tions. By means of a table of random numbers, the two lesions that would receive an application from tube A were determined (two of lesions 1, 2, 3, and 4). The other two lesions received an application from tube B.

15. After the two lesions that were to receive an application from tube A were determined, one was randomly selected to be a biopsy site; likewise, the two lesions that were to receive an application from tube B were selected randomly. Therefore, one of the two lesions from each treatment was scheduled to be biopsied but the code (tube A and tube B) was not broken until after biochemical analyses were completed. The two remaining lesions, one having received a glucocorticoid and one a control cream treatment, were not biopsied but were left to be visually evaluated 2 days later.
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17. The surgically removed treated or control tissue used as starting material in this report is designated by several terms which are meant to be synonymous. These terms are lesional epithelium, lesional epidermis, lesional tissue, and diseased tissue. By frozen section histology we estimate that keratinocytic epithelium (epidermis) occupies 80 to 95 percent of the specimen volume. The 80 to 95 percent range is the result of surgical technique, vertical lesional configuration, and the presence of non-keratinocytes (inflammation-associated cells, endothelial cells, fibroblasts, and collagen). Therefore, although 5 to 20 percent of the specimen volume is occupied by collagen and nonepithelial cells, the latter of which probably contain the arachidonic acid transformation cascade (Fig. 2), it seems probable that the data in Fig. 1 are derived mainly from lesional epithelium.
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23. Mild lesional pallor in 4 of 12 patients was still evident at 3 days. The degree of redness in 7 patients did not change between the initial application and day 3. There was pallor at day 1 but return to the initial degree of redness in 1 of the 12 patients. Lesional thickness as estimated by the examining finger remained decreased in 3 of 12 patients at day 3. No change in thickness occurred in 7 patients between the initial application and day 3. In 2 of the 12 patients, thickness was decreased at day 1 but returned to a thickness similar to that observed at the time of initial application.
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Thyroid Hormone Action: The Mitochondrial Pathway

Abstract. *The subcellular compartments have been investigated to compare proteins capable of binding triiodothyronine and thyroxine; specific binders have been found in cytosol, nuclei, and mitochondria from rat liver and kidney. The binding protein from the inner mitochondrial membrane had the highest association constant ($>10^{11}$ liters per mole), suggesting possible direct hormone action on the mitochondria. Binding of hormone analogs was found to be related to known physiological potency, and stereospecific discrimination between* L- *and* D-*thyroxine was observed. The saturable receptor was found in the mitochondrial membranes of rat liver, kidney, myocardium, and skeletal muscle but not in mitochondria from the unresponsive tissues: brain, spleen, and testis. Oxidative phosphorylation by mitochondrial vesicles from hypothyroid rats increased after the addition of physiological concentrations of triiodothyronine, which corroborated direct hormone action on mitochondria.*

Postulated models for steroid hormone action involve nuclear localization of the hormones after binding by a cytoplasmic receptor. A somewhat similar mechanism has seemed plausible for thyroid hormone action in view of reported binding of the thyroid hormones to the cell nucleus or to acidic nuclear proteins (*1*–*4*). We have been examining the subcellular binding of the thyroid hormones to the nucleus, cytoplasm, and the mitochondria as well, to determine the biological relevance of the previously described mitochondrial receptor (*5*) by comparing its binding characteristics to that of other known cellular binding sites and relating binding affinity and hormonal action.

