JCI The Journal of Clinical Investigation

Stimulation of hepatic mitochondrial alpha-glycerophosphate dehydrogenase and malic enzyme by L-triiodothyronine. Characteristics of the response with specific nuclear thyroid hormone binding sites fully saturated.

J H Oppenheimer, … , H L Schwartz, M I Surks

J Clin Invest. 1977;59(3):517-527. https://doi.org/10.1172/JCI108667.

Research Article

Experiments were designed to analyze the relationship of a single i.v. dose of triiodothyronine (T3), the level of plasma and hepatic nuclear T3 attained, and the tissue response as reflected in increased activity of hepatic mitochondrial alpha-glycerophosphate dehydrogenase (alpha-GPD) and cytosol "malic enzyme" (ME). These studied were carried out in euthyroid rats by varying the dose of T3 injected and the time at which the animals were killed and the enzyme levels measured. The plasma T3 concentration was determined and the fraction of nuclear sites occupied at any time t was calculated from the known plasma:nuclear relationship. As a first step, the analysis was confined to the limiting situation in which all nuclear sites were effectively saturated. The following additional information was required and obtained: A proportional relationship between the half-neutralizing volume of a specific antiserum to malic enzyme and the activity of malic enzyme was established, thus confirming previous reports that the increase in enzyme activity induced by T3 is due to increased enzyme mass. The absolute refractory period immediately after i.v. injection of T3, during which no enzyme response could be detected, was determined. This was shown to be 13.4 h for alpha-GPD and 8.2 h for ME. Lastly, the t1/2 of the enzyme decay after pulse injection of T3 was measured. This was similar for both enzymes, […]

Stimulation of Hepatic Mitochondrial α-Glycerophosphate Dehydrogenase and Malic Enzyme by L-Triiodothyronine

CHARACTERISTICS OF THE RESPONSE WITH SPECIFIC NUCLEAR THYROID HORMONE BINDING SITES FULLY SATURATED

JACK H. OPPENHEIMER, ENRIQUE SILVA, HAROLD L. SCHWARTZ, and MARTIN I. SURKS

From the Division of Endocrinology, Department of Medicine, Montefiore Hospital and Medical Center, and Albert Einstein College of Medicine, Bronx, New York 10467

ABSTRACT Experiments were designed to analyze the relationship of a single i.v. dose of triiodothyronine (T_3), the level of plasma and hepatic nuclear T_3 attained, and the tissue response as reflected in increased activity of hepatic mitochondrial α-glycerophosphate dehydrogenase (α-GPD) and cytosol "malic enzyme" (ME). These studies were carried out in euthyroid rats by varying the dose of T_3 injected and the time at which the animals were killed and the enzyme levels measured. The plasma T_3 concentration was determined and the fraction of nuclear sites occupied at any time t was calculated from the known plasma:nuclear relationship. As a first step, the analysis was confined to the limiting situation in which all nuclear sites were effectively saturated. The following additional information was required and obtained: A proportional relationship between the half-neutralizing volume of a specific antiserum to malic enzyme and the activity of malic enzyme was established, thus confirming previous reports that the increase in enzyme activity induced by T_3 is due to increased enzyme mass. The absolute refractory period immediately after i.v. injection of T_3, during which no enzyme response could be detected, was determined. This was shown to be 13.4 h for α-GPD and 8.2 h for ME. Lastly, the $t_{\frac{1}{2}}$ of the enzyme decay after pulse injection of T_3 was measured. This was similar for both enzymes, 2.8±0.6 (SD) days for α-GPD and 2.7±0.6 (SD) days for ME.

The results of these studies indicated that the extent of hepatic response appears limited by full occupancy of a set of intracellular receptor sites by T_3 which is in rapid equilibrium with the plasma hormone pool. The kinetic properties of the receptors, as functionally defined in these studies, resemble those associated with the recently described specific nuclear T_3 sites. These data per se are thus compatible with but do not prove a nuclear site of initiation of hormone effect. They do allow the development of an internally consistent mathematical model which permits prediction of enzyme response when the receptor sites are fully occupied for a given length of time after the i.v. injection of hormone.

A separate series of studies was carried out in thyroidectomized rats. The response characteristics of α-GPD were similar to those observed in euthyroid animals. In contrast, however, the early response of ME to pulse injections of T_3 was very much reduced in hypothyroid animals as compared to euthyroid animals in which nuclear sites were saturated for comparable periods. These findings raise the possibility that a factor required for the induction of malic enzyme but not α-GPD is deficient in the hypothyroid state.

INTRODUCTION

A major problem in clinical endocrinology is the definition of the interrelationship of hormone dose, plasma and receptor concentration, and the tissue response as a function of time. To develop an appropriate conceptual framework to analyze this problem with respect to the thyroid hormones in man, we have chosen to examine these variables in the rat. In the

The present address of Doctors Oppenheimer and Schwartz is the Section of Endocrinology and Metabolism, Department of Medicine, University of Minnesota, Minneapolis, Minn. 55455. Please address requests for reprints to Dr. Oppenheimer. Dr. Surks is the recipient of a Research Career Development Award, AM 19502-04, from the National Institutes of Health.

Received for publication 29 July 1976 and in revised form 22 November 1976.

following studies we have used as an index of thyroid response two hepatic enzymes, mitochondrial α-glycerophosphate dehydrogenase (α-GPD)[1] (L-glycerol-3-phosphate:cytochrome *c*-oxidoreductase [EC 1.1.99.5]) and soluble "malic enzyme" (ME) (L-malate:$NADP^+$ oxidoreductase [decarboxylating] EC 1.1.1.40). These enzymes have been extensively used as measures of thyroid hormone effect by other investigators (1–22). Although α-GPD is believed to be involved in intracellular hydrogen transport (23) and ME, in lipogenesis, fatty acid saturation, and elongation (5), the precise role of these enzymes in effecting thyroid hormone action at a cellular level is unknown. Nevertheless, the activity of both enzymes is markedly increased in hyperthyroid animals, achieving levels several-fold greater than those which exist under base-line conditions. Conversely, in hypothyroid animals enzyme activity is depressed. In the rat an excellent correlation between α-GPD activity and O_2 consumption has been established both with respect to the response of specific tissues (3) and the effect of various thyroid hormone analogues (15, 19).

Indirect evidence strongly suggests that the increase in α-GPD and ME activity after thyroid hormone treatment represents de novo enzyme synthesis. Inhibitors of protein synthesis both at the transcriptional and translational levels block the enzyme response (6, 8, 9, 13). The increase in enzyme activity appears to be a reflection of increased mass as demonstrated by the use of specific antisera to ME and measurement of increased rate of leucine incorporation into this enzyme (16, 18, 20, 22). Analogous studies with α-GPD have not been performed since specific antisera to this enzyme are not available. α-GPD is strongly bound to the inner mitochondrial membrane and has not yet been isolated.

These considerations make α-GPD and ME suitable models for the study of enzyme induction by thyroid hormones. We have previously reported that any i.v. dose that saturates hepatic nuclear receptor sites for a given period will result in a maximal accumulation of α-GPD during the interval (24). These studies also indicated that the number of sites does not vary with thyroidal status of the animal or with the plasma L-triiodothyronine (T_3) level of administered hormones, in agreement with in vitro studies (25, 26).

