The Effect of Thyroid Hormone on Bile Salt-Independent Bile Flow and Na^+, K^+-ATPase Activity in Liver Plasma Membranes Enriched in Bile Canaliculi

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ABSTRACT The relationship between bile salt-independent canalicular flow and ATPase activity in liver plasma membranes (LPM) enriched in bile canaliculi, was studied in control, hyperthyroid, and hypothyroid rats. Canalicular bile production was significantly increased in hyperthyroid rats (3.19±0.23 μl/min per g liver) compared to controls (2.27±0.24 μl/min per g liver), while it diminished in hypothyroid animals (1.58±0.17 μl/min per g liver). Although bile salt excretion was also increased in hyperthyroid animals (62.4±13.3 vs. 41.2±8.4 nmol/min per g liver), the stimulation in canalicular secretion was primarily related to enhancement of the bile salt-independent fraction of flow (2.47 μl/min per g liver in hyperthyroid rats vs. 1.67 μl/min per g liver in controls). LPM Na^+, K^+-ATPase activity doubled in hyperthyroid animals (21.5±5.8 vs. 10.7±3.1 μmol P_i/mg protein per h) while Mg^{++}-ATPase activity remained unchanged and 5′-nucleotidase activity increased to a small but significant extent. In hypothyroid rats, bile salt excretion remained unchanged from control values so that the reduced secretion was entirely secondary to an inhibition of bile salt-independent secretion (1.19 μl/min per g liver). Na^+, K^+-ATPase activity in LPMs from hypothyroid animals decreased by nearly 50% (5.4±1.6 μmol P_i/mg protein per h), although comparable reductions in the specific activity of Mg^{++}-ATPase and 5′-nucleotidase were also observed. Administration of L-thyroxine to hypothyroid animals restored both bile salt-independent canalicular secretion and membrane enzymes to control values within 2 and 4 days, respectively. Sodium dodecyl sulfate gel electrophoresis demonstrated no significant changes in LPM protein fractions from any of the treatment groups. These studies indicate that thyroid hormone has a parallel effect on bile salt–independent canalicular secretion and LPM Na^+, K^+-ATPase activity, supporting the hypothesis that Na^+ transport and Na^+, K^+-ATPase may be determinants of bile salt–independent canalicular flow.

INTRODUCTION

Canalicular bile formation is a complex process which is dependent on the osmotic effects of bile salt excretion as well as a bile salt-independent mechanism (bile salt-independent flow [BSIF]) [1] (1, 2). While canalicular excretion of bile salts results in a given fraction of bile per micromole of bile salts (3, 4), BSIF consists of a relatively constant portion of basal flow that ranges from 20% of canalicular secretion in the dog (5), to over 50-60% in the rabbit (6) or rat (7-9), and 30% in man (10, 11). BSIF may be induced by certain compounds such as phenobarbitol (7), and inhibited by other drugs, chemicals, or steroids (1). Several studies suggest that this secretion is related at least in part to the active transport of sodium (6, 9). If this hypothesis is true, BSIF might be regulated by the activity of Na^+, K^+-ATPase, a membrane-bound enzyme that controls the transport of sodium across many other cellular

Portions of this study were presented at the Annual Meeting of the American Federation for Clinical Research, May 1974. (1974. *Clin. Res.* 22: 363A.)

Dr. Layden is a recipient of a U. S. Public Health Service fellowship AM 01742, and Dr. Boyer is a recipient of an Academic Career Development Award from the National Institute of Arthritis, Metabolism, and Digestive Diseases (AM 70218). Dr. Layden's present address is Westside Veterans Administration Hospital, University of Illinois, Chicago, Ill. 60612.

Received for publication 17 July 1975 and in revised form 10 December 1975.

[1] *Abbreviations used in this paper:* BSIF, bile salt-independent flow; LPM, liver plasma membrane; SDS, sodium dodecyl sulfate; T_3, L-3,5,3′-triiodothyronine; T_4, L-thyroxine.

membranes (12-15). In support of this view, previous studies from this laboratory have demonstrated that Na^+, K^+-ATPase is present in fractions of liver plasma membranes that are enriched in bile canaliculi. Furthermore, the properties of this enzyme are consistent with a role in bile secretion (16, 17). In the present study, we have assessed the effects of thyroid hormone on the determinants of bile secretion in the rat, since a major biologic effect of triiodothyronine and thyroxine is the stimulation of sodium transport and plasma membrane Na^+, K^+-ATPase in a variety of tissues (18-21). In addition to studying the effect of thyroid hormone on determinants of bile secretion in vivo, we have also measured changes in the specific activities of enzymes in liver plasma membranes that may regulate biliary secretion. Results of these studies support the hypothesis that Na^+, K^+-ATPase and sodium transport are determinants of bile secretion and indicate that modulations in thyroid hormone levels profoundly influence BSIF in the rat.

METHODS

Animals and method of thyroid administration. Nonfasted 250- to 325-g male rats (Charles River Breeding Laboratories, Wilmington, Mass.) were used in all experiments. All animals were fed Purina lab chow ad lib. (Ralston Purina Co., St. Louis, Mo.) and were maintained in a constant temperature environment (22°C) with alternating 12-h light and dark cycles.

