparent plateau level between 5 and 7 days after the beginning of the experiment. The ratio of the maximal increment observed in the T_3-

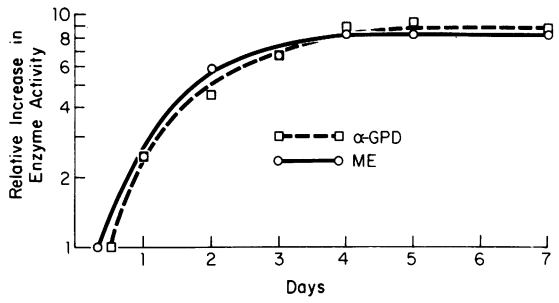

FIGURE 3 The relative increase in the activities of hepatic mitochondrial α-GPD and ME was measured initially in groups of euthyroid animals injected with a daily intraperitoneal dose of 200 μg T_3/100 g body wt. Each point represents the mean of levels of four animals in which both enzymes were measured concomitantly. Enzyme levels in euthyroid and hypothyroid animals killed at the same time were determined. The relative increase in enzyme activity was then determined from the following expression: $(E_t - E_{hypo})/(E_{eu} - E_{hypo})$, where E_t, the enzyme level achieved in T_3-treated animals at time t, E_{eu} the enzyme level in euthyroid untreated animals and E_{hypo}, the level of enzyme in hypothyroid animals. The dose of T_3 injected was such as to nearly saturate the available nuclear sites. If the relationship between responsivity and nuclear occupancy were linear one would anticipate a maximal relative increase in the induced enzyme activity of 2.13 (= 1/0.47). Since the fractional rate of enzyme decrease is not related to the thyroidal state of the animal, the finding of a maximal relative increase of 9 for ME and 8.5 for α-GPD points to an amplified response.

treated animals to the increment in euthyroid animals over base-line hypothyroid values was then determined. In the experiment illustrated in Fig. 3 this value was 8.5 for ME and 9.0 for α-GPD. An 11.7-fold increase for α-GPD was noted in the experiment not illustrated.

These experiments strongly suggested that the signal for enzyme induction undergoes major amplification as the nuclear receptor sites are saturated. This is clear from the following considerations. In the euthyroid state ≅50% of hepatic sites are normally occupied by T_3 as shown by in vivo displacement studies (20) and more recently by in vitro experiments with nuclear extracts (21). Furthermore, the fractional decrease in α-GPD and ME activity after the cessation of T_3 injection appears to be independent of the thyroidal state of the animal (11). Thus, if one were to assume that the responsivity of the enzyme induction is proportional to the occupancy of the nuclear sites one would anticipate, maximally, a doubling in the steady-state level of the enzyme achieved. The finding in our experiments that the maximal values were 9–12 times those which characterize the euthyroid state thus appears to be incompatible with the assumed linear relationship. As indicated above, the assumption that the activity of the enzyme measured is proportional to enzyme mass has been verified in the case of ME (11, 22–25). The studies of Goodridge with isolated chick hepatocytes (24, 25) also indicate that the effect of T_3 does not represent exclusively a secondary effect of T_3 mediated by some bloodborn agent such as growth hormone. Similarity in the response pattern between ME and α-GPD would strongly suggest that the correlation between enzyme mass and activity also holds for α-GPD. Recent electrophoretic data suggest that α-GPD induced by T_3 is structurally identical to the enzyme produced under the basal state (26).

Although the experiments illustrated in Fig. 3 point to an amplified response when the nuclear T_3 receptor sites are fully saturated, the quantitative inferences which can be drawn from these experiments are limited. The continuous saturation of the nuclear sites leads to extreme hyperthyroidism and at the end of 1 wk the animals are very sick. The influence of secondary factors resulting from a severely disturbed physiologic state cannot be excluded. To provide a more detailed quantitative analysis of the relationship between nuclear occupancy and responsivity it would have been desirable to achieve by constant infusion of T_3 a series of steady states characterized by progressively increasing occupancy levels of plasma and nuclear T_3. Unfortunately, such experiments are technically exceedingly difficult since constant infusions of at least 1 wk would be required to attain steady-state conditions with respect to the level of induced enzyme. Accordingly, we investigated the relationship between nuclear occupancy and response by analyzing data from conventional dose-response experiments in which animals were killed 24 h after the injection of increasing doses of intravenous T_3. Although these data were obtained under nonsteady-state conditions, it appeared possible to calculate the desired nuclear-response relationships. The analytic approaches used are described under Methods. In addition to the basic dose-response data, the supplementary information required for these calculations included an estimate of the lag time between the injection of T_3 and the onset of enzyme response, the relationship between the dose-injected and the plasma concentration as a function of time, the relationship between plasma T_3 and nuclear T_3 and lastly, the average enzyme level in euthyroid and hypothyroid animals. This experimental approach has the advantage of only minimally perturbing the system under study.

