Thyroxine-Induced Stimulation of Hepatic Cell Transport of Calcium and Magnesium

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ABSTRACT The effect of L-thyroxine on the bidirectional transport of calcium and magnesium in rat liver was assessed in vitro. An increase of 34% in the fractional coefficient for calcium influx was observed 24 hr after the administration of 500 μg of thyroxine. Chronic treatment with thyroxine for 1 and 3 wk at a dose of 750 μg/wk resulted in increases in calcium influx of 57 and 51%, respectively. Calcium efflux was increased irregularly, by 14–26%. Magnesium transport measured in a similar system was not altered by 24 or 48 hr of treatment with thyroxine, but continuation of treatment for 1–3 wk resulted in increases in magnesium influx of 47–49%. Magnesium efflux was not significantly affected. Neither increased cellular binding of divalent cations nor enhanced protein synthesis could be incriminated in the stimulatory effect of thyroxine on divalent cation transport. Actinomycin-D and D,L-ethionine, inhibitors of protein synthesis, stimulated calcium and magnesium transport in liver independently of the effects of thyroxine. These data present the possibility that certain actions of thyroid hormone may be mediated or modulated by associated, direct changes in the cellular transport and intracellular concentrations of divalent cations.

INTRODUCTION

Previous investigations of the effects of thyroid hormone on the metabolism of calcium and magnesium have been concerned primarily with abnormal plasma concentrations, urinary excretions, and metabolic balances of these divalent cations in hyper- and hypothyroid states, and with associated skeletal manifestations (1–8). Radiomagnesium turnover studies in humans indicate that thyroid hormone accelerates magnesium transport in soft tissues and may be responsible for the inverse relation between plasma magnesium and the functional state of the thyroid gland (9). A comparable stimulation of calcium transport in soft tissues by thyroid hormone is suggested by the rapidity of the initial decline of the plasma specific activity during radiocalcium turnover studies in humans with thyroid dysfunction (8, 10). In rats, the in vivo exchangeability of soft tissue calcium is increased by thyroxine (11). The present study was undertaken to make in vitro measurements of the effect of thyroxine on the bidirectional cellular transport of divalent cations. The data confirm the ability of thyroxine to stimulate the cellular transport of calcium and magnesium and therefore implicate an additional parameter relevant to the tissue actions of thyroid hormone.

METHODS

Mature male Sprague-Dawley rats (Charles River Breeding Laboratories, Inc., Wilmington, Mass. and Blue Spruce Farms, Inc., Altamont, N. Y.), 4–8 months old and weighing 350–500 g, were thyroparathyroidectomized and treated with L-thyroxine in the doses and periods indicated in the text. The hormone was administered subcutaneously, dissolved in a solution consisting of one part 0.01 N NaOH and three parts isotonic saline. Control rats received the vehicle alone. At the completion of the treatment period, the rats were decapitated and their livers sliced freehand to a thickness of 0.3 mm with a Stadie-Riggs blade.

Transport studies. The methods for the measurements of bidirectional transport of calcium and magnesium were described previously (12, 13). In brief, 5 g of fresh liver slices from each rat were placed in 50 ml of a buffered pH 7.4 medium containing 10–15 μCi of either ^{47}Ca or ^{28}Mg. For the radiocalcium transport studies, the buffered medium contained 148 mM Na^+, 5 mM K^+, 1.5 mM Ca^{++}, 128 mM Cl^-, 25 mM HCO_3^-, 1 mM $PO_4^{=}$, and 5.5 mM glucose. For the radiomagnesium transport studies, the buffered medium contained identical concentrations of Na^+, K^+, Cl^-, and HCO_3^-, but no Ca^{++} or $PO_4^{=}$. 2.5 mM Mg^{++} and 11 mM glucose were also present. The slices were incubated at

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37°C in a Dubnoff metabolic shaker at 90–105 oscillations per min with a gas phase of 95% O_2, 5% CO_2. After 30 min, the labeled slices were removed and washed twice for 5-min periods in nonradioactive medium. Samples of 2.5 g of washed, blotted slices were then reincubated in 25 ml of nonradioactive medium for 180 min. Periodically, 0.25-ml samples of medium were removed for counting. At the end of incubation, the slices were removed and blotted. The water, radioactivity, and divalent cation concentrations of the pre- and postincubation slices were determined. Divalent cation analyses and pH measurements were performed on pre- and postincubation samples of the medium. In all experiments, slices from a control rat and an L-thyroxine-treated rat were incubated side by side to achieve random distribution of the data.