Animals in control and hyperthyroid groups were studied when they reached a weight of 250–300 g. Animals were then made hyperthyroid with three 50-μg intraperitoneal injections of L-3,5,3'-triiodothyronine (T_3) (Sigma Chemical Co., St. Louis, Mo.)/100 g body wt which was dissolved in 0.5 mM NaOH and given at 48-h intervals as previously described (18). An additional group of animals were made hypothyroid by removal of the thyroid gland by Charles River Breeding Laboratories when animals weighed approximately 200 g. Thyroidectomized animals were then maintained on drinking water containing 1.0% calcium lactate, and were studied 4–5 wk after thyroidectomy when they weighed approximately 250–300 g. Basal metabolic rate was restored to control values in hypothyroid animals by administering L-thyroxine (T_4) (Sigma Chemical Co.) which was dissolved in 0.5 mM NaOH and administered intraperitoneally at a dose of 20 μg/100 g body wt for 2–4 consecutive days (22). Thus, at the time of study, age and weight were comparable in the control and hyperthyroid group and in the hypothyroid and hypothyroid-treated group. However, hypothyroid animals were approximately 2 wk older than control and hyperthyroid animals to achieve comparable body weights. All animals were studied or sacrificed between 8 and 10 a.m., 24 h after the last injection of thyroid hormone.

Studies of bile secretion, [^{14}C]erythritol clearance, and bile acid excretion. Groups of control, hyperthyroid, hypothyroid, and hypothyroid-treated animals (12 animals in each group) were anesthetized with intraperitoneal pentobarbital (5.0 mg/100 g body wt) to study the effects of thyroid hormone on bile secretion. Only one-half to one-third the dose was required to anesthetize hypothyroid animals. The abdominal cavity was then exposed by a midline incision and the bile duct was isolated and cannulated with a polyethylene cannula (Clay-Adams, Inc., Parsippany, N. J., PE-10). The tip of the cannula was positioned just below the bifurcation of the common bile duct and was firmly tied in place with a 5:0 silk suture. The renal pedicles were ligated to avoid loss of the injected isotopes and fluid loss was corrected by infusing 0.9% NaCl into the jugular vein with a Harvard Pump (Harvard Apparatus Co., Inc., Millis, Mass.) calibrated to deliver 0.04 ml/min. Body temperature was monitored with a rectal probe and maintained at 37.5°C by a heating lamp connected to a temperature control device (Yellow Springs Instrument Co., Yellow Springs, Ohio).

Estimates of canalicular bile flow were determined as previously described (8, 9). [^{14}C]Erythritol (3 μCi), mixed with 5 mg of unlabeled erythritol and dissolved in 0.6 ml of 0.9% saline, was injected into the jugular vein immediately after bile duct cannulation and renal pedicle ligation. After a 20-min stabilizing period, bile was collected at 15-min intervals for 90 min. Bile volume was measured in a 1-cm^3 calibrated plastic syringe. 0.5-ml heparinized blood samples were obtained at 52.5, 67.5, and 82.5 min for determinations of [^{14}C]erythritol in plasma. At the completion of the studies the liver was removed and the wet weight determined. 50-μl samples of bile and plasma were then added to 12 ml of dioxane and counted in a Nuclear-Chicago Isocap liquid scintillation counter (Searle Analytic Inc., Des Plaines, Ill.) to an error of $< 1.0\%$. Quenching was corrected by external standard ratios. Bile-to-plasma ratios of [^{14}C]erythritol were calculated for the last three periods of bile collection in each animal, since bile-to-plasma [^{14}C]-erythritol values did not vary significantly in individual experiments. Canalicular clearance was determined by disintegrations per minute bile per disintegrations per minute plasma times microliters per minute per grams liver or per kilograms body weight.

Bile acids were also determined in duplicate 5-μl samples of bile from each 15-min bile collection using the 3-hydroxysteroid dehydrogenase method (23). BSIF was determined as previously described by correlating bile salt excretion with [^{14}C]erythritol clearance in each 15-min bile sample and extrapolating the linear regression of this relationship to zero bile salt excretion (9, 10).

Isolation of liver plasma membranes (LPM) enriched in bile canaliculi. LPM were isolated from control, hypothyroid, and hyperthyroid rats by a modification of the discontinuous sucrose gradient method of Song et al. (24), as previously described from this laboratory (16). Livers were removed from decapitated animals at 5°C, placed on ice, and the hepatic veins were perfused with 1 mM $NaHCO_3$ buffer (pH 7.5) to remove erythrocytes. Liver tissue was then minced on ice with scissors, and homogenized in 4 vol of buffer, using 15 strokes in a loose-fitting Dounce homogenizer. The homogenate was filtered through a double layer of surgical gauze to remove particulate matter and then centrifuged at 1,500 *g* for 10 min to obtain a pellet of crude membranes. This membrane fraction was then diluted in 1 mM $NaHCO_3$ to a volume equal to the original volume of liver tissue. Sucrose, *d* 1.26 (70.74% wt/vol) was added to the membranes and gently stirred with a magnetic bar. 7- and 5-ml sucrose solutions with respective densities of 1.18 (48.45% wt/vol) and 1.16 (42.97% wt/vol), were then carefully layered over the sucrose membrane mixture and centrifuged in an angle rotor (Spinco 30) at 66,000 *g* for 1 h in a Beckman L2 ultracentrifuge (Beckman Instruments, Inc., Spinco Div., Palo Alto, Calif.). Canalicular membranes were recovered at the *d* 1:16 – *d* 1:18 interface and diluted twice with 4–5 vol of 1 mM $NaHCO_3$ buffer and recentri-

