

# RELATIONSHIP BETWEEN $\text{Na}^+$ -DEPENDENT RESPIRATION AND $\text{Na}^+ + \text{K}^+$ -ADENOSINE TRIPHOSPHATASE ACTIVITY IN THE ACTION OF THYROID HORMONE ON RAT JEJUNAL MUCOSA

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**ABSTRACT** Administration of three successive doses of triiodothyronine ( $\text{T}_3$ ) (50  $\mu\text{g}/100$  g body wt), given on alternate days to thyroidectomized and euthyroid rats, stimulated oxygen consumption ( $\text{QO}_2$ ) and  $\text{Na}^+$  transport-dependent respiration ( $\text{QO}_2(t)$ ) in the stripped jejunal mucosa, a preparation that consisted mostly of epithelial cells. The increase in  $\text{QO}_2(t)$  accounted for 57% of the increment in  $\text{QO}_2$  in the transition from the hypothyroid to the euthyroid state and for 29% of the increment in the transition from the euthyroid to the hyperthyroid state. Administration of  $\text{T}_3$  to hypothyroid rats also increased the yield of epithelial cells. Injection of  $\text{T}_3$  into thyroidectomized and euthyroid rats increased the specific activity (at  $V_{\text{max}}$ ) of the ( $\text{Na}^+ + \text{K}^+$ )-dependent adenosine triphosphatase (NaK-ATPase) in jejunal crude membrane preparations. No significant change was recorded in the activity of Mg-ATPase in the same preparation. The ratio of  $\text{QO}_2/\text{NaK-ATPase}$  and  $\text{QO}_2(t)/\text{NaK-ATPase}$  in the various thyroid states remained constant, indicating proportionate increases in the respiratory and enzymatic indices. The effect of administration of  $\text{T}_3$  to thyroidectomized rats on the number of NaK-ATPase units (recovered in the crude membrane preparation) was estimated by: (a)  $\text{Na}^+ + \text{Mg}^{++} + \text{ATP}$ -dependent binding of [ $^3\text{H}$ ]-ouabain to crude membrane fractions, and (b) the amount of the phosphorylated intermediate formed in the NaK-ATPase reaction from  $\text{AT}^{32}\text{P}(\gamma)$ . Estimates were obtained of the maximal number of [ $^3\text{H}$ ]ouabain binding sites ( $N_m$ ) and dissociation constants ( $K_d$ ).  $N_m$  for [ $^3\text{H}$ ]ouabain and NaK-ATPase specific activity increased to about the same extent after  $\text{T}_3$  administration to thyroidectomized rats, with no change in the apparent  $K_d$  values. The amount of phosphorylated intermediate formed in jejunal crude membrane preparations also increased significantly. Thus, thyroid hormone administration may increase the number of active  $\text{Na}^+$  pump sites

Preliminary reports of this work were presented at the Pacific Regional Conference on General and Comparative Endocrinology, July, 1973 and at the 56th Annual Meeting of the Endocrine Society, June, 1974.

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in the plasma membrane. The apparent increase in the number of  $\text{Na}^+$  pump sites also correlated with the hormone dependent increases in  $\dot{Q}\text{O}_2$  and  $\dot{Q}\text{O}_2(t)$ .

## INTRODUCTION

Ismail-Beigi and Edelman (1) proposed that increased energy expenditure for transmembrane active  $\text{Na}^+$ -transport (the  $\text{Na}^+$  pump) mediates a significant fraction of the thermogenic response to thyroid hormone. This hypothesis is supported by the following lines of evidence: In liver, skeletal muscle, and kidney, 30–100% of the thyroid-induced increments in oxygen consumption ( $\dot{Q}\text{O}_2$ ) could be attributed to increased utilization of energy by the  $\text{Na}^+$  pump (1–3). Oxygen consumption coupled to active  $\text{Na}^+$ -transport ( $\dot{Q}\text{O}_2(t)$ ), was defined as the ouabain-sensitive or  $\text{Na}^+$ -dependent respiration. A similar effect of thyroid status on the specific activity of  $(\text{Na}^+ + \text{K}^+)\text{-adenosine triphosphatase}$  (NaK-ATPase) (the biochemical expression of the  $\text{Na}^+$  pump) was observed in crude homogenates and partially purified cell membrane fractions from the same tissues (2–4). The specificity of the thyroid hormone effect on NaK-ATPase activity was indicated by the lack of comparable effects on other membrane bound enzymes, and on the NaK-ATPase from the adult rat brain, which is unresponsive to the thermogenic action of the hormone (2). Proportionate effects of  $\text{T}_3$  on  $\dot{Q}\text{O}_2(t)$  and NaK-ATPase activity of skeletal muscle were observed after hormone administration to hypothyroid and euthyroid rats (3). Similarly, in skeletal muscle from thyroidectomized rats injected with single doses of  $\text{T}_3$ , either 10, 50, or 250  $\mu\text{g}/100$  g body wt,  $\dot{Q}\text{O}_2(t)$  increased linearly with NaK-ATPase activity (3). The changes in NaK-ATPase activity after thyroid hormone administration correlated with effects on intracellular  $\text{K}^+$  and  $\text{Na}^+$  concentrations. Injection of  $\text{T}_3$  to either hypothyroid or euthyroid rats lowered the intracellular ratio of  $\text{Na}^+/\text{K}^+$  by 20–40% in liver slices in vitro and in diaphragm and heart in vivo with no effect on serum  $\text{Na}^+$  and  $\text{K}^+$  concentrations, and also increased the  $\text{Na}^+$  efflux rate constant in liver (5). The findings cited above are in accord with the inference that thyroid hormone enhanced  $\text{Na}^+$  transport activity in vivo.

Augmentation of NaK-ATPase activity by thyroid hormone could result from activation of a fixed number of enzyme sites or from an increase in the total number of sites. The latter effect would yield an increase in  $V_{\text{max}}$  of the enzyme and the former may increase the  $V_{\text{max}}$  or decrease the  $K_m$  or have both effects. In skeletal muscle, thyroid status did not alter the  $K_m$  for ATP and in the kidney neither the  $K_m$  for ATP nor the  $K_{1/2}$  for  $\text{Na}^+$  or  $\text{K}^+$  were affected (3, 6). In both tissues, however,  $V_{\text{max}}$  was significantly increased. In addition, Lo and Edelman (7) provided evidence of thyroidal regulation of the synthesis of the large subunit of NaK-ATPase derived from the rat renal cortex.

There are well documented effects of thyroid hormone on the structure, motility, and absorptive activity of the small intestine (8, 9). The effect of thyroid status on intestinal oxygen consumption, however, has not been clearly defined. It has been claimed that small intestine from hypothyroid rats has a normal  $\dot{Q}\text{O}_2$  (10) and that administration of thyroid hormone either increased (10, 11) or decreased (12) the  $\dot{Q}\text{O}_2$  of small intestine.

The present study was designed to provide information on the effect of thyroid hormone on jejunal epithelium, with respect to: (a) the fractional contribution of  $\dot{Q}\text{O}_2(t)$  to total  $\dot{Q}\text{O}_2$  of the upper part of the small intestine, (b) the jejunal NaK-ATPase; (c) the quantitative relationships between  $\dot{Q}\text{O}_2$ ,  $\dot{Q}\text{O}_2(t)$  and NaK-ATPase activity of this tissue; and (d) the

number of active NaK-ATPase units in jejunal plasma membrane preparations measured by high-affinity binding of [ $^3$ H]ouabain and incorporation of  $^{32}$ P derived from  $AT^{32}P(\gamma)$  into a phosphorylated intermediate.

