Title
The mechanism of the respiratory and transport effect of thyroid hormone

Permalink
https://escholarship.org/uc/item/8753q37r

Author
Liberman, Uri Aharon

Publication Date
1974

Peer reviewed|Thesis/dissertation

THE MECHANISM OF THE RESPIRATORY AND
TRANSPORT EFFECT OF THYROID HORMONE

by

Uri Aharon Liberman
M.D., Hebrew University and Hadassah Medical School, 1963

DISSERTATION

Submitted in partial satisfaction of the requirements for the degree of
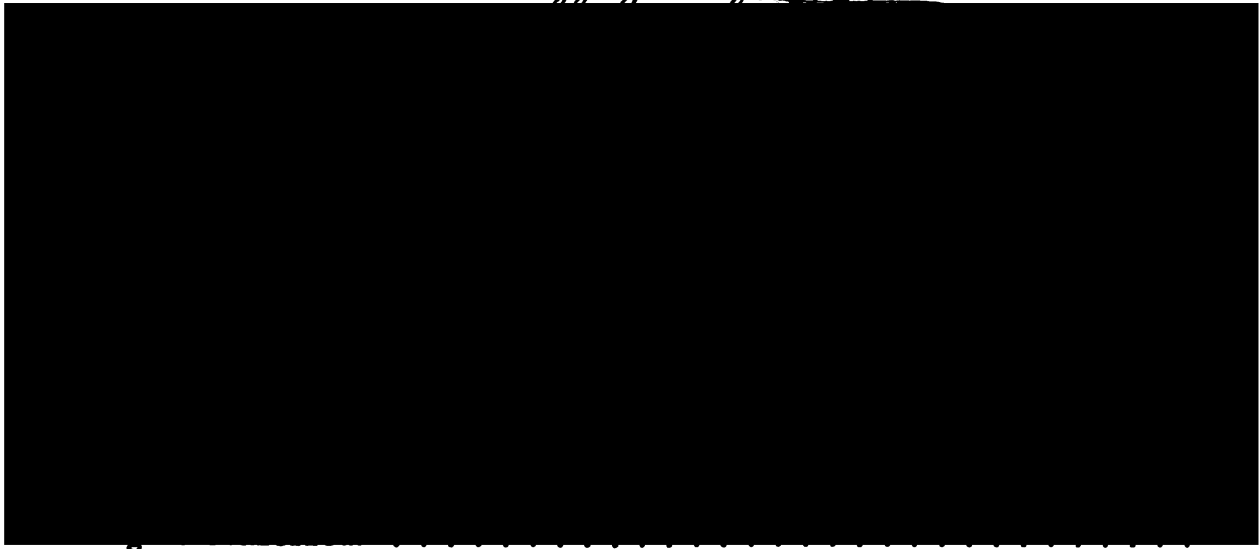
DOCTOR OF PHILOSOPHY

in

ENDOCRINOLOGY

in the

GRADUATE DIVISION

(San Francisco)

of the

UNIVERSITY OF CALIFORNIA

ACKNOWLEDGEMENTS

To Ayala

Table of Contents

ABSTRACT

The hypothesis that the thermogenic action of thyroid hormone is mediated mainly through energy expanditure in active transmembrane sodium transport was examined in the rat small intestinal epithelium, a tissue in which net transepithelial Na^+ transport occurs. The underlying mechanism for this effect was investigated.

It was shown that the stripped jejunal mucosa which contained mainly epithelial cells, was a thermogenic target organ for thyroid hormone action. Administration of T_3 to thyroidectomized and euthyroid rats stimulated oxygen consumption (QO_2) and Na^+-transport dependent respiration $QO_2(t)$. An appreciable fraction of the increment in QO_2 after thyroid hormone administration was due to increased energy utilization by the Na^+-pump of jejunal mucosa. Activation of the Na^+-pump accounted for 55% of the increment in QO_2 in the transition from the hypo- to euthyroid state and 30% in the transition from the euthyroid to the hyperthyroid state. T_3 treatment also increased the number of epithelial cells per unit length of small intestine. I postulated, therefore, that a part of the increment in the Na^+-pump independent respiration, after T_3 administration, might be due to energy required for morphogenesis.

Administration of thyroid hormone to thyroidectomized and euthyroid rats increased the specific activity of the membrane bound Na^++K^+ activated Mg^{2+} dependent adenosine triphosphatase (NaK-ATPase) in jejunal crude plasma membrane fractions. No comparable change, after the administration of the hormone, was recorded in the activity of another membrane bound enzyme - Mg^{2+} activated adenosine triphosphatase (Mg-ATPase) in the same preparations. Moreover, there was a constant ratio of

QO_2/NaK-ATPase and QO_2(t)/NaK-ATPase in the various thyroid states, as well as a close correspondence between the increase in enzyme activity and the increase in QO_2(t) after administration of T_3. These results, namely the proportional increases in QO_2 and NaK-ATPase activity, supported the inference of a causal relationship between Na^+-pump activity and the thermogenic response.

A filtration method for the measurement of specific binding of ^{3}H-ouabain to plasma membrane fractions was developed. A linear correlation between NaK-ATPase activity of a jejunal crude plasma membrane fraction and specific binding of ^{3}H-ouabain to this fraction was established. T_3 administration to thyroidectomized rats caused a significant increase in the number of specific ^{3}H-ouabain binding sites in jejunal crude plasma membrane fractions. By utilizing a Lineweaver-Burk plot, the reciprocal values of ^{3}H-ouabain bound to the plasma membrane fraction as a function of free ^{3}H-ouabain in the incubation media, estimates of maximal number of ^{3}H-ouabain binding sites (N_{max}) and dissociation constants (K_d) were obtained. I concluded that administration of T_3 to thyroidectomized rats increased the number of active Na^+-pump sites in the plasma membrane but with no apparent change in the characteristic properties of these pump sites.

Administration of thyroid hormone to thyroidectomized rats did not elicit any measurable changes in transepithelial potential difference (PD) and short-circuit current (I_{sc}) of the stripped jejunal mucosa as measured in vitro.

The results presented in this thesis support the sodium pump hypothesis in thyroid thermogenesis. Thyroid hormone probably induces an increase in the number of active Na^+-pump sites.

ABBREVIATIONS

ADP – adenosine-5'-diphosphate

ATP – adenosine-5'-triphosphate

C^o – degrees Celsius

Ca^{2+} – calcium ion

Cl^- – chloride ion

cyclic AMP – cyclic 3',5'-adenosine monophosphate

cm – centimeters

cpm – counts per minute

EDTA – ethylenediaminetetraacetic acid

Eq – equivalents

g – gram

^3H – tritium

hr. – hour

K^+ – potassium ion

L – liter

M – molar

m – milli

Mg^{2+} – magnesium ion

mg – milligram

mM – millimolar

ml – milliliter

mV – millivolts

Na^+ – sodium ion

O_2 – oxygen

O_{sm} – osmolal

^{32}P - radiactive phosphorus

T_3 - L-3,5,3'-triiodothyronine

T_4 - L-3,5,3',5'-tetraiodothyronine

TCA - Trichloroacetic acid

μ - micro

wt. - weight

$\sim$ - approximately

I. INTRODUCTION

A. Historical Background

The description of goitre dates back to the second and first millenium B.C. in accounts provided by Chinese, Hindu and Egyptian writers (90). The existence of endemic goitre and its association with endemic cretinism, was described in medieval Europe by many physicians living in or near the Alpine regions, e.g. Paracelsus and others (105). In 1850 Curling (35) reported the first necropsy findings of complete absence of the thyroid gland in two children with clinical cretinism; he used this term to describe a combination of idiocy and dwarfism. Thus, he provided the first evidence of the importance of the thyroid gland to growth and development in man. Gull's (60) in 1874 and Ord (122) four years later recorded that cretinoid changes could occur in adults, and suggested the term myxaedema to describe the condition. Kocher (86) and Reverdin (134) reported of myxodedema in some of their patients who had been subjected to a complete surgical thyroidectomy. In 1884 Schiff (140) demonstrated that thyroidectomy in animals partially reproduced the clinical syndrome of myxoedema, and that the symptoms could be prevented by grafting the thyroid gland of the same animal elsewhere in the body. In 1891, Murray (114) demonstrated that the administration of a glycerol extract of sheep's thyroid corrected the clinical picture of hypothyroidism. Thus it was proved that the thyroid gland secretes a substance which is essential for health.

The second distinctive feature of the thyroid gland on bodily function, is its effect on metabolic rate. In 1895 Magnus-Levy (104) demonstrated that hypothyroid patients consumed oxygen (QO_2) at a lower rate and hyperthyroid patients at a higher rate than normal subjects. Rohrer (137) in 1924, observed increased rates of oxygen consumption in vitro,
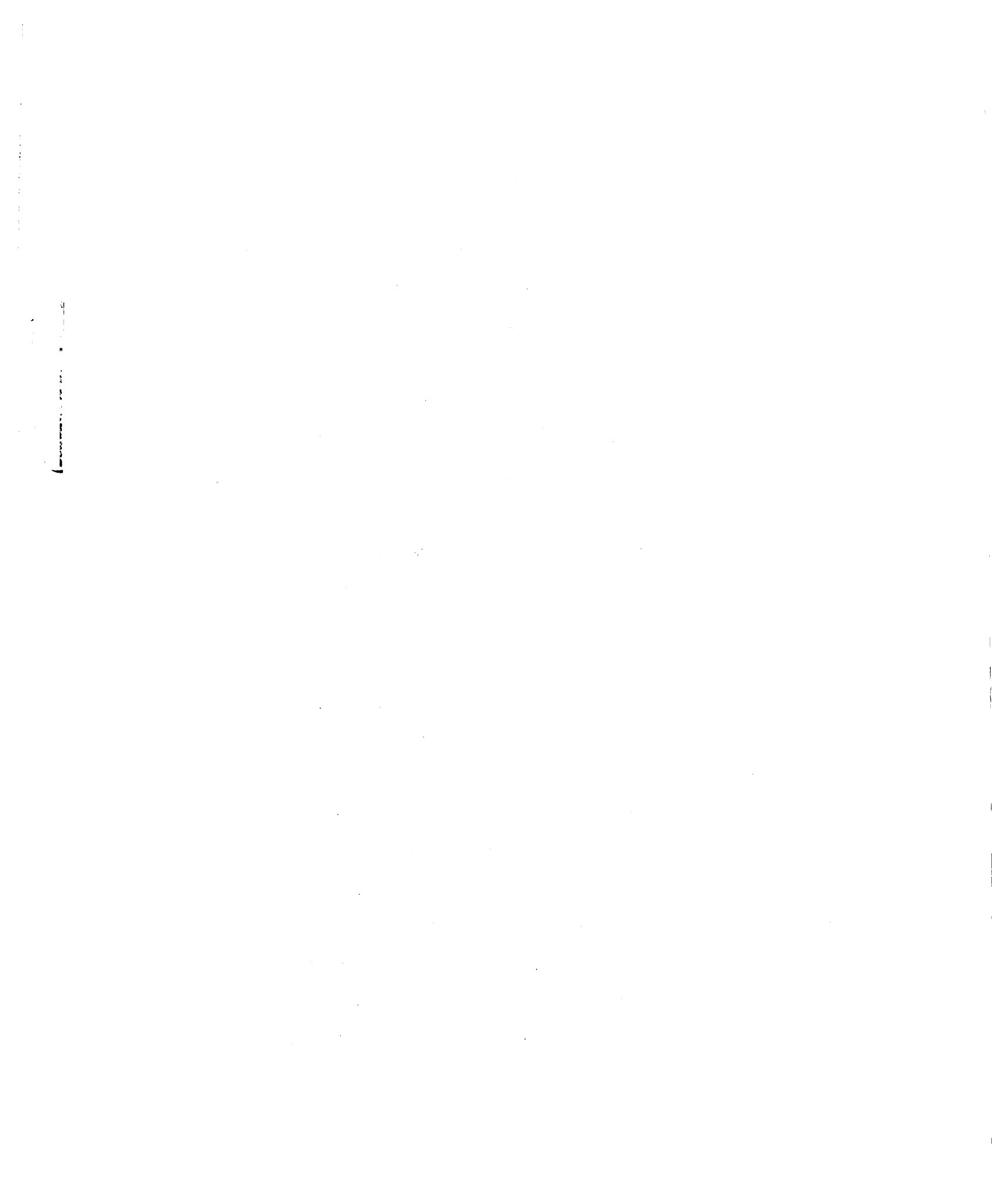
by liver, kidney and skeletal muscle excised from mice made hyperthyroid

by administration of desiccated thyroid. Three years later, Foster noted

that oxygen consumption of diaphragm obtained from thyroidectomized ani-

mals was abnormally low. Many reports followed that established that QO_2

of skeletal muscle, heart, diaphragm liver, kidney, salivary gland, gastric

mucosa and skin taken from hypothyroid, euthyroid or hyperthyroid animals

parallel total body QO_2 of the host (17). In contrast, brain, gonades,

spleen, thymus, gastrointestinal smooth muscle and lung were unresponsive

to the metabolic action of thyroid hormone (16). From these observations

it was concluded that the effect of thyroid on QO_2 of the whole body is

exerted through control of the rate of energy turnover in hormone-responsive

tissues. Since oxidative reactions account for almost all the heat pro-

duction in animal cells, this effect is referred to as thyroid thermogen-

esis.

The observations that the thyroid gland secretes an essential sub-

stance for health, evoked a search for the active principal and led to the

key discovery by Baumann (21) that the thyroid gland contains considerable

amounts of iodine. He also demonstrated that most of the iodine was con-

tained in a protein fraction, that could be hydrolized, and was effective

in relieving the signs of hypothyroidism both in man and in experimental

animals. Kendall (81) in 1914 separated and crystallized thyroxine (T_4).

Thirteen years later Harington and Barges (63) synthesized the hormone.

In 1951 Gross and Pitt-Rivers (57,58) identified another thyroid substance,

3,5,3'-triiodothyronine (T_3) in the circulation, and in the thyroid gland

(59). On a molar basis the new compound was 3-5 times more potent in

biological activity than T_4 (59). Recently deiodination of T_4 to T_3 in

peripheral tissues was observed (27,149) and raised the question of whether

T_4 is an active substance or a precursor to T_3 in target tissues.