TABLE I

Effect of Thyroid Hormone on Liver Weight, Body Weight, and Liver Weight/Body Weight Ratio

	Control	Hyperthyroid	Hypothyroid	Hypothyroid treated (2 days T_4)
	(20)	(20)	(28)	(15)
Body wt, *g*	290.8 ±20.7	286.1 ±18.1	266.0* ±27.0	273.7* ±19.3
Liver wt, *g*	11.2 ±1.6	10.0* ±1.0	7.6* ±1.1	7.1* ±0.7
Liver wt/body wt	0.038 ±0.004	0.035* ±0.003	0.028* ±0.003	0.026* ±0.002

Numbers of experiments are given in parentheses. Data are expressed as means±SD.
* Significantly different from control at $<0.01-<0.001$ by Student's *t* test.

fuged at 1,500 *g* for 10 min in a Sorvall refrigerated centrifuge (4°C) (Ivan Sorvall, Inc., Norwalk, Conn.). The final pellet was resuspended in distilled water to a final concentration of protein equalling 3–4 mg/ml.

Protein was determined by the method of Lowry et al. (25) using phenol reagent and crystallized bovine plasma albumin (Metrix, Armour Pharmaceutical Co., Chicago, Ill.) as the standard.

Phase and electron microscopy. Membrane preparations were periodically examined by phase and transmission electron microscopy to determine the purity and enrichment of the preparation obtained from control and experimental animals. Membrane pellets were fixed at 5°C for 2 h in 1% osmium tetroxide buffered with s-collidine (pH 7.4), embedded in Epon, then sectioned and stained with uranyl acetate and lead citrate. Samples were photographed with a Siemens Elmiskop IA electron microscope (Siemens Corp., Iselin, N. J.).

Plasma membrane ATPase determinations. Enzyme assays were determined in LPM from 8 control, 8 hyperthyroid, and 16 hypothyroid animals. Assays were also performed after T_4 was administered to eight hypothyroid animals for 2 days and to six hypothyroid animals for 4 days. It was necessary to combine livers from two untreated hypothyroid animals to obtain sufficient protein for the assays since the yield of LPM was substantially reduced in this group. LPM yield was restored to normal after only 2 days of T_4 so that membranes were isolated from individual livers in the hypothyroid-treated groups. ATPase assays were usually performed on the day of LPM isolation although enzyme activity was stable when stored overnight at 4°C. The assay conditions for measuring Na^+, K^+-ATPase in the LPM preparation have been described in detail previously from this laboratory (16). Total ATPase activity was assayed using 150–250 μg of LPM protein. The standard incubation media for total ATPase activity consisted of 150 mM NaCl, 20 mM imidazole buffer (Eastman Kodak Co., Rochester, N. Y.), and 5 mM KCl adjusted to pH 7.8 at 37°C. KCl was omitted from the incubation media to measure the specific activity of Mg^{++}-ATPase, and the difference between total ATPase- and Mg^{++}-ATPase-represented Na^+, K^+-ATPase. To initiate the reaction, 100 μl of a 0.125-M Mg Cl_2—disodium ATP (Sigma Chemical Co., A-3127) solution was added and allowed to incubate for exactly 15 min in a Dubnoff shaker at 37°C. Addition of 1 ml of trichloroacetic acid (TCA) (35%, wt/vol) terminated the reaction. The sample was centrifuged at 25,000 *g* for 10-min and 1.5-ml aliquots of the supernate were analyzed for inorganic phosphate by the Fiske-Subbarow method (26). All determinations were performed in duplicate or triplicate and enzyme protein was eliminated from control assays to monitor for spontaneous hydrolysis of ATP. The specific activity of ATPase was expressed as micromoles P_i per milligram LPM protein per hour.

Enzyme marker assays. 5'-Nucleotidase, a plasma membrane marker, was measured in various LPM isolates by the method of Song and Bodansky (27). To compare the purity of the various membrane preparations, enzyme markers of microsomes and mitochondria were also analyzed in the LPM fraction of control, hypothyroid-treated, and hyperthyroid animals. Sufficient protein was not available for these assays in hypothyroid animals. Succinate dehydrogenase (EC 1.3.99.1) (Shephard and Hübscher [28]) was assayed as a marker for mitochondria, and glucose-6-phosphatase (EC 3.1.3.9) (Nordlie and Arion [29]) was measured to assess microsomal contamination.

Sodium dodecyl sulfate (SDS)-acrylamide gel electrophoresis. Plasma membranes were isolated from control and experimental animals as described above and 175 μg was solubilized for 1 h at 65°C with 50 μl of a stock solution of 2% (wt/vol) SDS (Fisher Scientific Co., Pittsburgh, Pa.), 2% 2-mercaptoethanol (vol/vol) (J. T. Baker Chemical Co., Phillipsburg, N. J.), and 6 M urea (Fisher Scientific Co.) (30, 31). The solubilized proteins were separated on 5.5% acrylamide—6 M urea gels containing 0.1% SDS. Electrophoresis was performed in 0.1 M phosphate buffer (pH 7.4) at a constant current of 6 mA/gel. Gels fixed overnight in 10% TCA were stained with 0.25% Coomassie Blue for 2 h and destained in 10% acetic acid at 70°C. Gels were scanned at 555 nm in Gilford 2400 spectrophotometer (Gilford Instrument Laboratories, Inc., Oberlin, Ohio).

