ington, except that its rare known manifestations seem to be confined mainly to the western margin of the Columbia Plateau. Apparently the Early Boreal Tradition never gained here the importance it had for some 5500 years in the cultural development in British Columbia's interior.
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41. I thank the National Museum of Man (Ottawa), the British Columbia Provincial Government, the University of British Columbia Committee on Research, the Vancouver Foundation, and the late H. R. MacMillan for supporting part of my own field research in British Columbia; Mr. and Mrs. E. Manis (owners of the mastodon site) and R. D. Daugherty, C. Gustafson, and D. Gilbow (Washington State University Archaeological Research Center) for permission to examine the site and findings; and R. L. Carlson (director of the present Namu project) for permission to participate in the excavations of some of the earliest levels of the site. I particularly thank C. Gustafson for relaying the latest information on the age of the Manis mastodon site.

Thyroid Hormone Action at the Cellular Level

J. H. Oppenheimer

Summary. A large body of circumstantial evidence suggests that the basic unit of thyroid hormone action is the triiodothyronine nuclear receptor complex. This complex stimulates the formation, directly or indirectly, of a diversity of messenger RNA (mRNA) sequences. A generalized increase in mRNA as well as a disproportionate increase in a limited number of RNA sequences have been demonstrated. Regulation of thyroid hormone effects may be carried out largely at a local cellular level. Highly selective alterations in sensitivity to the triiodothyronine nuclear receptor complex may occur at specific target genes. Metabolic factors and hormones participate in such regulation. In a given tissue, alterations in the total number of receptor sites has not been shown to be useful as an index of thyroid hormone response, and local modulation of the response to the triiodothyronine receptor complex by a variety of factors other than triiodothyronine may be carried out at a postreceptor level.

The mechanism of action of the thyroid hormones triiodothyronine (T_3) and thyroxine (T_4) is of considerable interest in part because of the amazing diversity of thyroid hormone effects. These agents influence the metabolism of almost every class of foodstuff. They exert profound effects on many enzymes and on almost all organ systems, and they play an integral role in the complex biological processes involved in growth and differentiation (*1*). Since the studies of Magnus-Levy in 1895 (*2*), the action of thyroid hormones has also been traditionally linked with an augmentation in respiration. Recently, Edelman and coworkers (*3*) focused further attention on this association by showing that a large proportion of the T_3-induced increase in oxygen consumption in tissue slices is due to a stimulation of the sodium pump. This in turn is brought about by the hormonal induction of membrane-linked sodium and potassium-dependent adenosinetriphosphatase (*4*). In the intact animal, however, the contribution of the sodium pump to the T_3-induced increment in oxygen consumption may not be as high as in surviving slices (*5*). Regardless of these considerations, it is apparent that the multiple actions of thyroid hormone cannot be understood exclusively in terms of increased respiration.

The recent description of specific nuclear binding sites for thyroid hormone (*6*) has stimulated renewed interest in the initiating mechanism and has provided a potentially convenient method for investigating this problem at the molecular level. In this article I review recent progress in this area. First, I discuss the biochemical, biophysical, and binding characteristics of the specific nuclear T_3 sites and the kinetics relationship in intact animals between T_3 bound to these sites and T_3 bound to cytoplasmic and plasma proteins. Second, I summarize the evidence that supports, but does not necessarily prove, the concept that nuclear sites play a role as receptors in the initiation process. Last, I discuss some general principles and characteristics governing thyroid hormone action at the cellular level. These include (i) specificity of T_3 action at individual tissue loci, (ii) multifactorial and multihormonal modification of the T_3-gene interaction, and (iii) local cellular control of thyroid hormone action. These principles may be helpful in clarifying the complex relations between plasma hormone concentration and tissue indices of thyroid hormone action under various pathophysiological states.

Nuclear Binding Sites

Specific tissue binding sites for T_3 were first identified during kinetic studies in the rat designed to elucidate the relation between iodothyronine bound to plasma and to tissues (*7*). In experiments with tracer quantities of radioactively labeled T_4, the partitioning of T_4 between unfractionated tissue and plasma was not substantially influenced by the total dose of T_4 injected. Similarly, when radio-

The author is a professor in the Department of Medicine at the University of Minnesota, Minneapolis 55455. This article is adapted from the Edwin B. Astwood Lecture delivered to the 60th Annual Meeting of the Endocrine Society, 14 June 1978 in Miami Beach, Florida.

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actively labeled T_3 in tracer quantities was injected together with unlabeled T_3, the distribution of T_3 between plasma on the one hand and whole brain, kidney, and liver on the other did not vary greatly with the total dose of T_3 injected. In contradistinction, however, increasing doses of T_3 resulted in a precipitous decrease in the ratio of T_3 bound to anterior pituitary to T_3 in the plasma, a finding which pointed to specific T_3 binding sites in this tissue.

The possibility was considered that other tissues might also harbor specific T_3 binding sites, but that the number of such sites might be too small a proportion of the total exchangeable T_3 to allow detection in unfractionated tissue (*6*). Accordingly, subcellular fractionation of liver and kidney was performed 1/2 hour and 3 hours after rats were injected intravenously with ^{125}I-labeled T_3 in tracer quantities together with increasing quantities of unlabeled T_3. The percentage of radioactive T_3 in the total tissue that was bound to nuclei purified by centrifugation through 2.4*M* sucrose decreased progressively with increasing T_3. The ^{125}I-labeled T_3 displaced from the nuclei was distributed in a proportional fashion among the other subcellular fractions according to the law of mass action. These findings thus demonstrated specific binding sites for T_3 in rat liver and kidney nuclei. Similar experiments with labeled and unlabeled T_4 showed only a barely perceptible decline in the percentage of radioactive T_4 bound to nuclei with increasing doses of injected T_4. These were the first indications that the nuclear iodothyronine binding sites exhibited a substantially higher affinity for T_3 than for T_4.

Compartmental relationships of T_3 bound to nuclei, cytoplasm, and plasma were defined in the experiments illustrated in Fig. 1A (*8*). After tracer quantities of radioactive T_3 were injected intravenously, maximum values of plasma and cytoplasmic T_3 were established within the first 5 minutes. The rapid parallel decrease in T_3 radioactivity in both compartments illustrated the rapid interchange of iodothyronine between tissue and plasma as demonstrated previously (*9*). In the nucleus, radioactivity increases, reaches maximum 1/2 hour after injection, and thereafter decreases. The rate of change of radioactivity begins to approximate that observed in the cytoplasmic curve within 2 to 3 hours after injection. On the basis of these data and the known metabolic clearance rates of T_3, it was possible to calculate that virtually all of the T_3 delivered to the nucleus was returned to the cytoplasm as T_3 (*8*). Kinetic data suggesting a totally reversible process were supported by experiments in vitro that showed that T_3 was not metabolized when added to a nuclear suspension together with multiple cofactors (*10*).