We now report studies in which we have examined in detail the characteristics of T_3 induction both of mitochondrial α-GPD and cytosol ME when the putative receptor sites are fully saturated by a large dose of i.v. T_3. We believed that an analysis of the ME response in addition to that of α-GPD would be advantageous because of the distinctive subcellular locations of these enzymes and because the increase in ME activity could be directly related to enzyme mass. The results of these studies were found to provide additional support for the concept that enzyme induction is constrained by the occupation of a set of intracellular receptor sites with which T_3 in plasma is in rapid exchange. Our findings are compatible with but do not conclusively prove that these receptors are the nuclear T_3 sites under general discussion. Under any circumstance our formulations have enabled us to propose a heuristic model useful in the quantitation of enzyme induction by thyroid hormone.

METHODS

Male Sprague-Dawley rats (150–225 g) obtained from Charles River Breeding Labs, Wilmington, Mass. were used in these studies. Tap water and food (Wayne Lab-Blox, Allied Mills, Inc., Chicago, Ill.) containing 1.0 μg I/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 I/g) for 7 days and then injected i.p. with 100 μCi of Na ^{131}I (Mallinckrodt Inc., St. Louis, Mo.). The animals were weighed twice weekly and used experimentally when their weight 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 as described by Lee and Lardy (4) with the synthetic electron acceptors phenozine methosulfate and p-iodonitrotetrazolium violet. With varying aliquots of mitochondrial suspensions prepared from livers of both euthyroid and thyroid hormone-treated hyperthyroid rats, it was found that the reaction was proportional to protein in the range 50–115 μg per assay. The usual content of protein per assay was 70–95 μg. Although the assay was shown to be linear for at least 20 min, assays were generally terminated after 10 min. The intra-assay coefficient of variation was approximately 2%. The observed mean enzyme activity for separate groups of euthyroid rats measured in various assays was 0.107 OD/min per mg protein (range 0.094–0.126).

Hepatic ME activity was measured by the method of Ochoa (27) as modified by Hsu and Lardy (28). Enzyme activity was expressed as optical density per minute per milligram of protein at 240 μm. Proportionality to protein content was established by the use of varying dilutions of cytosol derived from the liver of hyperthyroid rats. The identity of the enzyme purified from liver of euthyroid and hyperthyroid rats has been established by Murphy and Walker (16). The reaction was shown to be linear for at least 7 min. Assays were carried out for 3–4 min. The intra-assay coefficient of variation for multiple assays on a single sample was less than 6.0%. Mean enzyme activity observed in separate groups of euthyroid rats was 0.0431 OD/min per mg protein (range 0.032–0.051).

To determine whether increased malic enzyme activity in T_3-treated rats was due to activation of preexisting enzyme or to increased enzyme mass, groups of euthyroid rats were injected with 15, 200, or 1,000 μg T_3/100 g body weight in saline and killed 24 h later. Cytosol was prepared from the livers. Aliquots of 150 μl of each cytosol were incubated with 1–30 μl of specific rabbit antimalic enzyme antiserum, kindly provided by and characterized by

[1] *Abbreviations used in this paper*: α-GPD, α-glycerophosphate dehydrogenase; ME, malic enzyme; T_3, triiodothyronine.

Dr. Jonathan J. Li (18). Samples were incubated for 30 min at 37°C and then for 1 h at 0°C. Tubes were centrifuged at 10,000 *g* for 20 min to remove precipitated protein. Aliquots representing 50 μl of original cytosol were taken from each supernate for assay of enzyme activity. The volume of antiserum required to inhibit initial enzyme activity by 50% was determined by plotting the enzyme activity as a function of the volume of antiserum.

Plasma concentrations of T_3 were measured by the radioimmunoassay procedure of Surks et al. (29). Protein content of mitochondrial preparations and cytosol were measured by the method of Lowry et al. (30).

The method of calculating nuclear occupancy was as follows. Previous studies had shown that binding of T_3 to nuclei is noncooperative (31, 32) and that a rapid equilibrium exists between T_3 bound to nuclear sites and plasma (33). The equilibrium relationship between p, the plasma T_3, and q, the fraction of nuclear sites occupied, could therefore be expressed by a simple rearrangement of the law of mass action as:

$$q = \frac{p}{p + p_{\frac{1}{2}}}, \quad (1)$$

where $p_{\frac{1}{2}}$ is the plasma concentration of T_3 when nuclear sites are half occupied. This relationship is implicit in the experimental studies relating plasma concentration to nuclear occupancy (24). Under physiological conditions with an average plasma concentration of 0.6 ng/ml, nuclear sites are 47% occupied (34). Thus, the value of $p_{\frac{1}{2}}$ can be calculated to be 0.67. Eq. 1 can therefore be used to determine q for any value of p.

RESULTS

Enzyme accumulation as a function of dose and time. Two experiments in euthyroid rats were conducted with a design analogous to those used in previous studies (24). A single large dose of T_3 (5,000 μg/100 g body weight) and a relatively smaller dose (200 μg/100 g body weight)[2] were injected intravenously into two groups of animals which were subsequently killed at the intervals designated in Fig. 1. In the study illustrated in Fig. 1 both the activities of cytosol ME and mitochondrial α-GPD were measured. In another experiment not illustrated here only the concentration of ME activity was measured. The results of the experiments described under Methods confirmed previously reported studies (16) indicating that the thyroid hormone-induced increase in ME activity was due to an increase in enzyme mass. The increment in the volume of antiserum required to neutralize 50% of the enzyme activity was indeed found to be proportional to the increment in enzyme activity induced by T_3 administration ($r = 0.93$). Fig. 2 documents both the plasma concentration of T_3 and the nuclear occupancy as a function of time in the experiment illustrated in Fig. 1. The curvilinear nature of the plasma disappearance curve on a semilog plot resembles results observed in previous studies (35). The results of the experiment in which ME only was measured were similar to those illustrated in Figs. 1 and 2.

In confirmation of the results of our previously

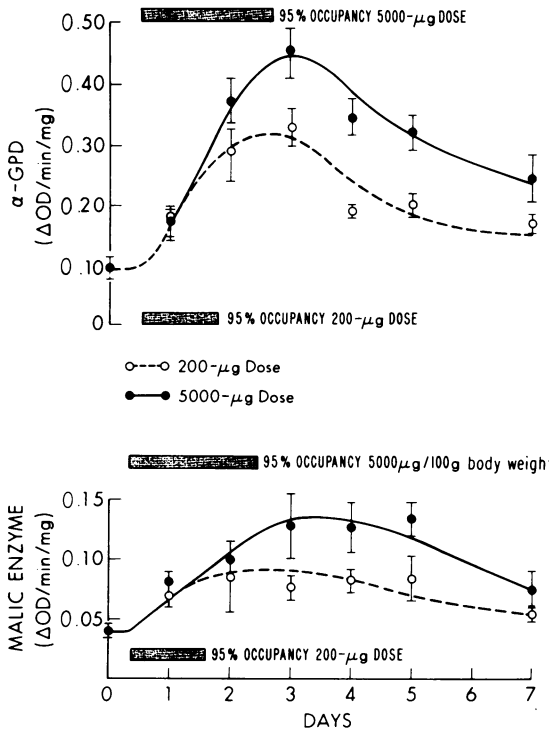

FIGURE 1 Response of mitochondrial α-GPD (Upper panel) and ME (Lower panel) in same groups of animals treated with 200 and 5,000 μg T_3/100 g body weight, respectively. Each point represents the mean value of four animals±SD. Duration of 95% nuclear occupancy is indicated by horizontal stippled bar. Because of the delay in the first appearance of enzyme (absolute refractory period) the time scale for occupancy was shifted to the right (13.4 h for α-GPD and 8.2 h for ME) (See Fig. 4). Statistically significant differences (analysis of variance) in the level of enzyme activity between animals treated with 200 and 500 μg/100 g body weight were observed for α-GPD on day 2 ($P < 0.005$) and days 3, 4, 5, and 7 ($P < 0.001$) and for ME, day 3 ($P < 0.001$), 4 ($P < 0.001$), and 7 ($P < 0.05$).