Male Sprague-Dawley rats weighing more than 300 g were obtained from Charles River Breeding Farms. Surgically thyroidectomized rats (Hormone Assay Laboratories) given 0.2 percent $CaCl_2$ in tap water were kept at least 1 month on a low-iodine diet, by which time hypothyroid stigmata had become obvious and serum triiodothyronine (T_3) and thyroxine (T_4) concentrations were in the hypothyroid range. Normal rat serum T_4 concentrations were (mean ± standard deviation) 4.8 ± 1.6 μg/dl in contrast to 1.1 ± 0.6 μg/dl in hypothyroid serums, and T_3 values were undetectable. Isolation and handling of nuclei and cytosol from rat liver and kidney have been described (*1*, *6*). Isolation of mitochondria and preparation of mitochondrial membrane protein were as described elsewhere (*5*), except that the initial homogenate was centrifuged at approximately 620*g* (corresponding to 2250 rev/min in the Sorvall SS-34 head). The mitochondrial membrane protein obtained after Triton X-100 treatment was partially purified. Gel filtration of mitochondrial membrane protein was carried out on a calibrated column of Sephadex G-200 (90 by 1.5 cm) at a flow rate of ~ 15 ml/hour with 0.05*M* tris-HCl buffer, *p*H 7.0, and 3-ml fractions were collected for determinations of absorbance and other analytic procedures. Electron micrographs were interpreted as verifying the high purity of the mitochondria. An electrophoretic study of mitochondrial membrane protein with added labeled T_3 (*5*) showed it to have a mobility greater than that of serum prealbumin, whereas nuclear protein had a mobility less than that of serum albumin, judging from scans of paper strips. Separation of inner and outer mitochondrial membranes was done by the method of Schnaitman and Greenawalt (*7*) and verified by determinations of monoamine oxidase, cytochrome oxidase, and malic dehydrogenase.

Samples of ^{125}I-labeled T_3 and T_4, provided by Abbott Laboratories, were checked for purity by paper electrophoresis and chromatography. Phosphate labeled with ^{32}P was purchased from Squibb. All other chemicals were obtained from commercial sources and were of the highest purity available.

Determination of binding constants. Hormone concentrations in the subcellular constituents studied were: cytosol, 2.3×10^{-2} to 300×10^{-2} μ*M*; intact nuclei or acidic nuclear protein (*1*), 0.75×10^{-4} to 10×10^{-4} μ*M*; and mitochondrial membrane protein, 0.75 to 10 p*M*. The total radioactivity in each tube was determined by counting for ½ to 2 minutes during brief removal from the incubation bath. Incubations were for 1 hour at 0°C for cytosol and mitochondrial preparations and at 37°C for nuclei. The volumes of the protein solutions were 0.4 ml plus 40 μl of isotope solution. The medium consisted of 0.05*M* tris-HCl and 0.01*M* EDTA, at *p*H 7.0 for mitochondria and at *p*H 7.4 for cytosol and nuclei. In studies of mitochondrial protein binding a general range of 1 absorbance (280 nm) unit per milliliter (~ 0.3 to 1 mg/ml) was sought. The actual protein concentrations were determined subsequently and

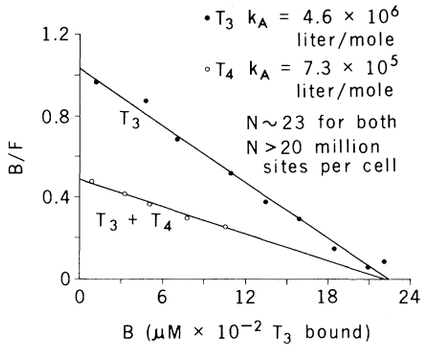

Fig. 1. Interaction between rat renal cytosol binding protein and ^{125}I-labeled T_3 with and without the nonradioactive competing ligand T_4 at 1.5 μM (Scatchard plot).

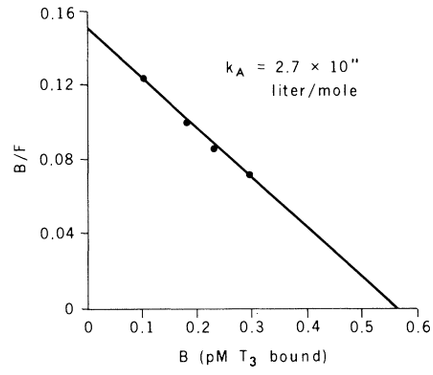

Fig. 2. Interaction between T_3 and rat liver mitochondrial membrane protein (Scatchard plot). Our preliminary estimate is that there are approximately 2000 primary mitochondrial binding sites per hepatic cell.

were found to vary from 0.05 to 0.42 mg/ml, without any detectable influence on the binding constants obtained, despite the almost tenfold range of protein concentrations employed.