Experiments in vivo (*11*) also allowed an estimation of the rate at which T_3 dissociates from the nucleus (Fig. 1B). Tracer T_3 was injected into animals and 1/2 hour later a loading dose of T_3 was administered, thus effectively blocking the reuptake of labeled T_3 by the nucleus. Serial measurements in groups of animals killed at progressive intervals indicated that 50 percent of the T_3 was dissociated from the nucleus in approximately 15 minutes ($t_{1/2}$ = 15 minutes). This is rapid in comparison to the rate at which T_3 is irreversibly cleared from plasma ($t_{1/2}$ = approximately 6 hours) (*12*).

The nuclear content of T_3 can also be measured in vivo (*8*). The nuclear to plasma (N/P) ratio is determined in groups of animals killed 1/2 hour after the injection of tracer T_3 together with increasing doses of unlabeled T_3. The 1/2-hour value was chosen for liver because the specific activity of the nuclear T_3 is

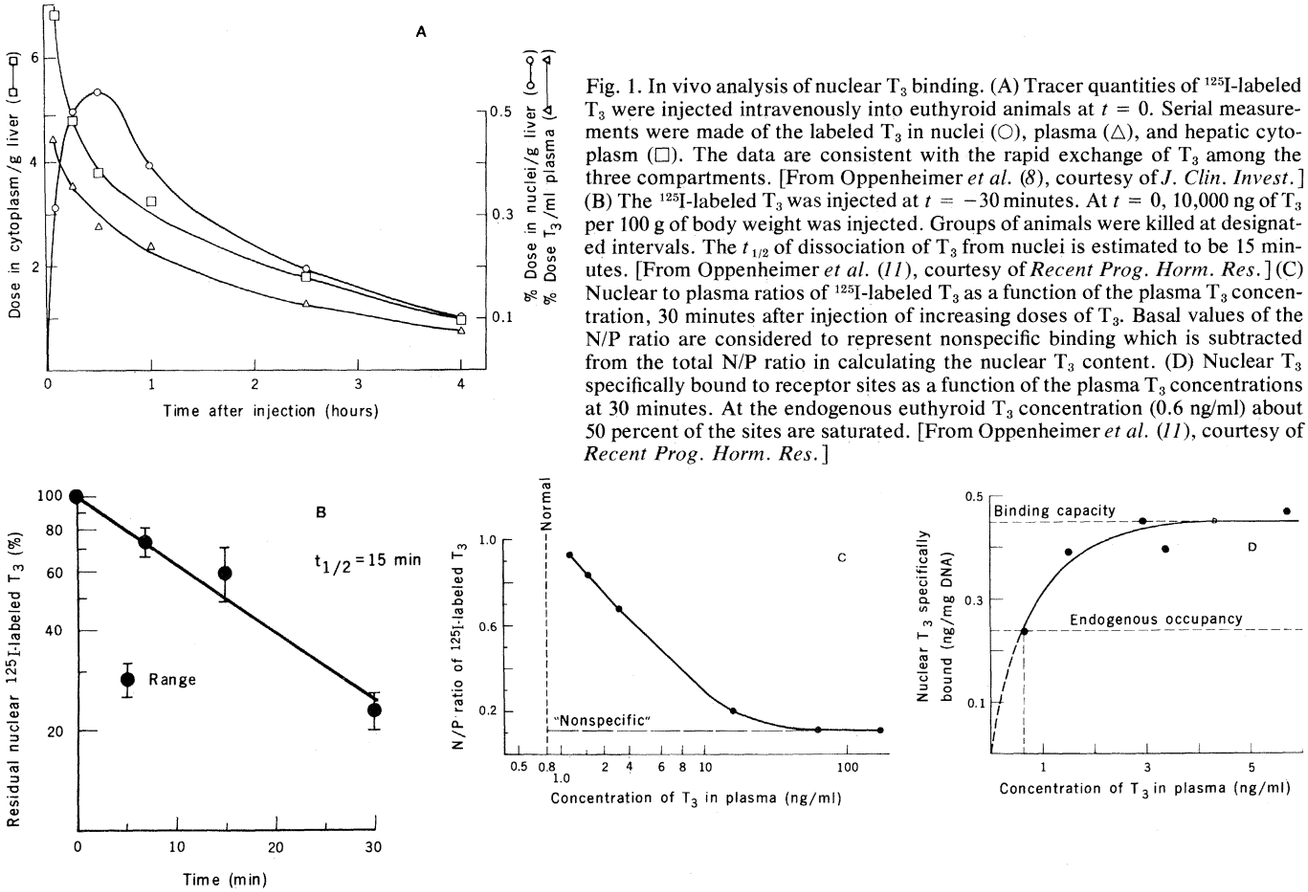


Fig. 1. In vivo analysis of nuclear T_3 binding. (A) Tracer quantities of ^{125}I-labeled T_3 were injected intravenously into euthyroid animals at t = 0. Serial measurements were made of the labeled T_3 in nuclei (○), plasma (△), and hepatic cytoplasm (□). The data are consistent with the rapid exchange of T_3 among the three compartments. [From Oppenheimer *et al.* (*8*), courtesy of *J. Clin. Invest.*] (B) The ^{125}I-labeled T_3 was injected at t = −30 minutes. At t = 0, 10,000 ng of T_3 per 100 g of body weight was injected. Groups of animals were killed at designated intervals. The $t_{1/2}$ of dissociation of T_3 from nuclei is estimated to be 15 minutes. [From Oppenheimer *et al.* (*11*), courtesy of *Recent Prog. Horm. Res.*] (C) Nuclear to plasma ratios of ^{125}I-labeled T_3 as a function of the plasma T_3 concentration, 30 minutes after injection of increasing doses of T_3. Basal values of the N/P ratio are considered to represent nonspecific binding which is subtracted from the total N/P ratio in calculating the nuclear T_3 content. (D) Nuclear T_3 specifically bound to receptor sites as a function of the plasma T_3 concentrations at 30 minutes. At the endogenous euthyroid T_3 concentration (0.6 ng/ml) about 50 percent of the sites are saturated. [From Oppenheimer *et al.* (*11*), courtesy of *Recent Prog. Horm. Res.*]

equal to that of plasma T_3 at that time. Figure 1C illustrates the N/P ratio as a function of the terminal plasma T_3 concentration determined by radioimmunoassay. The N/P ratio falls progressively until minimum values are observed when the concentration of T_3 is in excess of 30 nanograms per milliliter. At this level the N/P ratio is considered to reflect nonspecific binding. The product of the plasma T_3 concentration and the corrected N/P (= total N/P minus nonspecific N/P) yields an estimate of the nuclear T_3 content. As shown in Fig. 1D, a sharp plateau value can be generated with increasing doses of T_3. Of interest is that about 50 percent of the nuclear sites are occupied when tracer doses only are administered to a euthyroid animal.