[2] The doses of T_3 used in these and subsequent experiments are generally quite large in relation to the daily subcutaneous replacement dose of T_3 required to render the hypothyroid rat euthyroid, 0.34 μg/100 g body weight, and the daily production rate, 0.25 μg/100 g body weight based on a metabolic clearance rate of 17.6 ml/100 g body weight and an average plasma T_3 concentration of 0.6 ng/ml (35). Large doses are required to saturate the putative receptors and to elicit a maximal rate of hepatic enzyme induction during the period of observation. Because of the slow rate at which thyroid hormone effects are dissipated (see Discussion), smaller doses given over a prolonged period would have resulted in comparable increases in enzyme activity. The use of large doses appears to be justified by the findings in the present study that the effect of these doses can be understood completely by the variable duration of saturation of the receptor sites. In preliminary studies we have shown that the cumulative effect of smaller doses can be predicted from the model system developed here for single doses.

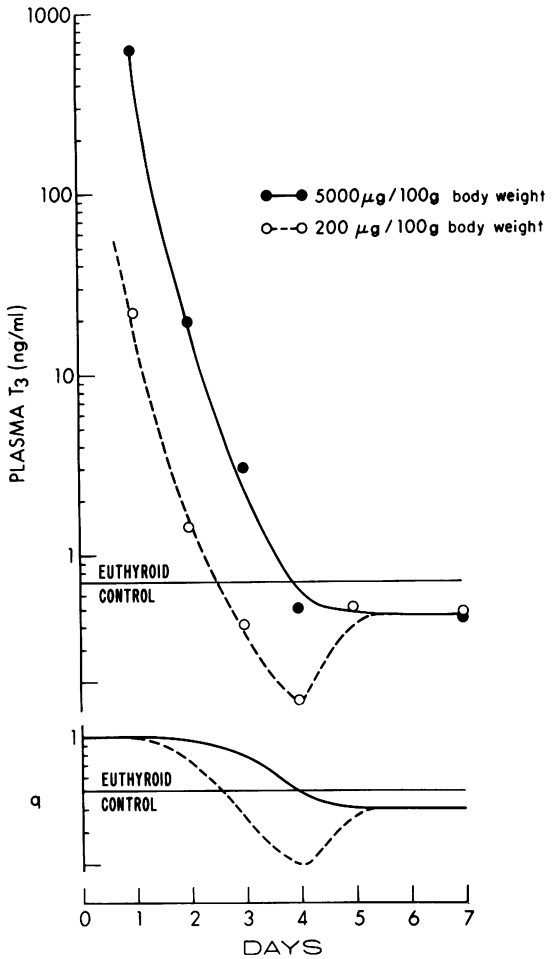

FIGURE 2 Plasma T_3 concentration in experiments illustrated in Fig. 1. Nuclear occupancy is calculated as described under Methods. Base-line concentration of T_3 in a group of simultaneously studied control animals was 0.71±0.24 ng/ml (SD) (n = 10). Dip in plasma T_3 concentration below base-line levels may represent temporary suppression of thyroid-stimulating hormone secretion.

published experiments, our findings again appear to demonstrate that the accumulation of the hepatic enzyme is independent of the dose, as long as the nuclear sites appear relatively saturated (>95%). In the experimental design used in these studies, 95% saturation occurred with the 200 μg/100 g body weight dose for approximately 1.5 days (Figs. 4 and 5). After desaturation of the sites, accumulation of new enzyme stopped and the total enzyme activity fell with the characteristic $t_{\frac{1}{2}}$ of about 3 days (see below). In the animals treated with a larger dose, 5,000 μg/100 g body weight, enzyme accumulation continued until the 2nd to 3rd day. Enzyme accumulation also ceased with the desaturation of the sites and the total level of enzyme again fell with the characteristic $t_{\frac{1}{2}}$ of about 3 days. These results are compatible with the formulation that enzyme induction is limited by the saturation of receptor sites. To test the validity of this hypothesis additional studies were undertaken in which the induction characteristics of α-GPD and ME were measured. These experiments will be described in the following sections.

Dose-response relationships at 24 h. Graded doses of T_3 (1, 5, 20, 50, 100, and 1,000 μg/100 g body wt) were injected i.v. into euthyroid animals and the levels of α-GPD and ME measured 24 h later. The increments in enzyme activity (α-GPD and ME) per milligram of protein above the control value in untreated euthyroid animals were determined. In each experiment the percent of the maximal response was calculated. The mean percentage increase at every dose is plotted in Fig. 3. A function describing the dose-response relationship was empirically fitted by application of the equation:

$$E = \frac{D}{D + D_{\frac{1}{2}}}, \qquad (2)$$

where E is the induced enzyme activity as expressed as a fraction of the maximal response, D is the dose of

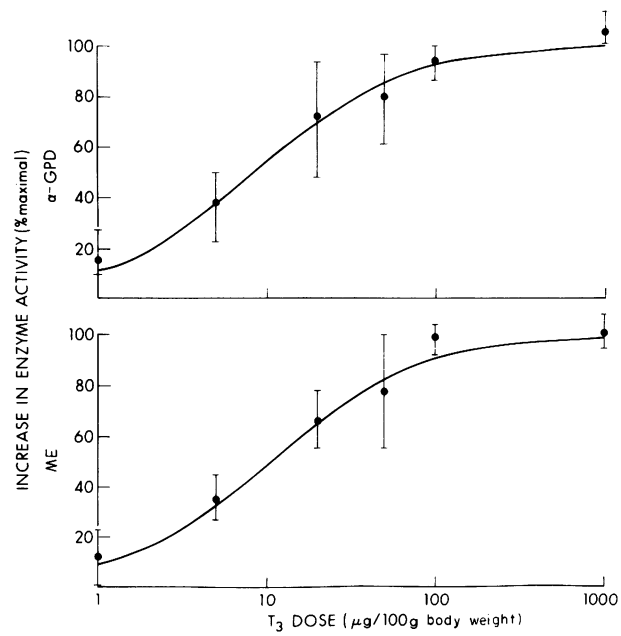

FIGURE 3 Mean percentage increment in mitochondrial α-GPD activity. Maximal response in each experiment was assumed to be the average elicited by the 100 and 1,000 μg/100 g dose. The data points represent the mean of four experiments, each consisting of four animals for each time point. Bars indicate range of results in the four experiments. For α-GPD (Upper panel) the estimating equation is E = D/D + 8.2, where E is the relative enzyme activity at 24 h (Maximum E = 1) and D = dose of T_3 in micrograms per 100 g of body weight. For ME (Lower panel), the corresponding estimating equation is E = D/D + 10.9.