Molecular weights were estimated by the electrophoresis of proteins with known molecular weights according to the method of Shapiro et al. (32). Immunoglobulin G (160,000), bovine albumin (67,000), chymotrypsin A (25,000), and myoglobin (17,800) were obtained from Schwarz/Mann (Div. of Becton, Dickinson & Co., Orangeburg, N. Y.) and electrophoresis was performed under identical conditions as described for membrane proteins.

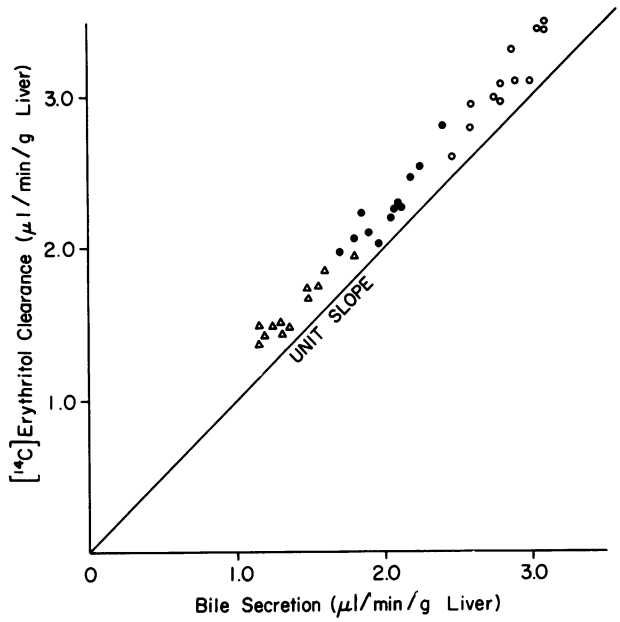

FIGURE 1 Biliary clearance of [^{14}C]erythritol as a function of total bile flow in control (●), hypothyroid (△), and hyperthyroid (○) animals.

RESULTS

Thyroid hormone effects on liver and body weight: Table I summarizes the effect of thyroid hormone on body weight, liver weight, and liver weight-body weight ratios. Hyperthyroid animals had significantly lower liver weights (10.0±1.0 g) than control animals (11.2±1.6 g) although differences in body weight were not significant. In hypothyroid animals, liver mass was substantially reduced and did not change significantly after 2 days of T_4. Although not depicted, the weight gain in hypothyroid and hypothyroid-treated animals was similar.

Bile secretion. Bile-to-plasma [^{14}C]erythritol ratios averaged 1.10±0.05 in control animals and were not significantly different in the hypothyroid or hyperthyroid state despite the wide range in biliary secretion (Fig. 1). If one assumes that erythritol enters the bile at the canaliculus and does not traverse the bile ducts (5-11), a ratio greater than one would suggest that some ductular reabsorption of water may have occurred in these experiments. However, the proximity of the bile-to-plasma ratio of [^{14}C]erythritol to unity and the absence of effect of thyroid hormone on this relationship suggests that the observed secretory changes resulted primarily from alterations in canalicular secretion. The alternative that erythritol diffuses freely across the ductules seems much less likely, in view of experimental evidence obtained in other species.

Fig. 2 illustrates the effect of thyroid hormones on [^{14}C]erythritol clearance and bile acid output. There was a significant reduction in [^{14}C]erythritol clearance in hypothyroid animals (1.58±0.17 μl/min per g liver) compared to control animals (2.27±0.24 μl/min per g liver). However, after 2 days of T_4 replacement, canalicular secretion increased significantly to 2.59±0.41 μl/min per g liver. No further increase was observed after 4 days of T_4 (2.72±0.31 μl/min per g liver). In contrast to hypothyroid animals, the hyperthyroid state was associated with a significant enhancement in [^{14}C]-erythritol clearance (3.19±0.23 μl/min per g liver). Since liver weights changed during thyroid hormone replacement or withdrawal, data were also expressed as a function of body weight to determine if similar secretory relationships existed (Table II). The only difference observed when data were expressed in terms of body weight was that 4 days of treatment with T_4 were required to restore [^{14}C]erythritol clearance to normal rather than 2. Bile salt excretion during the 90-min collection was significantly altered only in hyperthyroid animals, increasing from the control value of 41.2±8.4 to 62.4±13.3 nmol/min per g liver. Bile salt outputs in hypothyroid and hypothyroid-treated animals were similar to the control groups.

Bile salt-independent secretory function. Fig. 3 illustrates the relationship of bile salt excretion to [^{14}C]-erythritol clearance in control, hypothyroid, and hyperthyroid animals. For any rate of bile salt excretion, [^{14}C]erythritol clearance was significantly greater in hyperthyroid and lower in hypothyroid animals when compared to controls. The bile salt-independent component averaged 1.67 μl/min per g liver in control animals and was significantly lower in the hypothyroid state, averaging 1.19 μl/min per g liver. In contrast, the bile acid-independent component was significantly

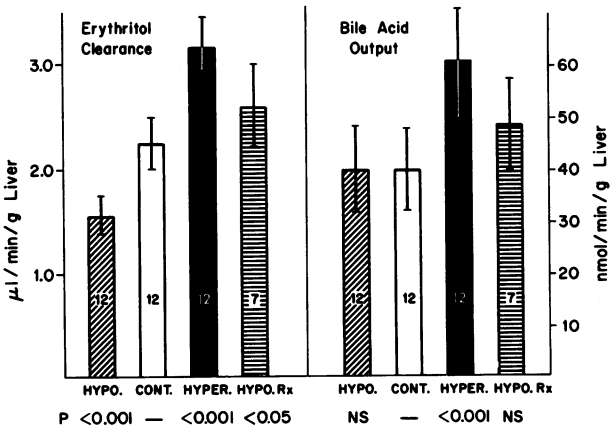

FIGURE 2 [^{14}C]Erythritol clearance is shown in left-hand panel and bile salt excretion is shown in right-hand panel in hypothyroid (Hypo), control (Cont), hyperthyroid (Hyper), and hypothyroid animals treated with 20 μg T_4/100 g body wt for 2 days (Hypo Rx). Number on each bar is the number of animals studied in each group.