Radioactivity measurements were made at constant geometry in a gamma scintillation spectrometer calibrated to eliminate emissions from the ^{47}Sc daughter of ^{47}Ca and the ^{28}Al daughter of ^{28}Mg. After counting, the liver samples were dried at 105°C for 48 hr and the water content was determined by differential weight. The dried slices and postincubation media were prepared for divalent cation analysis by homogenization in 10% trichloroacetic acid. Calcium analyses were performed by atomic absorption spectrometry in a Perkin-Elmer Corp. (Norwalk, Conn.) model 303. Magnesium analyses were performed by complexometric titration with EDTA after oxalate precipitation of the traces of calcium present in the extracts.

Calculations. The slices were assumed to contain interstitial and cellular fractions of divalent cation. After equilibration of the slice interstitium with the medium, exchange between the cellular and extracellular fractions could be expressed by a differential equation describing a closed two-compartment system,

$$\frac{d(C)_s}{dt} = -K_{sb}(C)_s + K_{bs}(C)_b, \qquad (1)$$

where $(C)_s$ = ^{47}Ca or ^{28}Mg concentration of slices (counts/minute per gram), $(C)_b$ = ^{47}Ca or ^{28}Mg concentration of medium (counts/minute per milliliter, K_{sb} = efflux transfer coefficient, and K_{bs} = influx transfer coefficient.

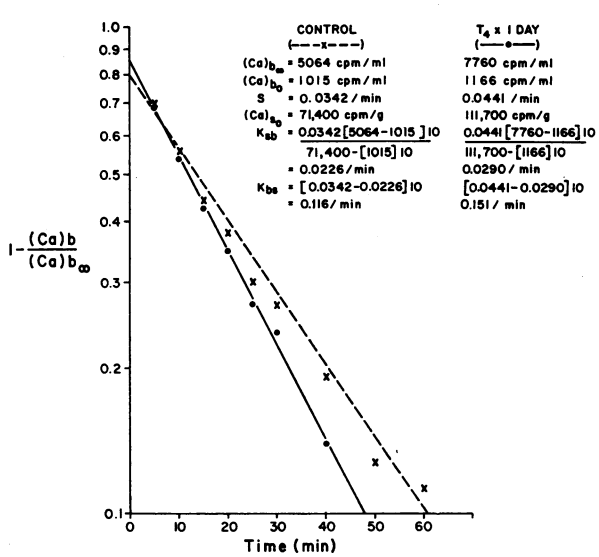

FIGURE 1 Representative plots of log $1-[(Ca)_b/(Ca)_{b\infty}]$ vs. linear time and the method of calculation of the calcium transport data.

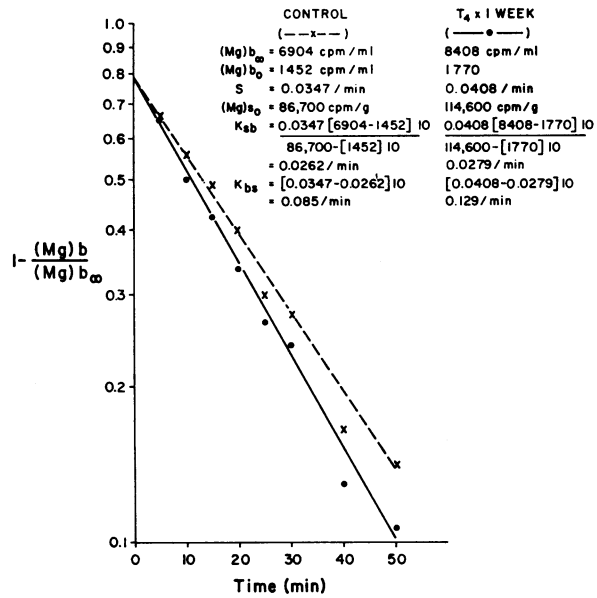

FIGURE 2 Representative plots of log $1-[(Mg)_b/(Mg)_{b\infty}]$ vs. linear time and the method of calculation of the magnesium transport data.