μg injected/100 g body weight, and $D_{\frac{1}{2}}$ the dose required to achieve half-maximal response. $D_{\frac{1}{2}}$ for α-GPD was estimated as 8.2 μg and that for ME 10.9 μg/100 g body weight. The mean $D_{\frac{1}{2}}$ for each enzyme was determined by averaging individual values for $D_{\frac{1}{2}}$ calculated from the paired experimental values E and D in Eq. 2.

Kinetics of appearance of enzyme activity. In any analysis of the induction phenomenon, it is essential to understand both the kinetics of enzyme appearance and enzyme disappearance after the injection of hormones. Fig. 4 illustrates the early enzyme activity accumulation after the injection of 3,000 μg T_3/100 g body weight, a dose designed to saturate the nuclear sites for the period of the experiment. The average result of three experiments is shown. In each, α-GPD and ME activities were measured and the increments in enzyme activity above base-line control levels were plotted. For α-GPD the initial refractory period lasted approximately 13.4 h. No significant increases in α-GPD activity were observed during this period. After the refractory period a sharp linear increase in enzyme accumulation occurred. The corresponding refractory period for ME appears to be shorter, 8.2 h. These experiments clearly indicate that even with full occupation of the nuclear sites, a defined time interval is required before appearance of new enzyme can be demonstrated. Presumably, this refractory period represents the time required for preliminary transcriptional and translational processes at the cellular level.

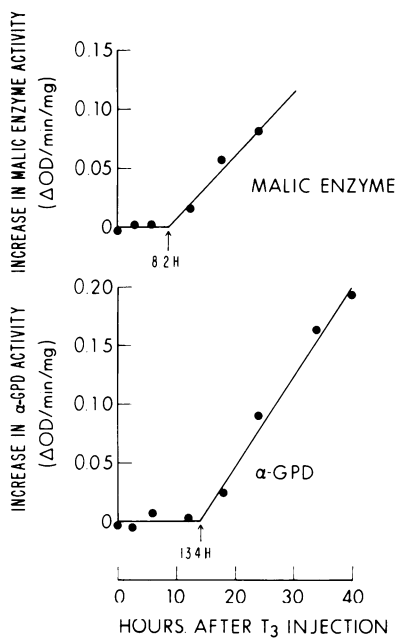

FIGURE 4 Kinetics of enzyme appearance in euthyroid animals in response to T_3 injection. During the period of observation the nuclear sites were completely saturated by the i.v. injection of 3,000 μg T_3/100 g body weight. Indicated are the average changes in enzyme activity from base-line euthyroid values as determined in simultaneous control animals. Upper panel: malic enzyme (ME). Lower panel: α-glycerophosphate dehydrogenase (α-GPD). In each panel the average of three experiments is represented, each consisting of four animals per time point. Intercepts with base-line value were determined by the method of least mean squares. An absolute refractory period of 8.2 h for ME and 13.4 h for α-GPD presumably represents the time necessary for transcriptional and translational events before the appearance of newly synthesized enzyme molecules. Thereafter a sharp and apparently linear increase in enzyme accumulation occurs.

TABLE I
Terminal Decay in Enzyme Activity after Single Pulse Injections of T_3

	Expt. no.	T_3 dose	$t_{\frac{1}{2}}$
		μg/100 g body weight	*days*
α-GPD	1	20	2.0
	2	20	3.2
	3	200	3.1
	4	200	2.3
	5	1,250	2.4
	6	1,250	2.9
	7	5,000	3.8
			Mean 2.8±0.6 (SD)
ME	1	200	2.9
	2	200	1.7
	3	200	3.3
	4	5,000	2.6
	5	5,000	2.8
			Mean 2.7±0.6 (SD)

Compilation of experiments in which the terminal decay of enzyme activity was determined. Half-decay times were estimated from least square analysis of terminal portion of enzyme decay curves of individual experiments.

Kinetics of enzyme decay. Decay characteristics of enzyme activity were examined in the experiments summarized in Table I. In individual studies, the log enzyme activity above euthyroid control levels was plotted as a function of time. Depending on the dose of T_3 injected, maximal enzyme activity was attained between 1 and 3 days after injection. The terminal portion of the decay curve was analyzed by the method of least squares. Results indicated that the average rate of decline for both enzymes was similar, 2.8±0.6 days (SD) for α-GPD and 2.7±0.6 days (SD) for ME. Moreover, the $t_{\frac{1}{2}}$ of decline in enzyme activity did not appear to be related to the dose of T_3 injected. When both enzymes were analyzed in the same animals, the rate of decline was indistinguishable. Further evidence that the dissipation of enzyme activity was not dependent upon the hormonal state

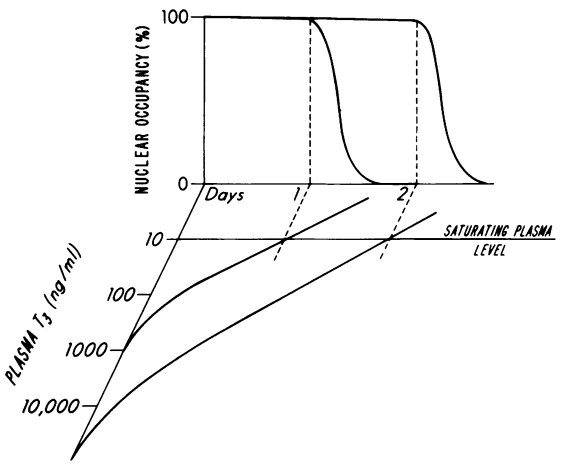

FIGURE 5 Schematic representation of proposed model. The assumption is made that a plasma concentration of T_3 can be defined such that the receptors are effectively saturated. With full occupancy of such sites for a period of time t, the tissue response to hormone is also assumed to be maximal when assessed at t. As is apparent from the exponential decrease in plasma T_3 concentration, progressively larger doses of T_3 are required to saturate these sites for longer intervals with pulse injections of hormone at $t = 0$. On the basis of available data the receptor sites have been provisionally identified in the illustration as "nuclear."

of the animal was derived from an experiment in which a group of rats was rendered hyperthyroid by the daily administration of 200 μg T_3/100 g body weight for 7 days. After the last T_3 injection the activity of α-GPD was measured over the subsequent 9 days. The terminal $t_{\frac{1}{2}}$ was 3.1 days, not significantly different from that of α-GPD in animals injected with a single dose of T_3 and with a substantially lower peak value of α-GPD.

Formulation of model. The data generated in the foregoing studies allow formulation of a provisional model of hepatocellular response to thyroid hormone administration as illustrated in Fig. 5. In essence, after pulse injection of T_3 into the intact animal the plasma concentration declines in a generally exponential fashion. We postulate a rapid exchange of T_3 between plasma and receptor. When the plasma concentration is sufficiently high, all receptors should be saturated and a maximal hormonal response would be expected. It should also be possible to define the saturating plasma concentration at which the receptor sites are nearly completely occupied. Moreover, it should be possible to determine whether or not the specific nuclear sites meet the criteria for receptors as defined by this model.

If this formulation is correct, then the saturating plasma concentration experimentally determined should be similar regardless of the time of the observation. For example, if enzyme activity is determined at 36 h rather than 24 h after T_3 injection, it is obvious that a substantially larger dose of T_3 would be required to achieve a terminal saturating plasma concentration. The accumulated enzyme activity would also be greater at 36 than at 24 h. Despite these differences, however, the model predicts that the plasma concentration at which maximal enzyme responses are observed would be the same at 24 and 36 h.