Table I summarizes the calculations which were performed to estimate the maximal theoretical increase in enzyme level to be attained with the application of a maximal stimulus for an indefinite period. Shown also is the calculated response factor f representing the ratio of the theoretical maximal enzyme response to the increment over hypothyroid levels achieved in the euthyroid state. The value of f is 14.6 for α-GPD and 14.3 for ME, values even higher than those calculated from the 7-day experiment with full nuclear satu-

TABLE I

Estimation of Maximal Response and Response Factor (f) in Euthyroid Animals Treated with Doses of T_3 which Saturate Specific Nuclear Sites

$$E_{max} = \frac{E_t - E_{eu}e^{-\lambda t}}{1 - e^{-\lambda t}} \text{ and } f = \frac{E_{max}}{E_{eu}} = \frac{R(1.0)}{R(0.47)},$$

where:

$$t = t' - \tau$$

$$E_t = E_t' - E_{hypo}$$

$$E_{eu} = E_{eu}' - E_{hypo}.$$

	α-GPD	ME
t', *days*	1.00	1.00
τ, *days*	0.558	0.342
t, *days*	0.442	0.658
λ, *day*	0.248	0.257
E_{hypo}, *OD/min/mg*	0.0277	0.00342
E_{eu}, *OD/min/mg*	0.0677	0.0307
E_t, *OD/min/mg*	0.164	0.0943
E_{max}, *OD/min/mg*	0.991	0.440
f	14.6	14.3

Abbreviations: E_{max}, the theoretically maximal increase in enzyme activity above hypothyroid base-line values which would be attained with saturation of the nuclear sites for an indefinitely long period and with maintenance of the initial response conditions; E_{eu}', observed enzyme activity in euthyroid animals; E_t', observed enzyme activity after saturation of nuclear receptor sites and maximal rate of enzyme induction for period t; E_{hypo}, enzyme activity of athyroidal animals; t', time of observation after pulse intravenous injection of T_3 at $t' = 0$; τ, lag-time after T_3 injection before maximal rate of enzyme induction is observed; λ, fractional rate of enzyme disappearance; $R(1)$ and $R(0.47)$, responsivity of the system when $q = 1.0$ and $q = 0.47$, respectively. All values for E_t, E_{eu}' and E_{hypo} represent the mean value of four groups, each consisting of four animals. These data as well as the values for λ and τ have been presented in the published companion paper (11). The equations used in the calculations have been developed in the text. Assuming that under physiological euthyroid conditions 47% of the specific nuclear sites are occupied (20) the value for f would be 2.13 in a linear system. The higher values for f thus emphasize the strongly nonlinear and amplified relationship between nuclear occupancy q and the responsivity of the system (R).

ration illustrated in Fig. 3. These results further emphasize the high degree of nonlinearity which characterizes the relationships between nuclear occupancy and the rate of hepatic enzyme induction.

Evaluation of factor f also permits detailed examination of the relationship between R and q in the interval between $q = 0.47$ and $q = 1.0$ in the euthyroid animals. A detailed description of the calculations performed are provided in Methods. Fig. 4 illustrates the highly curvilinear shape of $R(q)$ in the interval under consideration.