## METHODS

### *Animal Preparation*

All of the experiments were on male, Sprague-Dawley rats, body weights 130–180 g, maintained on Purina chow (Ralston Purina Co., St. Louis, Mo.) ad libitum. The thyroid gland was excised surgically; a special effort was made to leave the parathyroid glands intact, and the rats were used 3–4 wk post-surgery. Success in producing hypothyroidism 2 wk after surgery was judged by three criteria: (a) a body weight increase of less than 5 g per week, (b) a fall in the concentration of serum thyroxine-iodine to 30% or less of the euthyroid level ( $<1 \mu\text{g}/100 \text{ ml}$ ), and (c) a fall in the resting heart rate, measured with the electrocardiogram, to  $<50\%$  of that of the euthyroid control. Serum thyroxine-iodine concentrations were measured by the competitive binding method of Murphy and Pattee (13) by the Lazaroni Medical Laboratories, San Francisco, California. Na-L-3,5,3'-triiodothyronine ( $T_3$ ) was dissolved in  $5 \times 10^{-4} \text{ M NaOH}$ , to a final concentration of  $150 \mu\text{g}$  per milliliter, and frozen until used. Rats of about equal body weights and age were randomly assigned to hormone or control groups. Euthyroid or hypothyroid rats were injected subcutaneously with  $T_3$  ( $50 \mu\text{g}/100 \text{ g body wt}$ ), or an equivalent volume of the diluent on alternate days, for a total of three injections. The dosage and number of injections were based on the results of Tata (14) and recent work from our laboratory (2, 3, 15).

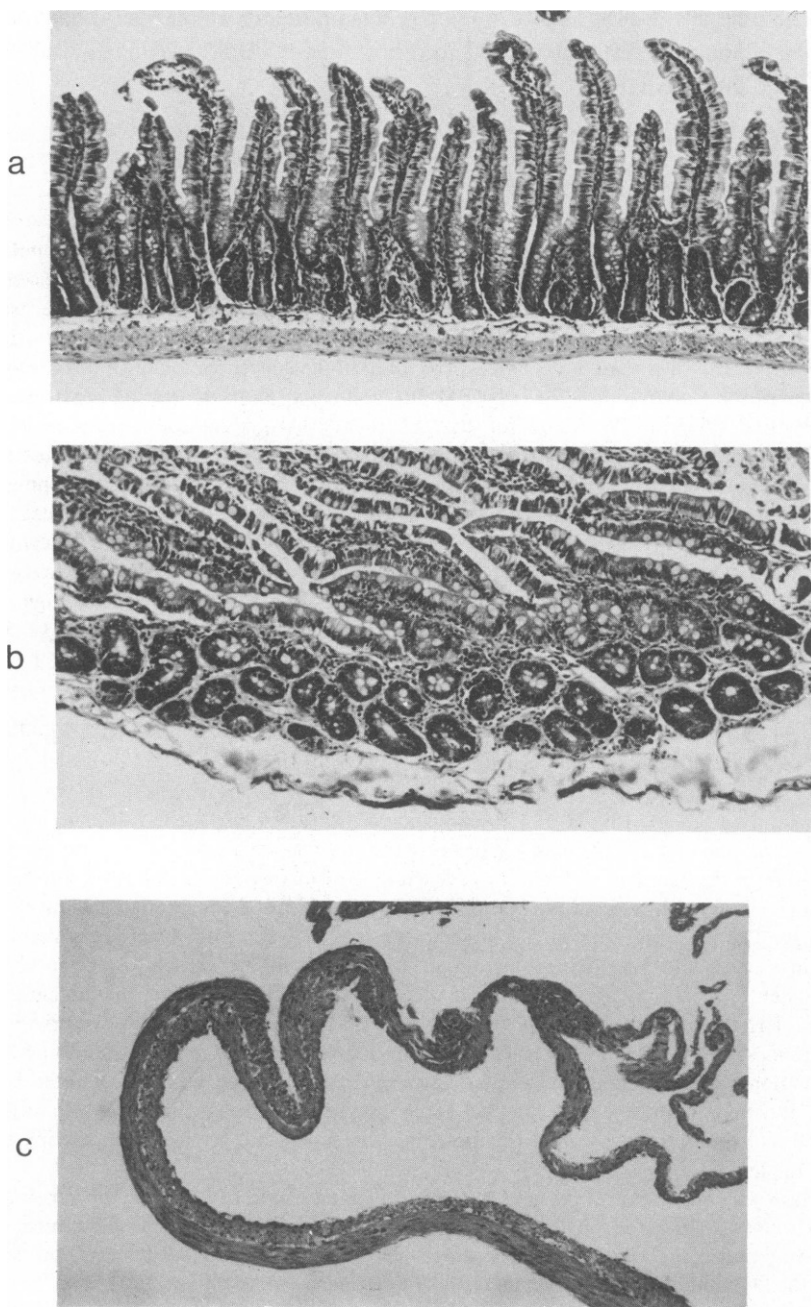
### *Oxygen Consumption ( $QO_2$ )*

24 h after the last injection, the rats (unanesthetized) were decapitated instantly with a guillotine, and a segment of small intestine about 50-cm length, starting from the pylorus, was removed and placed in ice-cold, oxygenated, modified  $\text{Na}^+$ -Ringer's solution of the following composition:  $\text{Na}^+$ , 135;  $\text{K}^+$ , 5;  $\text{Mg}^{++}$ , 0.5;  $\text{Ca}^{++}$ , 1.0;  $\text{Cl}^-$ , 139;  $\text{H}_2\text{PO}_4^-$ , 5.0; glucose, 10; (all in mM), pH 7.40, and osmolality, 290 mOsm/kg water. The first 10–12 cm of gut distal to the pylorus was discarded, the next section of about 10-cm length from the ligament of Treitz was cut out of the proximal end of the small intestinal segment and the lumen rinsed with ice-cold  $\text{Na}^+$ -Ringer's solution ( $20 \text{ ml} \times 3$ ), through a blunt needle with a 20-ml syringe. The intestinal section was slipped over a glass rod with a tapered point that had been prewetted in the ice-cold  $\text{Na}^+$ -Ringer's solution and placed in  $\text{Na}^+$ -Ringer solution chilled on ice ( $3\text{--}4^\circ\text{C}$ ). Under a magnifying glass, the fat and mesentery were stripped along the mesentery reflection with a pair of fine curved forceps. The serosa, and longitudinal and transverse layers of muscularis externa were loosened, starting at the point of the mesentery reflection, by working around the intestine in both directions. This was done until about 2–3 cm of the intestine was free of these layers at the mesentery reflection, and the reverse side had about 0.5 cm free. The remaining length of the intestine was stripped by loosening the layers of the mesentery reflection and progressively moving the stripped front down the length of the section.

To ascertain which anatomical portions of the intestine had been removed by the surgical procedure and to make sure that the structural integrity of the mucosal border had been maintained, histological sections were prepared. The stripping procedure yielded an intact mucosal preparation devoid of the serosa and the longitudinal and transverse layers of muscularis externa (Fig. 1).<sup>1</sup>

The stripped intestine was cut longitudinally with a sharp scalpel blade, the mucosal sheet was transferred to a large Petri dish, and cut into pieces transversely. The time of tissue preparation in the cold was less than 20 min. Tissue from two animals,  $T_3$ -injected and control, were prepared for each experiment. One piece of tissue (2.5–4.0 mg dry wt) was placed into a Warburg flask containing 2 ml of oxygenated  $\text{Na}^+$ -Ringer's solution (equilibrated with 100%  $\text{O}_2$  at 1 atm.) The  $\text{CO}_2$  was trapped by a

<sup>1</sup>We are grateful to Dr. J. Strum for the microscopy.



**FIGURE 1** Histology of the rat jejunum. Panel *a* is a cross section of an intact jejunum: magnification,  $\times 110$ . Panel *b* is a cross section of a stripped jejunum: magnification,  $\times 136$ . Panel *c* is a cross section of the serosa and muscularis externa from the preparation shown in panel *b*: magnification,  $\times 100$ .

small filter paper disk (Whatman #1; Whatman, Inc., Clifton, N.J.) moistened with 0.1 ml of 12% KOH in the side arm. Respiratory rates were measured with a Warburg respirometer (American Instrument Co., Inc., Silver Springs, Md.) at 15–30 min intervals for 90 min at 37°C (16). Sodium-independent respiration ( $QO_2'$ ) was measured in parallel incubations by addition of ouabain ( $10^{-3}$  M, final concentration).  $QO_2$  and  $QO_2'$  of small intestinal mucosa from each animal were determined in triplicate and the results averaged. Respiration linked to  $Na^+$  transport ( $QO_2[t]$ ) was computed as the difference between  $QO_2$  and  $QO_2'$ . After about 60 min in the respirometer, the  $QO_2$  of the jejunal mucosa from euthyroid and hyperthyroid rats consistently declined while the readings were stable in the hypothyroid  $\pm T_3$  segments. Thus only the initial respiratory rates (first 30 min) were used to estimate  $QO_2$  and  $QO_2'$ . At the end of the incubation, the tissues were removed from the flasks, transferred to tared aluminum cups, and the dry weights were determined gravimetrically after being heated to 91°C for 24 h. The pH of the bathing solutions were measured at the end of the experiment and were 6.9–7.1 after about 120 min of respiration in the flasks in which  $QO_2$  was determined and about 0.1–0.2 pH units higher in the flasks containing ouabain. In the few instances in which the pH was above 7.4, indicating a spill of KOH from the side arm, these results were eliminated. No consistent differences were noted in these final pH's as a function of the thyroid status of the tissues.