Comparison of dose-response relationships at 24 and 36 h. To test this formulation, the following experiment was performed. A group of rats was divided into two subgroups: one subgroup was killed 24 h after the i.v. injection of 1, 5, 20, 100, 250, and 1,000 μg T_3/100 g body weight; the second subgroup was killed 36 h after the injection of 5, 25, 100, 500, 1,000, and 5,000 μg T_3/100 g body weight. Both hepatic α-GPD and ME activities were determined and the concentration of T_3 in the terminal plasma sample was measured by radioimmunoassay. Results of this experiment indicated that 24 h after injection the maximal increment in α-GPD activity above base line (0.084 OD/min per mg) was achieved with the 100-μg dose, whereas the maximal increment at 36 h (0.200 OD/min per mg) was attained with the 250-μg T_3 dose. Maximal increments of ME above the base line at 24 and 36 h, 0.062 and 0.100 OD/min per mg protein, respectively, were achieved by doses roughly comparable to those required to achieve maximal increases in α-GPD.

A plot of the enzyme level attained as a function of the terminal plasma concentration (Fig. 6) confirmed the expectation from the model that for each enzyme maximal effects are achieved at the same level of plasma T_3 in both the 24-h and 36-h experiments. For α-GPD, the apparent saturating plasma concentration was 12 ng/ml, whereas for ME the corresponding value appeared to be higher, approximately 20 ng/ml. Another difference between the response of these enzymes was the ratio of the increment in enzyme activity at 24 h to that at 36 h. This ratio was 2.1 for α-GPD and 1.6 for ME. Both the difference in saturating plasma levels and the 36 h/24 h ratios can probably be attributed to the differences in the absolute refractory periods for α-GPD and ME described in the foregoing section (See Appendix for calculations).

The characteristics of the specific nuclear T_3 binding sites appear compatible with the behavior of the receptor sites as functionally defined by the model. Thus, previous data from our laboratory (33) indicate that there is a rapid exchange between plasma and nuclear T_3. Moreover, the concentrations of plasma T_3 which saturate the receptor site can also be shown to saturate the nuclear sites.

Response of hypothyroid animals. Since the studies described above were carried out only in

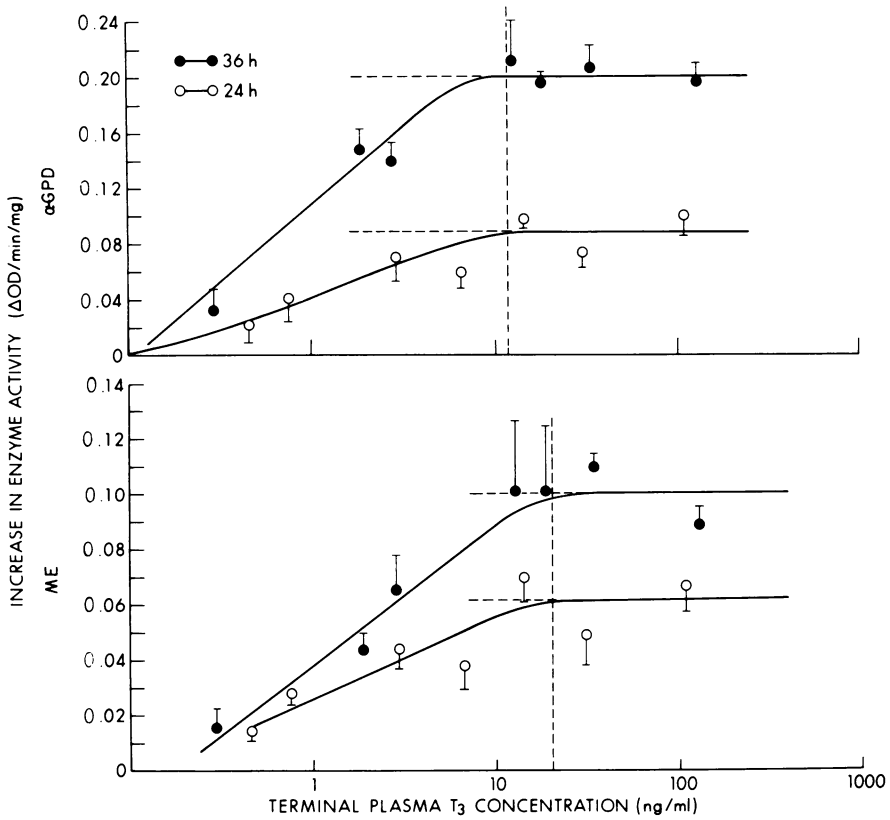

FIGURE 6 Relationship between increments in α-GPD (Upper panel) and ME (Lower panel) and terminal plasma T_3 concentrations in paired experiments carried out for 24 and 36 h. Graded i.v. pulse doses of T_3 were injected at $t = 0$. Maximal effects both at 24 and 36 h were attained at the same terminal plasma concentration, in accordance with the predictions of the model illustrated in Fig. 5. Differences between the two enzymes with respect to the saturating plasma concentration and the ratio of the enzyme level attained at 36 and 24 h can probably be explained by the difference in the absolute refractory period (See Appendix).

euthyroid animals, we believed that it would be equally important to determine the response characteristics in hypothyroid rats (Fig. 7). Injection of graded pulse doses of T_3 resulted in increases of α-GPD activity above hypothyroid base-line values which were practically indistinguishable from increments achieved by similar doses of T_3 in euthyroid animals above corresponding euthyroid base-line control levels. In contrast, the induction of ME by comparable doses of T_3 was very much reduced in chronic hypothyroid rats compared to the response in euthyroid animals. Nevertheless, when the results were expressed as a percentage of the maximal increment rather than as the absolute increase, the response characteristics of ME were generally comparable to those observed in euthyroid animals. Since the nuclear-plasma relationships in hypothyroid and euthyroid animals are similar (24), this would suggest that the response to T_3 in hypothyroid animals is also limited by nuclear occupancy.

Theoretically, the lesser absolute response of ME induction in hypothyroid animals could be attributed to factors other than the thyroid status per se. Thus, although the hypothyroid and euthyroid animals studied had similar body weights, hypothyroid animals were older, since approximately 8 wk were allowed to elapse before the hypothyroid status is achieved after thyroidectomy and radioiodine treatment. To rule out the possibility that factors other than hypothyroidism are responsible for the reduced induction rate of ME, a group of hypothyroid animals was injected with 200 μg T_3 at $t = 0$ and at $t = 48$ h to achieve full occupancy of the nuclear sites for a full 72-h period. Measurement of enzyme activity was carried out at the time intervals designated in Fig. 8. As anticipated from the previous experiments, the rate of increase in ME in the hypothyroid group was substantially reduced in relationship to the increase observed in the euthyroid animals. Of interest, however, was the progressive augmentation