From these studies in vivo, it was possible to calculate a number of important nuclear binding constants. Thus, the hepatic nuclear binding capacity was found to average approximately 1 picomole per milligram of DNA (*13*). If one assumes that there are 8 picograms of DNA per nucleus (*14*), one can estimate from Avogadro's number that there are some 4500 sites per nucleus. Since the free T_3 concentration is approximately $6 \times 10^{-12}M$, the physiological association constant of nuclear binding is approximately $4.7 \times 10^{11}M^{-1}$ (*8*). Furthermore, competition experiments in which both unlabeled T_3 and unlabeled T_4 are used to determine the relative displacement of radioactively labeled T_3 show that T_3 is bound some 10- to 20-fold more firmly than is T_4 (*8*). From the 15-minute $t_{1/2}$ of T_3 dissociation from the nuclear sites, it is possible to calculate a backward rate constant of 7.7×10^{-4} per second. This value, together with the association constant, yield a forward rate constant of $3.6 \times 10^{8}M^{-1}$ sec^{-1}, a high value suggesting that there are few barriers to penetration of the nucleus by T_3.

Analogous experiments in vivo, summarized in Table 1, have also allowed the assessment of binding characteristics of other tissues including kidney, heart, anterior pituitary, brain, spleen, and testis (*13*). The highest concentrations of binding sites were found in anterior pituitary and the lowest, in testis. Barker and Klitgaard (*15*) had reported that brain, spleen, and testis were not responsive to thyroid hormone administration, at least by the criterion of alteration of oxygen consumption with thyroid status (*15*). Extremely low concentrations of nuclear binding sites in testis and spleen provide a possible explanation for the apparent lack of responsivity of these tissues to thyroid hormones, but the intermediate abundancy of nuclear receptors in brain

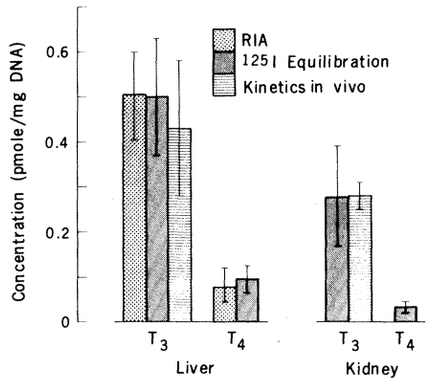


Fig. 2. Comparison of the nuclear T_3 and T_4 concentrations measured by radioimmunoassay, ^{125}I equilibration, and kinetic techniques in vivo. Bars indicate ± standard deviation. From 85 to 90 percent of nuclear iodothyronine appears to be in the form of T_3, and the rest in the form of T_4. [From Surks and Oppenheimer (*18*), courtesy of *J. Clin. Invest.*]

presents a problem in interpretation.

These data also facilitated an estimation of the percentage of total cellular T_3 bound to specific receptor sites (*13*). The results in Table 1 indicate that with the exception of the anterior pituitary, less than 15 percent of the total exchangeable T_3 is specifically bound to the nucleus. In the anterior pituitary, however, fully 52 percent of total exchangeable T_3 is specifically bound. This explains why specific binding in unfractionated anterior pituitary samples could be demonstrated (*7*). Our data also allowed calculation of the percentage of the total receptors that are occupied under physiological conditions. Despite the marked variation in the number of binding sites per milligram of DNA, the percentage of specific binding sites occupied in euthyroid animals (35 to 50 percent) does not change greatly from one tissue to another (*13*). Since T_3 in these tissues is presumed to be in equilibrium with plasma T_3, the effective association constant of nuclear binding in these tissues should also be similar. This in turn suggests the possibility that the sites are identical, a suspicion that has been strengthened by subsequent experiments demonstrating similar physicochemical characteristics of receptors from brain and liver (*16*). More recently, Silva and Larsen (*17*) have confirmed a 50 percent occupancy in the liver and kidney, but have claimed that 80 percent of the nuclear sites in the pituitary are occupied under physiological conditions. They attributed the higher fractional occupancy in the pituitary to local T_4 to T_3 conversion within this tissue. The T_3 arising from local conversion cannot be detected by kinetic methods relying exclusively on the injection of ^{125}I-labeled T_3 and the measurement of nonradioactive T_3 in the plasma. The newer findings, however, do not negate the concept that the association constants of nuclear sites in various tissues is similar.

The amounts of T_4 and T_3 in hepatic and renal nuclei have also been evaluated by radioimmunoassay and by paper chromatography of ethanolic extracts in animals equilibrated with ^{125}I (Fig. 2) (*18*). Results obtained with these techniques were compared to simultaneous estimates of nuclear content of T_3 by means of the in vivo displacement methods described above. Excellent agreement was found among all three methods. Approximately 85 percent of the total iodothyronine specifically bound to nuclei was in the form of T_3, and the remaining 15 percent in the form of T_4. Of special interest was that isotopic equilibration studies provided no evidence for the presence of specifically bound iodothyronines other than T_4 and T_3. If nuclear sites are the only point of initiation of thyroid hormone action and if, as it appears likely, equal concentrations of iodothyronine specifically bound for a given period yield equal effects, it follows that 85 percent of hormonal activity in the euthyroid rat liver and kidney is due to T_3. The remaining 15 per-

Table 1. Comparison of nuclear binding of T_3 in various rat tissues. Data from Oppenheimer *et al.* (*13*).

Tissue	Binding capacity (ng/mg DNA)	Estimated number of sites per nucleus*	Specific sites occupied in euthyroid rat (%)	Exchangeable tissue T_3 specifically bound to nuclear sites (%)
Liver	0.61	4515	47	12.9
Brain	0.27	1998	38	13.5
Heart	0.40	2960	44	15.4
Spleen	0.018	133	50	13.0
Testis	0.0023	17	-	3.0
Kidney	0.53	3923	35	9.0
Anterior pituitary	0.79	5847	48	52.6

*Estimated on basis of the assumption that each nucleus contains on the average 8 pg of DNA (*14*).