TABLE II

Effect of Thyroid Hormone on [^{14}C]Erythritol Clearance

	[^{14}C]Erythritol clearance	P*	P‡
	μl/min/kg body wt		
Control	81.5±9.3	—	<0.001
Hyperthyroid	109.6±10.3	<0.001	<0.001
Hypothyroid	44.6±4.5	<0.001	—
2 days T_4	63.2±12.8	<0.005	<0.001
4 days T_4	77.0±8.3	—	<0.001

* Compared with control.

‡ Compared with hypothyroid.

P value determined by Student's *t* test.

increased in the hyperthyroid state, averaging 2.47 μl/min per g liver. Even though bile acid output was also increased, the changes in canalicular secretion resulted primarily from increases in the bile salt-independent fraction. Increases in bile secretion observed after T_4 replacement in hypothyroid animals were also predominantly related to a stimulation in the bile salt-independent component (Fig. 4). The slope of the regression lines in hyperthyroid (0.0112) and hypothyroid animals (0.0109) were not substantially different than the slope in control animals (0.0149). Thus, each mole of bile salt resulted in an equivalent change

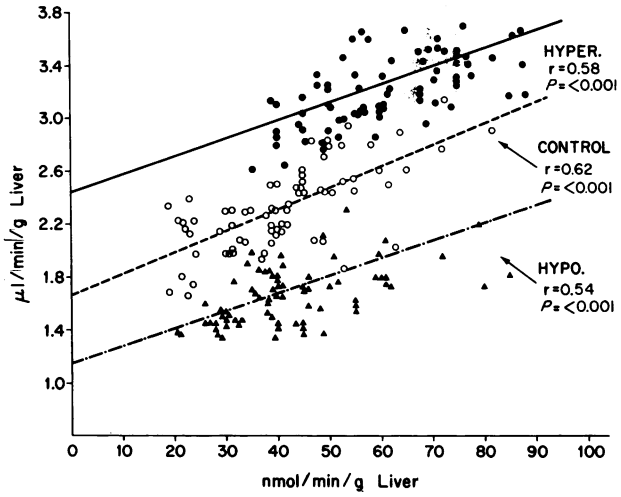

FIGURE 3 [^{14}C]Erythritol clearance as a function of bile salt excretion in 12 hyperthyroid (Hyper), 12 control (Cont), and 12 hypothyroid (Hypo) animals. Each point is obtained from a separate 15-min bile collection period. The regression line is calculated by the method of least squares and is defined by the equation $y = mx + c$, where y = [^{14}C]erythritol clearance, x = rate of bile salt secretion, m = slope of the regression line, and c = the y intercept. R = the correlation coefficients which were significant to $P < 0.001$. The values for each of the y intercepts (BSIF) were significantly different from one another ($P < 0.01$).

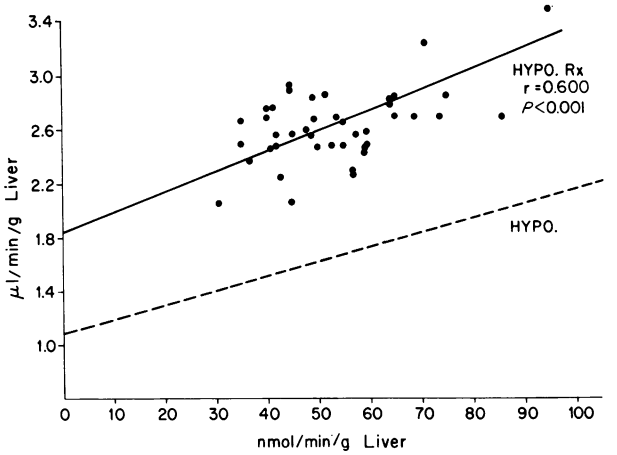

FIGURE 4 [^{14}C]Erythritol clearance as a function of bile salt excretion in hypothyroid animals treated with T_4 (20 μg/100 g body wt for 2 days) (Hypo Rx). The dashed regression line represents hypothyroid (Hypo) studies obtained from data in Fig. 3. Note the increase in the bile salt-independent fraction to control values.

in bile volume in all three study groups indicating that the osmotic effects of bile salts were presumably not altered in either hypothyroidism or hyperthyroidism.

Transmission electron microscopy of LPM. Electron microscopy indicated that the LPMs were enriched in canalicular structures and free of subcellular organelles in each group, although the canalicular membranes were disrupted more frequently in the hypothyroid animals.

Analysis of markers of subcellular organelles of LPM enzymes. Table III summarizes the results obtained from assay of subcellular enzyme markers and plasma membrane enzymes in the control, hypothyroid, hypothyroid-treated and hyperthyroid animals.