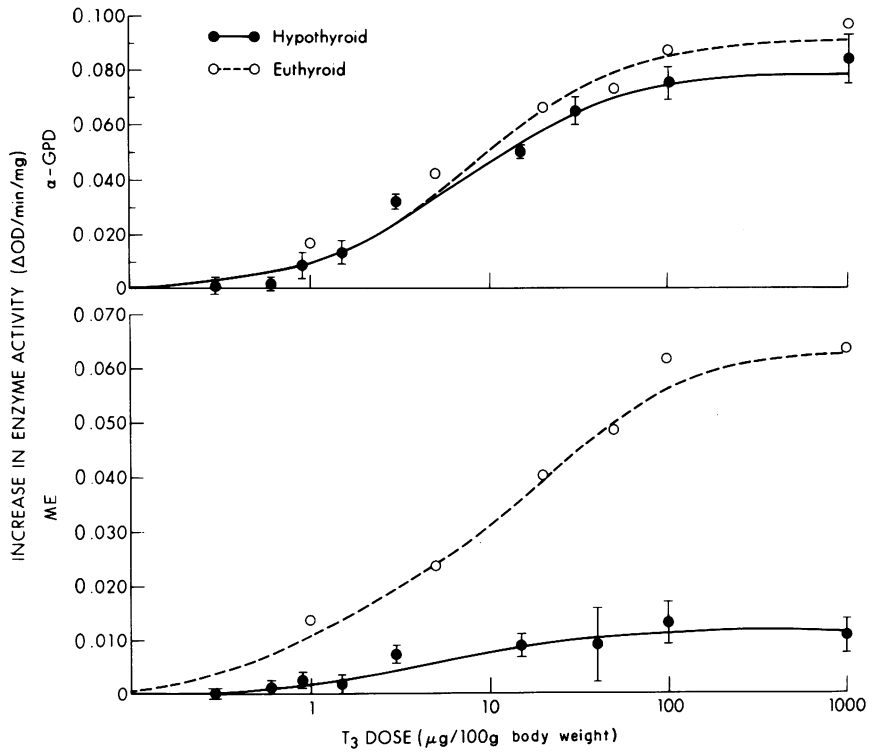

FIGURE 7 Increments in enzyme activity elicited in hypothyroid animals 24 h after the i.v. injection of graded doses of T_3. Indicated are the mean increases ±SD above hypothyroid base-line values in four animals. The open circles represent the mean increments observed in the studies of euthyroid animals illustrated in Fig. 1 (Upper panel: α-GPD; Lower panel, ME). These findings suggest that the increment in α-GPD is similar in euthyroid and hypothyroid animals but that ME induction by T_3 in hypothyroid animals is less than that observed in euthyroid animals.

in the rate of enzyme induction. At 48 h, the rate of increase in hypothyroid animals was identical to that observed in euthyroid animals treated with T_3. These results strongly suggest that the initially reduced rate of ME induction in hypothyroid animals is related to hypothyroidism per se and not to other factors such as the age of the animals.

DISCUSSION

Our findings indicate that the stimulus for new enzyme induction by T_3 is limited by the occupation of intracellular receptor sites by hormone in rapid equilibrium with plasma pools. A plasma concentration therefore exists which results in the effective saturation of these sites and the generation of a maximal stimulus for new enzyme induction. When appropriate account is taken of the refractory period, the saturating concentration can be shown to be independent of the time of experimental observation. Moreover, from our observations at 24 h and the $t_{\frac{1}{2}}$ of disappearance of enzyme activity, we were able to predict the level of enzyme activity at 36 h with full receptor occupancy (See Appendix). The internal consistency of our findings therefore supports the validity of the underlying assumption of our model and provides additional evidence for the existence of limited capacity sites which serve as points of initiation of those molecular processes which terminate in the characteristic effects of thyroid hormone at a cellular level. These findings per se do not prove that such sites necessarily correspond to the specific nuclear T_3 sites described by us (36). Nevertheless, since limited capacity sites with the requisite high affinity can be demonstrated by in vivo techniques only in the nuclear fraction and since T_3 bound to the nuclear sites is known to exchange rapidly with plasma T_3 (33), it would appear reasonable as an operational hypothesis to identify the nuclear sites with the receptors as kinetically defined. The rapid exchange results in a relatively constant ratio between the plasma and nuclear concentrations of T_3 and allows the establishment of a saturating plasma concentration when the receptors are fully occupied. Other data supporting the nucleus as the site of initiation of thyroid hormone action have recently been summarized (37).

The studies presented here suggest that the decay of induced hepatic enzymes after cessation of hormonal stimulus proceeds with an apparent terminal $t_{\frac{1}{2}}$ of 2.8±0.6 days (SD). This decay in enzyme proceeds much more slowly than the fall in the plasma and tissue concentrations of T_3 which occurs with an average $t_{\frac{1}{2}}$ of only about 7 h (35). Whether the slow decay in the enzyme effect is a reflection of the intrinsic $t_{\frac{1}{2}}$ of the α-GPD and ME molecules or represents the effect of a long-lived rate-limiting intermediate as we have previously suggested (38) remains an unsettled issue. Recent studies in our laboratory with α-amantin (39) favor the role of a rate-limiting intermediate. In this connection, it should be emphasized that the appearance and disappearance rate of new enzyme induced by T_3 cannot be considered synonymous with enzyme synthesis or degradation. Measurement of enzyme turnover would require labeling of the enzyme molecule.

The slow onset in thyroid hormone effect after the administration of T_3 has been noted by many observers and is widely recognized as one of the distinguishing characteristics of this hormone. This appears only minimally related to the delay in distribution since if sufficiently large doses of T_3 are administered nuclear receptor sites can be saturated within 1 h after the intravenous administration of the hormone (34). Rather, the delay in the appearance of thyroid hormone effects can be attributed to two factors: (*a*) An absolute refractory period required for the necessary preliminary nuclear and extranuclear processing before the appearance of new protein. In the euthyroid animal this refractory period for α-GPD is 13.4 h and for malic enzyme 8.2 h. It appears at least theoretically possible that the longer time required for α-GPD represents the additional time necessary for the assembly of α-GPD in the inner mitochondrial membrane; (*b*) The period required from the first appearance of the enzyme to the time that peak levels are achieved. If the sites are fully occupied it is apparent that this interval is determined exclusively by the $t_{\frac{1}{2}}$ of the decay of enzyme effect. Thus, with a $t_{\frac{1}{2}}$ of 2.8 days, we would expect that an additional 2.8 days from the first appearance of enzyme are required to achieve half-maximal inducible levels. With an absolute refractory period of 13.4 h, the half-maximal levels of enzymes would be achieved 3.4 days after the i.v. injection of hormones and 90% of the maximal effects would be achieved after nuclear saturation for 9.9 days. It is apparent that definition of the analogous kinetic parameters for man would be of considerable interest in developing a rational program of replacement treatment in hypothyroid patients.