B. The "Uncoupling" Hypothesis of Thyroid Thermogenesis.

In adult mammals and birds, thyroid hormones control oxygen consumption. Because most of the total QO_2 involves mitochondria, attention was focused on that organelle as the site of action of the hormone. 2,4 Dinitrophenol (DNP) is an agent known to increase metabolic rates in vivo and in vitro, and in this respect has a similar effect to thyroid hormones. Loomis and Lipman in 1948 (100) found that DNP dissociated oxidative phosphorylation from respiration. QO_2 of tissue suspensions may continue normally, or may even be stimulated, but no coupled phosphorylation of ADP to ATP takes place, causing a marked fall in the P/O ratio. These observations evoked a search for a similar uncoupling effect of thyroid hormones, as the possible mechanism of the thermogenic action of the hormones. In the early 1950's it was reported that the P/O ratio was low in mitochondria from thyroxine treated rats and that addition of the hormone to mitochondrial preparations caused uncoupling as well (66,91,115,119). Subsequent studies however, questioned the validity of the uncoupling hypothesis as the basic mechanism in thyroid thermogenesis. Decreased P/O ratios of mitochondria were observed only when very high concentrations of thyroxine, in the range of 10^{-5} to 10^{-4} were employed (66,91,115), at the same dosage of thyroxine, mitochondrial QO_2 was usually depressed (66,91,115). Thus the principal effect of the hormone was not expressed. No correlation between structure and activity was observed, concerning the uncoupling effect, i.e. stereo isomers and physiologically inactive compounds exert the same effect as L-thyroxine, (166) and no difference between the effect of T_4 and T_3 was documented, though the latter is about 5 times more potent in intact animals (59). It was found that T_4 in huge
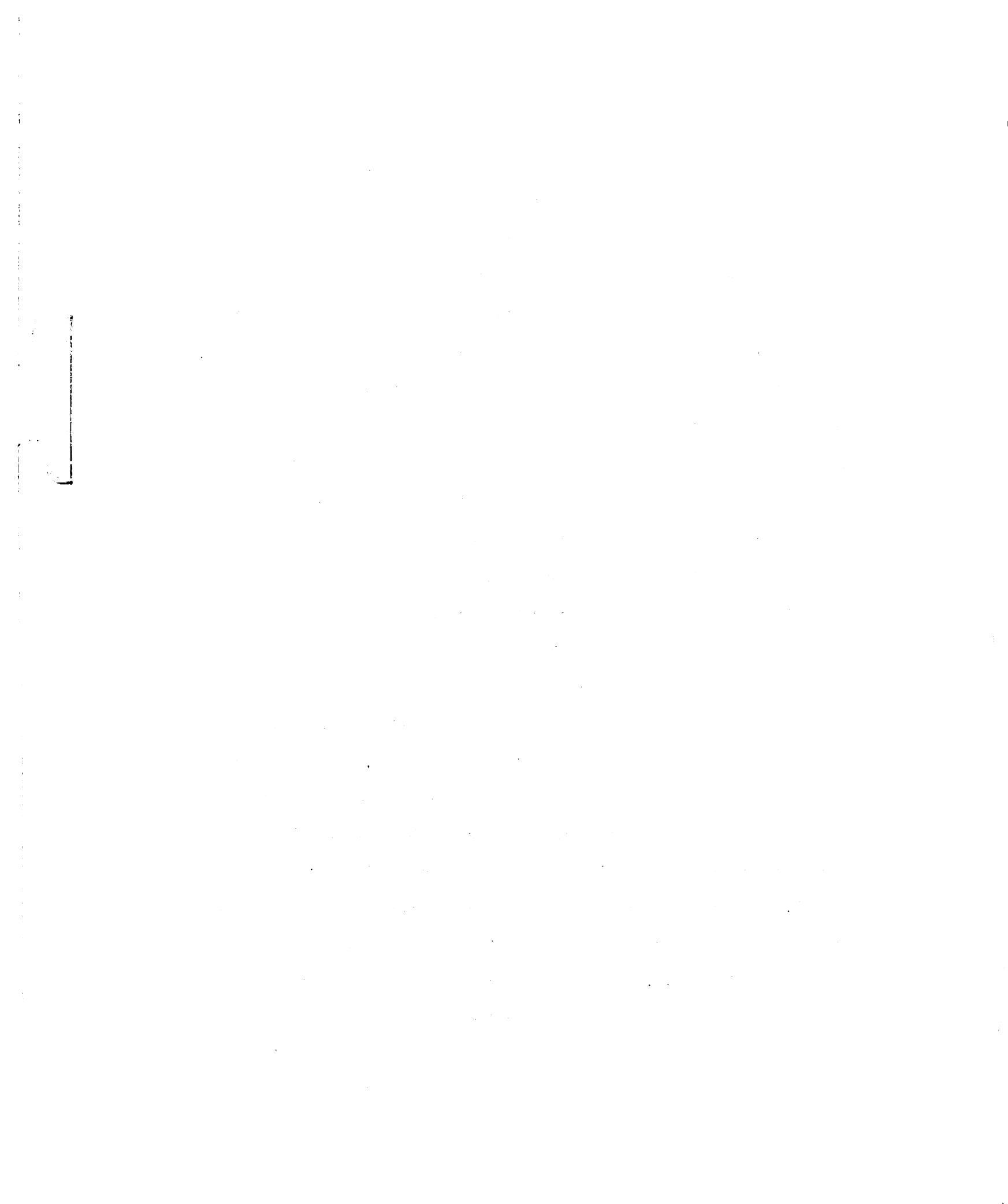
Concentrations resulted in osmotic swelling and structural derangement of mitochondria and it was suggested that the uncoupling was secondary to these changes (165,166). When lower, more physiological concentrations of thyroid hormones were employed, a thermogenic action could be recorded only after a 12 hour lag period and the effect peaked at 48-72 hours (75,169), no changes in oxygen consumption were recorded when T_3 was added in vitro either to tissues or isolated mitochondria (12). Furthermore, it was observed that administration of lower dosages of thyroid hormones in vivo produced an increase in tissue QO_2 and of isolated mitochondria with normal mitochondrial P/O ratios and respiratory control (45,167,169,174).

C. Sodium Pump Hypothesis.

It was shown mainly by Tata and his group that thyroid hormones increase the rates of RNA and protein synthesis sequentially in adult mammalian target tissues. The increase in respiratory activity as well as the effect of thyroid hormones on growth and development were blocked by inhibitors of protein and RNA synthesis, e.g. puromycin, actinomycin-D and 5—fluorouracil, when injected together with the hormone (83,168,175). It was suggested, therefore, that the effect of the hormones on metabolic activity was best explained by preferential synthesis of respiratory and phosphorylative units. This assumption has been supported by electron microscopic studies, that showed an increase in size, number and amount of cristae in skeletal muscle mitochondria, following thyroid hormone administration (38,61).

In homeotherms, cell content of energy rich nucleotides is exceedingly small compared to the rate of energy consumption. The turnover time of total content of adenosine triphosphate is about a minute or less in most mammalian tissues. Thus, ATP production in the cell is tightly

coupled to ATP consumption or the availability of the phosphate acceptor-ADP. Furthermore, it was found that under conditions when the supply of substrate and oxygen are ample the respiratory rate of mitochondria as well as intact cells depends on ADP concentration (85,92). It may be concluded therefore, that any process that increases ATP production will be accompanied by an increase in utilization of the energy rich nucleotides, and vice versa, any primary increase in ATP consumption will be followed, under controlled conditions, by an appropriate change in the rate of ATP production.

The observations cited above and the evidence of normal coupling between respiration and phosphorylation in mitochondria at various levels of thyroid hormone administration led Edelman and Ismail-Beigi (41,42) to suggest that energy utilization as a primary or secondary process is involved in the action of the hormone. Such an endergonic process should meet two criteria: a) The process should be common to all target tissues for thyroid thermogenesis and b) The process should have the capacity to use energy at a rate that is sufficient to account for the increase in QO_2 encountered in various thyroid states. A process that meets these requirements is transmembrane active Na^+ transport. In all mammalian tissues, the extrusion of Na^+ from the cell is against an electrochemical gradient and requires an input of energy. It has been estimated that under resting conditions from 20 to 45% of the total QO_2 is devoted to active sodium transport (72,185,188).

Experiments were conducted in three different tissues: liver, striated muscle and kidney and under three different thyroid conditions: hypothyroid, euthyroid and hyperthyroid states. It was observed that thyroid hormone administration to thyroidectomized or euthyroid rats caused an increase of

from 35 to more than 100% in QO_2 in these tissues and that 30 to 100% of these increments could be attributed to increased utilization of energy by the Na^+ pump (72,73). These observations supported the hypothesis that thyroid activation of Na^+ transport is an important mediator of the calorigenic response. Thyroidal stimulation of energy expenditure by the Na^+ pump could be a consequence of an action on one or more pathways.

A) An increase in the permeability of the cell membrane to Na^+ would increase intracellular Na^+ concentration and stimulate pump activity.

B) A decrease in the efficiency of the pump would obligate the use of more ATP per mole of Na^+ extruded. C) A primary effect on the mitochondria, leading to an increase in ATP synthesis could stimulate Na^+ transport by raising the ATP concentration at the pump site and D) A primary effect on the membrane-bound Na^+-pump, either by enhancing the activity of pre-existing pump sites or by an increase in the number of transport units could result in higher rates of Na^+ extrusion from the cell. It is possible that thyroid hormone will activate more than one mechanism. However, if one of these effects is dominant, a possible separation between two groups from the four proposed mechanisms could be achieved by determining changes in intracellular ion concentrations. It was found that administration of T_3 to either hypothyroid or euthyroid rats lowered the intracellular ratio of Na^+/K^+ by 20 - 40% in liver slices in vitro and in diaphragm and heart in vivo (74). These observations supported either mechanism c or d as the main pathway for thyroid hormone effect. Fletcher and Myant (48,49) previously found a fall in ATP content of liver cells from hyperthyroid rats. Recently, it was observed that administration of T_3 to euthyroid and thyroidectomized rats lowered the ATP/ADP ratio; though the effect was not statistically significant in the latter group
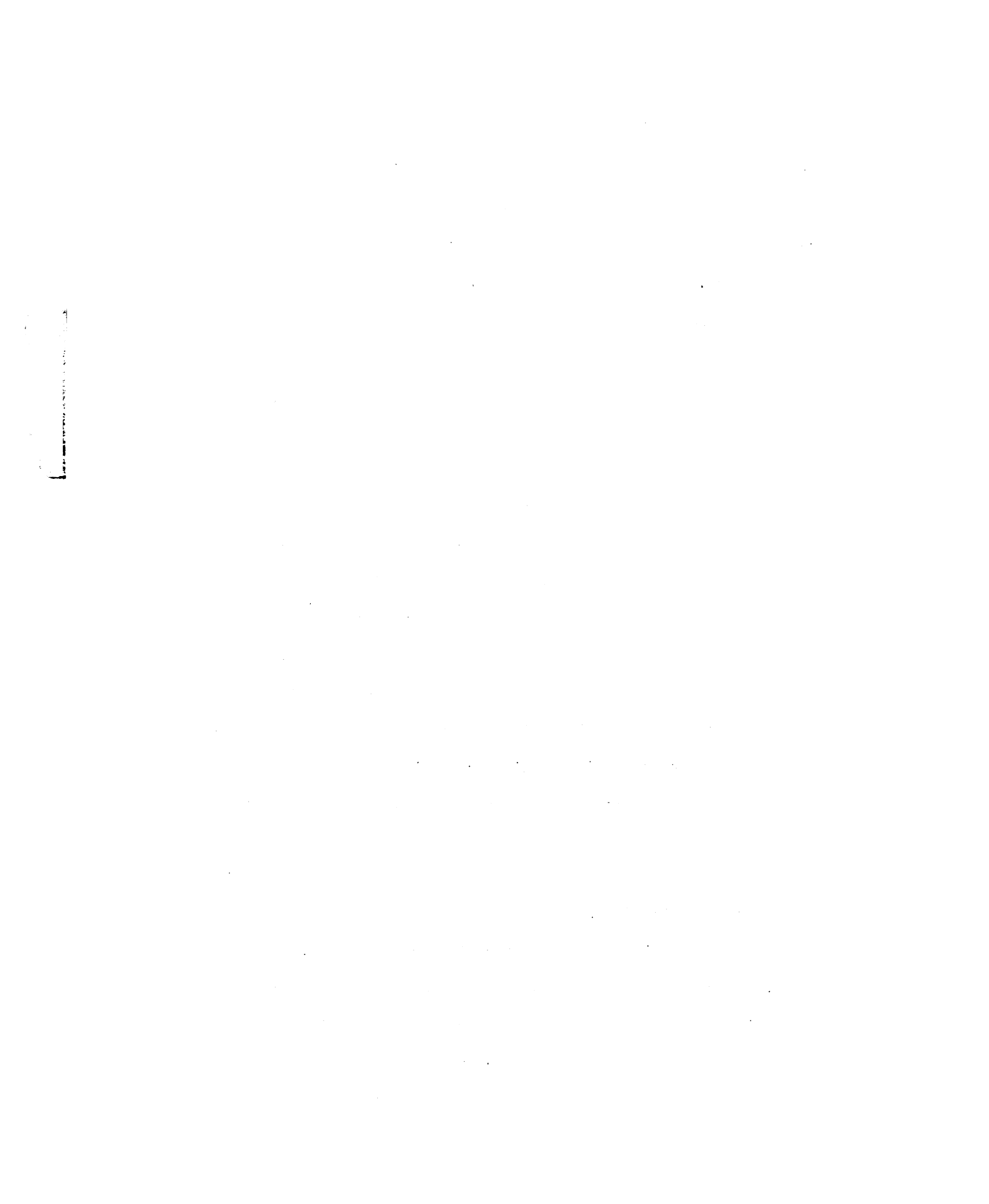
(76). These measurements of the adenine nucleotides were done on whole tissue and do not exclude the possibility of hormonal effects on ATP/ADP distribution or changes in the ATP/ADP ratio at the local pump site. These findings are, however, consistent with the hypothesis that thyroid hormone primarily acts on the Na^+ pump (as described under d above). The work of Skou (156) and others has established that the membrane bound Na^+ and K^+ activated Mg^{2+} dependent adenosine triphosphatase (NaK-ATPase) is the biochemical expression of the Na^+ pump. The effect of thyroid hormone on NaK-ATPase activity in crude homogenates and partially purified cell membrane fractions from liver and kidney was assessed in the different thyroid states. It was found that the specific activity of NaK-ATPase varied in thyroid states and in the same direction as the changes in oxygen consumption coupled to active Na^+ transport ($QO_2(t)$) (73). These observations provided additional support to the hypothesis of primary effects of thyroid hormone on Na^+ - pump activity.

NaK-ATPase determination in these experiments were performed under V_{max} conditions. The increase in specific activity of the enzyme following administration of T_3 could reflect therefore, either a qualitative or quantitative change in Na^+ - pumps. In order to differentiate between these possibilities, and to elucidate the mechanism of thyroid hormone regulation of the enzyme activity, a direct measurement of the number of active Na^+ - pumps in each of the different thyroid states is needed.

The experiments described above were performed on nonpolar cells; no net transport of ions occured under the steady state conditions. The amount of energy expended on active Na^+ transport was assessed indirectly by blocking Na^+ pump activity. Further information is needed on the effect of thyroid hormone on QO_2 and $QO_2(t)$ in tissues in which net Na^+

transport occurs and is measurable. Thus the existance of a quantita-tive relationship between active Na^+ transport and respiration could be evaluated. A tissue that seems to be suitable for these experiments is the small intestine. Active transmural Na^+ transport, from the lumen to the serosal capillary network is known to occur in the small intestine. An important role of thyroid hormones in the physiology of the gastroin-testinal tract has long been indicated by the frequent and major clinical symptoms of diarrhea in hyperthyroidism and constipation in hypothyroidism. The known effects of thyroid hormones on some physiological parameters will be discussed in the appropriate chapters.

The research project to be described was designed to provide infor-mation on: a) the effect of thyroid hormone on QO_2 and NaK-ATPase activ-ity of small intestine, b) the effect of the hormone on the number of Na^+ pump sites in the plasma membrane of intestinal epithelium, and c) the effects of thyroid hormones on the quantitative relationships between QO_2, $QO_2(t)$, NaK-ATPase activity, and net transepithelial Na^+ transport.
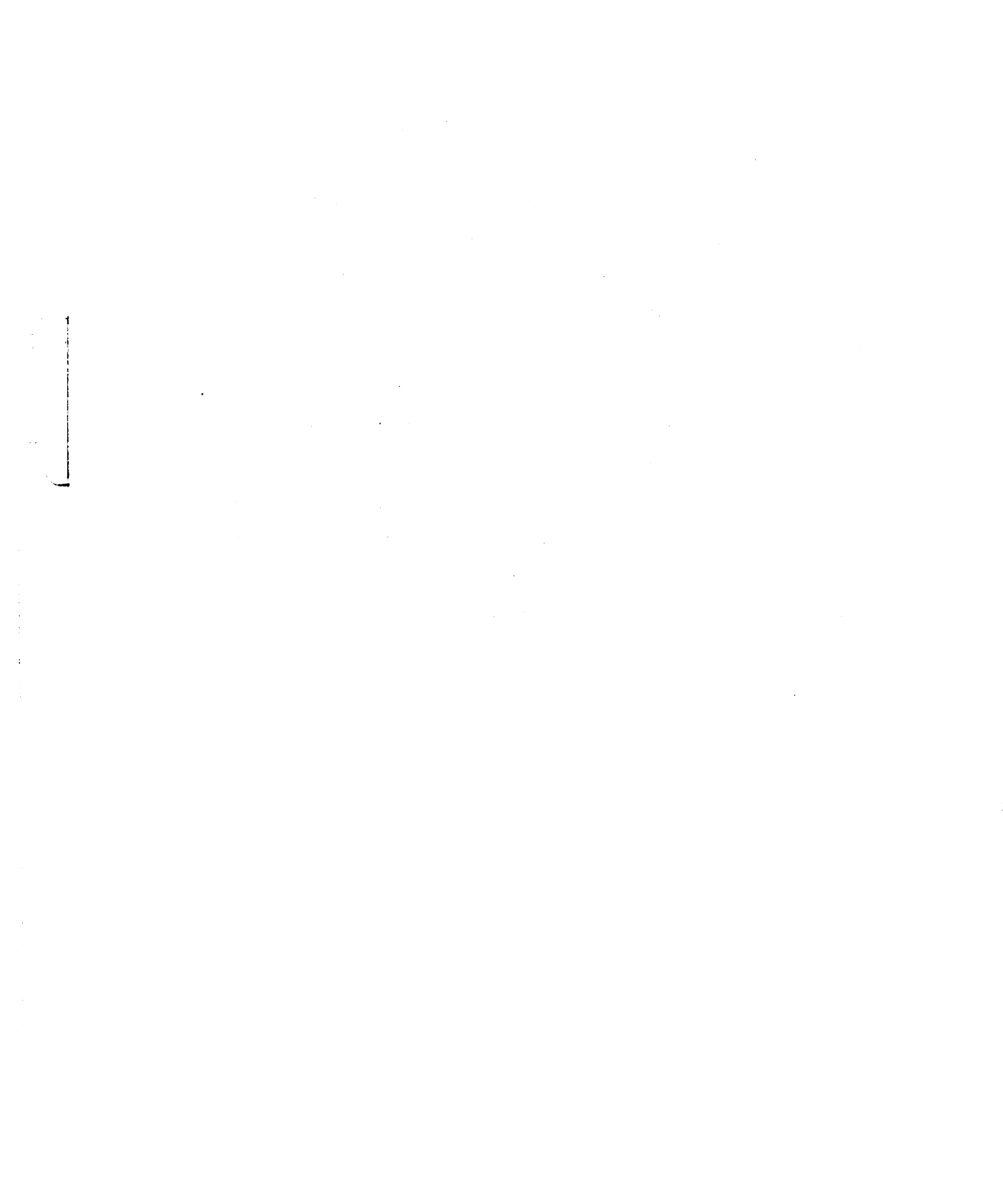
II. THE EFFECT OF T_3 ON QO_2 OF JEJUNAL EPITHELIUM

A. Introduction.

Conflicting reports on the effect of thyroid hormones on respiration of small intestine have appeared previously - Althausen in 1949 (9) reported that injections of T_4 stimulated glucose absorption and QO_2 of intestinal slices in vitro by 30 - 35%. Bronk and Parsons (29) found that jejunal rings taken from hypothyroid rats respired normally, but one injection of T_3 (30 µg subcutaneously) increased QO_2 by 26%. Seshardi (150), on the other hand, observed that injection of T_4 into euthyroid rats for 3 days (100 µg/100g body wt) decreased QO_2 of the upper part of the intestine in vitro.