The yield of LPM protein was significantly reduced in the hypothyroid state, averaging 0.075 mg protein/g liver compared to the control average of 0.297 mg protein/g liver. The yield of LPM protein, however, was restored to normal with 2 days of T_4 replacement.

The specific activity of glucose-6-phosphatase and succinate dehydrogenase, enzyme markers of subcellular organelles, was low in the LPM fraction in comparison with the specific activity of the LPM enzyme markers, 5′-nucleotidase and Mg^{++}-ATPase. These results are in agreement with earlier studies performed in this laboratory (16) which also demonstrated a 20-fold enrichment in LPM enzyme markers compared to the whole homogenate and low LPM:homogenate ratios for markers of subcellular organelles. Neither the hyperthyroid nor hypothyroid-treated state was associated with a substantial change in these ratios, although glucose-6-phosphatase and succinate dehydrogenase were not assayed in the hypothyroid group because of the low yield of protein.

TABLE III

Effect of Thyroid Hormone on Enzyme Markers in LPM

	Subcellular organelle markers and LPM protein yield		
	LPM protein	Glucose-6-phosphatase	Succinic acid dehydrogenase
	mg protein/g liver	*μmol P_i/mg protein per h*	*ηmol formazin/mg protein per h*
Control	0.297±0.15 (8)	0.526±0.22 (7)	2.8±1.2 (5)
Hyperthyroid	0.187±0.07* (8)	0.220±0.38 (3)	2.45±2.0 (3)
Hypothyroid	0.075±0.05* (8)	ND	ND
2 days T_4	0.294±0.09 (8)	0.130±0.10* (3)	3.2±2.3 (3)

	Membrane markers		
	Na^+, K^+-ATPase	Mg^{++}-ATPase	5′-Nucleotidase
	μmol P_i/mg protein per h		
Control	10.7±3.1 (8)	65.8±12.7 (8)	48.5±7.3 (8)
Hyperthyroid	21.5±5.8* (8)	64.3±15.5 (8)	59.4±13.2* (8)
Hypothyroid	5.4±1.6* (8)	39.6±4.3* (8)	30.9±3.2* (5)
2 days T_4	7.3±1.5* (8)	56.9±12.7 (8)	36.2±5.5* (6)
4 days T_4	11.1±2.3 (6)	67.7±18.8 (6)	50.2±10.5 (6)

* P = <0.05 – <0.001 by Student's *t* test.
All values compared to control.
ND, assay not performed due to low yield of membrane protein.
Number in parentheses is the number of individual LPM preparations studied.

In contrast to markers of subcellular organelles, the specific activity of the plasma membrane enzymes was markedly affected by variations in thyroid hormone status. Na^+, K^+-ATPase doubled in liver plasma membranes isolated from hyperthyroid animals (21.5±5.8 μmol P_i/mg protein per h), compared to control values (10.7±3.1 μmol P_i/mg protein per h) (Table III), while the specific activity of Mg^{++}-ATPase remained unchanged. 5′-Nucleotidase also increased slightly but significantly from 48.5±7.3 to 59.4±13.2 μmol P_i/mg protein per h in control and hyperthyroid animals, respectively. In plasma membranes isolated from hypothyroid animals, Na^+, K^+-ATPase activity (5.4±1.6 μmol P_i/mg protein per h) diminished significantly from control values. However, there also was a similar reduction in both Mg^{++}-ATPase and 5′-nucleotidase activity. Thus, the activity of all three LPM enzymes were decreased by 30-45% in the hypothyroid animals. T_4 replacement (20 μg/100 g body wt) progressively increased the specific activity of Na^+, K^+-ATPase from 5.4±1.6 to 7.3±1.5 at 2 days and 11.1±2.3 μmol P_i/mg protein per h 4 days after treatment (Table III). Furthermore, Mg^{++}-ATPase and 5′-nucleotidase also progressively and significantly increased during the 4 days of T_4 administration.

SDS-acrylamide gel electrophoresis. 12-13 polypeptide fractions with molecular weights ranging from 30,000 to 300,000 were identified in LPM from control animals when the gels were scanned at 555 nm. The number and appearance of identifiable LPM polypeptide fractions were similar to controls in hyper- and hypothyroid animals (Fig. 5).

DISCUSSION

Previous studies indicate that thyroid hormone alters bile secretory function by influencing both the rate of bile production and the synthesis and biliary excretion

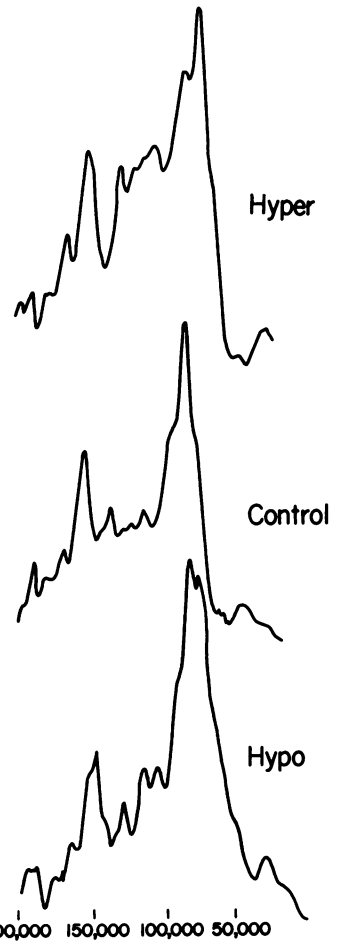

FIGURE 5 LPM proteins (175 μg) were separated by electrophoresis on 5.5% acrylamide 6-M urea gels containing 0.1% SDS. Gels were stained with Coomassie Blue and scanned at 555 nm. Molecular weights were estimated by the method of Shapiro et al. (32). 13 polypeptide fractions were identified in control (Cont), hyperthyroid (Hypo), and hyperthyroid (Hyper). Molecular weights ranged from 30,000 to 300,000. No qualitative differences in the protein fractions from the different treatment groups were observed.