### *Tissue Weights, Protein, and DNA Content*

Six segments of 1-cm length of the stripped upper small intestine were cut transversely into small pieces and the wet and dry weights measured as described above. The dry segments were homogenized in 4 ml of 2.5 mM Tris-EDTA (pH 7.4), using 20 full strokes in a teflon-glass Elvehjem-Potter homogenizer, at 2–3°C. An equal volume of ice cold 20% TCA was added to the homogenate and the samples were left overnight in the refrigerator. 12 h later the tubes were spun in an angle rotor, at 13,000 g for 10 min (Ivan Sorvall Inc., Norwalk, Conn.; model RC2-B). The pellet was resuspended in 2 ml ethanol-ether (3:1) and recentrifuged as above. The recovered pellet was dried and the nucleic acids were extracted with 2 ml of 5% TCA in a water bath at 90–95°C for 20 min. The extracts were iced and centrifuged at 13,000 g for 10 min. The supernatants were analyzed for DNA content by the method of Burton (17). The pellets were redried, then dissolved in 1N NaOH and analyzed for protein content by the method of Lowry et al. (18). The DNA (calf thymus DNA, Type V; Sigma Chemical Co., St. Louis, Mo.) and protein (crystalline bovine albumin, Armour Pharmaceutical Co., Phoenix, Ariz.) standards were treated in every respect the same as the unknown.

### *ATPase Assays*

A crude membrane fraction from jejunal mucosa was prepared by a modification of the method of Quigley and Gotterer (19, 20). About 60 cm of proximal small intestine was removed and put in ice-cold isotonic solution A, that contained 155 mM NaCl, 5 mM  $Na_2$  EDTA (pH 7.4). Two segments, 10 cm each, distal to the ligament of Treitz, were cleared of visible fat and mesenteric connective tissue and irrigated with ice-cold solution B, that contained 5 mM  $Na_2$  EDTA, 100 mM NaCl, and 50 mM *N*-acetyl-L-cysteine buffered to pH 7.4 with 1 M Tris-HCl, 12 ml  $\times$  3. These segments were rinsed again with 15 ml  $\times$  2 of solution A, everted on a blunt long needle, wiped with Microwipe paper (Scott Paper, Co., Philadelphia, Pa.) and cut along the side. These jejunal sheets were put in a chilled Petri dish containing 20 ml of 5 mM  $Na_2$  EDTA solution (pH 7.4 and osmolality 15 mOsm per kilogram water). The mucosa was removed by gentle scraping  $\times$  3 with a glass slide. Histological sections of the repetitive scrapings and the remaining intestine revealed that the scrapings contained epithelial cells exclusively, while the remaining intestine contained some epithelial cells, and intact submucosa, muscularis, and serosal layers. The wet weight of the scraped mucosa ranged from 700–850 mg per Petri dish (i.e., 20-cm length of jejunal mucosa). The scrapings (in 5 mM  $Na_2$  EDTA) were homogenized in a loose-fitting, teflon-glass Elvehjem-Potter homogenizer at 2–3°C by using 15 full strokes. In preliminary experiments it was found by light microscopy that after 15 strokes almost no intact epithelial cells remained. The homogenate was filtered through a double layer of bleached cheese-cloth (Marco-Marslaes Co., N.Y.) and then through a 132 mesh nylon cloth (John Stainer, Manchester, England). The filtrates were spun at 700 g for 10 min at 2–3°C. The supernatant was decanted and saved, and the

gelatinous pellet, containing crude brush border, unbroken cells, and nuclei was washed in 10 ml of 5 mM Na<sub>2</sub> EDTA. The supernatant of the second spin was collected and added to the previous one. The combined supernatants were then centrifuged at 10,000 g for 10 min, and the pellet was resuspended in 20 ml 2.5 mM Tris-EDTA (pH 7.4 and osmolality 10 mOsm per kilogram water) and recentrifuged at 10,000 g for 10 min. This procedure was repeated once more and the final pellet was resuspended in 2.5 mM Tris-EDTA. Quigley and Gotterer (19) recovered ~60% of total NaK-ATPase activity of unfractionated crude homogenates of rat jejunal epithelium in the 10,000 g pellet. The ATPase assays were completed immediately after preparation of this fraction. In each experiment, 2 or 4 preparations, control and T<sub>3</sub>-treated, were prepared simultaneously.

The assay medium for total ATPase activity contained a final concentration of NaCl, 100; KCl, 10; MgCl<sub>2</sub>, 5; Tris-HCl, 50; Tris-ATP, 5; (all in mM); pH 7.4. These concentrations are in the optimal range for the activity of this enzyme in the intestinal epithelium (19, 20). Mg-ATPase activity was determined during the first experiments by two methods; a complete medium + ouabain in a final concentration of 10<sup>-3</sup> M, and a medium lacking Na<sup>+</sup> and K<sup>+</sup>. The results obtained by the two methods for the first 85 determinations were identical: 26.5 ± 0.7 μmol Pi per milligram protein per hour in 10<sup>-3</sup> M ouabain and 26.3 ± 0.7 in (Na<sup>+</sup> + K<sup>+</sup>)-free medium. Thus, in the remaining experiments Mg-ATPase was measured as the ouabain resistant activity. The assay system contained 30–70 μg of enzyme protein in a final volume of 2 ml; the reaction was started by the addition of ATP and was maintained for 15 min at 37°C in a shaking water bath. Enzyme activity was linear with time (5–60 min), and protein concentration (35–200 μg protein per tube). The reaction was terminated by addition of 2 ml of ice-cold 10% (wt/vol) TCA. The mixture was centrifuged at 100,000 g for 10 min at 0–4°C, and the orthophosphate content of the supernatant was determined by the method of Fiske and Subbarow (21). Protein content of the TCA precipitate was determined by the method of Lowry et al (18). NaK-ATPase activity was computed as the difference between total activity and Mg-ATPase activity. All assays were performed in duplicate. In each set of assays, control tubes were included that contained the complete medium (including ATP) but with no added membrane preparations. These tubes were incubated along with the experimental tubes and the tissue preparation was added to the control tubes after the addition of TCA. All enzyme assays were corrected for the quantity of phosphate released in the presence of acid and the acid-treated tissue. This quantity was invariably small.