The solution of this differential equation has been outlined elsewhere (12). The efflux and influx transfer coefficients are given by the following equations:

$$K_{sb} = \frac{S(C)_{b\infty}\left(\frac{v}{m}\right)}{(C)_{so}}, \qquad (2)$$

$$K_{bs} = [S - K_{sb}]\left(\frac{v}{m}\right), \qquad (3)$$

where $(C)_{b\infty}$ = the ^{47}Ca or ^{28}Mg concentration of the medium at isotopic equilibrium, $(C)_{so}$ = the ^{47}Ca or ^{28}Mg concentration of the slices at the beginning of incubation, S = the slope of the straight line obtained when experimental values of $1-[(C)_b/(C)_{b\infty}]$ are plotted semilogarithmically against time, v = the volume of medium (25 ml), and m = the mass of liver slices (2.5 g).

In each experiment, a semilogarithmic plot of $1-[(C)_b/(C)_{b\infty}]$ against time yielded a linear relation between 10 min and the time required for 90% of equilibrium to be reached (Figs. 1 and 2), validating the assumption of a closed two compartment system. Between 0 and 10 min, a curve of greater slope was obtained which was assumed to represent equilibration of radioactivity initially in the slice interstitium with the medium. To eliminate interstitium-medium equilibration from consideration, the following equation was substituted for Equation 2:

$$K_{sb} = \frac{S[(C)_{b\infty} - (C)_{bo}]\left(\frac{v}{m}\right)}{(C)_{so} - (C)_{bo}\left(\frac{v}{m}\right)}, \qquad (4)$$

TABLE I

Transport Coefficients and Slice Calcium Concentrations in ^{47}Ca Transport Studies

	K_{bs}	K_{sb}	Initial slice calcium	Final slice calcium
	per min	*per min*	*mmoles/kg*	*mmoles/kg*
I. Control (7)	0.113±0.003	0.021±0.0001	2.58±0.20	4.44±0.21
T_4, 1 day (7)	0.151±0.004	0.024±0.001	2.82±0.10	4.38±0.28
	$P < 0.001$	$P = 0.02$	NS	NS
II. Control (10)	0.116±0.007	0.022±0.001	1.60±0.08	2.85±0.21
T_4, 1 wk (8)	0.182±0.014	0.026±0.002	2.06±0.31	4.07±0.36
	$P < 0.001$	NS	NS	$P < 0.01$
III. Control (7)	0.112±0.005	0.019±0.001	1.65±0.15	2.70±0.20
T_4, 3 wk (7)	0.169±0.020	0.024±0.003	1.84±0.23	3.13±0.36
	$P < 0.02$	NS	NS	NS

Values are means±SEM. T_4, L-thyroxine; K_{bs}, influx transfer coefficient; K_{sb}, efflux transfer coefficient.

where $(C)_{bo}$ = the apparent extracellular concentration of ^{47}Ca or ^{28}Mg at zero time. This value was determined by extrapolation of the linear portion of the curve to zero time and the use of the intercept value of $1 - [(C)_b/(C)_{b\infty}]$ to calculate $(C)_{bo}$. A digital computer program was devised to yield a least squares fit of values of $1 - [(C)_b/(C)_{b\infty}]$ against time between 10 min and 90% of equilibrium. The slope and intercept of this line were utilized to calculate K_{sb} and K_{bs}, employing Equations 3 and 4.

Divalent cation binding studies. Fresh rat livers were homogenized in a fourfold volume of iced, pH 7.4, Tris-buffered medium in glass tissue grinders. The buffered medium contained 119 mM Na^+, 5 mM K^+, 124 mM Cl^-, 10 mM Tris, and 0.1 mM glucose. The homogenates were centrifuged for 20 min at 4°C at a speed of 1400 *g*. The supernates were then filtered through glass wool and three 5-ml volumes of each supernate were transferred to screw-capped tubes. In succession, 2–5 μCi of ^{47}Ca or ^{28}Mg in a volume of 0.025 ml, and 1.0 ml of a suspension of Chelex-100 resin (Bio-Rad Labs, Richmond, Calif.) were added. The tubes were capped, shaken vigorously for 1 min, and then centrifuged for 5 min. A 1 ml sample of supernate from each tube was taken for counting. The results were expressed as per cent of added ^{47}Ca or ^{28}Mg retained by the liver supernates.