of bile acids. Bile secretion declines in thyroidectomized rats according to Gartner and Arias (33), although bile salt output remains unchanged in the initial 6 h of bile collection as reported by Eriksson (34) and Strand (35). In contrast, in the hyperthyroid rat there is an expansion of the bile acid pool, an increase in synthesis of chenodeoxycholic acid, and an increase in biliary excretion of bile salts (34, 35). Thyroid hormone also altered bile flow and bile acid excretion in the present studies. However, the major changes in bile secretion were related to alterations in BSIF, which increased by nearly 50% in hyperthyroid animals and diminished by 30% in the hypothyroid state (Fig. 3). Although bile salt excretion increased in hyperthyroid animals, it accounted for only 10% of the observed increment in bile flow (0.3 μl/min per g liver), and no changes in bile salt excretion were observed in the hypothyroid animals.

BSIF was also increased when hypothyroid animals were treated with T_4. Under these conditions, BSIF returned to control levels in 2 days without any significant change in caloric intake, bile salt output, and liver or body weight compared to the untreated hypothyroid animals. These findings indicate that thyroid hormone has a direct effect on BSIF, which is independent of changes in body or liver mass or dietary intake and illustrate that this fraction of canalicular secretion can be substantially modified by physiological changes in the level of this endogenous hormone in the rat.

Although it is not entirely clear how thyroid hormone influences BSIF, neither T_3 nor T_4 altered the volume of bile secreted per micromole of bile acid, indicating that thyroid hormone did not change the osmotic effect of bile acids. Since the biliary tract is the major route of excretion for both T_3 and T_4 (36), it is possible that a choleresis resulted from the osmotic effects of excretion of these solutes into bile. However, even if each hormone was completely eliminated in the bile in monomolecular form, and underwent an efficient enterohepatic circulation, the osmolarity of thyroid hormone would be negligible compared to bile acids. Thyroid hormone might stimulate secretion or reabsorption of bile peripherally at the level of the biliary ductules. However, this possibility also seems unlikely since [^{14}C]-erythritol is assumed to enter bile predominantly at the level of the canaliculus (37, 38), and bile-to-plasma ratios of [^{14}C]erythritol were similar to control ratios in either the hypothyroid or hyperthyroid state. The alternative that [^{14}C]erythritol passively diffuses across both the hepatocytes and ducts cannot be completely excluded in the rat, however. Nevertheless, the data in the present study are most consistent with the hypothesis that thyroid hormone affects bile secretion by modifying the specific activity of Na^+, K^+-ATPase in canalicular membranes, thereby regulating the rate of sodium excretion into bile.

Although a number of previous studies have suggested that BSIF might be mediated by the active transport of sodium (1, 6, 9), and the activity of Na^+, K^+-ATPase, conclusive evidence is still lacking. Nevertheless, cardiac glycosides inhibit the enzyme in canalicular-enriched LPM fractions in concentrations required to inhibit BSIF in the isolated perfused rat liver (16); temperature dependence of bile flow in this system and LPM Na^+, K^+-ATPase are also identical (16); substitution of sodium for lithium in the perfusate of the isolated perfused rat liver is associated with an impairment in bile secretion (39); several drugs and hormones such as ethynylestradiol (40, 41), rose bengal (42, 43) and icterogenin (44), have inhibitory effects on both BSIF in vivo, and LPM ATPase activities in vitro; and chronic ethanol administration, which is associated with enhanced BSIF (45), also stimulates Na^+, K^+-ATPase in liver slices in rats (46). All of these findings are consistent with a role for Na^+, K^+-ATPase in the regulation of BSIF.

Moreover, there is considerable evidence that the metabolic effect of thyroid hormone is exerted largely through the regulation of Na^+, K^+-ATPase activity in a variety of tissues including rat liver homogenate and LPM (18-21). Although thyroid hormone influences liver protein and RNA synthesis (47, 48), as well as glycolytic, mitochondrial, and microsomal enzyme systems (22, 49), changes in these metabolic functions do not account for the major change in oxygen consumption that is associated with alterations in thyroid hormone status. Recently, Ismail-Beigi and Edelman have presented evidence which suggests that a major portion of the thermogenic action of thyroid hormone is mediated through an effect on transmembrane cation transport, which can largely be accounted for by changes in ouabain-sensitive sodium transport (18-21). Furthermore, modulations in thyroid hormone levels preferentially affect the specific activity of Na^+, K^+-ATPase in liver and kidney homogenates and in LPM (18).