"Free" and "bound" fractions of intact nuclei were separated by centrifuging the nuclei at 10,000g and comparing the radioactivity in the nuclear pellet (bound) with that in the supernatant (free). In the case of the protein solutions, incubation was terminated by the addition of 0.2 ml of dextran-coated charcoal [Norit A and dextran (molecular weight, 60,000 to 90,000), both 10 g/liter], as previously described (*8–10*). The charcoal pellet (free hormone) and supernatant (bound hormone) were counted in a Packard Auto-Gamma spectrometer. The lowest additions of labeled T_3 sometimes resulted in values for bound hormone as low as 25 counts per minute (cpm) above background, so that prolonged counting was needed to achieve a statistical accuracy of about 5 percent. The association constant (k_A) was determined by plotting the ratio of bound to free hormone (B/F) against bound hormone (B) in a Scatchard plot (*11, 12*). Addition of 1000-fold or greater excess of "cold" hormone gave nonspecific binding, which was subtracted from each experimental point. The findings with dextran-coated charcoal were corroborated by data from classical equilibrium dialysis (*8, 9*) with cytosol, the only protein readily available in sufficient amount for replicate studies with 1-ml quantities in a dialysis bag.

To determine the association constant of the hormone analogs D-thyroxine (D-T_4), tetraiodothyroacetic acid (TA_4), and 3′-isopropyl-3,5-diiodo-L-thyronine (3′-isopropyl-3,5-T_2), we used the equation

$$k_B = \frac{1}{[B]}\left(\frac{k_A}{k_{A'}} - 1\right) \quad (1)$$

where k_A is the association constant for labeled T_3 or T_4 alone, $k_{A'}$ is the association constant for T_3 or T_4 in the presence of the competing (nonradioactive) ligand, [B] is the concentration of the competing ligand, and k_B is the association constant of the competing ligand for the primary site previously determined for labeled T_3 or T_4 alone.

We have used Eq. 1 to study binding by weaker competing ligands for primary binding sites (*13*), based on the formulation of Edsall and Wyman (*14*) for the effects of competition between different ligands for the same binding site. Such an experiment is illustrated in Fig. 1.

The number of binding sites was derived from the intercept on the abscissa and calculated as number of sites per cell, based on recovery studies and protein determinations, plus the value of 1×10^8 cells per gram (wet weight) of rat liver (*15, 16*).

Oxygen consumption was measured at 37°C in a Clark oximeter in both intact mitochondria and mitochondrial vesicles. Only preparations showing active respiration were used for studies of adenosine triphosphate (ATP) formation. Oxygen consumption was observed to increase within 1 to 2 seconds on addition of T_3 at final concentrations as low as 5 or even 0.5 μM, but not at lower concentrations. We also examined the effect of T_3 in vitro on oxidative phosphorylation in isolated mitochondrial vesicles prepared from hypothyroid rat liver by digitonin treatment (*7*) followed by 15 seconds of sonication. The mitochondrial vesicles were incubated with ^{32}P for 2 minutes at 37°C in a buffered medium containing 5 mM succinate, 1 mM phosphate, and 1 mM adenosine diphosphate (ADP) at pH 7.0. Incorporation of ^{32}P was measured in a liquid scintillation counter after extraction by Pullman's procedure for [^{32}P]ATP (*17*).

The data in Fig. 1 illustrate a binding study with rat renal cytosol, showing a primary class of binding sites. Table 1 shows mean values obtained in about 150 experiments with cytosol, nuclei, and mitochondria, which show successively greater binding affinity. The values of Table I and Fig. 2 represent experiments with partially purified mitochondrial protein material. In Sephadex G-200 gel filtration of mitochondrial membrane protein (*5*), the main protein peak emerges early, soon after the void volume. The peak begins to emerge a single tube after the position of the immunoglobulin G peak, determined by prior calibration of

Table 1. Association constants (k_A) for the interaction between cellular constituents and thyroid hormones or hormone analogs. Values are means ± standard error of the mean.