### *[<sup>3</sup>H]Ouabain Assay*

Four 10-cm segments of intestine, starting from the ligament of Treitz, were prepared from rats (body weight of 200–240 g), either control or T<sub>3</sub>-treated, and processed simultaneously. The final pellets of the 10,000-g spin from two rats treated identically were pooled together; thus the crude plasma membrane fractions (prepared as described in the preceding paragraph) from two rats were combined into a single pool, derived from four segments of jejunum taken from each animal. The preparations were kept overnight in the refrigerator at 1–3°C. NaK-ATPase assays were done on the day of preparation of the fraction and repeated the following day at the same time [<sup>3</sup>H]ouabain binding was measured. [<sup>3</sup>H]ouabain (13 Ci/millimole or 12.7 Ci/millimole) was used either undiluted with ouabain or diluted up to a ratio of 1:50 with ouabain. The incubation medium contained various concentrations of [<sup>3</sup>H]ouabain in NaCl, 100; MgCl<sub>2</sub>, 5; Tris ATP, 5; and Tris HCl, 50; (all in millimoles); pH 7.4, in a final volume of 1 ml. Various concentrations of plasma membrane protein were added to the tubes, and were pre-incubated for 3 min at 37°C; the reaction was started by the addition of Tris-ATP, and maintained for 5 min. In preliminary experiments, nonspecific binding was assessed by parallel incubations in media that either contained 10<sup>3</sup>–10<sup>6</sup>-fold excess unlabeled ouabain, or were devoid of NaCl, MgCl<sub>2</sub>, and Tris-ATP. As the results were the same with both methods in all subsequent experiments, parallel incubations in media free of NaCl, MgCl<sub>2</sub>, and ATP were used to correct for nonspecific binding. A filtration method (22) was used to separate bound and free [<sup>3</sup>H]ouabain: 200 microliter aliquots (in quadruplicate) were applied to 0.45-μ Millipore filters (Millipore Corp., Bedford, Mass.) premoistened with ice-cold 2.5 mM Tris-EDTA, pH 7.4, in a 10-place manifold (Hoefer Scientific Instruments, San Francisco, Calif.). Each aliquot was washed 3 times with 1.0 ml of ice-cold 2.5 mM Tris-EDTA under continuous vacuum. With this procedure the filter retains <0.2% of the

applied amount of [ $^3\text{H}$ ]ouabain, even in the presence of equivalent amounts of added albumin (22). The washed filters were dissolved in 10 ml of modified Bray's solution (xylene, 429 ml; p-dioxane, 1,284 ml; ethylene glycol monoethyl ether, 1,284 ml; PPO (2,5-diphenyl oxazole), 30 g; POPOP [(dimethyl-1,4-bis-2-(4-methyl-5-phenyloxazole)], 1.5 g; naphthalene, 240 g) and assayed in a Mark I, Nuclear Chicago liquid scintillation spectrometer (Searle Analytic, Inc., Des Plaines, Ill.). Quadruplicate 25  $\mu\text{l}$  aliquots of the incubation mixture (without filtration) were also assayed in 10 ml of the counting solution. Counting efficiencies (25% with a filter and 27% without) were monitored by the external-standard, channel ratio method. The concentration of the free [ $^3\text{H}$ ]ouabain in the filtrate was calculated as the difference between [ $^3\text{H}$ ]ouabain concentration in the incubation mixture and the total amount bound to the filter.

### *Phosphorylated Intermediate*

These assays were carried out at 0–4°C, as described elsewhere (7). In brief, the phosphorylated intermediate was generated in a 1.0-ml reaction mixture containing NaCl, 100;  $\text{MgCl}_2$ , 3; EDTA, 1; Tris-HCl, 100;  $\text{AT}^{32}\text{P}(\gamma)$  (1.5 Ci/mmol),  $1.5 \times 10^{-3}$ ; (all in mM): pH 7.4, and 75–150  $\mu\text{g}$  of crude plasma membrane protein, in Eppendorf vials (Fisher Scientific Co., Pittsburgh, Pa.). Tandem tubes contained 0.02 M KCl rather than 0.1 M NaCl, and served to measure nonspecific phosphorylation. The reaction was started by the addition of  $\text{AT}^{32}\text{P}(\gamma)$  and immediate mixing with a vortex mixer, and terminated by addition of 0.3 ml of ice-cold 30% TCA containing 1.5 mM ATP after 15 s of incubation. The mixture was then centrifuged at 17,000 g for 2 min in an Eppendorf centrifuge (model 3200). The pellet was resuspended in 1.0 ml of 30% TCA, 1.5 mM ATP, by sonication for 2 s at scale 3 in a sonifer cell disruptor, model W-1400 (Heat Systems-Ultrasonic, Inc., Plainview, N.Y.). The suspension was recentrifuged at 17,000 g for 2 min in the Eppendorf centrifuge. The process of decanting, resuspension (in 1.0 ml of 10% TCA), sonication, and centrifugation was then repeated. The resulting pellet was drained and dissolved in 0.5 ml of 1N NaOH. The entire solution was transferred to a counting vial containing 10 ml of Aquasol (New England Nuclear, Boston, Mass.) and 0.3 ml of concentrated HCl, and assayed for  $^{32}\text{P}$  content in a Mark II, Nuclear Chicago liquid scintillation spectrometer, with a 61% efficiency by the external-standard channel ratio method.

### *Statistical Calculations*

The data are presented as means  $\pm$  SE and evaluated for significance by the unpaired Student *t*-test. Regression lines were obtained by the least mean square method with the aid of a digital PDP-12 Computer (Digital Equipment Corporation, Marlboro, Mass.) and a Hewlett Packard 9100B calculator (Hewlett-Packard Co., Palo Alto, Calif.).

### *Materials*

All of the conventional reagents were analytical grade or spectroquality. Na-L-3,5,3'-triiodothyronine was obtained from CalBioChem-Behring Corp. (San Diego, Calif.); tris-ATP, ouabain, and EDTA from Sigma Chemical Co., and tris-base from Schwarz/Mann Div. (Orangeburg, N.Y.). Aquasol and uniformly labelled [ $^3\text{H}$ ]ouabain (G) were purchased from New England Nuclear Corp. (13 Ci per millimole, >97% pure), and  $\text{AT}^{32}\text{P}(\gamma)$  from Amersham-Searle Corp. (Arlington Heights, Ill.).

## RESULTS

### *Effect of $T_3$ on Oxygen Consumption*

In hypothyroid rats, administration of  $T_3$  increased jejunal  $\text{QO}_2$  and  $\text{QO}_2(t)$  (26% and 41%, respectively) (Table I). Ouabain-insensitive respiration ( $\text{QO}_2'$ ) increased by 17%, but this change did not quite achieve statistical significance. In euthyroid rats,  $T_3$  evoked changes of smaller magnitude: 15% in  $\text{QO}_2$ , 16% in  $\text{QO}_2'$ , and 12% in  $\text{QO}_2(t)$ . The 12% increase in  $\text{QO}_2(t)$ , however, did not achieve statistical significance. The respiratory rates of jejunal

TABLE I  
RESPIRATION OF STRIPPED JEJUNUM FROM THYROIDECTOMIZED  
AND EUTHYROID RATS ( $\pm T_3$ )

	Thyroidectomized			Euthyroid		
	$-T_3$	$+T_3$	$\Delta$	$-T_3$	$+T_3$	$\Delta$
$Q_{O_2}$	$11.8 \pm 0.4$	$14.8 \pm 0.53$	$3.0^*$	$14.4 \pm 0.34$	$16.5 \pm 0.43$	$2.1^*$
$Q_{O_2}'$	$7.8 \pm 0.4$	$9.1 \pm 0.55$	1.3	$9.4 \pm 0.36$	$10.9 \pm 0.53$	$1.5^*$
$Q_{O_2}(t)$	$4.0 \pm 0.3$	$5.7 \pm 0.51$	$1.7^*$	$5.0 \pm 0.40$	$5.6 \pm 0.60$	$0.6^*$
No. of rats	9	9		12	12	

$Q_{O_2}'$  is the ouabain-insensitive oxygen consumption.  $Q_{O_2}(t)$  is the difference between  $Q_{O_2}$  and  $Q_{O_2}'$ . Rats were injected with  $50 \mu\text{g}$  of  $T_3/100 \text{ g}$  body wt or an equal volume of the diluent on alternate days for a total of three doses. Results are given as mean  $\pm$  SE in units of microliter  $O_2$  per milligram dry weight per hour.

\* $p < 0.05$ .

mucosa from hypothyroid rats were lower than euthyroid values and were restored to normal by three injections of  $T_3$ . The increase in  $Q_{O_2}(t)$  accounted for 57% of the  $T_3$ -dependent increment in  $Q_{O_2}$  of jejunal mucosa of treated hypothyroid rats, and for 29% of the increment induced in treated euthyroid rats. The increase in  $Q_{O_2}'$  evoked by  $T_3$  in the euthyroid rats (71% of the total increase in  $Q_{O_2}$ ) was statistically significant ( $p < 0.05$ ).