Table 2. Findings compatible with a receptor role for nuclear T_3 binding sites.

1. Low capacity, high affinity characteristics (*6, 8*)
2. Identity as nuclear nonhistone proteins (*23*)
3. Apparent universality of distribution in thyroid hormone-responsive tissues (*13*)
4. Familial tissue resistance to thyroid hormones associated with defective nuclear binding of T_3 (*32*)
5. T_3 analogs: Correlation between nuclear binding and thyromimetic effect (*19*)
6. Occupancy-response relationships
   (i) Limitations imposed by full nuclear occupancy in enzyme induction in the intact animal (39)
   (ii) Polymerase activity (*43, 45*), nuclear kinase activity (*46*), alterations in nuclear proteins (*47*)
7. Increase in nuclear activity after T_3 administration
   (i) Formation rate and steady-state concentration of poly(a)-containing RNA (*44, 51*),
   (ii) Polymerase activity (*43, 45*), nuclear kinase activity (*46*), alterations in nuclear proteins (*47*)
   (iii) Specific product mRNA; hepatic α-2u globulin (*48, 49*) and pituitary growth hormone (*50*)

cent can be attributed to the intrinsic hormonal activity of T_4, that is, the biologic effect of T_4 which is not contingent on its peripheral conversion to T_3. Moreover, it appears that further degradation of T_3 does not result in the formation of thyroactive substances that contribute significantly to the maintenance of normal thyroid hormonal status.

Although the techniques for measuring binding capacity and affinity in vivo probably provide the most reliable physiological data, the techniques are expensive and cumbersome because of the large number of animals that must be used. Techniques for measuring binding constants in isolated nuclei have been developed independently by three laboratories (*10, 19, 20*). Moreover, an interaction in vitro of T_3 with solubilized nuclear extracts first demonstrated by Samuels *et al.* (*21*) can be effectively used in the measurement of nuclear binding (*21, 22*).

Although efforts to isolate the binding sites have not been successful to date, the biochemical and biophysical properties of the receptors have been partially characterized. The sites are associated with the chromatin fraction, and their characteristic extraction at an alkaline *p*H and their lack of preferential proteolysis with trypsin indicate that they belong to the class of nonhistone chromatin proteins (*23*). Latham *et al.* (*24*) have provided the most up-to-date report of their physicochemical characteristics. The Stokes radius of the receptor molecule is estimated to be 35 angstroms, the molecular weight 50,500, and the frictional ratio 1.4, a value that suggests slight asymmetry of the receptor molecule.

A considerable body of data indicates that a preliminary interaction of T_3 with cytosolic receptor sites is not required for penetration of the nucleus (*10, 25*). T_3 reacts reversibly with cytosolic binding proteins, but dissociates from these proteins prior to entering the nucleus. In contrast to the nuclear binding sites, cytosolic proteins exhibit a high capacity and low affinity. Moreover, the relative avidity with which cytosolic proteins bind various thyroid hormone analogs differs sharply from the relative binding spectrum by nuclear sites. Last, a cytosolic component is not required for the interaction of T_3 in vitro with nuclei obtained from hypothyroid animals in a strictly aqueous medium. For T_3, therefore, there appears to be no counterpart to the cytosolic receptors that are essential for the translocation of steroid hormones to the nucleus.

Initiation of Hormone Action at the Nuclear Site

The evidence summarized in Table 2 supports the concept that thyroid hormone sites act as true receptors involved in the initiation of thyroid hormone action. Such evidence, however, cannot be considered to be conclusive. Definitive proof of the relevance of these sites would require a totally reconstituted transcriptional system in vitro, in which the addition of the T_3 receptor complex could be shown to result in the generation of specific messenger RNA (mRNA) sequences coding for T_3-inducible proteins. Unfortunately, such experiments are not yet technically feasible.

Items 1 to 3 in Table 2 in support of a nuclear site of initiation have already been cited—that is, nuclear T_3-binding sites have attributes generally associated with receptors, namely, a low capacity and high affinity; they belong to the class of nonhistone proteins that are believed to play an important role in the regulation of gene expression (*26*); and they are present in all rat tissues examined to date which are known to be responsive to thyroid hormone. In addition to the tissues already discussed, recent studies have indicated that the receptor sites are found in rat lung and may participate in the synthesis of lung surfactants (*27*). Also, T_3 receptors have been demonstrated in other species including the mouse (*28*), tadpole (*29*), and man (*30*).

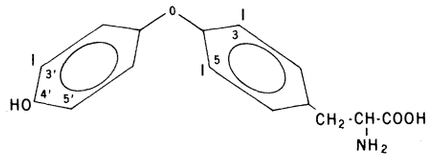
Because of the potential clinical importance of measurements of binding sites it would be useful to quantitate human nuclear binding sites in man. Tsai and Samuels (*30*) first demonstrated nuclear binding in lymphocytes from hypothyroid patients. The binding affinity determined in vitro was comparable to that observed with the nuclei of rat pituitary GH_1 tumor cells grown in tissue culture and in rat hepatocytes derived from intact animals. Difficulty, however, has been encountered in measuring nuclear receptors in lymphocytes from euthyroid individuals. This is probably related to the low concentration of such receptor sites in lymphoid tissue and the relatively high fractional nuclear occupancy in the euthyroid state. Recently, however, nuclear binding was examined in human hepatic and renal nuclei (*31*) and compared to nuclear binding in homologous rat tissues. Renal specimens were obtained at surgery, and human liver was obtained from cadavers maintained for transplant purposes. The physicochemical characteristics of nuclear binding sites from both species were remarkably similar. Chromatography on diethylaminoethyl Sephadex A-50, sedimentation profiles on 5 to 20 percent sucrose gradients, affinity constants determined in whole nuclei and solubilized receptors, as well as the relative nuclear binding spectrum of thyroid hormone analogs were indistinguishable in both species. Of interest was the observation that the binding capacity per milligram of DNA was similar in homologous tissues. Estimates based on nuclear binding capacity and the total content of nuclear T_3 as determined by radioimmunoassay of ethanolic extracts suggest that, as in the rat, approximately 50 percent of the nuclear sites in human tissues are occupied in the euthyroid state. These considerations support the concept that the basic kinetic principles governing the interrelations of plasma and nuclear T_3 in the rat are applicable to man.

The fourth item listed in Table 2 is based on clinical observations. Studies by Bernal *et al.* (*32*) have indicated that lymphocytes from a patient with tissue resistance to thyroid hormone display defective nuclear binding with an apparent marked diminution in affinity. Alterations were also observed in nuclear T_3

binding of fibroblasts cultured from this patient.