Constituent and tissue	k_A (liter/mole)				
	T_3	T_4	TA_4	D-T_4	3′-Isopropyl-3,5-T_2
Cytosol					
Liver	$2.3 \pm 0.6 \times 10^6$	$2.0 \pm 0.3 \times 10^5$			
Kidney	$3.3 \pm 0.4 \times 10^6$	$8.9 \pm 1.6 \times 10^5$	$4.9 \pm 1.3 \times 10^5$		
Nuclei					
Liver	$5.0 \pm 2.1 \times 10^8$	$1.9 \pm 0.9 \times 10^8$			
Kidney					
Mitochondria					
Liver	$1.9 \pm 0.2 \times 10^{11}$	$9.4 \pm 2.5 \times 10^{10}$	$2.2 \pm 1.4 \times 10^9$	$1.1 \pm 0.5 \times 10^{10}$	$2.4 \pm 0.5 \times 10^{12}$
Kidney	$2.0 \pm 0.4 \times 10^{11}$	$6.2 \pm 1.1 \times 10^{10}$	$5.3 \pm 3.3 \times 10^9$	1.3×10^{10}	$4.3 \pm 0.9 \times 10^{12}$

the column with human serum. This fraction appears to have a primary class of binding sites with an association constant for T_3 greater than 10^{11} liter/mole, the highest observed for any subcellular component (Fig. 2). This specific binding was observed in such fractions obtained from inner mitochondrial membrane. In contrast, material from outer mitochondrial membrane or whole unfractionated mitochondrial protein reveals an almost horizontal line on a Scatchard plot, signifying a large number of low-affinity, high-capacity, virtually unsaturable binding sites. Data from hypothyroid and from normal rats did not differ significantly.

Mitochondrial preparations from myocardium and from striated skeletal muscle showed highly specific binding similar to that observed with material from liver and kidney. However, mitochondria prepared by similar methods from rat brain, spleen, and testis (*18*) failed to show specific, saturable binding.

Table 1 shows that T_3 is bound with a higher association constant than T_4 for all subcellular moieties. The most extensive analog studies, carried out with mitochondrial protein, showed that T_4 was more firmly bound than TA_4, which was in turn more firmly bound than D-T_4. The highly active synthetic analog 3′-isopropyl-3,5-T_2 was the only compound with a higher association constant than T_3 itself.

As shown in Fig. 3, mitochondrial vesicles from hypothyroid rats were capable of oxidative phosphorylation, which was stimulated by the addition of T_3 in vitro. Since incubations lasted only 2 minutes, this represents a rapid effect, evident at the nanomolar level or even lower. The T_3 was added to the medium, which contained succinate as the substrate, and the mitochondrial vesicles were added at the start of the 2-minute period. No stimulation was observed in mitochondrial vesicles prepared from normal rats. Uncoupling of oxidative phosphorylation was observed with dinitrophenol (DNP) at $6 \times 10^{-6} M$, as well as T_3 at the unphysiologically high concentration of 100 μM (Fig. 3). The uncoupling by DNP is the anticipated effect on the incorporation of inorganic phosphate into ATP in a mitochondrial vesicle system carrying out oxidative phosphorylation.

Some reservations must be entertained regarding the binding constants obtained from Scatchard plots of the interaction between T_3 and the mitochondrial membrane protein, as shown in Fig. 2. The points illustrated in Fig. 2 represent occupancy of approximately one-half of the binding sites. Points representing greater T_3 additions (not illustrated) usually reveal a flattening of the curve. In no case was a single linear negative slope observed beyond the point of two-thirds occupancy. Flattening of the curve can be produced by the presence of other weaker classes of binding sites either on the major receptor protein itself or on other contaminating proteins. The data at hand do not permit discrimination between these possibilities. Nevertheless, it seems reasonable to accept the values listed as first-order approximations. Subtracting the contribution of secondary or higher-order classes of binding sites from the observed data would result in steeper slopes and hence higher association constants for the primary class. Thus, values for k_A slightly above 10^{11} liter/mole may be taken as minimal. These mitochondrial association constants are appreciably higher than those obtained for the nuclear receptors (*1–4*).