#### *Effects of Thyroid Status on Jejunal Mucosal Mass, Protein, and DNA Content*

4 wk after thyroidectomy, the wet and dry weights of segments of jejunum (stripped of the muscularis) were reduced to about 65% and 61%, respectively, of comparable euthyroid segments (Table II). Injection of  $T_3$  into hypothyroid rats ( $50 \mu\text{g}/100 \text{ g}$  body wt q. 48 h  $\times$  3) produced 19% and 21% increases in wet and dry weight, respectively, but no significant changes in weights were observed in the transition from the euthyroid to the hyperthyroid state. The constancy in the dry weight/wet weight ratio (Table II) and the corresponding protein/dry weight, DNA/protein ratios (Table III) implies that the use of dry weight as a reference standard for  $Q_{O_2}$  and  $Q_{O_2}(t)$  is a valid index of cell content regardless of thyroid status. Thus, the changes in  $Q_{O_2}$  and  $Q_{O_2}(t)$  (Table I) induced by thyroid hormone and expressed per unit mass of tissue can be ascribed to changes in respiratory rates per cell.

TABLE II  
WET AND DRY WEIGHTS OF STRIPPED JEJUNAL SEGMENTS FROM  
THYROIDECTOMIZED AND EUTHYROID RATS ( $\pm T_3$ )

	Thyroidectomized		Euthyroid	
	$-T_3$	$+T_3$	$-T_3$	$+T_3$
Wet weight	$78.2 \pm 4.6$	$92.9 \pm 6.1^*$	$111.5 \pm 5.3$	$112.8 \pm 4.0$
Dry weight	$10.9 \pm 1.0$	$13.2 \pm 1.0^*$	$15.7 \pm 0.8$	$16.1 \pm 0.6$
Dry weight/wet weight	$0.14 \pm 0.01$	$0.14 \pm 0.005$	$0.14 \pm 0.002$	$0.14 \pm 0.002$
No. of rats	6	6	8	8

Rats were injected with  $50 \mu\text{g}$  of  $T_3/100 \text{ g}$  body wt (q. 48 h  $\times$  3) or the diluent. Results are given as mean  $\pm$  SE in units of milligram per centimeter length.

\* $p < 0.05$ .



TABLE III  
 DRY WEIGHT, PROTEIN, AND DNA CONTENT OF STRIPPED JEJUNAL SEGMENTS  
 FROM THYROIDECTOMIZED RATS ( $\pm T_3$ )

Thyroid status	Protein	DNA	
	Dry weight	Dry weight	Protein
$-T_3$	$0.43 \pm 0.07$	$0.037 \pm 0.001$	$0.085 \pm 0.003$
$+T_3$	$0.43 \pm 0.09$	$0.036 \pm 0.002$	$0.087 \pm 0.002$

Rats were injected with 50  $\mu$ g of  $T_3$ /100 g body wt or an equal volume of the diluent on alternate days for a total of three doses. Results are given as mean  $\pm$  SE, as ratios of the respective weights.  $N = 9$  rats in each group; 6 pieces of jejunum from each rat. None of the differences ( $\pm T_3$ ) are statistically significant.

#### *Effects of Thyroid Status on ATPase Activities*

As summarized in Table IV,  $T_3$  augmented jejunal mucosal NaK-ATPase specific activity (i.e., units per milligrams protein) by 71% in hypothyroid rats and by 27% in euthyroid rats. In contrast, Mg-ATPase specific activity either decreased or did not change in response to  $T_3$ . It is noteworthy that administration of the same dose of  $T_3$  yielded higher NaK-ATPase activities in the thyroidectomized than in the euthyroid rats (13.0 vs 10.3). These results suggest that prolonged hypothyroidism may sensitize the intestinal mucosa to the action of  $T_3$ .

The jejunal mucosa responded to  $T_3$ , as noted above for the stripped jejunum, with an increase in wet weight of mucosa (about 23%) recovered per unit length of intestine with no change in the wet weight/dry weight ratio. In view of the constancy of the ratios of the various parameters of mass (Table III), the significant increases in NaK-ATPase activity per milligram protein of the crude membrane preparation probably reflect increases in the cellular content of this enzyme.

The crude membrane preparation used in the present studies recovers ~60% of the total enzyme content of the primary homogenate of euthyroid rat jejunal mucosa (19). One possible explanation for the apparent increases in NaK-ATPase activity in response to  $T_3$  would be changes in recovery of the enzyme (redistribution) rather than changes in cellular content. The differential effects of  $T_3$  on Mg-ATPase vs NaK-ATPase activities and the magnitude of the increases (particularly in the hypothyroid case), however, favor an effect on

TABLE IV  
 ATPASE ACTIVITY OF JEJUNAL CRUDE PLASMA MEMBRANE PREPARATION FROM  
 THYROIDECTOMIZED AND EUTHYROID RATS ( $\pm T_3$ )

	Thyroidectomized			Euthyroid		
	$-T_3$	$+T_3$	$\Delta$	$-T_3$	$+T_3$	$\Delta$
Mg-ATPase	$35.3 \pm 1.1$	$29.3 \pm 1.4$	$-6.0^*$	$22.6 \pm 0.8$	$22.4 \pm 0.7$	$-0.2$
NaK-ATPase	$7.6 \pm 0.5$	$13.0 \pm 0.7$	$5.4^*$	$8.1 \pm 0.3$	$10.3 \pm 0.5$	$2.2^*$
No. of rats	13	13		29	29	

Rats were injected with  $T_3$  50  $\mu$ g/100 g body wt or an equal volume of the diluent on alternate days for a total of three doses. Results are given as mean  $\pm$  SE in units of micromoles Pi per milligram protein per hour.

\* $p < 0.05$ .

cellular content or on the activity of NaK-ATPase. Moreover, in previous studies,  $T_3$  had the same effect (as noted herein) in crude homogenates of liver and kidney. Nevertheless, it is conceivable that thyroid status could change the sedimentation properties of some of the particulates and thereby alter the distribution of the enzyme among the subcellular fractions. Further studies are needed to rule out this possibility definitively.

### *The Relationship Between [ $^3\text{H}$ ]Ouabain Binding and NaK-ATPase Specific Activity*

Five sets of experiments were performed. In each set, 4–6 samples of varied protein concentrations, from the same jejunal crude membrane fraction pool, were added to a final volume of 1 ml of the reaction medium. [ $^3\text{H}$ ]ouabain was added to a final concentration of  $2.6 \times 10^{-6}$  M (from the same stock solution). The amount of plasma membrane protein applied ranged from 40–240  $\mu\text{g}$  per filter. Two of the pools of the jejunal plasma membrane fraction were prepared from hypothyroid rats, and three from euthyroid rats. As shown in Fig. 2, there was a highly significant linear correlation between specific binding of [ $^3\text{H}$ ]ouabain and total NaK-ATPase activity applied to the filter with a correlation coefficient of 0.91. These results suggest that augmentation of NaK-ATPase activity by  $T_3$  is a consequence of an increase in the number of  $\text{Na}^+$  pump units in that both populations lie along the same regression line. To explore the validity of this inference, we completed a more extensive set of studies, including an assessment of the effect of thyroid status on the affinity of [ $^3\text{H}$ ]ouabain for the specific sites.

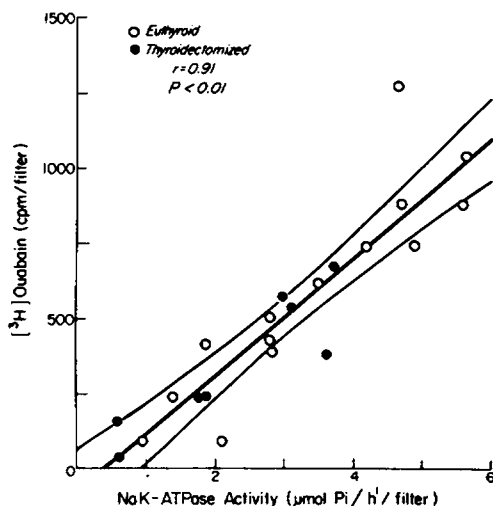


FIGURE 2 Specific binding of [ $^3\text{H}$ ]ouabain to jejunal crude plasma membrane fractions. The jejunal crude plasma membrane fractions were prepared from euthyroid ( $\circ$ ), and thyroidectomized rats ( $\bullet$ ). Each preparation was assayed simultaneously for NaK-ATPase activity and for specific binding of [ $^3\text{H}$ ]ouabain at a concentration of  $2.6 \times 10^{-6}$  M. Nonspecific binding of [ $^3\text{H}$ ]ouabain was estimated in parallel incubations devoid of  $\text{Na}^+$ ,  $\text{Mg}^{++}$ , or ATP. From 40 to 240  $\mu\text{g}$  of protein (4–6 aliquots from each preparation) were applied per filter and the binding of [ $^3\text{H}$ ]ouabain is expressed as a function of the corresponding amount of the enzyme per filter. The outer lines define the 95% confidence intervals of the regression line.