The studies to be described were designed to assess the effect of thyroid hormone on QO_2 and $QO_2(t)$ of the upper part of the small intestine.

There is no practical method to measure directly the part of the respiration that is devoted to active sodium transport $QO_2(t)$. Thus, I used a "difference" method: The rate of oxygen consumption per unit weight (QO_2) was determined simultaneously with tissue respiration after the sodium pump activity was inhibited [sodium-transport independent respiration (QO_2')]. The calculated difference between QO_2 and QO_2' was taken as the sodium transport - dependent respiration $QO_2(t)$.

B. Materials and Methods

1. Animals and tissue preparation.

Male, Sprague-Dawley rats, from a local supplier (Simonsen Labs Inc. Gilroy, Calif.) body weight 130-180 grams, were maintained on Purina chow ad libitum. A hypothyroid state was achieved by surgical thyroidectomy, a special effort was made to leave at least two parathyroid glands intact.

The animals were used 3-4 weeks post surgery. Three criteria were used as measures of thyroid status 1) growth rate; all rats were weighed twice a week 2) serum thyroxine-iodine; blood samples were taken by percutaneous or open cardiac puncture and T_4 levels measured by the competitive binding method of Murphy and Patee (112,113) (Lazaroni Medical Laboratories, San Francisco, California) and 3) heart rate, measured by electrocardiogram. The last two tests were performed on selected groups rather than as a routine. The animals were anesthetized with an intra-peritoneal injection, 8 mg/100g body wt., of Inactin Na^+ ethyl-(1-methyl-propyl)-malonyl-thio-urea (Promonta, Hamburg, Germany). Na-L-3,5,3 - tri iodo-thyronine (Cal biochem, San Diego Calif.) was dissolved and diluted in $5x10^{-4}$ M NaOH at a final concentration of 150 µg/ml and frozen until used. A control solution of $5x10^{-4}$ M NaOH was prepared at the same time. Rats of about equal body weights and age were randomly assigned to hormone or control groups. Euthyroid or thyroidectomized rats were injected subcutaneously with T_3 50 µg/100 g body weight, or an equivalent volume of the diluent on alternate days, for a total of three injections. The dosage and number of injections were chosen on the basis of the work of Tata (169) and recent work from our laboratory (72). The animals were sacrificed 16-24 hours after the last injection (the reason for this time schedule will be discussed later). The rats were killed by decapitation, and the abdomen opened by a midline incision. A segment of small intestine about 60 cm. length, starting from the pylorus, was taken out and placed in ice cold, oxygenated, modified Na^+ - Ringers solution. The composition of the Na^+ Ringers solution was as follows: Na^+-135, K^+-5.0, Mg^{++}-0.5, Ca^{++}-1.0, Cl^--138, $H_2PO_4^-$ -5.0, Tris base-5.0, glucose - 10 (all in mM), pH -7.4 and osmolality 290 mOsm/kg water. The first 10-12 cm. of

gut distal to the pylorus were 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 Na^+-Ringers solution 20 ml x 3, through a blunt needle with a 20 ml syringe. The intestinal section was then slid onto a glass rod with a tapered point that had been prewetted in the ice cold physiological solution, and the intestine and rod were placed in Na^+-Ringer solution chilled on ice (3-4°c). Under a magnifying glass, the fat and mesentery were pulled 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 and 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 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 of stripped rat gut preparation were prepared. The tissues were fixed in formalin solution and stained with hematoxylin-eosin. Three photomicrographs, representative of the observations made, were taken of the intestine of a single rat. Figure 1a-II is a cross section of rat jejunum on which no mechanical manipulations had been performed. Figure 1b-II shows a section of rat jejunum that had undergone the stripping procedure and Figure 1c-II the stripped portion. As can be seen, the stripping procedure yields an intact mucosal preparation from which the serosa, and the

Figure 1 - II

a. Cross section of rat jejunum on which no mechanical
    manipulation have been performed. X 110.

b. Cross section of a stripped rat jejunum. X 136.

c. Serosa and muscularis externa layers from the
    preparation in Figure 1b - II. X 100.

a
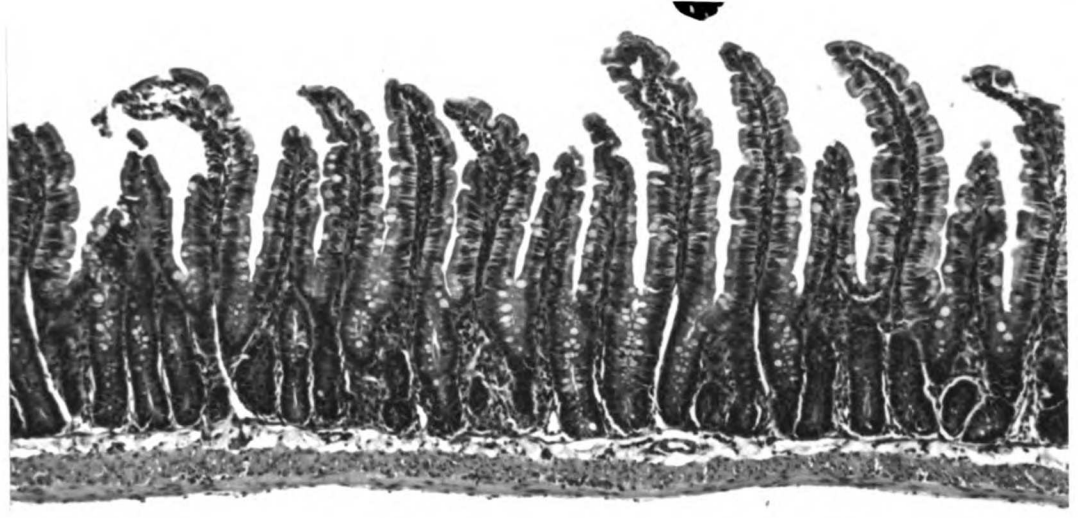
b

c

longitudinal and transverse layers of muscularis externa had been removed.

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 transversally. The relatively uniform and undamaged pieces were selected for measurements of QO_2. Tissue from two animals, T_3 injected and control, were prepared for each experiment. The time of tissue preparation, in the cold, for each experiment was approximately 20 minutes.

2. Oxygen consumption measurements.

One piece of tissue (2.5-4.0 mg dry weight) was placed in a Warburg flask containing 2.0 ml of oxygenated Na^+-Ringers solution with or without ouabain. To trap the CO_2 produced during the experiment, a small filter paper disc (Whatman #1) with 0.1 ml of 12% KOH was placed in the side arm. Respiratory rates were measured with a Warburg respirometer (Aminco, Silver Springs Md.). During the first 5 minutes the flasks were gassed with 100% O_2, the connectors to the oxygen tank were then closed and equilibration continued for an additional 20 minutes. Respiratory rates were determined at 37°c (182). Manometer readings were taken at 15 or 30 minutes intervals for 90 minutes. QO_2 of each specimen was determined in triplicates and the results were averaged.

There is good evidence that ouabain (Strophantin-G) is a specific inhibitor of Na^+ transport (185,188). It was shown recently, by comparing QO_2 in Na^+-free media to that obtained in the presence of a maximally effective concentration of ouabain (10^{-3}M) and to a combination of ouabain and Na^+-free media, that ouabain inhibits selectively and completely Na^+-pump activity in several tissues (11,42,72,73). I decided therefore to use ouabain in a final concentration of 10^{-3}M to inhibit Na^+ transport

in this series of experiments. After about 60 minutes in the respirometer
the QO_2 of the jejunal mucosa consistantly declined: If oxygen consump-
tion during the first 30 minutes (25 to 55 minutes in the respirometer)
is taken as 100% QO_2 for euthyroid segments was 96.5% in the second 30
minutes period and 91.5% in the third period. Expressed in the same way
QO_2 for segments of intestine from euthyroid rats injected with T_3, were
86.4% of the initial rate in the second period and 79.6% in the third per-
iod. It is of interest to note that the readings were stable for two
periods (total 60 minutes) in the hypothyroid $\pm$ T_3 segments. For calcu-
lations however, only the initial respiratory rates were used (i.e. QO_2
measured during the first 30 min). At the termination of the experiment,
the tissues were removed from the flasks and heated in preweighed alumi-
num cups for 24 hours at 91-95°c to obtain the dry weight. The pH of the
bathing solutions were measured. In the few instances in which the pH
was above 7.4, indicating a spill of KOH from the side arm into the flask,
these results were eliminated. pH values followed usually, oxygen consump-
tion measurements, ranging between 6.9-7.1 (after about 120 minutes of
respiration in the closed system) for the flasks in which QO_2 was deter-
mined and were about 0.1-0.2 pH units higher in the flasks containing oua-
bain (QO_2' measurements).

3. Protein and DNA measurements.

I noted that there were visible changes in the thickness of intes-
tinal segments obtained from rats in various thyroid states. A series of
experiments were designed in order to measure wet and dry weights of je-
junal segments from hypothyroid, euthyroid and hyperthyroid rats, and to
determine the effect of T_3 on protein and DNA content of gut mucosa from
hypothyroid rats. Segments of 1 cm length of the upper small intestine

were cut and the wet and dry weights measured as mentioned before. Dry segments of stripped jejunum were homogenized in 4 ml of 2.5 mM Tris-EDTA (pH-7.4), using 20 full strokes in 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. Twelve hours later the tubes were spun in an angle rotor, at 13.000xg for 10 minutes (Sorvall superspeed refrigerated centrifuge model RC2-B). The supernatant was discarded carefully, the pellet resuspended in 2 ml ethanol-ether (3:1) and centrifuged as above. The supernatant was discarded again and the pellet dried at room temperature. Nucleic acids were extracted with 2 ml of 5% TCA in a water bath, 90-95%c for 20 minutes. The extraction was stopped by cooling the tubes on ice. Centrifugation was performed as mentioned above, the supernatant was collected for nucleic acid determination according to the method of Burton (30) and the pellet after being dried at room temperature was dissolved in 1N NaOH. Protein content was determined by the method of Lowry et al (102). DNA standards were prepared from a stock solution containing 0.2 mg DNA/ml in 5 mM NaOH, the source of which was a Calf Thymus DNA, Type V, sodium salt, highly polymerized (sigma). Protein standards were prepared from crystallin Bovine Albumin (Armour Pharmaceutical Company, Chicago Ill.), precipated with cold TCA. The DNA and protein standards were treated in every respect the same as the unknown.

All results are calculated as mean $\pm$ SE. The values were obtained from the Student "t" test for unpaired or when appropriate for paired populations (161). "p" values lower than 0.05 were taken as statistically significant.

C. Results

1. Effect of thyroid state on physiological parameters.

Three criteria were used a) Growth rate, as measured by body weight.
It was observed that the growth rate of hypothyroid rats is extremely
low, less than 5 g per week, at 1-2 weeks after thyroidectomy as compared
to the normal growth rate of about 35 g per week. Any "hypothyroid" rat
gaining more than 15 g/week during the third week after thyroidectomy was
discarded. Administration of T_3 to hypothyroid rats restored the normal
rate of growth. Administration of T_3 to euthyroid rats was accompanied
by a decrease in rate of growth to about 25 g/week. b) Serum thyroxine-
iodine. The following observations were made: (Table 1-II) 1) Serum
thyroxine iodine in hypothyroid rats was about 30% of the euthyroid level.
2) One week after thyroidectomy hormone levels in blood fell below nor-
mal and remained constant for at least 6 weeks. It is noteworthy, that
in 8 rats in which thyroid gland ablation was achieved by I^{131} treatment,
Dr. Y. Asano and I documented plasma iodine thyroxine levels of 0.66 ± 0.18
mg/100ml; thus, in the same range as for thyroidectomized animals.
3) Injection of T_3 to euthyroid rats caused a decrease in serum thyroxine
levels, presumably by blocking Thyrotropin (TSH) secretion (71). 4) T_3
treatment of hypothyroid animals increased serum thyroxine levels in pro-
portion to the dose injected. This change is due to contamination of the
T_3 preparation prepared from biological sources with T_4. The manufacturers
claim that the preparation contains no more than 10% T_4, was confirmed by
our finding of about 7-8% of T_4 in T_3 stock solutions. c) Heart rates,
were measured in anasthetized animals with the aid of an electrocardio-
graph. The results are summarized in Table 2-II. Throidectomy caused a
significant decrease of about 48% in heart rates of the animals, while

Table 1-II Serum Thyroxine-Iodine Levels in Various Thyroid States.

Thyroid state	Euthyroid		Hypothyroid				
Time after surgery			1 week		4 weeks		6 weeks
T_3 Treatment ug/100 g B.W.	none	50x3	none	50x1	none	50x3	none
Serum Thyroxine	1.97	0.80	0.49	1.02	0.71	1.77	0.65
Iodine in mg/100ml*	± 0.15	± 0.25	± 0.10	± 0.15	± 0.06	± 0.15	± 0.28
	(13)	(5)	(13)	(5)	(8)	(6)	(5)

* mean ± SE number of rats in each group in brackets

Table 2-II Effect of Thyroid Status on Heart Rate.*

	Thyroidectomized	Euthyroid
	Heart rate (beats /2 seconds)	
Control	8.4 ± 0.2	16.1 ± 0.4
+ T₃	16.3 ± 0.5	19.8 ± 0.5
	7.9	3.7
p	< 0.001	< 0.001

Mean ± SE n=7 for the thyroidectomized groups, n=8 for the euthyroid and n=5 for the euthyroid +T₃ groups. Rats were injected with 50 g T₃/100g body weight or diluent on alternate days (x3) and the measurements were made 48 hours after the third injection.

injection of T_3 into hypothyroid rats restored heart rate, to euthyroid levels. In the transition from euthyroid to hyperthyroid state, the heart rate increased by about 23%.

From these observations it was concluded that: 1) Thyroidectomy created a state of hypothyroidism comparable to I^{131} thyroid ablation. 2) Weight change is a sufficient criterion to determine thyroid status. 3) Changes in growth and heart rates of hypothyroid rats after T_3 treatment support our claim that the dose of T_3 injected resulted in responses within the 'physiological' range.

2. Effects of T_3 on oxygen consumption.

Respiratory rates of stripped jejunum from hypothyroid rats with and without thyroid hormone administration are summarized in Table 3-II. T_3 produced significant increases of 26% and 41% in QO_2 and $QO_2(t)$ respectively; the 18% increase in the ouabain-insensitive respiration was statistically insignificant. In euthyroid rats (Table 4-II), the changes evoked by T_3 administration were of smaller magnitude, increases of 12-16% in QO_2, QO_2' and $QO_2(t)$ were demonstrated. A summary of the respiratory rates in the various thyroid states is given in Table 5-II. It should be noted that the respiratory rates of small intestinal epithelium from hypothyroid rats were lower than euthyroid values. Three injections of T_3 to hypothyroid rats increased QO_2, QO_2' and $QO_2(t)$ to euthyroid levels, while in the transition from euthyroid to hyperthyroid state only a relatively small increase in total respiration and ouabain-independent respiration was observed.

Quantitative estimates of the relationship between QO_2 and $QO_2(t)$ were calculated from the data of Tables 3-II and 4-II and are presented in Table 6-II. In hypothyroid, euthyroid and hyperthyroid states, about

Table 3-II Oxygen Consumption (QO_2) of Small Intestine of

Thyroidectomized Rats $\pm$ T_3 (50 µg per 100 g body weight)*

Ouabain	Thyroidectomized	Thyroidectomized + T_3		p	/TX+
0	11.77 ± 0.43	14.79 ± 0.53	3.02	< 0.001	0.26
10^{-3}M	7.76 ± 0.42	9.13 ± 0.55	1.37	> 0.05	0.18
QO_2(t)**	4.01 ± 0.31	5.66 ± 0.51	1.65	< 0.025	0.41

*Mean ± SE (n = 9) expressed as µl/mg dry weight/hr.