RESULTS

Mean values for the transfer coefficients for calcium influx (K_{bs}) and efflux (K_{sb}) and the changes in liver slice concentrations of calcium during incubation are shown in Table I. Control values for K_{bs} ranged from 0.110 to 0.116 per min and for K_{sb}, 0.019 to 0.022 per min. 24 hr after a single subcutaneous injection of 500 μg of L-thyroxine, K_{bs} and K_{sb} were increased by 34 and 14%, respectively. When the administration of L-thyroxine was continued for 1 wk at a dose of 250 μg three times per wk, K_{bs} and K_{sb} were increased by 57 and 18%, respectively. The continued administration of L-thyroxine at the same dose level for 3 wk caused increases in K_{bs} and K_{sb} of 51 and 26%, respectively.

Mean values for the transfer coefficients for magnesium influx (K_{bs}) and efflux (K_{sb}) and the changes in liver slice concentrations of magnesium during incubation are shown in Table II. Control values for K_{bs} ranged from 0.075 to 0.087 per min and for K_{sb}, 0.018 to 0.022 per min. In contrast to the rapid effect of thyroxine on calcium transport, a single dose of 500 μg of L-thyroxine 24 hr previously did not stimulate the influx (K_{bs}) or efflux (K_{sb}) of magnesium. The administration of 350 μg of L-thyroxine on two successive days also failed to stimulate magnesium transport. However, the administration of 250 μg of L-thyroxine three times during a 1 wk period increased K_{bs} by 47% but K_{sb} was unaltered. A comparable increase in K_{bs} of 49% was observed after 3 wk of L-thyroxine administration at the same dosage schedule.

In an attempt to elucidate the mechanism of stimulation of divalent cation transport by L-thyroxine, the binding of divalent cations by liver homogenates from rats treated with L-thyroxine was studied. As used, the Chelex-100 resin bound all but 1–3% of ^{47}Ca and 0.5–1% of ^{28}Mg added to 5-ml portions of buffered medium. The consistent finding of greater than 3% retention of ^{47}Ca and 1% retention of ^{28}Mg by the liver supernates (Table III) indicated that the tissue homogenates competed with the resin for binding ^{47}Ca and ^{28}Mg. Liver supernates from rats given either a single dose of L-thyroxine of 750 μg or 250 μg three times during 1 wk retained 8.5–9.5% of added ^{47}Ca compared to 9.0–10.0% retention by control liver supernates. Hence, no enchancement of hepatic binding of calcium was observed in L-thyroxine-treated rats. Mea-

TABLE II

Transport Coefficients and Slice Magnesium Concentrations in ^{28}Mg Transport Studies

	K_{bs}	K_{sb}	Initial slice magnesium	Final slice magnesium
	per min	*per min*	*mmoles/kg*	*mmoles/kg*
I. Control (9)	0.081±0.002	0.018±0.001	6.50±0.50	6.25±0.40
T_4, 1 day (11)	0.083±0.003	0.017±0.001	6.70±0.35	6.55±0.35
	NS	NS	NS	NS
II. Control (5)	0.085±0.003	0.020±0.002	8.65±0.80	9.80±1.15
T_4, 2 days (6)	0.088±0.002	0.020±0.001	9.95±1.15	8.50±0.25
	NS	NS	NS	NS
III. Control (8)	0.087±0.003	0.022±0.002	6.35±0.20	5.85±0.30
T_4, 1 wk (8)	0.128±0.006	0.023±0.003	7.30±0.75	6.00±0.35
	$P < 0.001$	NS	NS	NS
IV. Control (10)	0.075±0.004	0.018±0.002	6.70±0.30	6.10±0.30
T_4, 3 wk (10)	0.112±0.006	0.020±0.003	6.75±0.20	6.55±0.40
	$P < 0.001$	NS	NS	NS

Values are means±SEM. T_4, L-thyroxine; K_{bs}, influx transfer coefficient; K_{sb}, efflux transfer coefficient.

surements of hepatic binding of magnesium utilized livers from rats treated with L-thyroxine, 250 μg three times per wk for 2 and 3 wk (Table III). The retention of ^{28}Mg by the liver homogenates ranged from 2.5 to 3.4% whether or not L-thyroxine was administered, indicating that L-thyroxine also did not alter hepatic binding of magnesium.