### Effect of Thyroid Status on Specific Binding of [ $^3\text{H}$ ]Ouabain

Pairs of thyroidectomized rats were injected with the diluent or  $\text{T}_3$  ( $50\text{ }\mu\text{g}/100\text{ g body wt q. } 48\text{ h} \times 3$ ). The crude jejunal membrane fractions of two donor rats treated identically were pooled ( $N = 9$  pools in each group) and all pools were incubated in varying concentrations of [ $^3\text{H}$ ]ouabain ( $2.4\text{--}19 \times 10^{-6}\text{ M}$ ). To ensure equilibrium in the binding of [ $^3\text{H}$ ]ouabain, at these concentrations, the time-course of binding was measured at 1, 2, 5, 7, 10, and 15 min of incubation ( $n = 6$  experiments). No difference in total or specific binding was detected during this interval. 5 min was chosen as the incubation period to remain well within the time needed for equilibration. The concentrations of [ $^3\text{H}$ ]ouabain used in the present study were restricted to  $<2 \times 10^{-5}\text{ M}$  to avoid errors introduced by high nonspecific values. At these concentrations, ( $<2 \times 10^{-5}\text{ M}$ ) specific binding was 50% or more of total binding of [ $^3\text{H}$ ]ouabain.

The results in Fig. 3 show a curvilinear dependence of specific [ $^3\text{H}$ ]ouabain binding on ouabain concentration. At all concentrations, [ $^3\text{H}$ ]ouabain binding was significantly greater ( $p < 0.05$ , nonpaired  $t$ -test) in the  $\text{T}_3$ -treated fractions.

The parameters of [ $^3\text{H}$ ]ouabain binding were evaluated by Scatchard analysis (23). If specific [ $^3\text{H}$ ]ouabain binding is to a single, homogenous class of sites, a plot of the bound/free [ $^3\text{H}$ ]ouabain ratio versus bound [ $^3\text{H}$ ]ouabain should yield a linear regression relationship. Individual Scatchard plots were made of the results obtained with each of the pools of crude membrane and two representative Scatchard plots are shown in Fig. 4. In 16 of the 18 pools, the correlation coefficient of linearity was statistically significant ( $p < 0.05$ ) and in the remaining two (one in each group, control and treated with  $\text{T}_3$ ),  $p = 0.1$ . The estimates of the

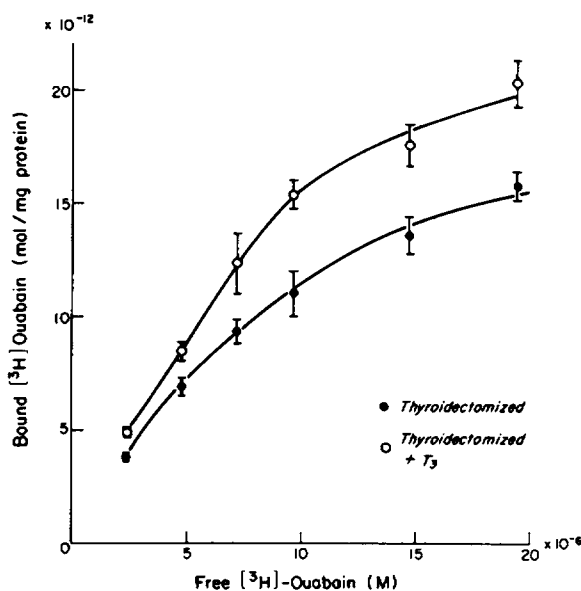


FIGURE 3 Specific binding of [ $^3\text{H}$ ]ouabain as a function of the free [ $^3\text{H}$ ]ouabain concentration. Thyroidectomized rats were injected either with the diluent ( $\bullet$ ) or  $\text{T}_3$  ( $50\text{ }\mu\text{g}/100\text{ g body wt q. } 48\text{ h} \times 3$ ) ( $\circ$ ). The crude jejunal plasma membrane fractions were incubated in  $2.4 \times 10^{-6}\text{--}1.9 \times 10^{-5}\text{ M}$  [ $^3\text{H}$ ]ouabain with  $\text{Na}^+$ ,  $\text{Mg}^{++}$ , and ATP in the medium. Aliquots were incubated in medium devoid of  $\text{Na}^+$ ,  $\text{Mg}^{++}$ , or ATP, to correct for nonspecific binding. The points and vertical lines represent the mean  $\pm$  SE  $N = 9$  pools (two rat jejunums per pool) in each group.

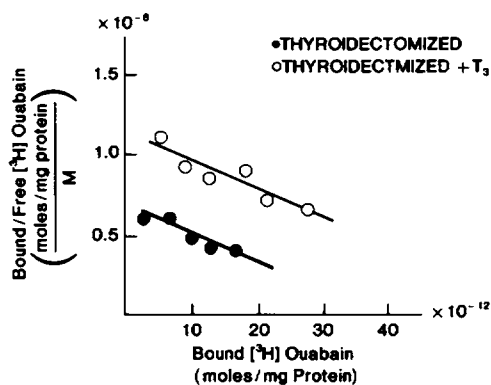


FIGURE 4 Scatchard analysis (23) of specific binding of [ $^3\text{H}$ ]ouabain to jejunal crude membrane fractions. These results are two representative individual experiments taken from the groups shown in Fig. 3. The binding parameters are summarized in Table V.

equilibrium dissociation constants ( $K_d$ ) and the total number of binding sites ( $N_m$ ) were computed individually for each pool of the crude jejunal membrane fraction and these results are summarized in Table V.  $T_3$  elicited proportionate and significant increases in  $N_m$  and NaK-ATPase activity but had no significant effect on the  $K_d$ 's. The means of the turnover numbers were indistinguishable, implying no change in the properties of this enzyme on treatment with  $T_3$ . It should be noted, however, that the SE's of the mean ( $\sim 13\%$  of the mean value) are sufficiently high as to admit of the possibility that some change in the intrinsic activity of the enzyme might be undetected by this analysis.

In the binding studies with [ $^3\text{H}$ ]ouabain, the medium contained NaCl,  $\text{MgCl}_2$ , and ATP, but no  $\text{K}^+$ . Addition of  $\text{K}^+$  to the medium decreases the affinity of the enzyme for [ $^3\text{H}$ ]ouabain without changing maximal binding capacity (4). Potassium exerts a corresponding increase in the half-maximal concentration ( $K_i$ ) for inhibition of NaK-ATPase by ouabain (4). In rat small intestinal epithelial crude membrane preparations, Quigley and

TABLE V  
EFFECT OF  $T_3$  ON SPECIFIC BINDING OF [ $^3\text{H}$ ]OUABAIN AND NaK-ATPASE ACTIVITY

Thyroid status	$K_d$ $\times 10^{-5} \text{ M}$	$N_m$ picomoles per milligram protein	NaK-ATPase micromoles Pi per milligram protein per hour	Turnover number $\text{min}^{-1}$
Diluent	$1.8 \pm 0.2$	$30.7 \pm 2.5$	$11.4 \pm 0.9$	$6580 \pm 850$
$T_3$	$2.0 \pm 0.2$	$43.3 \pm 3.9$	$15.9 \pm 1.0$	$6420 \pm 800$
% $\Delta$	11	41*	39*	-2

Thyroidectomized rats (36 in all) were injected either with the diluent or  $T_3$  ( $50 \mu\text{g}/100 \text{ g body wt q. } 48 \text{ h} \times 3$ ). Crude jejunal membrane fractions from two rats treated identically were pooled and assayed for specific [ $^3\text{H}$ ]ouabain binding (concentration range  $2.4\text{--}19 \times 10^{-6} \text{ M}$ ) and NaK-ATPase activity at  $V_{\text{max}}$ . The binding parameters were computed by the method of Scatchard (23), as shown in Fig. 4. The turnover number was computed under the assumption of one enzyme site per [ $^3\text{H}$ ]ouabain binding site. All results are given as mean  $\pm$  SE.  $N = 9$  pools in each group.