Discussion. Mitochondrial binding of the various hormone analogs was in the sequence anticipated from physiological activity: 3′-isopropyl-3,5-diiodo-L-thyronine > T_3 > T_4 > TA_4 > D-T_4. The stereospecificity of discrimination between L-T_4 and D-T_4 is considered particularly significant. However, the correspondence between firmness of binding and biological activity was not precisely linear. Thus, 3′-isopropyl-3,5-T_2 is probably not more than two or three times as potent as T_3, but it is bound more than ten times as firmly. On the other hand, T_3 is bound two to three times as firmly as T_4, proportional to their respective potencies in the maintenance of athyreotic myxedema in the euthyroid state. Although T_4 is converted to T_3 in vivo (*19–21*) it seems reasonable to consider that T_4 possesses intrinsic hormonal activity.

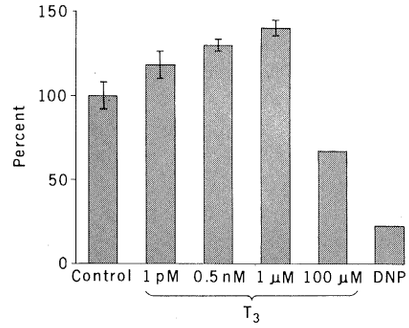

Fig. 3. Triiodothyronine stimulation of mitochondrial oxidative phosphorylation. Submitochondrial vesicles were prepared from the mitochondria of hypothyroid rats (see text). Incubation with and without added T_3 in the concentrations designated was carried out in 0.05*M* tris-HCl buffer with 0.075*M* sucrose and 0.05*M* KCl at *p*H 7.0 for 2.0 minutes in the presence of 5 m*M* succinate, 1 m*M* ADP, and tracer [^{32}P]phosphate in 1 m*M* phosphate buffer shaken in a water bath at 37°C. The control column shows the mean ± standard error of the mean for ^{32}P incorporation in quadruplicate incubation flasks. The first three T_3 columns represent the means for duplicate flasks ± deviations. The last two columns represent single incubation flasks; similar findings were obtained in several such experiments. The results were interpreted as indicating significantly (P = or < .03) increased [^{32}P]ATP formation in the physiological T_3 range 0.5 n*M* to 1 μM, but marked diminution at the high T_3 level of 100 μM, signifying uncoupling of oxidative phosphorylation which was almost as pronounced as with the known uncoupling agent DNP at $6 \times 10^{-6} M$. At 1 p*M* T_3, the slight increase in [^{32}P]ATP was not statistically significant. The 1 p*M* values in the majority of such studies exceeded the control mean slightly.

Extrapolation of the findings to the situation in vivo will require further quantitative data. Our preliminary estimate is approximately 2000 specific saturable mitochondrial binding sites per hepatic cell, which should be compared with the number of nuclear sites—about 15,000 (our estimate) or about 5000 to 8000 (*1–4*).

It might be supposed that at normal T_3 concentrations the specific mitochondrial binding sites are largely saturated. However, the existence of an excess of nonspecific, low-affinity, high-capacity sites in the outer mitochondrial membrane must be taken into account. Making the speculative assumption that the intracellular free T_3 concentration approximates that of the circulating plasma, it may be estimated that approximately half of the mitochondrial receptor sites would be occupied.

The concept of a direct thyroid hormone effect on mitochondria has been supported by numerous previous reports (*22–27*), especially the findings of Bronk (*22*), which have suggested immediate effects on oxidative phosphorylation. This concept is in no way incompatible with stimulation of nuclear transcription (*1–*

4), which is regarded as a probable sustained effect after a lag required for activation of protein synthesis. The enhanced sodium-potassium adenosine triphosphatase mechanism or "sodium pump" (28) is regarded as a major final pathway of energy expenditure.