+TX = thyroidectomized rats.

**QO_2(t) denotes the ouabain sensitive QO_2.

Table 4-II Oxygen Consumption (QO_2) of Small Intestine of Euthyroid Rats

$\pm$ T_3 (50 µg per 100 g body weight)*

Ouabain	Euthyroid	Euthyroid + T_3	Δ	p	Δ/Euthyroid
		(µl oxygen per mg dry weight per hour)			
0	14.41 $\pm$ 0.34	16.48 $\pm$ 0.43	2.07	< 0.005	0.14
10^{-3}M	9.37 $\pm$ 0.36	10.86 $\pm$ 0.53	1.49	< 0.05	0.16
$QO_2(t)$+	5.04 $\pm$ 0.40	5.62 $\pm$ 0.60	0.58	NS	0.12

*Mean $\pm$ SE (n = 12) expressed as µl/mg dry wt/hr.

+$QO_2(t)$ denotes the ouabain sensitive QO_2.

Table 5-II QO_2 of Small Intestine of Thyroidectomized and

Euthyroid Rats $\pm$ T_3 (50 µg per 100 g body weight)[*]

Ouabain	Thyroidectomized	Thyroidectomized + T_3	Euthyroid	Euthyroid + T_3
		(µl oxygen per mg dry weight per hour)		
0	11.77 ± 0.43	14.79 ± 0.53	14.41 ± 0.34	16.48 ± 0.43
10^{-3}M	7.76 ± 0.42	9.13 ± 0.55	9.37 ± 0.36	10.86 ± 0.53
QO_2(t)	4.01 ± 0.31	5.66 ± 0.51	5.04 ± 0.40	5.62 ± 0.60

[*]Mean ± SE (n = 9 and 12 for thyroidectomized and euthyroid respectively)

Table 6-II   Fraction of Total QO_2 Dependent on Na^+ Transport.*

	Hypothyroid	Hypothyroid + T_3	Euthyroid	Euthyroid + T_3
$QO_2(t)/QO_2$	0.34	0.38	0.35	0.34
$\Delta QO_2(t)/\Delta QO_2$		0.55		0.28

* Values calculated from the data in Tables 3-II and 4-II. $QO_2(t)/QO_2$ represents the fraction of QO_2 attributable to active Na^+ - transport. The ratio $QO_2(t)/$ QO_2 represents the fraction of the T_3 - induced increase in QO_2 that is attributable to an increase in $QO_2(t)$.

35% of total oxygen consumption is devoted to active Na^+ transport. The increase in $QO_2(t)$ accounted for 55% of the T_3 -dependent increment in QO_2 in hypothyroid tissue, and only 28% in small intestine from euthyroid rats. This implies that in the transition from euthyroid to hyperthyroid state, about 70% of the T_3 effect on respiration was not dependent on the ouabain-inhibitable component of Na^+ transport. As mentioned before, administration of T_3 to hypothyroid rats caused a visible increase in the thickness of the intestine. It is possible therefore, that a fraction of the increase in QO_2 is involved in energy requirements for morphogenesis.

3. **Effects of thyroid states on small intestinal mass, protein and DNA content.**

As shown in Table 7-II, 4 weeks after thyroidectomy, the wet and dry weights of segments of jejunum was reduced to 64.7% and 60.8% of a comparable euthyroid piece of intestine. T_3 injection in hypothyroid rats (50 μg/100g body wt x 3) produced a 22% and 29% increase in wet and dry weights respectively, but the gut weighed only about 78% of a comparable segment from euthyroid rats. No significant change in wet and dry weight was observed, in the transition from euthyroid to hyperthyroid state. The changes in wet and dry weights were proportional in the different thyroid states, thus the dry weight expressed as a fraction of wet weight was the same for the various groups of animals (Table 7-II). These results imply that thyroid hormone had its major effect on small intestinal mass and not on water content. To further investigate this effect, measurement of protein and DNA content of stripped jejunal homogenate from hypothyroid rats ± T_3 were performed, and the results are summerized in Table 8-II. Although differences in the absolute values of dry weight, protein and DNA content between the two populations were observed the

Table 7-II Wet Weight and Dry Weight of Pieces (1.25 cm length) of Stripped

Small Intestine from Thyroidectomized and Euthyroid Rats $\pm$ T$_3$

(50 μg/100 g body weight)*

	Weight Weight mg	Dry Weight mg	Dry Weight % of wet weight
Thyroidectomized (6)	78.24 $\pm$ 4.61	10.91 $\pm$ 1.04	13.8 $\pm$ 0.9
Thyroidectomized (6) +T$_3$	92.92 $\pm$ 6.10	13.22 $\pm$ 0.99	14.2 $\pm$ 0.5
Euthyroid (8)	111.51 $\pm$ 5.27	15.69 $\pm$ 0.79	14.1 $\pm$ 0.2
Euthyroid (8) +T$_3$	112.10 $\pm$ 4.01	16.08 $\pm$ 0.59	14.3 $\pm$ 0.2

*Mean $\pm$ SE. In brackets number of rats in each group.

Table 8-II Dry Weight, Protein and DNA Content of Pieces of Stripped Small Intestine

from Thyroidectomized Rats $\pm$ T$_3$ (50 µg per 100 g body weight)*

	Dry Weight % wet weight	Protein % dry weight	DNA % dry weight	% protein
Thyroidectomized	9.41 $\pm$ 0.30	43.26 $\pm$ 0.69	3.68 $\pm$ 0.10	8.52 $\pm$ 0.31
Thyroidectomized + T$_3$	9.02 $\pm$ 0.53	43.06 $\pm$ 0.85	3.62 $\pm$ 0.20	8.70 $\pm$ 0.19
	n.s.	n.s.	n.s.	n.s.

*Mean $\pm$ SE. The number of rats in each group = 9. From each animal

6 pieces of intestine were taken.

changes in these parameters were proportionate. Thus the amount of pro-
tein per unit dry weight of small intestine, and the DNA content per unit
dry weight or protein were unchanged.

I concluded, therefore, that administration of thyroid to hypothyroid
rats, for the most part, increased the number of cells per unit length of
the upper small intestinal mucosa. Furthermore, these results indicate
that a part of the increment in ouabain-insensitive QO_2, after thyroid
hormone administration, might be due to energy requirements for morphogen-
esis. Since in the various thyroid states the ratios of dry/wet weight,
protein/dry weight, and DNA/protein did not differ from control values,
the changes in QO_2 and $QO_2(t)$ induced by thyroid hormone can be ascribed
to changes in respiratory rates per unit mass of tissue (i.e. on a per
cell basis).

D. Discussion

Two main reasons prompted us to use a preparation that contains
mainly epithelial cells. 1. Smooth muscle layers of the small intestine
which contribute to about one third of the dry weight are not a target
organ for the calorigenic action of thyroid hormones (16). Moreover, it
was shown by several investigators that the existing gradient in the res-
piratory activity of the intestine from jejunum to ileum, was a function
of mucosal tissue activity with almost no change in the QO_2 of the muscle
layers (15,28,36,190). Thus differences in rates of respiration corre-
lated solely with the abundance of the epithelial layer. 2. It was ob-
served that the thickness of the preparation employed was the major fac-
tor determining the rate of oxidative metabolism in rat jejunum and rab-
bit ileum (15,28,51). QO_2 of rat jejunal mucosa was calculated to be 30%
greater than the value observed for the intact intestinal wall (28),

while a three-fold increase in respiration of the stripped mucosa in comparison to the whole thickness tissue was documented for the rabbit ileum (51). It seemed that the stripping procedure increased oxygen availability for mucosal cells especially the intervillar and crypt cells that are located deeper in the epithelium. Stripped jejunal and ileal epithelia were used for measurements of transport rates (117,191) and a substantial increase in salicylate transport rates, in comparison to everted sacs, was observed (191). I felt therefore, that the stripped small intestine was a viable and physiological preparation for measurements of changes due to thyroid hormone action.

As shown in Figure 1b-II, the stripping procedure yielded a preparation that was mainly composed of epithelial cells and submucosa. The respiratory rates observed for euthyroid segments were in the same range as reported in the literature for similar preparations (15,28,77). Our results demonstrated clearly that thyroid hormone had an effect on oxygen consumption of jejunal epithelium. QO_2 of small intestine from hypothyroid rats was lower than that from the euthyroid rats, while T_3 elicited an increase in respiration both in hypothyroid and euthyroid states. Due to the rapid decline in QO_2 of hyperthyroid tissue in the Warburg Respirometer the QO_2 values observed for this group were probably underestimated.

In discussing the measurement of $QO_2(t)$, it must be emphasized that this value was estimated indirectly and the validity of the estimates relies on the following assumptions: 1. that ouabain inhibited Na^+-transport completely and specifically, and 2. that secondary changes in intracellular electrolyte composition did not affect QO_2. In a series of experiments performed recently in our laboratory by Ismael-Beigi and Edelman, it was shown that ouabain ($10^{-3}M$) depressed QO_2 in liver slices

to almost the same level as removal of Na^+ from the medium (42,72). The former investigators as well as Asano, Liberman and Edelman (11) found that QO_2 of rat diaphragm in the presence of ouabain was equivalent to the values obtained when the tissue was bathed in Na^+ free media. Moreover, the addition of ouabain to liver, muscle and kidney slices incubated in Na^+-free media did not depress QO_2 further (42,72). Frizzell et al (51) reported recently that in the stripped rabbit ileum replacement of Na^+ by choline or the presence of 10^{-4} M ouabain reduced QO_2 to the same degree. Based on these observations I concluded that ouabain caused a specific and complete inhibition of Na^+ transport. As a consequence of inhibition of the Na^+ - pump by ouabain in a Na^+-Ringers medium, changes in intracellular composition will occur, cells gain Na^+ and lose K^+. Intracellular Na^+ will decrease when the pump is inhibited by using a Na^+-free medium. The fact that similar QO_2 values were obtained by using the two experimental procedures serves to rule out a major influence of intracellular Na^+ on respiration per se. However, with both techniques there is a fall in intracellular K^+ concentration. Recently, Asano, Liberman and Edelman (11) measured the effect of various intracellular K^+ concentrations on QO_2 of diaphragm. Intracellular K^+ concentrations were modified by supplementing Na^+-free Ringer's solutions with different K^+ concentrations; Na^+ free Ringer's solutions were prepared by substituting isomolar concentration of sucrose or choline-chloride for NaCl. It was found that while intracellular K^+ ranged from about 10% of the normal level in Na^+ Ringer + ouabain, to a full restoration of intracellular K^+ content in sucrose-Ringer + 40 mEq/L KCl, there were no significant changes in QO_2' measured in the same preparations. These observations implied that res-

piration was not dependent on intracellular K^+ concentration over the observed range. These results are in accord with other reports on liver slices (72) and kidney mitochondria (23). The experimental method of estimating $QO_2(t)$ therefore, was probably reliable.

As mentioned in the Introduction, in mammalian tissues about 20-45% of the resting QO_2 is expended in transmembrane active Na^+-transport (186,187). In the stripped jejunum we observed that about 35% of the resting QO_2 was coupled to Na^+-transport, thus the Na^+ pump in this tissue constitutes one of the major energy consumers and is, therefore, one of the main heat producers. My results show that activation of the Na^+ pump accounts for more than 50% of the increase in QO_2 in the transition from hypo- to euthyroid state, and about 30% of the increase in the transition from the euthyroid to the hyperthyroid state. It is probable, however, that $QO_2(t)$ as well as QO_2 of small intestine from hyperthyroid rats was underestimated because of the time-dependent fall in respiration in the Warburg Respirometer. That an appreciable fraction of the increased QO_2 after thyroid hormone administration, is due to increased energy expenditure by the Na^+-pump of the jejunal mucosa is obvious, however. The upper small intestinal mucosa behaviour is, therefore, similar to other target organs in response to the action of thyroid hormone.

In addition, a significant increase in Na^+-transport independent respiration of jejunal strips was recorded after T_3 treatment. It is possible that a fraction of the increase in ouabain-insensitive respiration was involved in energy requirements for morphogenesis. As mentioned before, T_3 treatment increased the mass of jejunal mucosa mainly by an increase in the number of cells per unit length. An additional possibility is that some of the increase in QO_2 after thyroid hormone administra-

tion is due to energy consumed by transport mechanisms that are not oua-

bain inhibitable. Munck (111) reported that in the absence of bicarbo-

nate and an actively transported sugar or amino acid, short circuited

preparations of rat jejunum actively secreted Cl^- and that Cl^- transport

accounted for approximately two thirds of the short circuit current.

Therefore, the possibility that, in the jejunum, thyroid hormone activates

more than one transport mechanism deserves further study.

III. THE EFFECT OF T_3 ON ATPase ACTIVITY OF JEJUNAL EPITHELIUM.

A. Introduction

Skou, in 1957, first demonstrated in a crab nerve particulate fraction, a Mg^{2+} activated ATPase, the activity of which was increased considerably upon addition of Na^+ and K^+ to the assay medium (156,159). Since then a considerable body of evidence on the involvement of the membrane-bound Na^+ + K^+ activated Mg^{2+} dependent ATPase (NaK-ATPase) in active transport across biological membranes has been accumulating. Bonting, Caravaggio and Howkins (24) found the activity to be present in 21 tissues isolated from 10 different species, which possessed a cardiac glycoside-sensitive transport system. Post et al (125) and Dunham and Glynn (40) showed a close correspondence of several properties between the enzyme and the transport system and concluded that the NaK-ATPase was directly associated with the active transport of Na^+ and K^+ across the erythrocyte cell membrane. Assuming therefore, that the NaK-ATPase is a biochemical expression of the Na^+-pump, additional information considering the thermogenic effect of thyroid hormone on jejunal mucosa can be obtained by measuring the effect of the hormone on this membrane-bound enzyme.

B. Materials and Methods

   1. Tissue preparation.

Euthyroid and thyroidectomized rats were used. The preparation of the rats and the protocol for T_3 injection were as described under Methods in chapter II. A crude plasma membrane fraction from jejunal mucosa was prepared with some modifications, by the method of Quigley and Gotterer (128). About 60-70 cm of proximal small intestine was removed and put in ice cold isotonic solution A, containing 0.9% NaCl - 5mM Na_2, EDTA (pH-

7.4). The first 10 cm segment from the pylorus to the ligament of Treitz was discarded, and the next 2 segments of 10 cm length each were used. The intestine was irrigated first with an ice cold isotonic rinsing solution B, 12 ml x 3. Rinsing solution B contained 5mM -Na$_2$ EDTA, 100 mM-NaCl and 50 mM N-acetyl-L-cysteine buffered to pH 7.4 with 1M Tris HCl. The intestine was rinsed again with 15 ml x 2 of solution A, everted on a blunt long needle, wiped on Microwipe paper(disposable wipers) and cut along the side. The jejunal sheet thus obtained was put in a chilled Petri dish containing 20 ml of a 5 mM Na$_2$ EDTA solution (pH -7.4 and os-molality 15 mOsm/kg water). The mucosa was removed by gentle scraping x 3 with a glass slide. Histological sections of three repetitive scrap-ings and the remaining piece of intestine are shown on Figure 1-III. As can be seen clearly, these scrapings contain epithelial cells exclusively, while the remaining intestine, Figure 1d-III, contains epithelial cells and intact submucosa, muscularis and serosal layers. The full thickness cross section of a normal mucosa is shown in Figure 1a-II as a guide to the normal histology. Two pieces of intestinal segments were scraped in each Petri dish. The wet weight of the scraped mucosa ranged from 700-850 mg. The scrapings (in 5mM Na$_2$ EDTA) were homogenized in a loose fitt-ing teflon-glass Elvehjem-Potter homogenizer, at 2-3°c 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 Cheesecloth (Marco, Marsales Co., New York), and then through a 132 Mesh Nylon cloth (John Stanier, Manchester, England). The filterates were spun at 700xg for 10 minutes (Sorvall model RC2-B). The supernatant was decanted and saved, and the gelatinous pellet, containing crude brush border, unbroken

Figure 1 - III

a - c. Cross sections of small intestinal scrapings (details in text).

a. First scraping. X 85.

b. Second scraping. X 107.

c. Third scraping. X 83.

d. A cross section of the remaining layers of epithelium, submucosa and muscularis after above scrapings. X 84.