Perhaps one of the most important considerations underpinning the nuclear initiation hypothesis is item 5 in Table 2, namely, the correlation between nuclear binding of analogs and their estimated thyromimetic effects. When account is taken of the known metabolic and distributive characteristics of such analogs, an almost perfect correlation can be drawn between hormonal activity and nuclear binding as determined in the whole animal (*33, 34*), isolated nuclei (*19, 20*), or solubilized sites (*21, 35*). On the basis of some 40 analogs thus far examined (*19*), the following general principles can be inferred with respect to the relation between structure, nuclear binding, and biologic activity (Fig. 3). For maximum binding and biologic activity, 4′ hydroxylation is required as well as 3′ substitution either by a halogen or by a bulky nonhalogen such as an isopropyl group. Compounds in which the rings are constrained in a distal position are invariably more active by both criteria than compounds in which these rings are in a proximal conformation. Compounds in which there is a single substitution in the 3′ position are more active than compounds in which substitutions occur both in the 3′ and 5′ positions. Substitution of the ether oxygen by a methylene or sulfur group results in active compounds both by nuclear displacement as well as by biologic activity criteria. A marked decrease in activity is noted after the removal of iodine from the inner ring. Thus, reverse T_3 is almost completely inactive. Nevertheless, halogen-free compounds in which methyl groups have been substituted for all the iodine groups show low but definite residual thyromimetic and nuclear displacement activity.

Exceptions to the correlation between nuclear binding and thyromimetic activity can be explained either by the metabolic or distributional characteristic of the analog. Thus, T_4, which exhibits low nuclear but considerable thyromimetic activity, derives most of its biologic potency from peripheral conversion to T_3 (*36*). The acetic acid analog of T_3, triac, which is bound more strongly to nuclear sites than is T_3 has only 1/6 to 1/3 of the biologic activity of T_3. This discrepancy can be explained by the fact that triac is more tightly bound to plasma proteins and more rapidly metabolized than is T_3 (*33, 37*). Thus, daily injections of small amounts of triac result in only a comparatively short residence time of triac on the nuclear site. Duration of contact between analog and receptor is an important determinant



1. Phenolic ring requirements
   4′ OH group
   3′ Substitution: halogen or nonhalogen
2. Activity of distal > prox. conformation
3. Activity of 3′ > 3′, 5′ substitution
4. Ether linkage: Substitution of O by (a) methylene or (b) sulfur results in active compounds
5. Decreased activity after substitution of halogens in inner ring
6. Halogen-free compounds have low but significant activity

Fig. 3. General requirements for T_3 analog binding to nuclear sites and biologic activity. Based on analysis of 40 compounds by Koerner *et al.* (*19*).

of analog potency, because some time is required for the accumulation of a long-lived intermediate that is essential in the generation of the biologic test parameter applied in the bioassay (*38*).

A sixth finding (Table 2) that supports the relevance of the nuclear sites in the initiation of thyroid hormone action is the correlation between nuclear occupancy and biologic response. The mitochondrial enzyme α-glycerophosphate dehydrogenase (α-GPD; E.C. 1.1.99.5) and the cytosolic enzyme malic enzyme (E.C. 1.1.1.40) are widely used as indices of thyroid hormone effects in rat liver. The hepatic response appears to be limited by the occupation of sites of limited capacity containing T_3 that is in rapid equilibrium with plasma stores of hormone (*39*). Samuels and co-workers (*40*) have investigated the relation between T_3 occupancy and the rate of synthesis of growth hormone by GH_1 cells. In contrast to the lack of influence of thyroidal status on the steady-state concentration of nuclear sites in the intact animal (*41*), Samuels *et al.* (*40*) have shown a dose-dependent depletion of T_3 receptors in GH_1 cells grown in tissue culture medium in the presence of varying concentrations of T_3. Nevertheless, even with a maximum concentration of T_3 in the culture medium, only about 50 percent of the sites were depleted. On this basis, Samuels has classified GH_1 receptors into depletable and nondepletable subsets. Occupation of the depletable sites appears linearly related to the rate of growth hormone synthesis. The reason for the difference between the steady-state stability of hepatic receptors in the intact animal and the T_3 dose-dependent depletion of receptor sites in GH_1 cells remains unexplained. Both sets of data, however, support the quantitative relation between nuclear occupancy and response.

If T_3 interacts with nuclear sites in a biologically meaningful fashion, one would anticipate characteristic biochemical changes in the nuclei after T_3 binding by the receptor (item 7 in Table 2). In fact, the first suggestion that T_3 exerts its effect through stimulation of nuclear processes was made by Tata *et al.* (*42, 43*) in the mid-1960's on the basis of observations of early nuclear events after the injection of T_3 into thyroidectomized animals. These data have now been widely confirmed and extended. An increase in the rate of formation of polyadenylate [poly (A)]-containing heterogeneous nuclear RNA (hnRNA) and mRNA (*44*) has been reported, as well as an increased nuclear RNA polymerase activity (*43, 45*) and nuclear protein kinase activity (*46*), and alterations in composition of nuclear proteins (*47*). In addition, two specific mRNA species coding for T_3-inducible proteins have been shown to increase in response to T_3: α-2u globulin, an exportable hepatic protein of male rats (*48, 49*), and pituitary growth hormone (*50*).

Dillmann *et al.* (*44*) confirmed the early observations of Tata indicating increased incorporation of labeled orotic acid into nuclear RNA after T_3 injection (*43*). Moreover, they excluded the possibility that this phenomenon was due to an alteration in specific activity of precursor uridine triphosphate by direct measurements of specific activity. The rate of formation of poly (A)-containing RNA and its precursor hnRNA was also measured. Euthyroid animals showed an approximately 60 percent increase in formation of both classes of poly (A)-containing polynucleotides. A substantial increase in the rate of orotic acid incorporation into nuclear RNA was observed as early as 6 hours after injecting hypothyroid animals with doses of T_3 designed to saturate the nuclear sites for the period of the experiment. Normalization in the rate of formation of mRNA and its precursor was apparent at 24 hours. The significance of sequential changes in RNA polymerase activity remains an enigma. Although RNA polymerase I activity, which is responsible for the assembly of ribosomal RNA, increases as early as 12 hours, significant augmentation in RNA polymerase II activity, which is responsible for the assembly of mRNA precursor, is not apparent until 24 hours after injection (*45*). Either present methods of detection of RNA polymerase II are

too crude to allow early detection or else polymerase activity is not rate-limiting in mRNA formation.