Our findings confirm that thyroid hormone has a significant effect on Na^+, K^+-ATPase activity in rat LPM since Na^+, K^+-ATPase doubled in LPM from the hyperthyroid rats, while Mg^{++}-ATPase remained unchanged. We also observed small increments in 5′-nucleotidase in contrast to the findings of Ismail-Beigi and Edelman (18), which might be related to differences in techniques of membrane isolation since Ismail-Beigi and Edelman used the Neville procedure (50). However, the major effect on the LPM enzymes in the

hyperthyroid animals was on Na^+, K^+-ATPase, which is in agreement with the observation of Ismail-Beigi and Edelman. Since the major effect of thyroid administration is on Na^+, K^+-ATPase in the LPM, the parallel changes in BSIF and LPM Na^+, K^+-ATPase activity provided further indirect support for the concept that BSIF may be mediated by sodium transport.

More generalized effects on LPM enzyme activity were observed in the hypothyroid animals since Mg^{++}-ATPase and 5′-nucleotidase activity decreased to a similar extent as Na^+, K^+-ATPase. These findings indicate that hypothyroidism caused diffuse effects on LPM enzymes which may have resulted from a reduction in hepatic protein synthesis. Other data from this laboratory support this contention since [^{3}H]leucine incorporation into the LPM fraction is decreased in the hypothyroid state (51). Although these findings demonstrate that a deficiency of thyroid hormone results in a broad spectrum of effects on LPM proteins, the observation that Na^+, K^+-ATPase activity was also reduced is still consistent with a role for this enzyme in the genesis of BSIF.

T_4 replacement, for 2 and 4 days in hypothyroid animals, progressively stimulated the activity of Na^+, K^+-ATPase as well as other LPM enzymes. Although there was a slight increase in Na^+, K^+-ATPase activity after 2 days of T_4 replacement, normal enzyme values were achieved only after 4 days of T_4 administration despite the observation that BSIF returned to normal within 2 days. This temporal dissociation between achievement of control Na^+, K^+-ATPase activity and BSIF in the hypothyroid-treated studies suggests that other undefined factors may contribute to the restoration of BSIF. However, enzyme activity assayed in vitro does not always reflect in vivo conditions. For instance, thyroid hormone may initially stimulate enzyme velocity or turnover rate, which would not be detected in this type of assay. Also, since the yield of LPM protein increased fourfold and the incorporation of [^{3}H]leucine into the LPM fraction was restored to normal with 2 days of T_4 replacement (51), an increase in Na^+, K^+-ATPase specific activity of greater magnitude may well have been obscured or diluted by a substantial increase in synthesis of new membrane proteins. Thus, several factors may account for the temporal dissociation between Na^+, K^+-ATPase specific activity and BSIF in the studies in the hypothyroid-treated animals.

The present studies do not clarify whether thyroid hormone has a specific effect on Na^+, K^+-ATPase activity or whether changes in enzyme activity are mediated indirectly through other physiological effects of the hormone. It is possible that thyroid hormone directly stimulates the turnover and synthesis of Na^+, K^+-ATPase or alters the phospholipid composition within the membrane so that an increase in enzyme activity occurs (19). Unfortunately, SDS gel electrophoresis of canalicular-enriched plasma membranes did not demonstrate any qualitative changes in protein bands obtained from either hyperthyroid or hypothyroid animals. Furthermore, studies of the turnover of these protein fractions indicate that changes in protein synthesis and degradation occur in many different polypeptide fractions (51).

Other effects of thyroid hormone may indirectly influence Na^+, K^+-ATPase. In the studies of Katz and Lindheimer (52), thyroid hormone was found to influence both sodium excretion in the urine and kidney microsomal Na^+, K^+-ATPase activity. Rather than demonstrating a direct effect of the hormone on the enzyme, changes in Na^+, K^+-ATPase appeared to be secondary to the effects of thyroid hormone on renal blood flow and the flux of sodium which led to secondary adaptive changes in enzyme activity. Although hepatic blood flow is also likely to be influenced by the thyroid status of the animal, phenobarbitol, which greatly increases hepatic blood flow (53), did not result in increased specific activity of Na^+, K^+-ATPase in our canalicular-enriched LPM preparation (17). It is conceivable that the increased flux of bile acids modified the structure of the LPM so that Na^+, K^+-ATPase activity in hyperthyroid rats was increased. However, the increase in bile acid output is largely chenodeoxycholic acid (34, 35) and studies from our laboratory indicate that this bile acid produces progressive inhibition of Na^+, K^+-ATPase in control LPM at concentrations from 0.1 to 5 mM (54). Cyclic AMP stimulates Na^+ transport in other tissues, but although adenyl cyclase activity is increased in some organ systems by thyroid hormone (55), the enzyme is not affected by thyroid hormone in rat LPM (56). Furthermore, dibutryl cyclic AMP diminishes the specific activity of Na^+, K^+-ATPase in rat LPM (57). It is also possible that Na^+, K^+-ATPase might be stimulated in LPMs by an increased passive movement of Na^+ that could result from an increase in the intracellular concentration of Na^+. However, this hypothesis has been rejected by Ismail-Beigi and Edelman (20) who showed that the intracellular concentration of Na^+ in liver tissue from the rat actually declined in the hyperthyroid animals compared to controls. Finally, it is possible that thyroid hormone either increases the surface area or alters the three-dimensional structure of the bile canaliculus. However, we have examined this structure with scanning electron microscopy in both hyperthyroid and hypothyroid animals, and can find no significant differences in the appearance, size, and number of the microvilli; or the dimensions of the canalicular space.

ACKNOWLEDGMENTS

This research was supported by U. S. Public Health Service grant AM 17153.

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