c
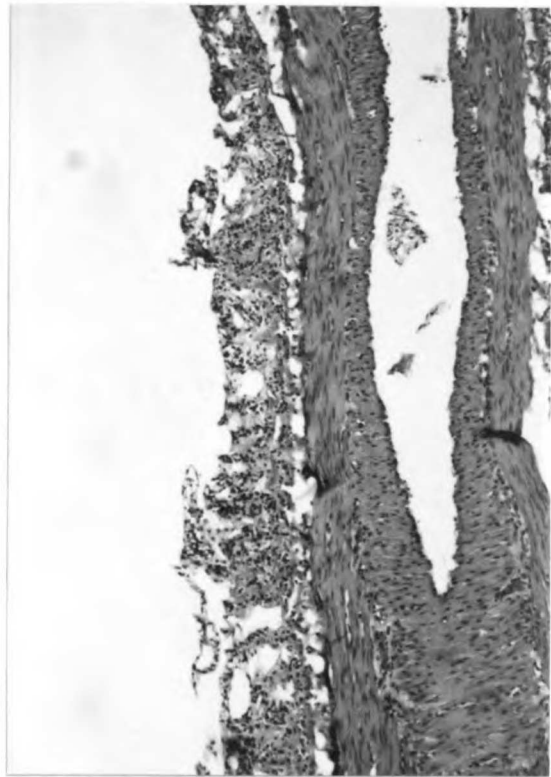
d
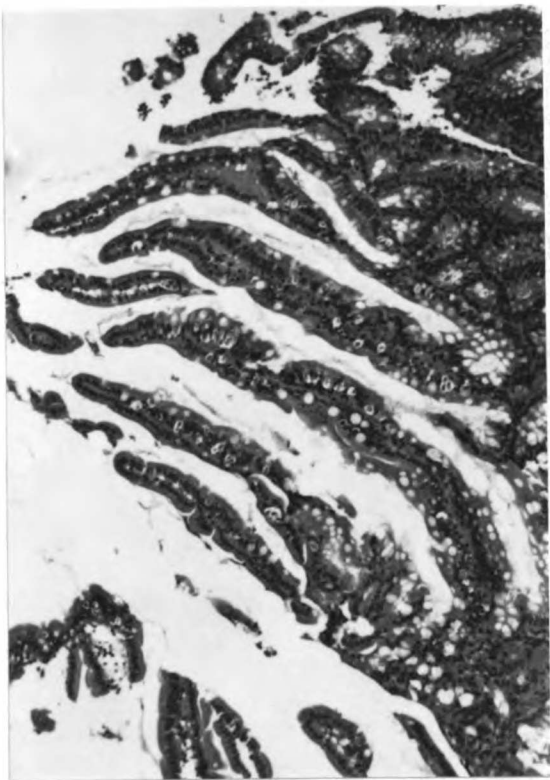
a
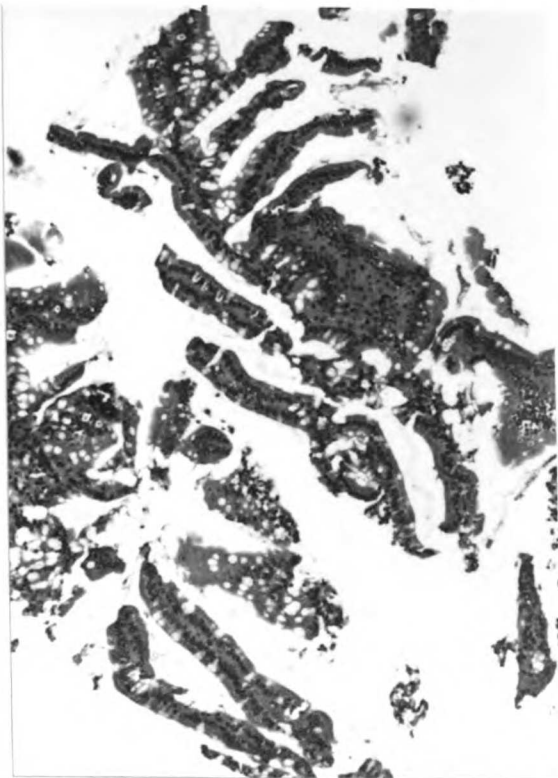
b

cells and nuclei (50,128) was washed in 10 ml 5 mM Na_2 EDTA. The super-
natant of the second spin was collected and added to the previous one.
The combined supernatants from the 700xg spins was then centrifuged at
10,000xg for 10 minutes, this supernatant was decanted and the pellet
washed in 20 ml 2.5 mM Tris-EDTA (pH-7.4 and osmolality 10 mOs/1kg water).
The final 10,000xg pellet was resuspended in 2.5 mM Tris-EDTA. In each
experiment 2 or 4 preparations, control and T_3 treated were prepared si-
multaneously.

Quigley and Gotterer (128) measured the activity of various marker
enzymes in the different fractions obtained in their preparative method
of jejunal epithelium homogenates. The marker enzymes used were: alka-
line phosphatase, invertase, glucose-6-phosphatase, cytochrome oxidase
and NaK-ATPase. When the activity of these marker enzymes recovered in
the 10,000xg pellet was measured and expressed as % of the total activity
in the homogenate, the following results were obtained: alkaline phos-
phatase and invertase ∿10%, glucose-6-phosphatase ∿26%, cytochrome
oxidase ∿75% and NaK-ATPase ∿60%. By placing the 10,000xg pellet on a
discontinuous sucrose gradient, Quigley and Gotterer obtained 75% of the
total NaK-ATPase activity in the 10,000xg pellet and very little cyto-
chrome oxidase activity. The M-1 fraction contained a considerable amount
of the 5'-nucleotidase activity of the intestinal mucosal cell homogenate.
This enzymatic activity was also found in a plasma membrane preparation
isolated from rat liver (43). In electron microscopy studies the M-1
fraction consisted mainly of empty vesicles much smaller in diameter than
mitochondria (128). From these observations, it was concluded that the
10,000xg pellet contained membrane vesicles with a high content of NaK-
ATPase. These vesicles were distinct from mitochondria that were abundant

in the fraction. The 10,000xg pellet was contaminated with some brush
border and microsomal membranes as judged by the activity of alkaline
phosphate and invertase as markers for brush border, and glucose-6-
phosphatase - as a marker for the microsomal fraction. The importance of
the plasma membrane of the intestinal mucosa, exclusive of the brush
border, as a mediator in active sodium transport has been discussed by
several investigators (34,145). It might be anticipated that this mem-
brane would contain a NaK-ATPase of high specific activity. It could be
assumed therefore, that the membrane vesicles with the high NaK-ATPase
content observed in the 10,000xg pellet were plasma membrane vesicles.

For the experiments to be described, I used the 10,000xg pellet.
Based on the studies of Quigley and Gotterer (128) and our own observa-
tion (to be discussed later), it is obvious that the 10,000xg pellet con-
tains mitochondria as well as plasma membrane vesicles. I prefer, there-
fore, to designate this fraction as a "crude plasma membrane preparation."

In a separate series of experiments Na^+-deoxycholate (DOC) (Sigma,
St. Louis, Miss) in concentrations from 0.3 mg/ml to 1.6 mg/ml was added
to the Petri dishes. Thus the scraping and homogenization procedures
were carried out in the presence of DOC. The DOC was diluted and prac-
tically eliminated from the plasma membrane fraction by additional wash-
ings of the 10,000g pellet with 2.5 mM Tris-EDTA.

2. ATPase assay.

Since the NaK-ATPase activity is always accompanied by Mg-ATPase ac-
tivity, a differential assay is necessary. Total ATPase activity is
measured in a complete medium while the Mg-ATPase is determined in the
same medium + ouabain, or in a medium lacking Na^+ and K^+. The calculated

difference between the total and Mg-ATPase activities is taken as the NaK-ATPase activity. The complete medium I used, contained a final concentration of Na^+-100, K^+-10, Mg^{2+}-5, Tris-HCl-50 and Tris-ATP-5 (all in mM), pH-7.4. These concentrations are in the optimal range for the activity of the preparation (127). Mg-ATPase activity was determined during the first experiments by two methods i.e. a complete medium + ouabain in a final concentration of 10^{-3} M and a medium lacking Na^+ and K^+. The results obtained by the two methods for the first 85 determinations were identical, 26.48 $\pm$ 0.69 μmol Pi/mg Protein per hr for the first procedure and 26.29 $\pm$ 0.65 when only Mg^{2+} was present. Therefore, Mg-ATPase activity in the remaining experiments was measured as the ouabain uninhibitable fraction. The reaction mixture contained 30-70 μg of protein in a final volume of 2 ml and was started by the addition of ATP. The incubation was maintained for 15 minutes in a 37°c shaking water bath. In preliminary experiments, the linearity of enzyme activity with time (5-60 minutes), and with protein concentration (35-200 μg protein per tube) was established. The time of incubation and ATP concentration were calculated to be such that less than 10% of the ATP was consumed during the reaction. The reaction was terminated by addition of an equal volume of ice cold 10% (w/v) TCA. After centrifugation at 10,000xg for 10 minutes, 2 ml of the supernatant were collected. The liberated inorganic phosphate was measured by the modified Fiske and SubBarow method (47,101), and the protein content by the method of Lowry et al (102). All assays were performed in duplicates. In each assay set, control tubes containing a complete medium but without a crude plasma membrane preparation were incubated, and the enzyme added after the addition of TCA. This control permitted correction for inorganic phosphate present in the tissue and for

the slow non-enzymatic hydrolysis of ATP. Reagent blanks and inorganic phosphate standard were included in each experiment and served to convert optical density into micromoles of inorganic phosphate released per mg protein per hr incubation at 37°c.

3. Time course of thyroid induced changes in NaK-ATPase.

Tata et al (169,174) found that changes in basal metabolic rate after a single injection of 25-40 µg T_3 to thyroidectomized rats were recorded only after a latent period of 20-30 hours, with a peak stimulation at about 70 hours. The time-response effect of the same dose of T_3 on QO_2 of mitochondria from liver and muscle, and on glucose-6-phosphatase and NADPH-cytochrome c reductase in liver microsomes was similar, though an increase in these parameters could be detected after 12 hours and peaked at about 48-60 hours (174). Recently, Ismail-Beigi and Edelman (75) observed that after a single injection of 50 µg of T_3 to thyroidectomized rats an increase in liver QO_2, $QO_2(t)$, and NaK-ATPase became apparent 12 hours after administration of the hormone, peaked at about 48 hours, and fell almost to the initial values after six days. However, the intestinal epithelium may present a different picture as the mucosal cell turnover time for mature rats is 2.1 days (33,99). A preliminary experiment, tracing the effect of a single injection of 50 µg T_3 on NaK-ATPase from jejunal crude plasma membrane was carried on. The experiment was executed in collaboration with Miss Irene Uyeyama who worked as a summer student in our laboratory. Thyroidectomized animals were injected 12, 24, 48 and 72 hours before being sacrificed. Plasma membranes were prepared as described above, and the epithelial scrapings were homogenized in DOC (1 mg/ml).

(4 rats)

The mean values for NaK-ATPase specific activity in the various time periods after T_3 administration is presented in Figure 2-III. No change

NaK – ATPase
SPECIFIC ACTIVITY
μmoles Pᵢ/hr per mg protein

- Thyroidectomized
○ Thyroidectomized + T₃
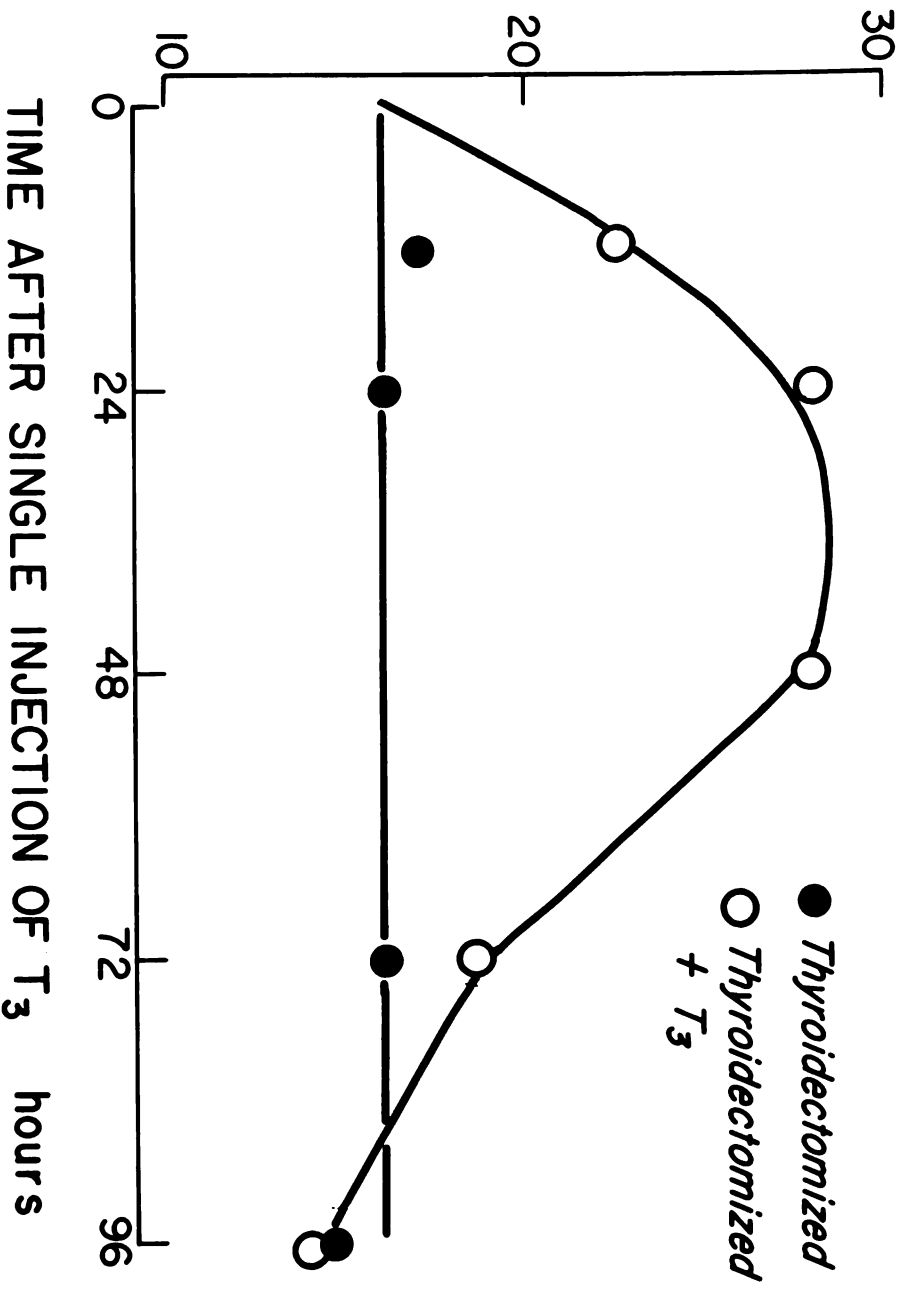
Figure 2-III. TIME-COURSE OF THE EFFECT OF A SINGLE INJECTION OF T₃ (50 μg/100 g BODY WT.) ON JEJUNAL NaK-ATPASE SPECIFIC ACTIVITY. MEAN OF 4 RATS.

TIME AFTER SINGLE INJECTION OF T₃ hours

in NaK-ATPase specific activity was depicted in the control group. In
the animals treated with thyroid hormone a clear trend was evident. As
in other tissues, a response was recorded 12 hours after T_3 administra-
tion, that peaked at about 24 to 48 hours, but in contrast to liver, the
enzyme levels were almost back to control values 72 hours after injection
of the hormone; probably reflecting the rapid turnover of intestinal
epithelial cells. No changes were recorded in Mg-ATPase levels with re-
spect to time after T_3 injection and in comparison to the control group.

As a consequence of these time-course observations, the animals, in
all my experiments, were sacrificed 24 hours after the last injection of
the hormone.

C. Results and Discussion.

1. ATPase activity in the upper and lower parts of the small intestine.

In a recent review of the literature Booth (25) concluded that the
proximal part of the small intestine was the major area for absorption of
dietary constituents. This finding correlates with the high respiratory
rate of the proximal part of the intestine as compared to more distal
parts (15,28,36,190). Furthermore, a decrease in the mitochondrial con-
tent and succinic dehydrogenase activity of the mucosa from jejunum to
ileum was observed (15), implying that mucosal cells have differing res-
piratory potentials depending on their position along the intestine. It
was shown that Na^+-flux in rat jejunum is higher than in the ileum; max-
imum transport occuring in the second quarter of the intestine (20,31,32).
It was therefore of interest to test if those differences are reflected
in the ATPase activity of the different regions.