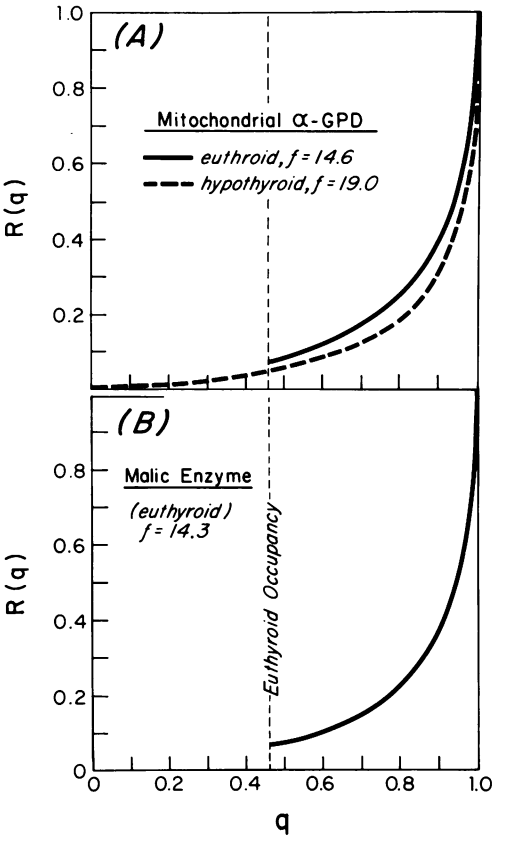

FIGURE 4 Relationship between the responsivity R and the fractional nuclear occupancy as determined for α-GPD (A) and ME (B). In euthyroid animals, the relationship between the limits of $q = 0.47$ (euthyroid occupancy) and $q = 1$, (full occupancy) is determined. In the hypothyroid animals, the relationship is calculated from $q = 0$ to $q = 1$. Methods for these calculations are described in the text. The term f is defined as $R(1)/R(.47)$ and is a measure of the degree of amplification of the signal for new protein appearance. With a linear system f would assume a value of 2.13 (= 1/0.47). The curvilinear and amplified relationship between the occupancy of the putative nuclear receptor sites and the responsivity is illustrated by the configuration of the curves generated and the large values of f calculated.

Since the complicating effects posed by endogenous levels of T_3 are eliminated in hypothyroid animals, fewer assumptions are required to evaluate R as a function of q in the more comprehensive interval from $q = 0$ to $q = 1.0$. The primary dose-response data are illustrated in Fig. 2C and the relationship between plasma concentration as a function of time and dose injected in Fig. 1A. The results of the calculations which are illustrated in Fig. 4A again emphasize the highly amplified relationship between q and R. The response factor f of 19 exceeds those calculated above for α-GPD and ME in euthyroid animals. On the basis of the available data, it is difficult to be certain whether or not these differences are biologically meaningful.

No effort was made to define the relationship between R and q with respect to the ME response in hypothyroid animals. As we have previously shown (11), the response of ME in hypothyroid animals is relatively small and the error of measurement large. Previous experiments with full nuclear occupancy have also suggested that the rate of T_3 induction of ME in hypothyroid animals increases as a function of time. For ME in the hypothyroid state, therefore, $R(q)$ may not be independent of t.

To test the adequacy of the model expressed in Eq. 1, efforts were made to compare the theoretically predicted and experimentally determined dose-response relationship 36 h after the injection of T_3. By application of the function $R(q)$ as illustrated in Fig. 4 and the known plasma disappearance curve of T_3 the expected dose-response relationship at 36 h could be calculated. The results of such calculations are illustrated in Fig. 5. General agreement between the observed and predicted response for α-GPD is apparent, although the fit probably was not optimal. In the case of ME, however, the observed response clearly appears to fall short of the theoretical prediction for submaximal doses. Again, it is difficult to be sure whether or not this discrepancy represents an intrinsic limitation of the model or is a function of the inherent experimental and biological variation in the limited data base. On the other hand, note should be taken of the excellent simulation of the ME response after the injection of 5,000 μg of T_3 (Fig. 6).