The binding of thyroid hormone by the proteins of the cytosol differs strikingly from postulated models for steroid hormone action wherein the cytosol receptor-hormone complex is "translocated" into the nucleus, apparently to stimulate the transcription of specific parts of the genetic message. In contrast, the specific T_3 binding sites on cytosol proteins, which number more than 20 million per rat liver cell, may simply hold the hormone inside the cell, facilitating interaction between a minute moiety of intracellular free T_3 and the effector loci (29).

The model we propose consists of an integrated sequence of actions of thyroid hormone at the cellular level, including direct effects on the mitochondria in addition to stimulation of nucleic acid and protein synthesis by way of receptors in the nuclear chromatin. It does not exclude further possible routes of thyroid hormone action, including effects on the plasma membrane (30, 31), and incorporation of the hormone molecule into tyrosine sites in proteins (32). However, on the basis of the available data, it would be most unlikely that binding of thyroid hormone by receptors in the nuclear chromatin could account for the entire spectrum of biological actions, however important such a mechanism may prove to be in growth and differentiation. The lag time before increased protein synthesis (33) shows that such a pathway must represent a sustained rather than an immediate or initial hormone action. In contrast, the activation of mitochondrial energy metabolism would seem a reasonable candidate for the first effects observable, such as increased oxygen consumption soon after administration of T_3 to thyroidectomized rats or to myxedematous human subjects.

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Induction of Suppressor T Cells in Systemic Lupus Erythematosus by Thymosin and Cultured Thymic Epithelium

Abstract. *Patients with systemic lupus erythematosus lacked suppressor T cell function. Suppressor cell activity was induced in cells from many of these patients by incubation with thymosin or cultured thymic epithelium. These results suggest that thymic manipulation may be a useful therapeutic modality in this disease.*

Systemic lupus erythematosus (SLE) is a serious autoimmune disease in humans. The reasons for the aberrant immune responses in this disorder are unknown. Several investigators suggest that a loss of immunoregulatory suppressor T cells is important in the pathogenesis of SLE (1, 2). In NZB/NZW mice, an excellent animal model of SLE, Krakauer *et al.* (2) provided direct evidence of loss of suppressor cell function with age. We have investigated suppressor T cell activity in human SLE and report that patients with this disease lack suppressor T cells and that suppressor T cell activity is induced in vitro in cells from these patients by incubation with thymosin or cultured thymic epithelium (CTE).

Suppressor T cell activity was determined by assessing the effect of concanavalin A (Con A)–treated lymphocytes on the one-way mixed leukocyte

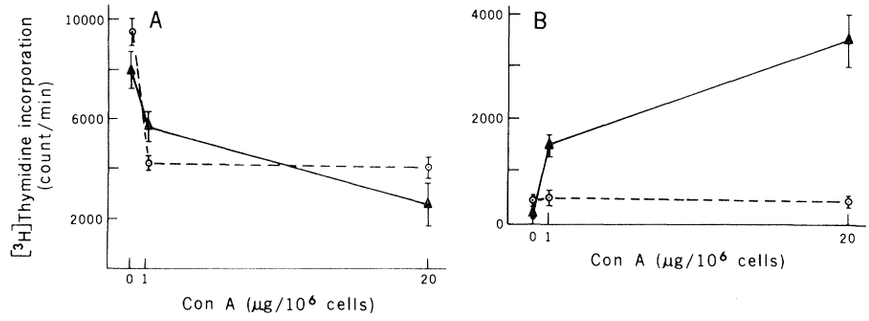

Fig. 1. Assays in normals. (A) The suppressor cell assay was performed as described (3). Representative data from one individual (autologous suppressor assay) are shown; they were similar in all 15 control patients. The allogeneic suppressor assay with normal suppressor cells gave similar results. Each point represents the mean counts per minute ± standard deviation of triplicate determinations. (○) Suppressor cells first incubated with Con A for 24 hours. (▲) Suppressor cells first incubated with Con A for 48 hours. (B) The Con A proliferation assay was performed as described (11). Representative data from one individual are shown but were similar in all 15 control patients. (○) Cells incubated with Con A for 24 hours. (▲) Cells incubated with Con A for 48 hours.