Two segments of 10cm length each starting at the ligament of Treitz
were taken as representing the jejunum, two segments of the same length
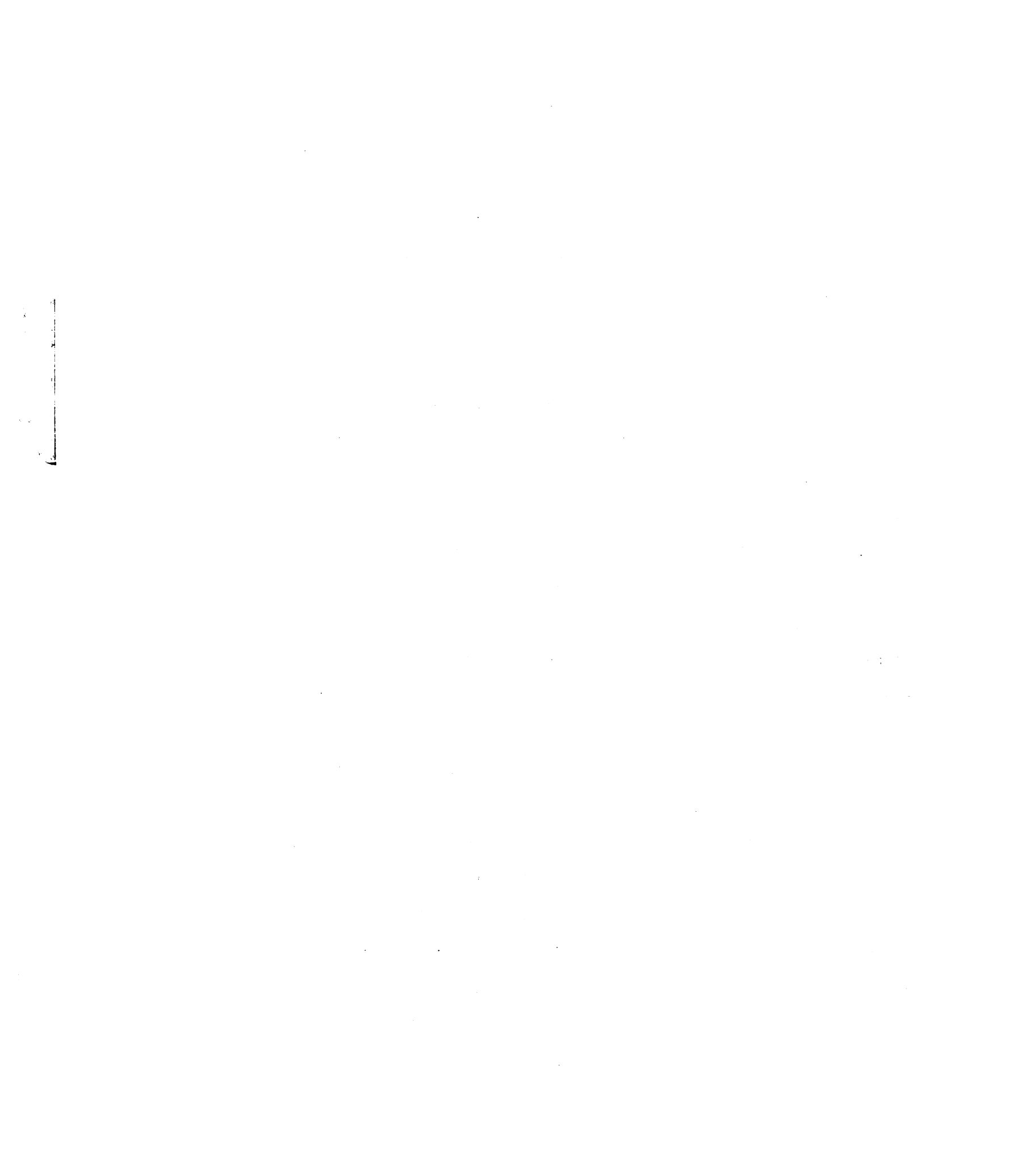
starting at the ileocecal junction and cephaled represent the ileum. The

epithelial mucosa was scraped and homogenized in 5 mM Na_2 EDTA + 1mg/ml

of DOC. Preparation and enzyme assay were performed as described in

Methods. In order to evaluate possible differences in the number of cells

scraped from each region or the protein content, DNA and protein were de-

termined in samples from the crude homogenate.

Table 1-III summarizes the specific activity data. NaK-ATPase spe-

cific activity in the ileum was about 30% less than in the jejunum. I

also found that the crude plasma membrane protein content per segment of

intestine was about 40% less in ileum than in jejunum (Table 2-III).

These differences may be due to one or more factors affecting the upper

part of the small intestine. 1) An increase number of cells per unit

length. 2) An increase in protein content per cell, and 3) an increase

yield of crude plasma membrane per cell mass. As shown in Table 2-III,

protein content per unit length of the crude homogenate is lower by about

32% in the ileum, than in the jejunum and DNA content is about 8% less;

this figure is statistically insignificant. It is of interest that the

relative differences in the protein content of the plasma membrane frac-

tion and the crude homogenate are similiar. Therefore, the yield or the

amount of protein in the plasma membrane fraction per mg of crude homo-

genate protein is in the same range, though somewhat lower for the ileum.

On the other hand if plasma membrane protein is calculated per mg DNA

the figure for the ileum is 35% lower than for the jejunum. Though the

possibility of a decrease in the number of cells per unit length in the

ileum was not completely excluded, these results imply that ileal epi-

thelium contains less protein per cell than the jejunum. The differences

in ATPase between jejunum and ileum are magnified when enzyme activity is
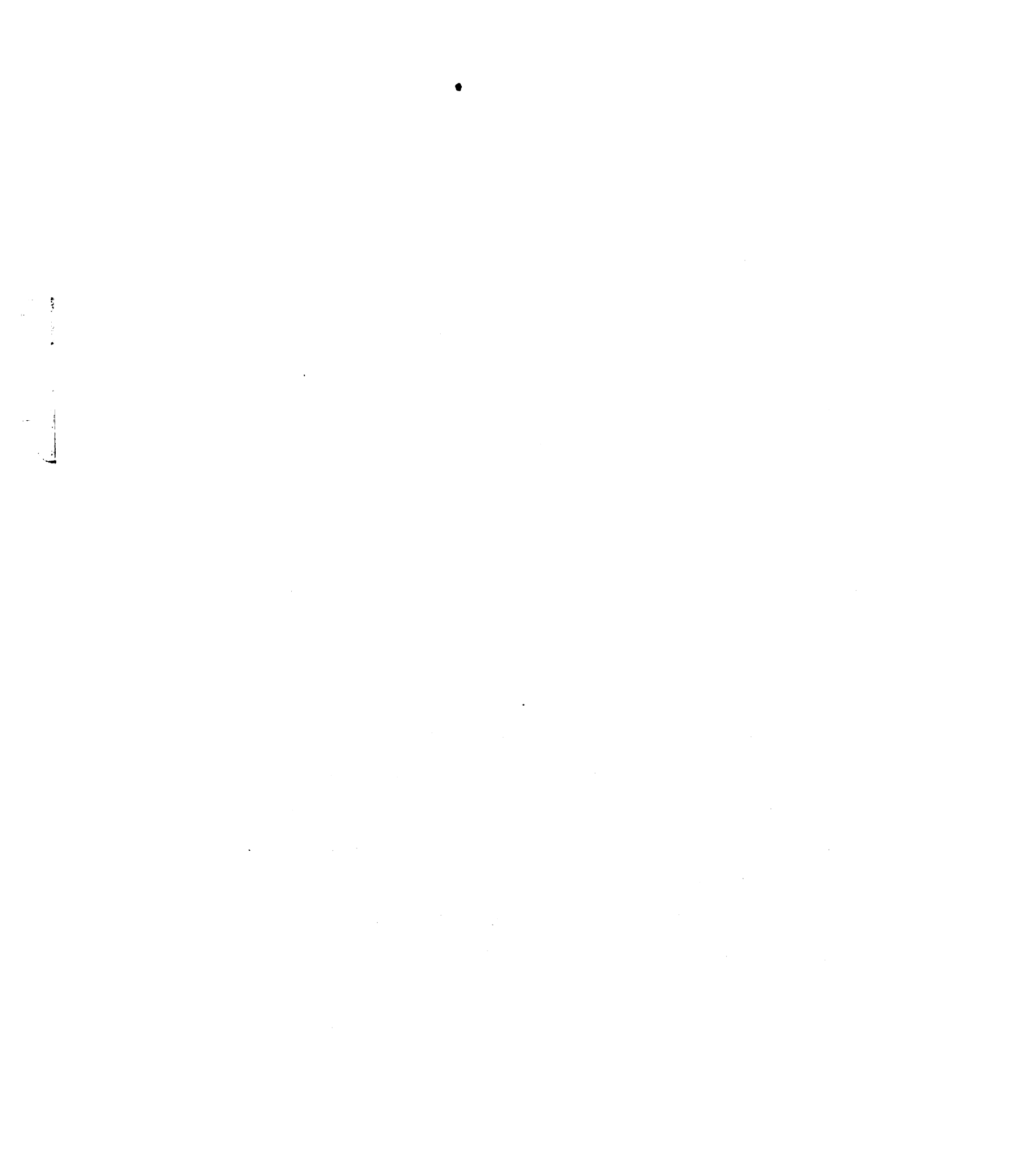
Table 1-III   ATPase Specific Activity of Jejunal and Ileal Crude Plasma

Membrane Fractions from Euthyroid Rats.[*]

	Mg-ATPase	NaK-ATPase
	μmole Pi/hr per mg protein	
Jejunum	42.19 ± 7.10	34.81 ± 4.69
Ileum	50.25 ± 6.54	24.58 ± 0.89
Δ	-8.06	10.23
p	N.S.	< 0.05

[*] Mean ± SE (n=7 and 8 for jejunum and ileum respectively).

Table 2-III Protein and DNA Content of Crude Homogenates and Total Plasma Membrane Protein of Segments of Jejunum and Ileum.*

	Total Protein in Plasma Membrane Fraction	Total Protein in Crude Homogenate	Total DNA in Crude Homogenate
		mg/segment of 10cm length	
Jejunum	1.679 ± 0.141 (7)	25.967 ± 0.772 (6)	1.893 ± 0.077 (6)
Ileum	1.000 ± 0.104 (8)	17.640 ± 0.595 (6)	1.748 ± 0.117 (6)
Δ	0.679	8.327	0.145
p	< 0.005	< 0.001	N.S.

*Mean $\pm$ SE. n in brackets.

calculated per mg DNA or unit length of intestine (Table 3-III). The
ileum contains 60% less NaK-ATPase than jejunum when expressed per mg
DNA. On the basis of DNA content as the reference mass, Mg-ATPase
content of ileum is about 30% less than that of jejunum but this
difference did not reach statistical significance.

Our demonstration that there is a substantial decrease in NaK-ATPase
from the distal small intestine as compared to proximal segments may be
taken as in agreement with the decrease observed in Na^+-flux and oxygen
consumption of the small intestine, in the aboral direction (15,20,28,31,
32,36,190). While this work was in progress, Hafkenscheid (68) reported
similar results i.e. that the NaK-ATPase specific activity decreased from
duodenum to ileum in the rat. Hafkenscheid's experiments were carried
out on crude homogenates with low specific activity: NaK-ATPase activity
was about 10-15% of total ATPase activity, and no information about DNA
content and ATPase activity per segment was supplied. Thus our results
are in agreement and add some additional information.

2. Effect of fasting on ATPase activity.

Several studies have shown that fasting alters the intestinal absorp-
tion patterns of compounds such as D-glucose, amino acids and some drugs
(37,65,82,118). It was of interest therefore to measure the effect of
fasting or availability of food on ATPase activity. For this purpose
food was taken out of the cages in the afternoon of the day before the
animals were sacrificed. Thus the period of starvation was about 14-16
hours. Since rodents are nocturnal animals, the actual fasting interval
was about 26-28 hours. The control group was fed ad libitum.

The effect of an overnight fast on ATPase activities is depicted in
Table 4-III. The animals used are in the same age group and it seems

Table 3-III   ATPase Activity per Unit Length of Jejunum and Ileum.[*]

	Mg-ATPase	NaK-ATPase
	μmole Pi/hr per total protein of 10 cm length segment	
Jejunum	137.90 ± 21.67	59.49 ± 9.17
Ileum	96.18 ± 10.47	24.08 ± 1.68
	41.72	35.41
p	> 0.05	< 0.005

[*]Mean $\pm$ SE.

Table 4-III Effect of Brief Fasting on ATPase Activity of Jejunal Crude Plasma Membrane Fractions from Euthyroid Rats $\pm$ T_3 (50 µg/100g body weight).*

| | Euthyroid | | Euthyroid + T_3 | |
	Mg-ATPase	NaK-ATPase	Mg-ATPase	NaK-ATPase
	µmole Pi/hr per mg protein			
Unstarved	24.85 $\pm$ 1.83 (6)	9.93 $\pm$ 0.90	26.75 $\pm$ 2.12 (4)	11.40 $\pm$ 1.02
Starved	22.37 $\pm$ 1.00 (21)	7.59 $\pm$ 0.31	21.48 $\pm$ 0.73 (21)	9.37 $\pm$ 0.46
Δ	2.48 (10%)	2.34 (24%)	5.27 (20%)	2.03 (18%)
P	NS	< 0.005	< 0.025	> 0.05

*Mean $\pm$ SE (n in brackets) For details see text.

that even a short fast causes a decrease of about 20% in NaK-ATPase as well as in Mg-ATPase specific activities.

Fasting produces a decrease in intestinal mass (109,118); McManus and Isselbacher (109) showed that after starvation overnight, the fasted rats had a lower intestinal weight and DNA content than fed rats. These findings were attributed to a decrease in the number of mucosal cells after fasting. Hopper et al (70) demonstrated that fasting evoked a continual decrease in the number of cells in the germinal crypts of the intestinal epithelium and that this change was apparent as early as the end of the first day of fasting. These investigators (70) noted that with a cell turnover time of 2.1 days for mucosal cells in mature rats (33,99), even short periods of fasting could have detrimental effects on mucosal cell number.

The finding of a decrease in ATPase activity after a short fast may be a result of a reduction in the number of epithelial cells per unit intestinal length. However, the changes were recorded as a decrease in the specific activity of the enzymes, which implies, assuming constant protein per cell, that the newly dividing cells would have higher enzyme content per unit cell than older cells. Alternatively, the changes in enzyme content may reflect a decrease in the number of enzyme units per cell. It was shown that 72 hours of fasting reduced the activity of the glycolytic and pentose phosphate pathway enzymes, in intestine, liver, muscle, and adipose tissue (151,180) and that glycolytic activity and hexokinase activity decreased significantly in the mucosa of small intestine of rats after 24-36 hours of starvation (151,162). On refeeding isocaloric amounts of dietary components, only glucose brought about an increase in hexokinase activity (151), and intravenous administration of glucose did

not alter intestinal hexokinase activity (151). While fasting led to a reduction in the activities of glycolytic enzymes in the small intestinal mucosa it brought about an increase in gluconeogenic enzymes (10,163). Alloxan diabetes in rats was associated with hypertrophy of the jejunal mucosa and elevated activity of glycolytic enzymes in the intestinal epithelium (180). Eliminating the increased mucosal weight of diabetic rats by pair feeding or a 24 hours fast, did not change the elevated levels of the jejunal glycolytic enzymes (180). Based on these findings and if the following observations are taken into account: a) Fasting leads to changes in enzyme content in tissues in which cell renewal rate is much slower than that of intestinal epithelium. b) Fasting leads to increased activity of gluconeogenic enzymes. c) Elevated enzyme activities in diabetic jejunum can be dissociated from mucosal hypertrophy, and d) Only the specific substrate induces the depressed enzyme activity. The changes in intestinal enzyme activity may reflect not only the number or age of cells but also the availability of the substrate for the particular enzyme. This conclusion has a direct bearing on the direct relationship to the effect of thyroid hormone on small intestinal mucosa. My observations and others (94) indicate that thyroid hormone increases the mass of the small intestine. Furthermore, administration of T_4, like glucose feeding, increases hexokinase activity of the intestine (66). It can be argued that thyroid hormone increases the number of newly dividing cells which in turn have a higher content of NaK-ATPase per cell. The finding that T_3 induced a similiar increase in NaK-ATPase activity in plasma membranes prepared from fasting as well as fed rats, however, contradicts this hypothesis.