\* $p < 0.025$ .

TABLE VI  
NaK-ATPase ACTIVITY AND FORMATION OF THE PHOSPHORYLATED  
INTERMEDIATE OF JEJUNAL MUCOSA ( $\pm T_3$ )

Thyroid status	NaK-ATPase	Phosphorylated intermediate
	micromole Pi per milligram protein per hour	picomole $^{32}\text{P}$ per milligram protein
$-T_3$ (6)	$13.9 \pm 1.3$	$44.5 \pm 4.6$
$+T_3$ (5)	$18.7 \pm 1.0$	$68.4 \pm 4.0$
% $\Delta$	35*	54*

Thyroidectomized rats were injected with  $T_3$  ( $50 \mu\text{g}/100 \text{ g body wt q. } 48 \text{ h} \times 3$ ) or the diluent. The crude plasma membrane fraction of jejunal epithelium was assayed simultaneously for NaK-ATPase activity (at  $V_{\max}$ ) and for  $(\text{Na}^+ + \text{Mg}^{2+})$ -dependent,  $\text{K}^+$ -sensitive incorporation of  $^{32}\text{P}$  from  $\text{AT}^{32}\text{P}(\gamma)$ . Results are given as mean  $\pm$  SE. The number of rats is indicated in parenthesis.

\* $p < 0.05$ .

Gotterer (24) obtained a  $K_i$  of  $10^{-4} \text{ M}$  ouabain in the presence of  $20 \text{ mM K}^+$ . Our estimate of the  $K_d$  of  $2 \times 10^{-5} \text{ M}$  [ $^3\text{H}$ ]ouabain (in  $\text{K}^+$ -free media), therefore, is in acceptable agreement with the  $K_i$ .

#### *Effect of $T_3$ on the Yield of the Phosphorylated Intermediate of NaK-ATPase*

In the presence of  $\text{Na}^+$  and  $\text{Mg}^{2+}$ ,  $\text{AT}^{32}\text{P}(\gamma)$  phosphorylates NaK-ATPase; this intermediate is hydrolyzed in the presence of  $\text{K}^+$  (25). At maximal concentrations of ATP, the quantity of  $(\text{Na}^+ + \text{Mg}^{2+})$ -dependent  $\text{K}^+$ -sensitive phosphoprotein is a measure of the total number of NaK-ATPase enzyme units in the system.

Thyroidectomized rats were treated with  $T_3$  ( $50 \mu\text{g}/100 \text{ g body wt q. } 48 \text{ h} \times 3$ ) or diluent. NaK-ATPase activity was measured under  $V_{\max}$  conditions simultaneously with the quantity of phosphorylated intermediate formed at  $1.5 \times 10^{-6} \text{ M AT}^{32}\text{P}(\gamma)$ , in crude jejunal membrane fractions. This concentration yields  $>90\%$  of  $N_{\max}$  value in rat kidney membrane fractions (6).  $T_3$  increased NaK-ATPase activity by 35% and the amount of the phosphorylated intermediated by 54% (Table VI).

#### DISCUSSION

Conflicting reports on the effect of thyroid hormones on respiration of small intestine have appeared previously. Bronk and Parsons (10) found that jejunal rings taken from hypothyroid rats respired normally, that one injection of  $T_3$  ( $30 \mu\text{g}$ ) increased  $\text{QO}_2$  by 26%, but in euthyroid rats high doses of  $T_3$  decreased  $\text{QO}_2$  of the upper part of the rat intestine, in vitro. Althausen (26), however, reported that the injections of thyroxine stimulated glucose absorption and  $\text{QO}_2$  of rat intestinal slices by 30–35%. Recently, Levin and Syme (11) noted that hypothyroidism induced by 6-*n*-propyl-2-thiouracil depressed  $\text{QO}_2$  of jejunal sacs by 36% and treatment of euthyroid rats with  $T_3$  stimulated jejunal  $\text{QO}_2$  by 39%. The failure to obtain a consistent dependence of intestinal  $\text{QO}_2$  on thyroid status in some of these earlier studies may have resulted from differences in the experimental conditions used in the various studies. The factors that significantly affect estimates of intestinal  $\text{QO}_2$  include: (a) the proportion of smooth muscle and connective tissue to epithelium, since  $\text{QO}_2$  correlates with the abundance

of the epithelial layer and smooth muscle does not respond calorigenically to thyroid hormone (27), (b) the ambient  $pO_2$ , since intestinal  $QO_2$  is highly dependent on  $pO_2$  (28), and (c) the presence of glucose in the medium since uptake of  $Na^+$  from the lumen is glucose dependent (11). For example, Levin and Syme (11) noted that hypothyroidism reduced jejunal  $QO_2$  only at high  $pO_2$  and with glucose in the medium. Our results, using 100%  $O_2$  and 10 mM glucose in the medium, confirm the dependence of jejunal mucosal  $QO_2$  on thyroid status. The  $QO_2$  of the stripped jejunum was lower in hypothyroid than in euthyroid rats and administration of  $T_3$  to both groups, increased  $QO_2$  of this preparation.

Two methods have been used to estimate the fraction of the  $QO_2$  coupled to  $Na^+$  transport (i.e.,  $QO_2(t)$ ); specific inhibition of the  $Na^+$  pump with cardiac glycosides, such as ouabain, and incubation in  $Na^+$ -free media (1-4, 29). A variety of findings support the validity of these estimates: (a) The cardiac glycosides are specific inhibitors of NaK-ATPase activity and are without effect on  $Mg^{++}$ - or  $Ca^{++}$ -dependent ATPases (4). (b) In  $Na^+$ -free media ouabain has no effect on  $QO_2(t)$  (1). (c) Ouabain and  $Na^+$ -free media yielded the same estimates of  $QO_2(t)$  regardless of thyroid status (3). (d) Changes in  $QO_2(t)$  elicited by thyroid hormone correlated linearly with changes in NaK-ATPase activity (3). (e) The activation energy, estimated from Arrhenius plots, for  $QO_2(t)$  was three-fold higher than that of  $QO_2$  and was the same as the activation energy for NaK-ATPase in both euthyroid and hyperthyroid liver (30). In contrast the activation energy of Mg-ATPase was less than one-third that of  $QO_2(t)$ .

On the basis of ouabain-inhibitable respiration, the  $T_3$ -dependent increase in  $QO_2(t)$  accounted for 55% of the increase in jejunal mucosal  $QO_2$  in the transition from the hypothyroid to the euthyroid state and for 30% of the increase in the transition from the euthyroid to the hyperthyroid state (Table I). A  $T_3$ -dependent increase in the ouabain-insensitive respiration ( $QO_2'$ ) is also evident in both transitions and may derive from the energy requirements for the growth response. The results in Tables II and III are consistent with the interpretation that thyroidectomy decreased and  $T_3$  administration increased the mass of the jejunal mucosa. This effect has also been reported previously (9, 31). The plausibility of this explanation (metabolic demands of tissue growth) is debatable, however, in that almost complete inhibition of protein synthesis had no significant effect on the  $QO_2$  of rat liver (32). Another possible mechanism for the effect of thyroid status on  $QO_2'$  is an increase in active  $Cl^-$  transport, since under some conditions, transmucosal  $Cl^-$  transport contributes about two-thirds of the short-circuit current across the rat jejunum (33).