Recently, Towle *et al.* (*51*) have shown a 60 percent increase in total mRNA per milligram of DNA in the livers of euthyroid rats compared to the mRNA content in hypothyroid rats. This increase is comparable in magnitude to the augmentation in the rate of mRNA and hnRNA formation noted above. These data also suggest that the rate of fractional turnover of mRNA is not influenced by the thyroidal status of the animal, at least in the transition from the hypothyroid to the euthyroid state. No alterations, however, could be detected in the composition of the poly(A)-containing mRNA population in livers from hypothyroid and euthyroid animals either by an analysis of mRNA size as determined by centrifugation through sucrose gradients or by studies of the kinetics of hybridization of complementary DNA (cDNA) with mRNA from euthyroid and hypothyroid animals. In such hybridization studies, it was possible to estimate that there were approximately 11,000 different RNA sequences which by computer analysis could best be divided into three groups: those with sequences present in highest abundancy, that is, with the largest number of copies per sequence; those with an intermediate abundancy; and those with the lowest abundancy (Table 3). In euthyroid animals and in hypothyroid animals treated with T_3, the number of copies of each class was proportionately increased in comparison to the hypothyroid group; however, no significant shift occurred in the relative distribution of mRNA abundancy classes. These findings thus suggest that T_3 exerts a relatively generalized effect on the hepatic genome.

A selective increase in mRNA for α-2u globulin (*48*, *49*) and a disproportionate increase in the activities of α-GPD and malic enzyme with T_3 treatment would suggest that in addition to the relatively generalized effects noted, T_3 must stimulate a limited number of sequences in a selective fashion. Limitations inherent in the sequence complexity analysis will not allow detection of the stimulation of a few low-abundancy sequences of high complexity. It should be emphasized, however, that neither the general nor the specific mRNA stimulation by T_3 necessarily implies that T_3 influences the involved genes directly. The possibility that T_3 exerts its effect indirectly has not been excluded and should receive serious consideration. Thus one could postulate that an increase in mRNA for a particular gene is brought about by the product of another gene which is primarily stimulated by T_3. In the case of pituitary growth hormone, however, Samuels and co-workers (*52*) were not able to observe any increase in protein species other than growth hormone.

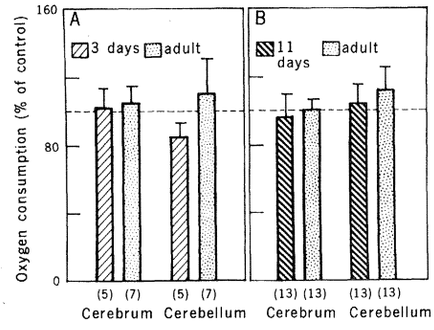


Fig. 4. Injection of T_3 in two dosage regimens [(A) 25 μg of T_3 per 100 g per day for 3 days and (B) 100 μg of T_3 per 100 g per day for 4 days] failed to elicit an increase in oxygen consumption either in the neonatal or in adult rat brain. Oxygen consumption was measured as microliters of oxygen consumed per minute per milligram of tissue. Bars indicate ± standard deviation of the mean. Numbers of experiments are shown in parentheses. The nuclear binding capacity of neonatal cerebrum approximates that of adult liver which responded to T_3 in simultaneous experiments with a 30 to 40 percent increase in oxygen consumption. [From Schwartz and Oppenheimer (*22*), courtesy of *Endocrinology*]

The evidence summarized here cannot be used to exclude the possibility that there are extranuclear sites of initiation of thyroid hormone action. A number of investigators have explored this possibility. A report (*53*) of high affinity, low capacity sites in solubilized mitochondrial extract has not been confirmed (*54*). Several investigators have also reported that thyroid hormones alter the rate of intracellular transport of certain compounds independent of any stimulation of protein synthesis (*55*). Nevertheless, on the basis of currently available evidence, it would appear that these systems operate optimally at T_3 concentrations above the range which characterizes biologic fluids, and a strict correlation between phenomena in vivo and in vitro have not been documented. Additional characterization of these systems would be desirable.

Table 3. Comparison of sequence complexity of poly(A)-RNA from the liver of euthyroid and hypothyroid animals. Calculations are based on the relative rates of hybridization of total poly(A)-RNA with cDNA and a determination of the total poly(A)-RNA content. The results indicate that the distribution of the three components of mRNA do not differ materially in liver from euthyroid and hypothyroid animals, but that the number of copies of each RNA sequence increases significantly in the euthyroid state. A relative generalized effect of T_3 on the genome is therefore suggested. [From Towle *et al.* (*51*)]

Component	Fraction of cDNA hybridized	Number of different sequences	Copies per cell
Liver from euthyroid animals			
1	27	5.7	18,600
2	37	350	430
3	29	11,300	10
Liver from hypothyroid animals			
1	27	8.5	7,500
2	40	400	240
3	26	9,800	6

Some General Principles of Thyroid Hormone Action

There are a number of patterns of thyroid hormone action that may provide additional insight into the mechanisms of these hormones. The recognition of such patterns may also be helpful in explaining the clinical paradox posed by the "low T_3 syndrome."

The principle of specificity in thyroid hormone action at its target site deserves emphasis. This is reflected as species specificity, tissue specificity, and specificity in the nuclear-occupancy response characteristics. It is well known, for example, that hepatic α-GPD fails to respond to T_3 in human (*56*) and guinea pig (*57*) liver, despite the fact that this enzyme is an excellent index of thyroid hormone action in the rat. Moreover, it is apparent that T_3 fails to stimulate either α-GPD or malic enzyme in brain (*58*); neither does T_3 augment enzyme activity in heart (*59*) despite the fact that the structure of malic enzyme appears to be identical in these tissues (*60*).

As mentioned previously, brain also fails to respond to T_3 with the characteristic increase in respiration. The finding of an intermediate number of nuclear receptor sites per milligram of DNA (*13*) has made it difficult to determine whether the failure to observe such responses can be related to a reduction in nuclear receptor sites. Recent studies by Schwartz and Oppenheimer with the developing rat (*22*) may have clarified this issue. In a study of the ontogenesis of brain receptors, a brisk increase in the concentration of T_3 nuclear receptors was noted from 2 days prior to birth to the second day after birth. At this time, the concentration of receptor sites per milligram of DNA as well as the content of receptors per gram of tissue is approximately equivalent to the corresponding

value in adult liver. Were the failure to respond to T_3 with increased oxygen consumption due solely to a diminished number of receptor sites one might anticipate that the neonatal brain, in contradistinction to the adult brain, would respond with a definite increase in oxygen consumption. In point of fact, a report to this effect was published in 1956 by Reiss *et al.* (*61*). Nevertheless, as indicated in Fig. 4, T_3 failed to increase oxygen consumption either in the neonatal or in the adult animal despite the use of multiple dosage regimens including the one used by Reiss *et al.* (*61*). Since many of the well-established T_3-induced changes in brain cytoarchitecture occur in rats within the first 14 days after birth, it is reasonable to assume that the neonatal brain is sensitive to T_3 and to conclude that oxygen consumption is not an appropriate parameter for measuring thyroid hormone action in this tissue. Each tissue appears to respond to T_3 in an entirely characteristic fashion.