3. Effect of T_3 on ATPase.

As noted with the stripped intestine preparation, an increase in the
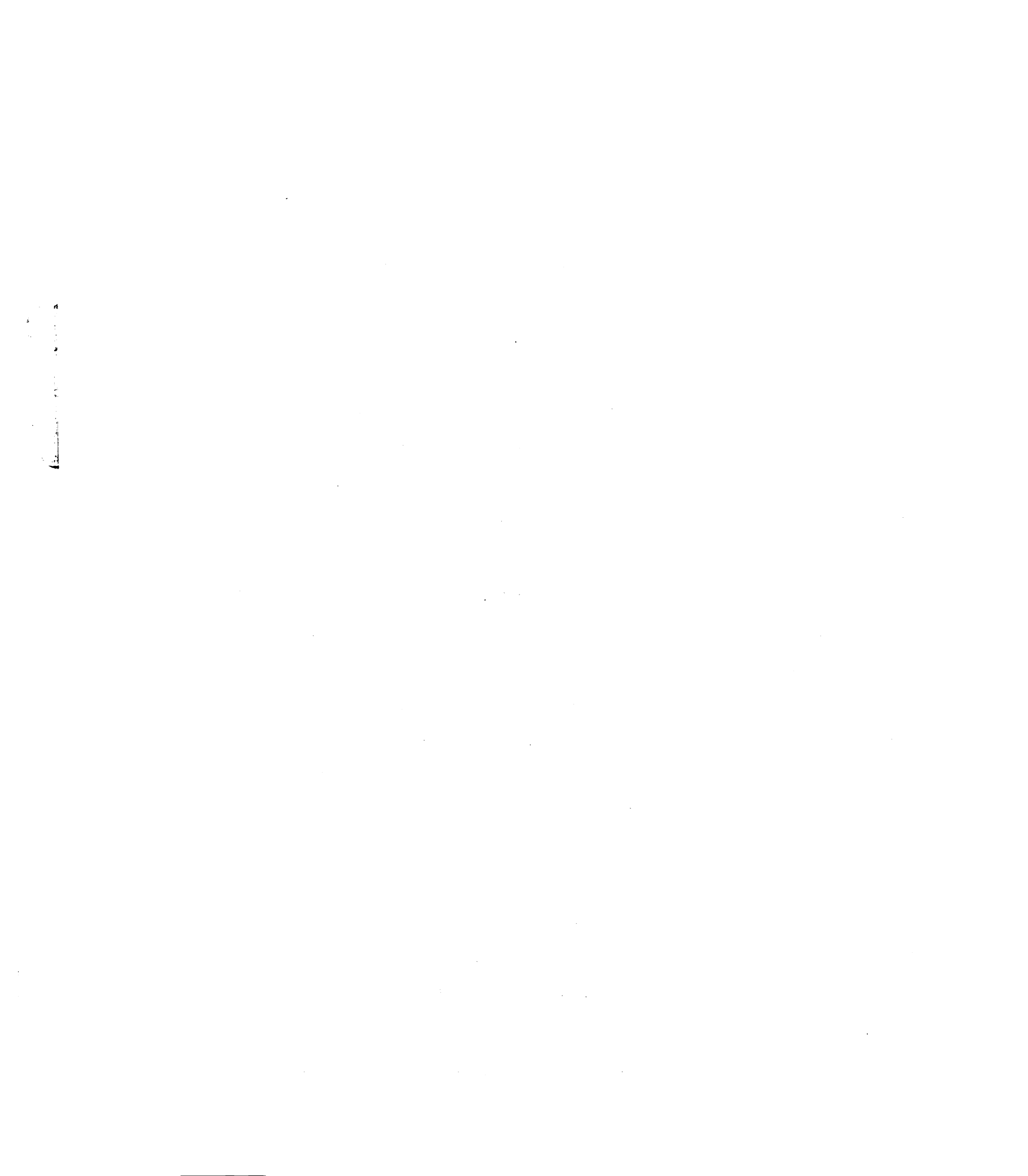
weight of the scraped jejunal mucosa after T_3 administration to hypothyroid animals, was observed (Table 5-III). A proportional increase in the wet and dry weight of about 23% and 32% respectively, was found. No change was noted in the ratio of dry to wet weight, indicating again that the hormone probably increased the cellular mass of the tissue. As mentioned in Chapter II, administration of thyroid hormone to hypothyroid rats did not change the DNA content per unit dry weight or protein. Thus the experimental results expressed as ATPase activity per my protein, can be taken, most probably, as representing enzyme activity per unit cell. The effect of injection of T_3 into hypothyroid rats on Mg-ATPase and NaK-ATPase activity in jejunal crude plasma membrane preparations are summarized in Table 6-III. A highly significant increase in NaK-ATPase specific activity $\sim$ 72% was observed; in contrast Mg-ATPase specific activity decreased by about 17%. In the transition from the euthyroid to the hyperthyroid state, NaK-ATPase was elevated significantly by about 26% and no change in Mg-ATPase was observed (Table 7-III). It is noteworthy that the specific activity of the NaK-ATPase in euthyroid rats was in the range of the hypothyroid preparation; enzyme values for hyperthyroid rats were lower than those observed in hypothyroid + T_3. The possibility of a direct comparison between thyroidectomized and euthyroid rats is somewhat complicated by the possibility of secondary effects due to the prolonged athyroid state. The ATPase results given in Table 7-III are in the same range as mentioned in the literature for rat (22,64,128,136) and rabbit jejunum (135). Mg-ATPase specific activity levels were more than two times greater than NaK-ATPase values. As shown in the electronmicrograph of the 10,000 g pellet, Figure 3-III, the preparation consists of membrane vesicles and sheets as well as mitochondria. Some of the Mg-ATPase activity recorded therefore, may

Table 5-III Wet and Dry Weight of Scraped Mucosa of Jejunum from

Thyroidectomized Rats $\pm$ T$_3$ (50 µg/100 g body weight)[*]

	Wet Weight mg. per 10cm	Dry Weight length	Dry Weight % of wet weight
Thyroidectomized	336.5 $\pm$ 13.2	35.6 $\pm$ 2.6	10.5 $\pm$ 0.4
Thyroidectomized + T$_3$	412.6 $\pm$ 22.4	47.0 $\pm$ 1.9	11.5 $\pm$ 0.5
Δ	76.1	11.4	1.0
p	< 0.01	< 0.005	> 0.1

[*]Mean $\pm$ SE. (n = 8 and 7 for the thyroidectomized and thyroidectomized + T$_3$ respectively).

Table 6-III   ATPase Activity of Jejunal Crude Plasma Membrane Fractions

from Thyroidectomized Rats $\pm$ T$_3$ (50 µg per 100 g body weight)[*]

	Mg-ATPase	NaK-ATPase
	µmoles Pi/hr per mg protein	
Thyroidectomized	35.30 $\pm$ 1.13	7.59 $\pm$ 0.53
Thyroidectomized + T$_3$	29.27 $\pm$ 1.38	13.04 $\pm$ 0.71
Δ	-6.03 (-17%)	5.45 (72%)
p	< 0.01	< 0.001

[*]Mean $\pm$ SE (n = 13)

Table 7-III   ATPase Activity of Jejunal Crude Plasma Membrane Fractions

from Euthyroid Rats $\pm$ T_3 (50 µg per 100 g body weight)[*]

	Mg-ATPase	NaK-ATPase
	µmole Pi/hr per mg protein	
Euthyroid	22.55 $\pm$ 0.83	8.13 $\pm$ 0.32
Euthyroid + T_3	22.36 $\pm$ 0.68	10.28 $\pm$ 0.51
Δ	-0.19	2.15 (26%)
p	n.s.	< 0.001

[*]Mean $\pm$ SE (n = 29)

Figure 3 - III

An electronmicrograph of the "crude plasma membrane
preparation" (details in text).   X 9,700.
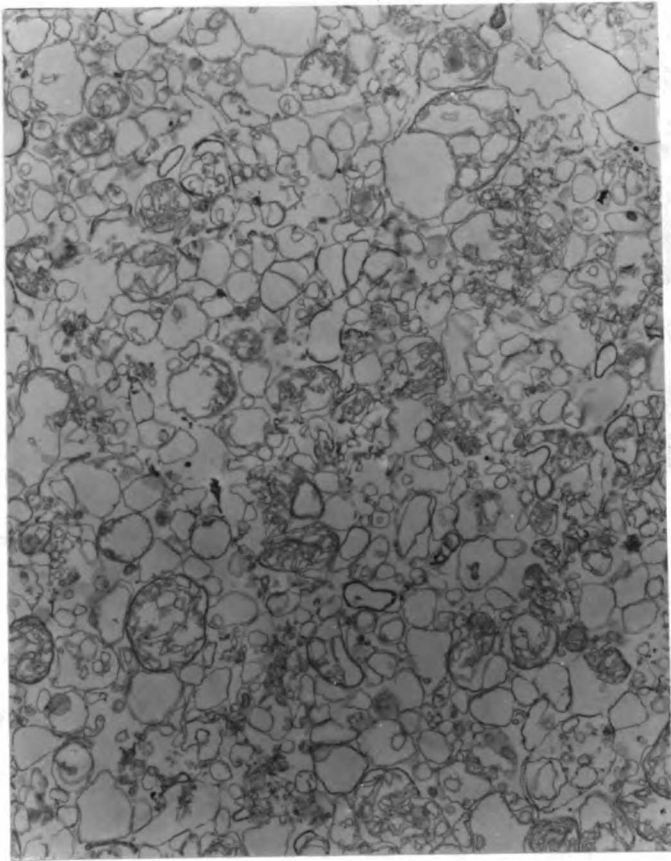
represent enzymes that are not attached to the cell membrane. On the other hand, numerous studies documented the absence of NaK-ATPase from mitochondria obtained from a variety of tissues (18,106,128,187). Thus, the values obtained for NaK-ATPase activity reflect mainly plasma membrane activity. About a 10-fold increase in NaK-ATPase specific activity was achieved by discontinuous sucrose gradient. I felt however, that further purification by this method would lead to losses in recovery of total enzyme activity and therefore the crude plasma membrane was preferable for the purpose of detecting changes in enzyme activity at the tissue level.

The ratios of QO_2/NaK-ATPase were 1.55 in hypothyroid, 1.73 in euthyroid, and 1.60 in hyperthyroid mucosae. A similiar constant relationship was found for Na^+ transport-dependent respiration and NaK-ATPase activity (QO_2(t)/NaK-ATPase); 0.53 in hypothyroid, 0.62 in euthyroid and 0.55 in hyperthyroid mucosae. A porportional increase in QO_2(t) and NaK-ATPase was observed after T_3 administration to hypothyroid and euthyroid rats. T_3 produced a greater rise in NaK-ATPase then in QO_2(t); 72% and 41% respectively in hypothyroid rats, 26% and 12% in euthyroid rats, (Figure 4-III). The differences between the groups are probably significant, because they are calculated from experimental results with a standard error of ∿5% (Tables 6-III, 7-III, 3-II and 4-II). The absolute and relative increase in enzyme activity and QO_2(t) were higher in hypothyroid rats, but the ratio ΔQO_2(t)/ ΔNaK-ATPase was about the same in both groups (i.e. ∿ ½).

These data clearly show that thyroid hormone produces an increase in the specific activity of the membrane bound enzyme NaK-ATPase in jejunal mucosa. This effect seems to be specific as a decrease or no change in the specific activity of Mg-ATPase, another membrane-bound enzyme was re-
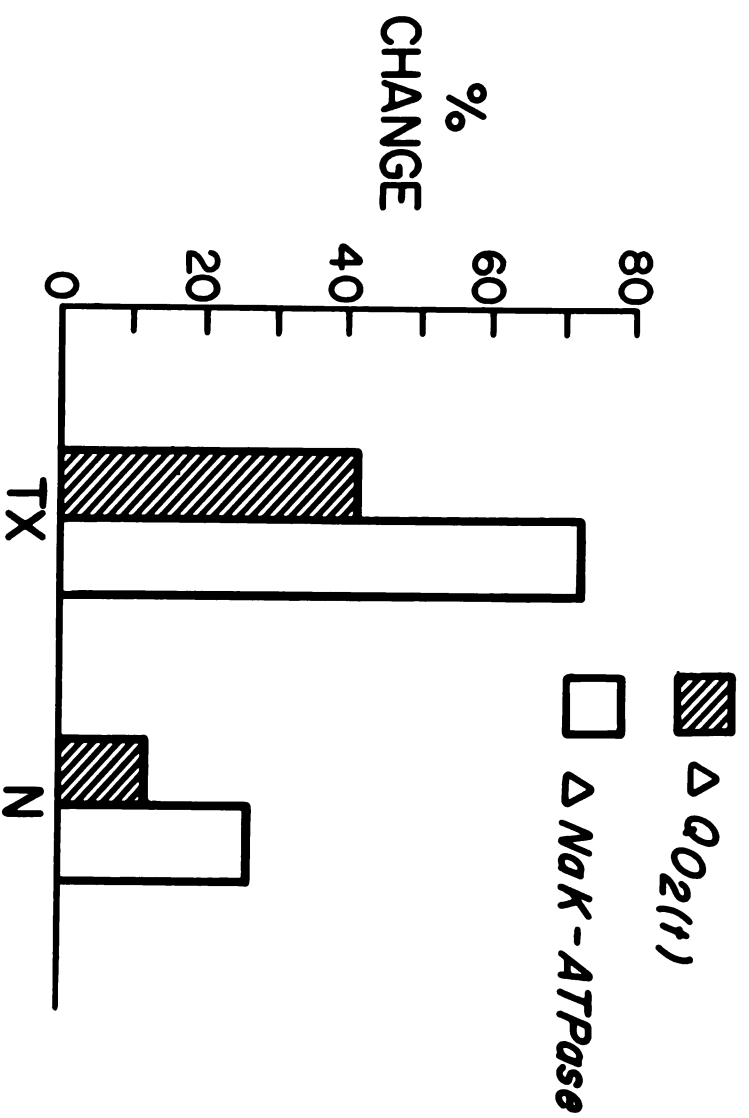
Figure 4-III. THE EFFECT OF ADMINISTRATION OF T₃ X 3 (50 µg/100 BODY WT.) TO
THYROIDECTOMIZED AND EUTHYROID RATS ON QO₂(t) AND NaK-ATPASE
SPECIFIC ACTIVITY OF JEJUNAL EPITHELIUM. THE RESULTS ARE
EXPRESSED AS % CHANGE IN COMPARISON OF T₃-TREATED AND CONTROL
TISSUES.

corded; although Mg-ATPase activity is not restricted to the plasma membrane. The constant ratios between QO_2/NaK-ATPase and $QO_2(t)$/NaK-ATPase in the various thyroid states as well as the close correspondence between the increase in enzyme activity and the increase in $QO_2(t)$ after administration of T_3 to hypothyroid and euthyroid rats, support the inference of proportional increases in QO_2 and in Na^+ pump activity. Moreover, it further suggests that stimulation of the Na^+ pump activity is an important pathway in thyroid thermogenesis.

It was shown recently by Ismail-Beigi and Edelman (73) that thyroid hormone increased NaK-ATPase activity in liver and kidney, tissues responsive to the thermogenic action of the hormone but not in the adult mammalian brain, an unresponsive tissue (47). The developing brain of immature post-natal mammals responds to thyroid hormone both thermogenically and morphogenitically (131,183). Ablation of the thyroid gland in the neonatal rat retarded the normal rise in brain NaK-ATPase activity, as well as the accumulation of K^+ and extrusion of Na^+ that occurred during normal maturation (183). An increase in QO_2 was observed in normal rats during the same period (179). These observations point again to a close relationship between oxygen consumption and Na^+-pump activity measured as NaK-ATPase and reflected by changes in intracellular K^+ and Na^+ concentrations. The specificity of the action of thyroid hormone on NaK-ATPase was indicated by the lack of comparable effects on other membrane bound enzymes (11,42,73,78). In the present study, no effect was observed on Mg-ATPase from intestinal crude plasma membrane preparation, as was the case in homogenates or plasma membrane preparations from liver, kidney (11) and striated muscle (73) in previous studies. In addition, administration of T_3 to hypothyroid and euthyroid rats did not change the activity of
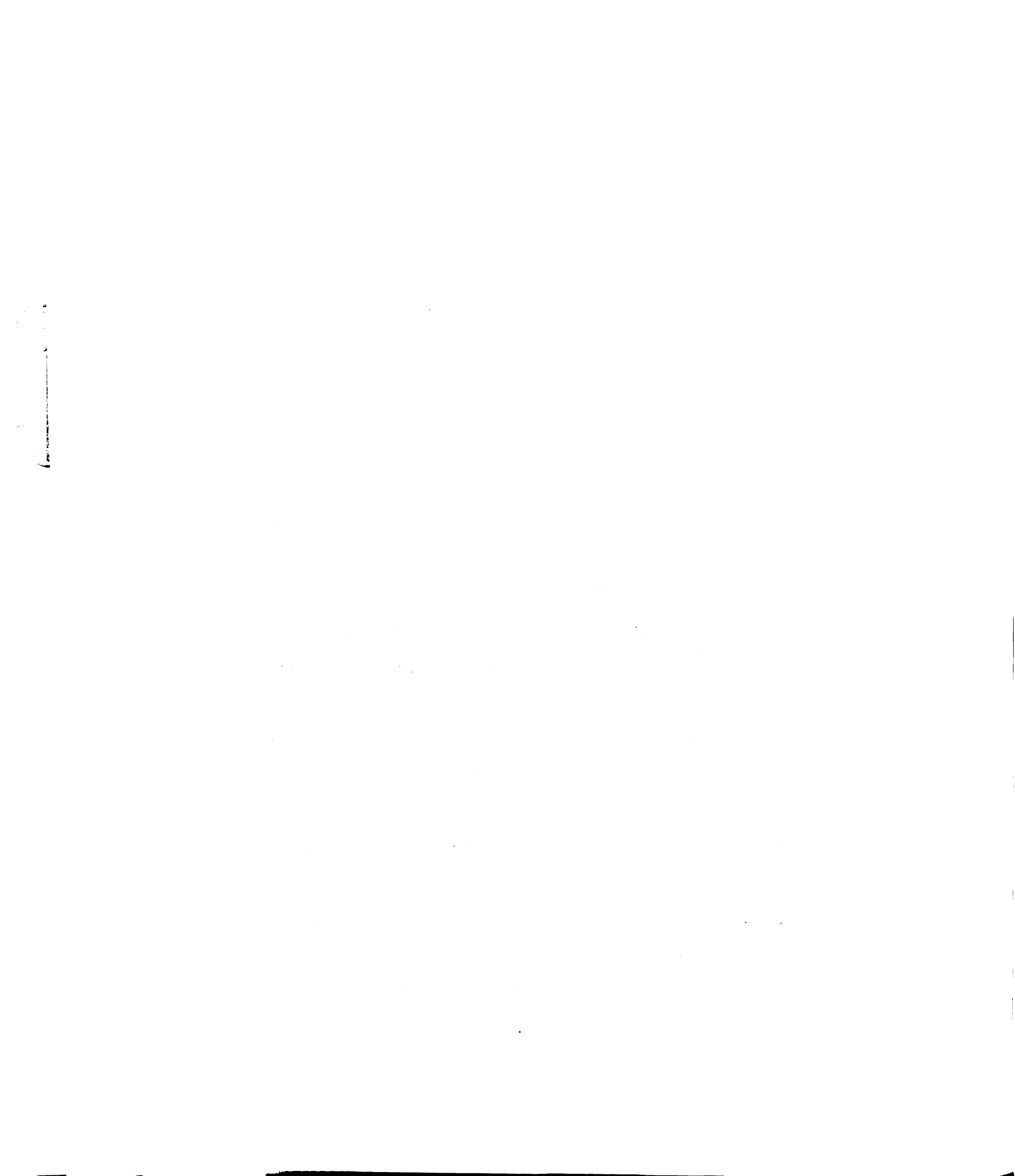
5'-nucleotidase and adenyl cyclase of liver plasma membrane preparations (42,78).