The possibility that L-thyroxine-induced stimulation of divalent cation transport involved protein synthetic activity was explored with actinomycin-D and D,L-ethionine. The intraperitoneal administration of actinomycin-D, 50 μg/100 g body weight, 4 hr before a single dose of 500 μg of thyroxine did not inhibit the stimulatory effect of the latter on calcium transport. In fact, K_{bs} was increased by 28% to 0.166±0.009, with actinomycin-D compared to a value of 0.130±0.004 with thyroxine alone. The explanation for this was determined in a subsequent experiment in which a single dose of actinomycin-D but no thyroxine was given to thyroparathyroidectomized rats. Measurements of calcium transport 9–36 hr later indicated a mean increase in K_{bs} of 32% to 0.145±0.008, compared to a value of 0.110±0.007 for control rats. In a third experiment in which the effects of lower doses of actinomycin-D were explored, 25 μg/100 g body weight increased K_{bs} by 37% to 0.152±0.007, whereas a dose of 12.5 μg/100 g body weight yielded data similar to the controls (0.113±0.009; 0.111±0.010).

D,L-Ethionine at intraperitoneal doses of 30 and 60 mg/100 g body weight daily for 3 or 4 days increased K_{bs} by 20–30%, to 0.157±0.009 and 0.143±0.004, respectively, compared to a control value of 0.119±0.007.

TABLE III

Binding of ^{47}Ca and ^{28}Mg by Liver Homogenates

	Amount ^{47}Ca remaining in supernate	Amount ^{28}Mg remaining in supernate
	%	%
I. Control (16)	9.03±0.76	
T_4, 1 day (14)	9.46±0.74	
	NS	
II. Control (19)	9.97±0.61	
T_4, 1 wk (14)	8.47±0.70	
	NS	
III. Control (7)		2.59±0.34
T_4, 2 wk (6)		2.52±0.24
		NS
IV. Control (5)		3.44±0.27
T_4, 3 wk (8)		3.07±0.13
		NS

Values are means±SEM. T_4, L-thyroxine.

The coadministration of D,L-ethionine at these dose levels and of 500 μg thyroxine daily yielded data (0.167 ±0.011) similar to that of thyroxine alone (0.168 ±0.011), so that a possible inhibitory effect on L-thyroxine-induced stimulation of calcium transport was not discernible. A further reduction in the dose of D,L-ethionine to 15 mg/100 g body weight caused no increase in K_{bs} compared to controls (0.119±0.004) and the administration of D,L-ethionine with thyroxine did not inhibit the stimulatory effect of the latter on calcium transport (0.132±0.004, 0.137±0.004).

Studies of the effect of actinomycin-D and D,L-ethionine on magnesium transport required the administration of actinomycin-D at least three times, 4 hr before each injection of thyroxine. The majority of rats failed to survive the 7 day period of treatment and only two successful experiments were obtained. In neither case was there any indication that actinomycin-D inhibited thyroxine-induced stimulation of magnesium transport, but the effect of actinomycin-D alone was not studied. D,L-Ethionine in an intraperitoneal dose of 60 mg/100 g body weight for 1 or 2 days appeared to increase K_{bs} and K_{sb} by 10–30%, but the number of studies was too small to make statistical comparisons.

DISCUSSION

These studies provide a direct demonstration of the ability of thyroxine to stimulate the transport of calcium and magnesium in liver, and are consonant with radioisotope turnover studies in humans indicating that thyroid hormone stimulates the turnover of divalent cations in soft tissues as well as in the skeleton. The stimulatory effect is largely on influx, but small increases in efflux are also seen. Nevertheless, the total divalent cation content of the liver slices at the beginning and end of incubation are similar to those of the control groups. Possibly, the induced stimulations of influx and efflux are not sufficiently disparate to cause a discernible change in divalent cation content during the time period studied. Since the cellular distributions of calcium and magnesium are discontinuous and multiple equilibria exist within the cell, the transport studies measure the rate of exchange between the extracellular phase and a rapidly exchanging cellular fraction, but provide no direct information as to the size of the rapidly exchanging cellular fraction nor quantitative data as to the slower rates of exchange between the rapidly exchanging cellular fraction and other intracellular fractions. Consequently, whether the induced stimulation of transport by thyroxine results in an altered distribution of divalent cation intracellularly cannot be readily answered.