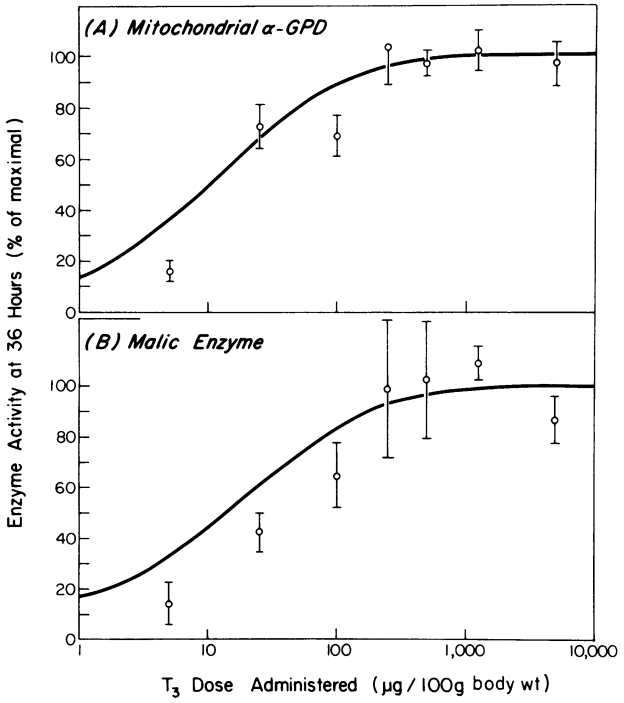

FIGURE 5 On the basis of $R(q)$ calculated for both α-GPD and ME in initially euthyroid animals (Fig. 4), the known fractional decay in enzyme effect λ, the plasma disappearance curve of T_3 (Fig. 1), the known relationship between plasma and nuclear specifically bound T_3, and the known lag periods in α-GPD and ME response, the dose-response relationships for α-GPD and ME 36 h after i.v. injection of T_3 were determined. Enzyme activities actually observed are indicated. Heavy line, computer-generated theoretical curve. Each point represents the mean of values from four animals, simultaneously assayed for α-GPD and ME. Bars indicate ±SE. All values are expressed as the percent of maximally generated enzyme activity. As previously pointed out (11), the maximal response is well predicted when the nuclear sites are saturated. The results for α-GPD show reasonable agreement between the theoretically generated curve and the observed values, but there appears to be a consistent overestimation of response in the case of ME in the lower dose range employed. The basis of this discrepancy has not been defined.

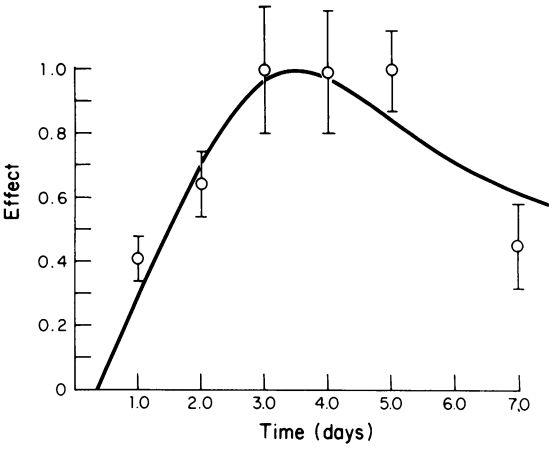

FIGURE 6 Simulation of ME response with computer model. At $t = 0$, 5,000 μg of T_3 was injected into euthyroid animals. Each point represents the mean of four animals and 1 SD is indicated by bars. Increases in enzyme activity above euthyroid base line are normalized to maximal response of 1.0. Excellent agreement is apparent between observed response and computer-generated predictions.

DISCUSSION

These studies clearly indicate that in the case of α-GPD and ME the instantaneous rate of new enzyme induction and nuclear occupancy is not linearly related but shows a marked degree of amplification with increasing concentrations of nuclear T_3. This conclusion is based on three independent methods of estimating the ratio of the rate of induction when sites are fully saturated to the rate of induction when the sites are 47% saturated as under physiological conditions. The first method involves the direct measurement of the enzyme activity after continuous saturation of the nuclear sites for a 7-day period; the second, is based on an estimation of the maximal response predicted from the initial rate of hormone induction and the $t_{1/2}$ of the decay in enzyme activity; and the third, is based

on a numerical solution of the differential equation which appears to describe the rate of new enzyme induction. The estimated values of this ratio range from 9 to 19, greatly in excess of the values expected from a linearly responsive system, 2.13 (=1.0/0.47).