In previous studies (1-3, 34, 35),  $T_3$  induced significant increases in NaK-ATPase activity with little change in Mg-ATPase activity in crude homogenates and partially purified membrane fractions of rat liver, kidney, and skeletal and cardiac muscle—all well characterized target organs for thyroid thermogenesis. Our results now establish a similar effect of thyroid status on the specific activity of NaK-ATPase, without a corresponding increase in Mg-ATPase in a crude jejunal membrane fraction (Table IV). In fact, administration of  $T_3$  to the hypothyroid rats decreased Mg-ATPase activity to some extent. If the  $T_3$ -dependent increase in  $QO_2(t)$  is a consequence of an increase in total  $Na^+$  pump activity, proportionate effects on  $QO_2(t)$  and NaK-ATPase (measured at  $V_{max}$ ) may be evident. As predicted, proportionate changes in  $QO_2$  and NaK-ATPase, and in  $QO_2(t)$  and NaK-ATPase were obtained in the various thyroid states:  $\Delta QO_2(t)/\Delta NaK\text{-ATPase}$  ratio was 0.3 in the

transition from the hypothyroid to the euthyroid state and 0.27 in the transition from the euthyroid to the hyperthyroid state, despite the considerable differences in the magnitude of the effects in these groups.

The significance of the  $T_3$  dependent increase in NaK-ATPase rests on the validity of this assay in the intestinal epithelium, a tissue rich in a variety of other phosphatases. In a detailed study of the properties of rat intestinal epithelial NaK-ATPase, Quigley and Gotterer (20) found that activation by  $Na^+$  and  $K^+$ , the  $K_{1/2}$  for  $K^+$ , the order of effectiveness of replacement of  $K^+$  with other monovalent cations, the absolute requirement for  $Mg^{2+}$ , specificity of ATP as a substrate, and specific inhibition by ouabain was very similar to these properties of NaK-ATPases in a wide variety of other tissues. In our first 85 assays we used ( $Na^+ + K^+$ )-free solutions and ouabain ( $10^{-3}$  M) additions in parallel on crude plasma membrane fractions from hypothyroid and  $T_3$ -treated ( $50 \mu g/100$  g body wt q. 48 h  $\times$  3) rats. The Mg-ATPase estimates were the same with both methods regardless of thyroid status. In preliminary experiments on crude plasma membranes of rat jejunum, we obtained the following results: (a) Addition of ouabain to a medium containing only  $Mg^{2+}$  ( $Na^+ + K^+$ -free) had no effect on the estimated Mg-ATPase activity. (b) Addition of NaCl (100 mM) alone or of KCl (10 mM) alone to a medium containing MgCl (5 mM) and no  $Ca^{2+}$ , had no effect on ATPase activity. The possibility of other phosphatases contributing significantly to the estimated NaK-ATPase activity is remote. The  $Ca^{2+}$ -sensitive ATPase of rat small intestinal epithelium is inhibited by ethacrynic acid but not by ouabain (36, 37). At the optimal pH of 9.1, alkaline phosphatase of rat small intestinal epithelial crude plasma membranes has a maximal activity of  $4.4 \mu mol$  Pi per milligram protein per hour and in the same preparation, at a pH of 7.1,  $Mg^{2+} + Na^+ + K^+$ -ATPase activity was  $31 \mu mol$  Pi per milligram protein per hour (19). Since alkaline phosphatase has negligible activities, i.e.,  $<0.4 \mu mol$  Pi per milligram protein per hour, at a pH  $<8.0$ , it is unlikely that the contribution of alkaline phosphatase to the estimates of NaK-ATPase at a pH of 7.4 (as used in our assays) could account for more than 5% of the activity.

The increase in the specific activity of NaK-ATPase induced by thyroid hormone could have resulted from activation (an increase in the turnover number) of the enzyme or an increase in the number of units. This issue was addressed by two independent methods: specific binding of [ $^3H$ ]ouabain, and the formation of the phosphorylated intermediate from  $AT^{32}P(\gamma)$ . For the former purpose, we used rapid filtration on Millipore filters, at  $\sim 4^\circ C$  to separate free from bound [ $^3H$ ]ouabain; a rapid method was needed because of the lability of the complex (38, 39). This method was sufficiently sensitive to detect specific and reproducible binding at a [ $^3H$ ]ouabain concentration as low as  $1.5 \times 10^{-9}$  M (22). This assay also gave a linear correlation between specific [ $^3H$ ]ouabain binding and NaK-ATPase activity when the quantity of enzyme applied to the filter was varied (Fig. 2).

The results obtained by both specific [ $^3H$ ]ouabain binding and ( $Na^+ + Mg^{++}$ )-dependent formation of the phosphoprotein suggest that the increase in NaK-ATPase activity on administration of  $T_3$  to thyroidectomized rats is a consequence of the acquisition of additional enzyme units in the jejunum (Figs. 2, 3). The invariance in the [ $^3H$ ]ouabain  $K_d$  (Table V) and in the turnover number of the NaK-ATPase suggests that the additional units have the same properties as in the hypothyroid state. In thyroidectomized rats, administration of  $T_3$  elicited significant increases in  $V_{max}$  of skeletal muscle and renal cortical NaK-ATPase, with

no change in the  $K_m$  for ATP in both tissues, and no change in the  $K_{1/2}$ 's for  $\text{Na}^+$  or  $\text{K}^+$  in the renal cortex (6). The turnover number of the jejunal mucosal NaK-ATPase is within the range of that reported previously in various tissues from many different species (40). Lin and Akera (41) measured the number of NaK-ATPase units in particulates of deoxycholate-treated homogenates, with [ $^3\text{H}$ ]ouabain. In rat liver, kidney, and skeletal muscle, thermogenically responsive tissues,  $\text{T}_3$  increased the concentration of this enzyme 60–100%. In contrast,  $\text{T}_3$  had no effect on the abundance of NaK-ATPase units in the brain (adult rat), a tissue which is not thermogenically dependent on thyroid status.

Although the results obtained in the present studies on jejunal epithelium also implicate acquisition of new  $\text{Na}^+$  pumps as the basis for  $\text{T}_3$  dependent increases in NaK-ATPase activity, two caveats should be noted: First, there may have been undetected effects on the distribution of the enzyme among subcellular fractions, and second, the relatively high SE's in the estimates of the turnover number (Table V) may have obscured additional effects on intrinsic enzyme activity. The evidence of  $\text{T}_3$ -regulated proportionate increases in NaK-ATPase activity and the number of enzyme units, however, is in accord with the findings obtained in rat renal cortex (7). In these studies, Lo et al (7) found that the increase in the number of  $\text{Na}^+$ -transport units was matched by equivalent increase in incorporation of amino acids into the large subunit of the enzyme, with no change in the rate of degradation of this subunit. These results provide clear evidence of de novo induction of the enzyme and by inference of the  $\text{Na}^+$  pump. The recent findings of high-affinity nuclear receptors for  $\text{T}_3$  and of induction of specific mRNA's by the hormone (42–44) suggest that augmentation of NaK-ATPase activity may also derive from induction of the specific mRNA.

The increase in NaK-ATPase of the jejunal mucosa may be an expression either of the acquisition of new cells containing an unusually high complement of  $\text{Na}^+$  pumps or of the induction of these units in the pre-existing population of epithelial cells. The former possibility is raised by the observation that prolonged administration (42 d) of thyroxine to thyroidectomized rats increased the mitotic index of intestinal crypt cells by ~33% (45). Brief periods of treatment with  $\text{T}_3$  (i.e., <3 d), however, did not produce significant changes in intestinal epithelial morphology (9), and the increase in NaK-ATPase in response to  $\text{T}_3$  is significant in 12 h (22). The effects of  $\text{T}_3$  on jejunal mucosal  $\text{QO}_2$ ,  $\text{QO}_2(t)$ , and NaK-ATPase are also qualitatively and quantitatively similar to those observed in skeletal muscle, a nondividing tissue (3). It remains for future studies, however, to define the precise mechanism responsible for thyroidal regulation of the  $\text{Na}^+$  pump content of jejunal epithelium.

It is a pleasure to acknowledge the competent technical assistance provided by Steven Wendelin, Joan Graf, and Allison Meader.

This work was supported by United States Public Health Service grants HL-06285 and HL-05725 from the National Heart, Lung, and Blood Institute.

Received for publication 3 May 1978 and in revised form 6 February 1979.

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