The studies reported here have been restricted largely to the kinetics of the induction of enzyme

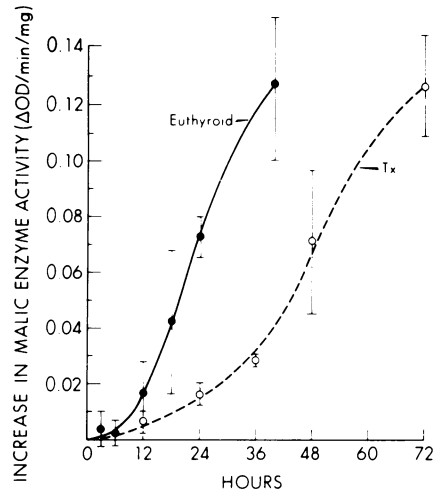

FIGURE 8 A group of euthyroid and thyroidectomized (Tx) animals were each injected with 3,000 μg T_3 at $t = 0$. Thyroidectomized rats killed at 72 h received a second i.v. injection at $t = 48$ h. This treatment regimen ensured full nuclear saturation for the duration of the experiment. Each point represents the mean increment in ME activity above either euthyroid or hypothyroid baseline levels in four animals. Bars represent±SD. Note the progressive acceleration in the rat of enzyme induction in the thyroidectomized group so that at 48 h the rate of induction of ME in thyroidectomized animals appears identical to that in euthyroid animals.

associated with full occupancy of the receptors. A detailed analysis of the occupancy-response relationships when the receptors are less than fully saturated is of substantial interest. Preliminary data from our laboratory (40) suggest that the relationship between nuclear occupancy and the rate of hepatic enzyme induction is nonlinear and that the signal for protein synthesis undergoes progressive amplification as more sites are occupied.

Our studies in hypothyroid rats suggest major differences between the mechanisms governing induction of ME and α-GPD by T_3. For α-GPD, the response characteristics of hypothyroid rats were almost identical to those observed in euthyroid animals. Full occupancy of the receptor for the same time intervals resulted in a comparable increase in enzyme activity. In contrast, for similar periods of full nuclear occupancy after T_3 injection, the increase in ME was markedly reduced in hypothyroid animals as compared to that in euthyroid rats. Only after the euthyroid status is attained does the rate of appearance of new enzyme approximate that observed in the euthyroid animal. The basis of the discrepancy between the response of α-GPD and ME is unclear. A possible explanation is that the hypothyroid rat lacks one or more cofactors necessary for the full response of ME but not for α-GPD. With a deficiency

of such co-factors, the rate of ME induction is submaximal in the hypothyroid animals. Decrease in food intake in hypothyroid rats could also be a factor. Correction of the hypothyroid state leads to a normalization of the rate of ME induction.

APPENDIX

According to the proposed model, the rate of change of enzyme activity at any time is described by the following equation:

$$\frac{dE}{dt} = R - \lambda E, \tag{1A}$$

where E is the induced enzyme activity at any time (t), R is the instantaneous rate of apparent enzyme production at full occupancy, and λ is the fractional rate of enzyme decline after the cessation of thyroid hormone stimulation (= ln 2/2.8 days = ln 2/67.2 h = 0.0103/h). Eq. 1A can be solved by standard techniques and from the limiting conditions that at $t = 0$, $E = 0$ (since E is defined as the induced rather than the absolute enzyme activity), it can be shown that

$$E = \frac{R}{\lambda}(1 - e^{-\lambda t}). \tag{2A}$$

From Eq. 1A we can calculate E at 36 and 24 h. Since there is an absolute lag period of 13.4 h for α-GPD and 8.2 h for ME, as demonstrated in the previous section, and since the equilibration between T_3 in plasma and the nucleus is rapid (33), it follows that changes in α-GPD accumulation observed at 24 h represent results of events occurring up to 10.6 h (=24−13.4 h) and for ME up to 15.8 h (=24−8.2 h). Similarly, the enzyme changes observed at 36 h reflect nuclear events terminating 13.4 and 8.2 h earlier for α-GPD and ME, respectively. Thus, from Eq. 2A we can calculate the theoretical ratio of induced α-GPD activity at 36 h and at 24 h from the expression:

$$\frac{E_{36}}{E_{24}} = \frac{1 - e^{-(0.0103)(36-13.4)}}{1 - e^{-(0.0103)(24-13.4)}} = 2.0. \tag{3A}$$

This value appears to be in good agreement with the observed ratio of 2.1. Similarly, for ME:

$$\frac{E_{36}}{E_{24}} = \frac{1 - e^{-(0.0103)(36-8.2)}}{1 - e^{-(0.0103)(24-8.2)}} = 1.7. \tag{4A}$$

Again, this value accords with the observed ratio of 1.6.

The apparent saturating plasma level of T_3 is also related to the lag time in response. Since the observed levels of α-GPD and ME represent the culmination of events which have occurred at 13.4 and 8.2 h, respectively, previously at the nuclear level and since the model assumes that the interaction of T_3 with its receptor results both in the induction of ME and α-GPD, it follows that:

$$\frac{[T_3]_{24,\,GPD}}{[T_3]_{24,\,ME}} = \frac{e^{8.2k}}{e^{13.4k}}, \tag{5A}$$

where $[T_3]_{24,\,GPD}$ represents the concentration of plasma T_3 at 24 h corresponding to the lowest dose required to elicit a maximal α-GPD response at 24 h and k is the fractional disappearance rate of T_3. Since $k = 0.092$ (35), it follows that

$$\frac{[T_3]_{24,\,GPD}}{[T_3]_{24,\,GPD}} = 0.62. \tag{6A}$$

This is in excellent agreement with the observed ratio of the saturating concentrations for these enzymes 12/20 = 0.6. Similarly, one can also calculate that the actual plasma concentration required to saturate the nuclear sites is 44.5 ng/ml. The internal consistency of these data and the agreement between calculated and experimentally observed values further support the underlying assumption of the model proposed.

ACKNOWLEDGMENTS

This investigation was supported by grants AM 15421-16, AM 19812-1, and CA 16463-02 from the National Institutes of Health.

REFERENCES

1. Lee, Y. P., A. E. Takemori, and H. A. Lardy. 1959. Enhanced oxidation of alpha-glycerophosphate dehydrogenase by mitochondria of thyroid-fed rats. *J. Biol. Chem.* **234:** 3051–3054.
2. Tepperman, H. M., and J. Tepperman. 1964. Patterns of dietary and hormonal induction of certain NADP-linked liver enzymes. *Am. J. Physiol.* **206:** 357–361.
3. Ruegamer, W. R., G. H. Newman, D. A. Richert, and W. W. Westerfeld. 1965. Specificity of the α-glycerophosphate dehydrogenase and malic enzyme response to thyroxine. *Endocrinology.* **77:** 707–715.
4. Lee, Y-P., and H. A. Lardy. 1965. Influence of thyroid hormones on L-α-glycerophosphate dehydrogenases and other dehydrogenases in various organs of the rat. *J. Biol. Chem.* **240:** 1427–1436.
5. Tepperman, H. M., and J. Tepperman. 1965. Effect of saturated fat diets on rat liver NADP-linked enzymes. *Am. J. Physiol.* **209:** 773–780.
6. Tarentino, A. L., D. A. Richert, and W. W. Westerfeld. 1966. The concurrent induction of hepatic α-glycerophosphate dehydrogenase and malate dehydrogenase by thyroid hormone. *Biochim. Biophys. Acta.* **124:** 295–309.
7. Lee, K-L., and O. N. Miller. 1967. Induction of mitochondrial α-glycerophosphate dehydrogenase by thyroid hormone: Comparison of the euthyroid and the hypothyroid rat. *Arch. Biochem. Biophys.* **120:** 638–645.
8. Sellinger, O. Z., and K-L. Lee. 1964. The induction of mitochondrial α-glycerophosphate dehydrogenase by thyroid hormone: Evidence for enzyme synthesis. *Biochim. Biophys. Acta.* **91:** 183–186.
9. Lee, K-L., and O. N. Miller. 1967. Studies in triiodothyronine-induced synthesis of liver mitochondrial α-glycerophosphate dehydrogenase in the thyroidectomized rat. *Mol. Pharmacol.* **3:** 44–51.
10. Hemon, P. 1967. L'activité alpha-glycérophosphate oxydase du foie de rat au cours du développement. *Biochim. Biophys. Acta* **132:** 175–178.
11. Hemon, P. 1968. Malate dehydrogenase (decarboxylating) (NADP) and α-glycerophosphate oxidase in the developing rat. *Biochim. Biophys. Acta.* **151:** 681–683.
12. Wise, E. M., Jr., and E. G. Ball. 1964. Malic enzyme and lipogenesis. *Proc. Natl. Acad. Sci. U. S. A.* **52:** 1255–1263.
13. Schapiro, S., and C. J. Percin. 1966. Thyroid hormone induction of α-glycerophosphate dehydrogenase in rats of different ages. *Endocrinology.* **79:** 1075–1078.
14. Ruegamar, W. R., W. W. Westerfeld, and D. A. Richert. 1964. α-glycerophosphate dehydrogenase response to thyroxine in thyroidectomized, thiouracil-fed and temperature-adapted rats. *Endocrinology.* **75:** 908–916.
15. Young, J. W. 1968. Effects of D- and L-thyroxine on