The observed amplification can be interpreted in a number of ways. One possibility is that the sites for initiation of induction of these hepatic enzymes are not the nuclear sites which are being quantitated. Although this possibility cannot be rigorously excluded it seems highly unlikely on the basis of the corollary data reviewed in the Introduction, especially those studies which specifically indicate that ME and α-GPD induction is inhibited by agents such as actinomycin D and α-amanitine known to block transcription (27–31). Further, hepatic α-GPD and ME are excellent indices of thyroid hormone action at the cellular level (32, 33) and a strong correlation between the activity of thyroid hormone analogues and nuclear binding has been established (34). Lastly, our previous studies clearly establish that thyroid hormone action is initiated at a set of receptor sites for T_3 which is in rapid equilibrium with hormonal pools in plasma (35). This has been clearly shown for the nuclear binding sites under consideration but in order to demonstrate a linear relationship between nuclear receptor sites and the rate of initiation it would be necessary for the receptor sites to have an association constant for T_3 one order of magnitude lower than that measured for the nuclear sites in vivo. Limited capacity binding sites meeting such specifications have not been recognized in displacement studies. It seems highly likely, therefore, that the induction of the hepatic enzymes measured is initiated at the nuclear sites in question. Moreover, preliminary studies indicate that occupancy and the accumulation of pituitary growth hormone are related in an approximately linear fashion (36) in agreement with the findings by Samuels et al. (3) in the GH_1 tissue culture system. Since nuclear T_3 receptor sites of various tissues appear to have similar characteristics, the amplification of the hepatic enzyme response probably does not reflect an intrinsic property of the receptor which might lead to site-site interactions. Rather, amplification is probably due to an as yet unidentified post-receptor mechanism.

It may be of interest to speculate about such mechanisms. The quantitative similarity in the response characteristics of α-GPD and ME suggest a common molecular basis for amplification. Moreover, the fact that ME is cytosolic and α-GPD mitochondrial in location provides no basis for the supposition that the amplification mechanism has a mitochondrial locus. A possibility which does deserve consideration is that thyroid hormone may stimulate other hormonal factors which in concert with T_3 may serve to augment the transcription of the common target gene. Such multihormonal control has recently been demonstrated for α_{2U} globulin and for pituitary growth hormone (5, 7, 37). Another possibility which deserves consideration is that thyroid hormone may stimulate mRNA not only for the specific proteins measured but also for elements in the translational machinery. In this connection, Matthews et al. (38) have suggested that thyroid hormone increases the efficiency of translation. A combined increase in translational and transcriptional factors could then result in amplification.

A third possibility is based on the consideration that malic enzyme is a tetramer consisting of identical subunits (39). Some studies have indicated that in their assembly into a complete protein at least the initial interaction of the subunits is a random process (40). One could, therefore, speculate that the rate of formation of the complete enzyme might be related to a fourth power function of the concentration of the subunits. If so, then the ratio f, the ratio of the rate of induction at full occupancy to the rate of induction of 47% occupancy, would be 20.5. If occupancy actually were 55% the calculated ratio would be 10.9. Since these values are in general agreement with the range of ratios determined experimentally, further attention should be directed to testing of this hypothesis by examining occupancy-response relationships of other proteins with defined subunit structure. Unfortunately, the subunit composition of α-GPD is unknown but it is interesting that pituitary growth hormone, which appears to be linearly related to occupancy, is a monomer.

Attention should also be directed to the possible pathophysiological and clinical significance which the amplification process may play in determining the tissue response to excess thyroid hormone. Amplification would appear to provide a mechanism for expressing the tissue response to T_3 over an extended range of plasma hormone concentrations despite the fact that approximately one-half of the sites already appear to be occupied in the physiological state. A linear relationship between nuclear occupancy and response would lead to plateau levels in nuclear occupancy and tissue response with substantially smaller increments in plasma T_3 concentration than in the case of an amplified relationship. Manifestations of clinical hyperthyroidism thus may be related primarily to those responses which undergo amplification.

The mathematical approach used in this study for analyzing nonsteady-state phenomenon may be of general interest. The model developed appears adequate at least as a first approximation in predicting the course of tissue response to the administration of T_3 in euthyroid animals. Additional studies, however, are required more fully to test this model. In the hypothyroid state, the response of ME suggests that the function $R(q)$ is dependent on t and that a modification of the basic equation proposed above may be required to de-

scribe this response. Our studies illustrate that it may be difficult or impossible experimentally to achieve steady-state conditions. Under such circumstances, the analysis of nonsteady-state data may be useful in evaluating the necessary biologic response parameters.

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