The mechanism of stimulation by thyroxine was investigated at two possible loci of action. The possibility

that the facilitated inward diffusion of calcium and magnesium was accelerated by an enhanced intracellular binding of divalent cation was found to be untenable despite the demonstration that parathyroid hormone stimulation of calcium influx in a similar system is associated with increased calcium binding (14). Studies directed at the possibility that thyroxine stimulated the synthesis of an intracellular or membrane protein involved in divalent cation transport were abortive because of the inherent ability of the inhibitory agents actinomycin-D and D,L-ethionine to stimulate calcium, and possibly, magnesium transport. By reducing the doses of these inhibitory agents, it was possible to prevent the stimulation of calcium transport, and at the reduced dose, D,L-ethionine did not inhibit the stimulation of calcium transport induced by thyroxine. Unfortunately, it is not known whether this dose of D,L-ethionine (15 mg/100 g body weight) is effective in inhibiting protein synthesis. Actinomycin-D has also been found to cause hypocalcemia in intact and thyroparathyroidectomized rats.[1] These data are especially relevant to the finding that mithramycin, an analogue of actinomycin-D, can cause hypocalcemia and positive calcium balance in humans (15), and the effects of these inhibitory agents on the transport of soft tissue as well as skeletal calcium must be taken into account in considering their mechanism of action and therapeutic efficacy.

In view of these negative studies regarding the mechanism of stimulation, other possibilities will have to be sought. Aikawa and Reardon (16–18) have reported that magnesium transport in soft tissues is stimulated by thyroxine, dinitrophenol, and salicylates, suggesting that an uncoupling of oxidative phosphorylation is involved. However, there is no convincing evidence that uncoupling of oxidative phosphorylation plays a significant role in other peripheral actions of L-thyroxine in vivo (19). Recently, certain actions of thyroxine have been ascribed to the production of cyclic-AMP in peripheral tissues (20). In the rat liver slice, the in vitro addition of cyclic-AMP and of its dibutyryl derivative causes no more than 20% stimulation of calcium transport, and little or no stimulation of magnesium transport (14).[2] It is therefore unlikely that the effects of thyroxine on divalent action transport in the present study are mediated via cyclic-AMP production. A third alternative involves the ability of thyroid hormone to stimulate carbohydrate metabolism in perfused liver (21). High energy intermediates from increased carbohydrate metabolism could conceivably be the energy source for thyroxine-stimulated transport, whereas they are not involved in basal divalent cation transport in liver (12, 13). On the other hand, the carbohydrate effects could be secondary to changes in intracellular calcium mediated by thyroxine (22). Studies of thyroxine-induced stimulation in the presence of inhibitors of intermediary metabolism would be of interest to elucidate this issue.

A common cellular transport mechanism for calcium and magnesium in soft tissues such as liver, as well as in gastrointestinal mucosal and renal tubular cells, has been proposed previously (12, 13) because of the quantitatively similar transfer coefficients for the two cations, the ability of either cation to inhibit the transport of the other, and the similar effects of various manipulations of the chemical and physical characteristics of the incubation medium. However, subsequent studies have indicated that parathyroid hormone and cyclic-AMP stimulate calcium but not magnesium transport in this system (14). In addition, the present study indicates quantitatively similar stimulations of calcium and magnesium transport by thyroxine, but significant differences in the time required for stimulation to appear. In earlier experiments, a given manipulation usually had a greater stimulatory or inhibitory effect on calcium than on magnesium transport (12, 13). Thus, if a common transport mechanism is involved, it appears to favor calcium and is more easily perturbated with respect to calcium than magnesium. However, these comparisons do not rule out the possibility that thyroxine may have totally dissimilar modes of action in stimulating the transport of the two divalent cations.

Calcium and magnesium exert potent regulatory effects on a variety of cellular processes and their effects can be either complementary or antagonistic. The present study supports the possibility that certain actions of thyroid hormone on peripheral tissues may be mediated or modulated by associated changes in the cellular transport of divalent cations. Valcana and Timiras (23) have reported that the brain tissue of hypothyroid neonates has abnormally low concentrations of ATPase, magnesium, and potassium, a normal content of calcium, and high amounts of sodium, chloride, and water. It is tempting to speculate that the influence of thyroid hormones on cellular transport and concentrations of divalent cations may determine the permeability characteristics and the ATPase activity of cells. Such an effect would be of extreme importance since much of the energy expenditure attending thyroid hormone activity involves a stimulation of ATPase activity and maintenance of the cellular pumps for univalent cation transport (24).

[1] Chausmer, A. B. Unpublished observations.
[2] Unpublished observations.

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