The relation between nuclear occupancy and the rate of response also appears to vary with the specific parameters examined (Fig. 5). In the case of α-GPD and malic enzyme, three independent techniques have shown that the relation between nuclear occupancy (q) and the instantaneous rate of responsivity (R) is a highly nonlinear amplified function (*62*). With α-GPD and malic enzyme, it is apparent that when 50 percent of the sites are occupied, the rate of response is only approximately 1/16th that which occurs when all of the sites are fully saturated. On the other hand, when similar nonsteady-state analytic methods are used, an entirely different pattern emerges when the rate of accumulation of pituitary growth hormone is plotted as a function of nuclear occupancy (*63*). Although this function is sigmoid, the overall relationship is substantially more linear than the corresponding relation between hepatic nuclear occupancy and the rate of enzyme induction. When the pituitary nuclear sites are approximately 52 percent occupied, the pituitary responsivity is 50 percent of the maximum value. The relation between pituitary occupancy and response therefore is in essential agreement with the basic findings reported by Samuels *et al.* in the GH_1 tissue culture systems (*40, 52*).

A second general characteristic of T_3 action is the multihormonal and multifactorial control of T_3-gene interactions. Thus Roy and Dowbenko (*49*) have shown that the hormonal requirement for the production of the α-2u globulin mRNA includes not only T_3 but cortisol, 5α-dihydrotestosterone, and growth hormone as well. Similarly, Samuels *et al.* and Baxter and co-workers (*64*) have examined the synergistic interaction of cortisol and T_3 in the synthesis of the specific mRNA for growth hormone by GH_1 cells.

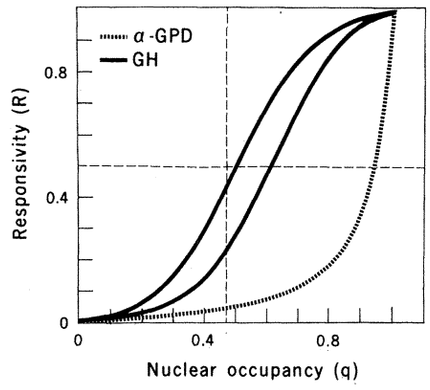
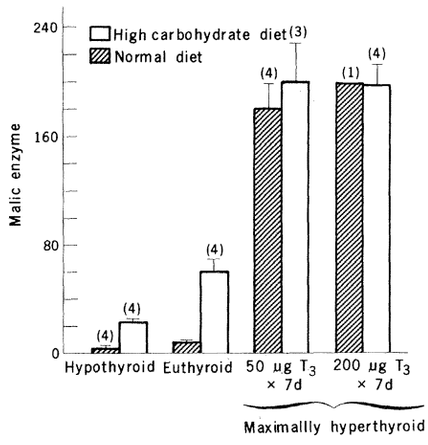


Fig. 5. Differences between the nuclear occupancy and responsivity relationships of hepatic mitochondrial α-GPD (*62*) and pituitary growth hormone accumulation (*63*). Responsivity is defined as the instantaneous rate of change of hepatic α-GPD or pituitary growth hormone (*GH*) accumulation. A highly amplified function characterizes the α-GPD response, whereas the growth hormone response is sigmoid in shape but substantially more linear than the α-GPD function. The dashed vertical line represents the normal euthyroid fractional occupancy and the dashed horizontal line, half-maximum responsivity. [From Coulombe *et al.* (*63*), courtesy of *J. Clin. Invest.*]

In addition, metabolic factors may modify the influence of thyroid hormone action on the expression of selected genes. The effect of partial hepatectomy, starvation, glucagon administration, and high carbohydrate administration on enzyme induction by T_3 has been examined. Dillmann *et al.* (*65–67*) have shown that partial hepatectomy results in: (i) a decrease in the number of T_3 receptors in liver; (ii) a reduction in the baseline amount of malic enzyme; (iii) a decrease in the increment in malic enzyme activity in response to a dose of T_3 designed to saturate the nuclear sites. In addition, it was shown previously by Leffert and Alexander (*68*) that after partial hepatectomy the concentration of plasma T_3 decreases. In contrast, the baseline concentration of α-GPD as well as the response of this enzyme to administration of T_3 is unimpaired or even slightly increased. In starvation, the behavior of malic enzyme, α-GPD, and nuclear T_3 receptor sites resembles the response pattern observed after partial hepatectomy (*65, 69*). The concentration of circulating T_3 also decreases (*70*). Experimentally induced diabetes in the rat is also accompanied by a diminution in nuclear receptor sites (*67*) as well as by a decrease in malic enzyme responsivity to T_3 administration (*71*). Since partial hepatectomy, starvation, and diabetes are all associated with increases in glucagon, the possibility that glucagon might mediate the observed alterations was considered. Administration of glucagon in what is generally considered to be a pathophysiological dose resulted both in a reduction in nuclear receptor concentration as well as in an inhibition of T_3-induced increases in malic enzyme (*66*).

Although glucagon in many ways mimics the effect of the other stimuli, insufficient quantitative data are available to conclude that glucagon is the only or even the predominant factor responsible for the observed changes in partial hepatectomy, starvation, and experimental diabetes. Moreover, it is not clear that the effect of glucagon in blocking enzyme induction is necessarily mediated by the observed reduction in receptor sites (*72*). Thus, if glucagon is infused at a constant rate of 5 μg per kilogram of body weight per hour for a period of 72 hours, the anticipated reduction in binding capacity is observed by 20 hours. A subsequent rebound in receptor concentration, however, was noted, possibly reflecting the recently documented "down regulation" of glucagon receptor sites (*73*). If T_3 is injected at 48 hours, while the glucagon infusion is still proceeding and after the T_3 receptor concentration has returned to normal, the malic enzyme response remains effectively blocked. Clearly, a reduction in nuclear receptor sites by glucagon cannot be considered to be the exclusive factor responsible for inhibiting the induction of malic enzyme, and the possibility that glucagon inhibits the expression of thyroid hormone action at a point beyond the receptor level must be considered. Whether such interaction occurs at a pretranslational or at a translational level remains to be determined (*74*).