enzymes in liver and adipose tissue of rats. *Am. J. Physiol.* **214:** 378–383.
16. Murphy, G., and D. G. Walker. 1974. Enzyme synthesis in the regulation of hepatic "malic" enzyme activity. *Biochem. J.* **144:** 149–160.
17. Lee, K-L., O. Z. Sellinger, and O. N. Miller. 1967. Induction of mitochondrial α-glycerophosphate dehydrogenase by thyroid stimulating hormone and thyroid hormone analogues. *Proc. Soc. Exp. Biol. Med.* **126:** 169–173.
18. Li, J. J., C. R. Ross, H. M. Tepperman, and J. Tepperman. 1975. Nicotinamide adenine dinucleotide phosphate-malic enzyme of rat liver. Purification, properties, and immunochemical studies. *J. Biol. Chem.* **250:** 141–148.
19. Westerfeld, W. W., D. A. Rickert, and W. R. Ruegamar. 1965. New assay procedure for thyroxine analogues. *Endocrinology.* **77:** 802–811.
20. Goodridge, A. G. 1975. Hormonal regulation of the activity of the fatty acid synthesizing system and of the malic enzyme concentration in liver cells. *Fed. Proc.* **34:** 117–123.
21. Short, S. H., and W. R. Ruegamar. 1966. Effects of route of administration and dietary hemoglobin on the liver mitochondrial α-glycerophosphate dehydrogenase response to various thyroxine analogs. *Endocrinology.* **79:** 90–96.
22. Goodridge, A. G., and T. G. Adelman. 1976. Regulation of malic enzyme synthesis by insulin, triiodothyronine, and glucogen in liver cells in culture. *J. Biol. Chem.* **251:** 3021–3022.
23. Bücher, T., and M. Klingenberg. 1958. Wege des Wasserstoffs in der lebendigen Organisation. *Angew. Chem. Int. Ed. Engl.* **70:** 552–570.
24. Oppenheimer, J. H., H. L. Schwartz, and M. I. Surks. 1975. Nuclear binding capacity appears to limit the hepatic response to L-triiodothyronine (T_3). *Endocrinol. Res. Commun.* **2:** 309–325.
25. Surks, M. I., D. H. Koerner, and J. H. Oppenheimer. 1975. In vitro binding of L-triiodothyronine to receptors in rat liver nuclei. Kinetics of binding, extraction properties, and lack of requirement for cytosol proteins. *J. Clin. Invest.* **55:** 50–60.
26. Spindler, B. J., K. M. MacLeod, J. Ring, and J. D. Baxter. 1975. Thyroid hormone receptors, binding characteristics and lack of hormonal dependency for nuclear localization. *J. Biol. Chem.* **250:** 4113–4119.
27. Ochoa, S. 1955. "Malic" enzyme. *Methods Enzymol.* **1:** 739–753.
28. Hsu, R. Y., and H. A. Lardy. 1969. Malic Enzyme. *Methods Enzymol.* **13:** 230–235.
29. Surks, M. I., A. R. Schadlow, and J. H. Oppenheimer. 1972. A new radioimmunoassay for L-triiodothyronine. Measurements in thyroid disease and in patients maintained on hormonal replacement. *J. Clin. Invest.* **51:** 3104–3113.
30. Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall. 1951. Protein measurement with the Folin phenol reagent. *J. Biol. Chem.* **193:** 265–275.
31. Koerner, D., M. I. Surks, and J. H. Oppenheimer. 1974. *In vitro* demonstration of specific triiodothyronine binding sites in rat liver nuclei. *J. Clin. Endocrinol. Metab.* **38:** 706–709.
32. Samuels, H. H., J. S. Tsai, J. Casanova, and F. Stanley. 1974. Thyroid hormone action: In vitro characterization of solubilized nuclear receptors from rat liver and cultured GH_1 cells. *J. Clin. Invest.* **54:** 853–865.
33. Oppenheimer, J. H., H. L. Schwartz, D. Koerner, and M. I. Surks. 1974. Limited binding capacity sites for L-triiodothyronine in rat liver nuclei. Nuclear-cytoplasmic interrelationships, binding constants, and cross-reactivity with L-thyroxine. *J. Clin. Invest.* **53:** 768–777.
34. Oppenheimer, J. H., H. L. Schwartz, and M. I. Surks. 1974. Tissue differences in the concentration of triiodothyronine nuclear binding sites in the rat: Liver, kidney, pituitary, heart, brain, spleen and testis. *Endocrinology.* **95:** 897–903.
35. Goslings, B., H. L. Schwartz, W. Dillmann, M. I. Surks, and J. H. Oppenheimer. 1976. Comparison of the metabolism and distribution of L-triiodothyronine and triiodothyroacetic acid in the rat: A possible explanation of differential hormonal potency. *Endocrinology.* **98:** 666–675.
36. Oppenheimer, J. H., D. Koerner, H. L. Schwartz, and M. I. Surks. 1972. Specific-nuclear triiodothyronine binding sites in rat liver and kidney. *J. Clin. Endocrinol. Metab.* **35:** 330–333.
37. Oppenheimer, J. H. 1975. The initiation of thyroid hormone action. *N. Engl. J. Med.* **292:** 1063–1068.
38. Oppenheimer, J. H., M. I. Surks, and H. L. Schwartz. 1972. 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. *J. Clin. Invest.* **51:** 2796–2807.
39. Dillmann, W., H. L. Schwartz, E. Silva, M. I. Surks, and J. H. Oppenheimer. 1975. Effect of alpha-amanitin on the induction of hepatic enzymes by triiodothyronine. Excerpta Med. Int. Congr. Ser. 378. *Thyroid Res.* 326–330.
40. Oppenheimer, J. H., E. Silva, H. L. Schwartz, M. I. Surks, and N. Gutfeld. 1976. Nonlinear relationship between hepatic enzyme response and nuclear occupancy by triiodothyronine (T_3): Possible role of an amplifying process in the production of "toxic" manifestations by thyroid hormone. Program of 58th Annual Meeting of the Endocrine Society. (Abstr. 90) 101.