These findings take on a special significance in view of many previously reported studies that indicate that both carbohydrates and thyroid hormone influence the induction of malic enzyme as well as related lipogenic enzymes (*75*). We have recently measured the influence of the thyroidal state on the malic enzyme induction by a high carbohydrate diet (Fig. 6) (*76*). Hypothyroidism markedly inhibits the induction of malic enzyme by a high carbohydrate diet. If animals are rendered maximally hyperthyroid, administration of carbohydrate provides no additional increment in the concentration of malic enzyme. These observations are compatible with the



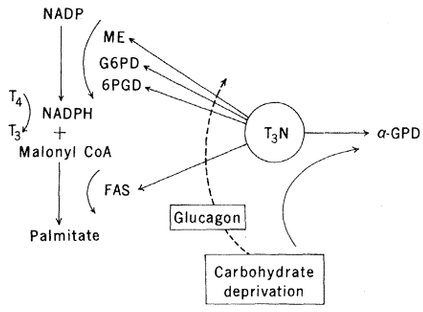


Fig. 6 (left). The interaction of thyroid hormone and a high carbohydrate diet in the induction of malic enzyme. Malic enzyme was measured as nanomoles of nicotinamide adenine dinucleotide phosphate reduced (to NADPH) per minute per milligram of protein. Hypothyroid, euthyroid, and animals rendered maximally hyperthyroid by T_3 injections (50 μg per day or 200 μg per day for 7 days) were given either a diet high in carbohydrate or a normal diet of laboratory chow. With maximum doses of T_3, the carbohydrate diet did not result in a significant increment in induction compared to regular chow diet. In hypothyroid animals a high carbohydrate diet resulted in a substantially smaller increment in malic enzyme than in euthyroid animals. These findings are compatible with the view that both T_3 and a factor induced by the carbohydrate are essential for lipogenic enzyme induction. With hyperthyroidism, maximum rates of induction are attained. Bars indicate ± standard error. Numbers of experiments are given in parentheses. Fig. 7 (right). Schematic representation of selective aspects of T_3 influence in rat liver. The T_3 nuclear complex, *T_3N*, stimulates a variety of enzymes either directly involved in the synthesis of fatty acids, such as fatty acid synthetase (*FAS*), or enzymes responsible for the generation of NADPH which is required for fatty acid synthesis [malic enzyme (*ME*), glucose 6-phosphate dehydrogenase (*G6PD*), and 6-phosphogluconate dehydrogenase (*6PGD*)]. As indicated by Balsam and Ingbar (*77*), NADPH is also essential in the intracellular conversion of T_4 to T_3. Glucagon may be a factor in inhibiting T_3 stimulation in states of carbohydrate deprivation. In carbohydrate deprivation the steady-state concentration of α-GPD, however, is maintained and the response of α-GPD to saturating doses of T_3 is preserved or actually augmented. Since the nuclear binding capacity and nuclear T_3 content are reduced, starvation appears to augment the effects of T_3 on α-GPD. The mechanism of this sensitization has not been defined. *Malonyl CoA*, malonyl coenzyme A.

concept that the induction of malic enzyme may require both the stimulus provided by the nuclear T_3 complex and a stimulus generated by the high carbohydrate diet.

These results can also be integrated in the following fashion (Fig. 7). As is well recognized, carbohydrate deprivation reduces and carbohydrate administration accelerates the rate of fatty acid synthesis (*75*). A number of enzymes appear to be involved in a coordinated way. Other lipogenic enzymes, such as the fatty acid synthetase complex and the NADP-linked hexose monophosphate shunt enzymes behave in an analogous manner to malic enzyme with respect to the thyroid hormone–carbohydrate interplay. In the liver, glucagon may well be an important factor blocking fatty acid synthesis in starvation. In other tissues, insulin may play a critical role in the regulation of lipogenesis. In states of carbohydrate deprivation, the T_3 signal appears to be effectively blocked with a marked inhibition in the synthesis of the lipogenic enzymes. With a decrease in malic enzyme and hexose monophosphate shunt enzymes, the generation of reduced nicotinamide adenine dinucleotide (NADPH) will also be decreased. Since Balsam and Ingbar (*77*) have recently shown that NADPH is required in the conversion of T_4 to T_3 in hepatic cytosol, a decrease in NADPH-generating enzymes may be causally related to the lower circulation of T_3 in starvation. Cessation of lipogenesis may be biologically important to the starving animal in conserving the limited energy supply for more essential biochemical processes.

It is important to emphasize, however, that in states of starvation, certain responses of T_3 are either unaffected or actually augmented. As an example, in the face of decreased concentration of plasma T_3 and decreased nuclear T_3 receptor content, the basal level of α-GPD is uninfluenced in starved animals. Moreover, the response of α-GPD to a dose of T_3 designed to occupy fully the nuclear sites is either unimpaired or actually increased. If the response of α-GPD to T_3 is expressed in relation to the reduced nuclear T_3 content, the sensitivity of the α-GPD response in starvation and partial hepatectomy is uniformly enhanced. The disparity in response between malic enzyme and α-GPD could serve to explain the clinical paradox posed by the "low T_3 syndrome," which is characteristic of patients with nonthyroidal disease and patients subjected to carbohydrate deprivation (*78*). Such individuals exhibit a marked reduction in the free T_3 concentration in plasma, appear clinically euthyroid, exhibit normal basal thyrotropin concentrations in plasma, and show a normal pituitary thyrotropin response to the administration of thyrotropin-releasing hormone. By analogy to the fasted rat, it appears possible that in the low T_3 syndrome some T_3 responses such as that of malic enzyme are blocked, but others, analogous to the response of α-GPD in the rat, are sensitized. Sensitization of selected parameters might then lead to the preservation of clinical euthyroidism. Sensitization of pituitary thyrotropin inhibition by T_3 would explain the normal levels of circulating thyrotropin and the normal response to TRH. Regardless of these speculations, the observed discrepancies in the response of malic enzyme and α-GPD to T_3 in the rat serve to illustrate the principle of local cellular control in the expression of thyroid hormone action. The thyroid hormone signal appears capable of being locally modulated, either amplified or attenuated. In fact, in a given cell, the expression of thyroid hormone may be concomitantly amplified and attenuated depending on the specific gene product assayed. The molecular basis of such local modulation remains to be defined.

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