4. Effect of deoxycholate on ATPase activity.

The NaK-ATPase system is firmly associated with subcellular particles. Under proper conditions incubation of subcellular fractions from brain, heart and kidney with detergents such as deoxycholate, sodium dodecyl sulfate and Lubrol gives rise to a marked increase in the specific activity of NaK-ATPase (79,89,153,158). In order to increase NaK-ATPase specific activity from jejunal crude plasma membrane, the effect of deoxycholate (DOC) on this preparation was examined.

The plasma membrane fraction was incubated with DOC for 30 minutes and samples of the incubation media were taken out for enzyme analysis at 5-10 minute intervals, as described by Jorgensen and Sloan (158). This procedure did not yield any increase in NaK-ATPase specific activity; on the contrary, some decrease in enzyme activity was observed. As an alternative method, DOC in various concentrations was added to the scraping-homogenization media of 5 mM Na_2 EDTA (for details see Methods section of this chapter). Four segments of small intestine, 10 cm length each were used. Two alternate pieces were scraped and homogenized in the solution containing DOC while the other two segments were scraped and homogenized in 20 ml 5 mM Na_2 EDTA. In each experiment therefore, the DOC effect was correlated with a control preparation taken from the same jejunum. After homogenization and filtration, the DOC was diluted by 2 additional washings of the 10.000xg pellet. I calculated that in the final plasma membrane suspension, DOC concentration was below 0.01 mg/ml and in the enzyme assay system it was below 0.5 µg/ml which was about 100-fold lower than the concentration that inhibits the enzyme reaction (158). Enzyme

assays were done as described under Methods.

The results of a dose response curve, relating DOC concentration to NaK-ATPase specific activity are shown in Figure 5-III. It is evident that a maximal increase in NaK-ATPase specific activity was obtained in the range of 0.6-1.0 mg/ml of DOC, while higher concentrations have a smaller effect and practically no effect when 1.6 mg/ml of DOC is applied. In the forthcoming experiments, DOC was used in a concentration of 1mg/ml. The effect of DOC on ATPase activities of jejunal crude plasma membrane fraction of euthyroid rats is given in Table 8-III. In these experiments, DOC caused a highly significant increase of about 300% and 90% in NaK-ATPase and in Mg-ATPase specific activity, respectively. NaK-ATPase activity accounted for about a third of the total ATPase activity, in control preparations increased to $\sim$ 50% of the total after DOC. The NaK-ATPase values of intestine of euthyroid rats in this experiment were within the range of values reported for hypothyroid rats injected with T_3 (see section 2 chapter III). The increase in Mg-ATPase activity recorded in Table 8-III is in contrast to the results or obtained after detergent treatment in other studies. However, the crude plasma membrane fraction I obtained, contained mitochondria. Siekevitz et al. reported that DOC, in a concentration of 1mg/ml, caused almost a complete disintegration of mitochondria and increased the activity of Mg^{2+} dependent ATPase (155). During several sets of experiments I found that total protein content of the crude plasma membrane fraction was lower by about 15-25% in the DOC treated segments as compared to control preparations. Similiar observations were made by Jorgensen and Skou (79) who found that after high speed centrifugation of DOC treated microsmal fractions, 44% of the total protein remained in the supernatant whereas all of the NaK-ATPase activity

NaK – ATPase
SPECIFIC ACTIVITY
μmoles Pi / hr per mg protein
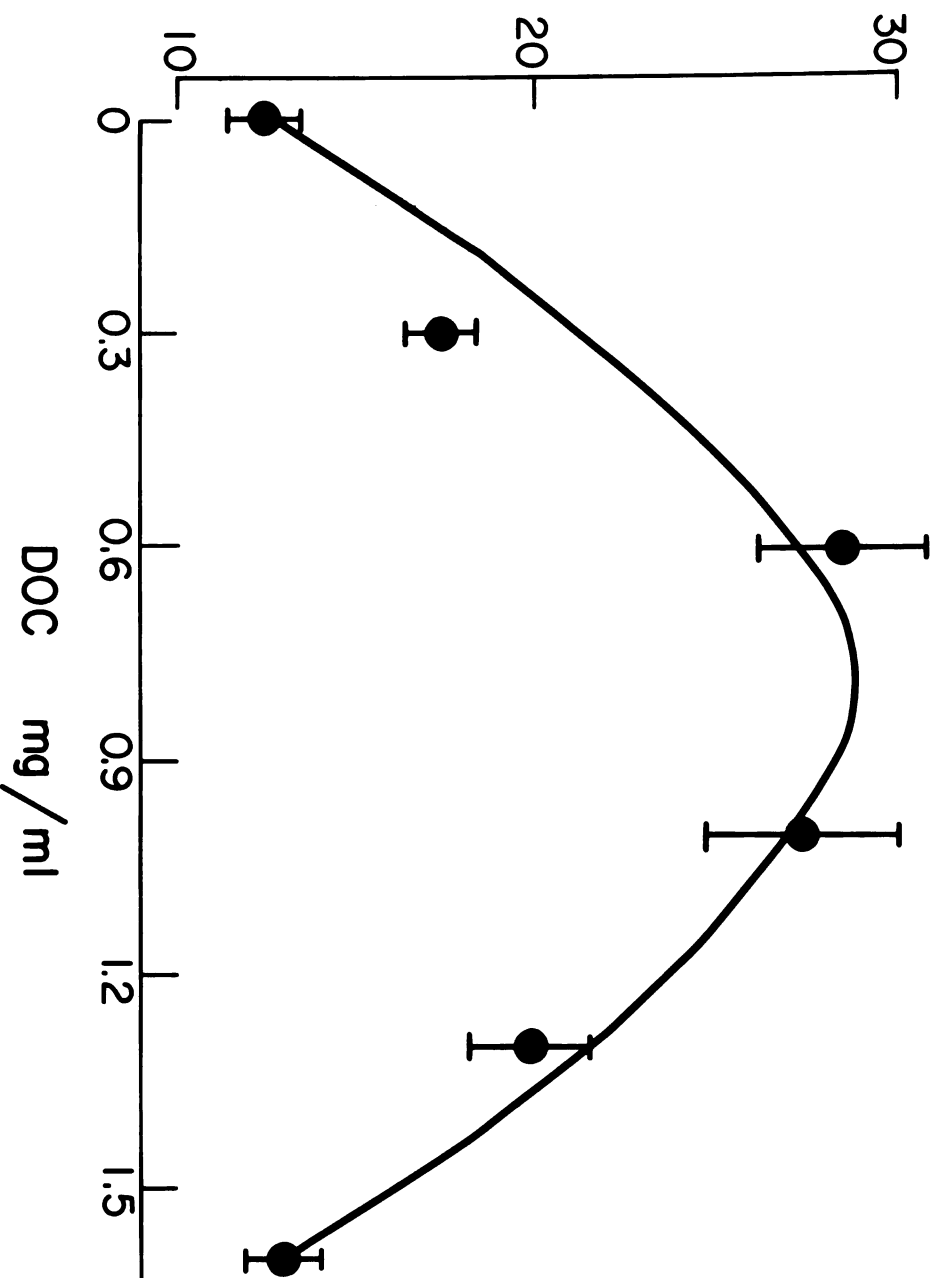
DOC   mg / ml

Figure 5-III.   EFFECT OF VARIOUS DOC CONCENTRATIONS ON JEJUNAL NaK-ATPASE SPECIFIC ACTIVITY.

Table 8-III Effect of DOC on ATPase Activity of Jejunal Crude Plasma

Membrane Fractions from Euthyroid Rats.[*]

Deoxycholate (DOC) mg/ml	Mg-ATPase	NaK-ATPase	Mg-ATPase/
	moles Pi/hr per mg protein		NaK-ATPase
0	26.10 ± 1.79	12.61 ± 1.16	2.1
1	47.48 ± 2.69	46.43 ± 2.50	1.0
Δ	21.38 ± 3.05	33.82 ± 2.75	
p	< 0.001	< 0.001	
Ratio + DOC/-DOC	1.92 ± 0.18	3.99 ± 0.38	

[*]Mean $\pm$ SE (n = 13)

was recovered in the sediment. However, the decrease in protein content of ⩘ 20% in the jejunal preparation after DOC treatment can account for an increase of no more than 33% in specific activity but could not explain an increment of 200-300%. To determine if the presence of DOC in the homogenization media increased cellular disintegration, samples of the homogenate, after filtration through Cheesecloth and Nylon Mesh (see Methods), were taken for protein and DNA determinations. An increase of about 23% and 26% in protein and DNA content per unit length of intestine was observed in the DOC treated homogenate in comparison to controls from the same rats. The ratio of protein per DNA in the homogenate was the same with and without DOC. These results indicate that the detergent caused a more efficient destruction of cells during homogenization. On the other hand, DOC treatment reduced the yield of protein in the plasma membrane fraction. Thus the ratio of protein in the membrane fraction to protein or DNA in the homogenate was about 30% less in the DOC-treated preparations than in the controls.

Another method of increasing NaK-ATPase specific activity in plasma membranes or microsomal fractions is "aging" of the fractions. The preparation is left overnight or up to 7 days in -5 to 0°c (38,72) or incubated in 37°c for 3-5 hours (128). Changes in NaK-ATPase activity as a result of aging and/or DOC are summarized in Table 9-III. An increase of about 45% in NaK-ATPase specific activity was recorded after 24 hours of aging while DOC treatment at the time of preparation caused an increment of more than 200%. Aging of the DOC treated specimen resulted in a further increase of about 8% (though statistically insignificant). Even after aging of both preparations, the specific activity of the enzyme treated with the detergent was almost 1.5 fold higher than the control.

Table 9-III   Effect of Aging (24 hours in 2-4°c) and DOC on NaK-ATPase

Activity of Jejunal Crude Plasma Membrane Fractions from

Euthyroid Rats.*

Deoxycholate (DOC) mg/ml	t_o	t_{24}	Δ	p
0	14.78 ± 1.70	21.32 ± 1.34	6.54 ± 2.59	< 0.05
1	46.55 ± 2.18	50.04 ± 3.07	3.49 ± 1.70	> 0.05
	31.77	28.72		
p	< 0.001	<0.001		

*Mean ± SE (n=7). t_o - in time of preparation. t_{24} - after 24 hr of aging.

Aging did not cause any significant change in Mg-ATPase activity.

The mechanism responsible for the increase in NaK-ATPase after aging or detergent treatment has not yet been determined precisely. The detergent removes protein and lipid from the membrane. This in turn may increase the permeability of the membrane vesicles created during the shearing of cells to substrates (i.e. ATP, Na^+, K^+) or may prevent the formation of membrane vesicles. Either of these mechanisms would provide free access of substrate and activators to their respective enzyme sites in the membrane. DOC treatment will remove, in addition, inactive protein from the fractions containing the NaK-ATPase.

In summary: A simple procedure for preparation of high specific activity NaK-ATPase from jejunal mucosa is described. The time of preparation is short, about 4 hours and doesn't involve special techniques such as sucrose density gradient centrifugation that consumes time and decreases the total yield. There is a 7 to 10 fold increase in specific activity over the original homogenate (128) and about 2-3 fold from the crude plasma membrane that is not treated with DOC. The specific activity of this preparation is about 2/3 of the activity reported for the M' fraction obtained by discontinuous sucrose gradients by Quigley and Gotterer (128) and about a 1/3 of the specific activity of the M-I fraction after aging for 4 hours at 37°c, documented by the same investigators.

IV. THE EFFECT OF T_3 ON THE NUMBER OF ACTIVE NaK-ATPase SITES

A. Introduction

The increase in the specific activity of NaK-ATPase at the V_{max}, induced by thyroid hormone might be due to an increase in the turnover number of the enzyme, the number of substrate molecules transformed per unit time by a single enzyme molecule, or to an increase in the number of active Na^+ pumps in the target tissue. Recently Asano, Liberman and Edelman (11) demonstrated an increase in V_{max} of the NaK-ATPase enzyme in rat skeletal muscle membrane fractions after T_3 administration; the apparent K_m for ATP, however, was not changed. This finding supports indirectly the hypothesis of an increase in the number of NaK-ATPase sites in the membrane as the mechanism underlying the effect of thyroid hormone. Firmer evidence would be provided by direct counting of the number of active Na^+ pumps in the cell membrane before and after T_3 treatment. The experiments to be described were designed to provide an answer to this problem.

It is more accurate to refer to the NaK-ATPase as a system rather than a single enzyme, since the hydrolysis of ATP occurs in more than one step. An analysis, therefore, of the NaK-ATPase membrane transport system will provide a basis for experimental evaluation of the number of Na^+ pump sites (or NaK-ATPase sites) in plasma membrane fractions. Skou (157) demonstrated that in addition to the hydrolysis of ATP, the NaK-ATPase system catalyses an ATP-ADP exchange reaction. Since the reaction was independent of the presence of inorganic phosphate, he concluded that a high energy intermediate, an enzyme-phosphate, was involved in the overall reaction. The phosphorylated intermediate was then demonstrated by exposing

NaK-ATPase preparation to ATP-x-32p in the presence of Mg^{2+} and Na^+ (44,69,126). The incorporation of ^{32}P was rapid and complete within a matter of seconds at low temperature. Addition of K^+ released the bound ^{32}P as inorganic phosphate (44). The label was also chased by the addition of unlabeled ATP (124,126); this suggested that the phosphorylation step is reversible. In a comparative study with six different tissues from eleven species, Na^+ activated phosphorylation and K^+ stimulated dephosphorylation of the plasma membrane fraction was detected in all cases (13). Although NaK-ATPase activity in these tissues varies over a 400-fold range, the ratio of phosphorylation and of dephosphorylation to NaK-ATPase activity varied only 2-fold. After peptic digestion the phosphorylated enzyme preparations yielded identical ^{32}P-labelled peptides upon electrophoresis. These findings support the inference that the phosphorylated protein is a functional intermediate in the enzyme system and that the mechanism is similiar in various species and tissues. The dephosphorylation step is stimulated by K^+ and inhibited by ouabain (14,26,139). A close relationship between K^+ activated phosphatase activity and the NaK-ATPase system was also established (24). Albers et al (44) and Post et al (124) reported evidence for two forms of the phosphorylated intermediate, however conflicting results were obtained with other techniques (1,96). From this brief discussion it is obvious that one way to count the number of active Na^+-pumps is to measure the incorporation of ^{32}P from ATP-^{32}P under conditions favoring the formation and stabilization of the phosphorylated intermediate.

Another method for measuring active Na^+-pump sites is based on the close association between the NaK-ATPase transport system and a cardiac glycoside receptor. Both sodium pump activity of intact cells and ATP

hydrolysis catalyzed by Na^+ and K^+ in broken membrane preparations (i.e. containing NaK-ATPase activity) are inhibited by cardioactive glycosides (56,157,187). The glycoside receptors may be identical to active sites of the enzyme, or reside on part of the enzyme, or on some membrane component closely associated with the pump. A close physical relationship was indicated by experiments in which a solubilized active NaK-ATPase was prepared, that maintained its sensitivity to cardiac glycosides (39,96,108). In intact membrane preparations cardiac glycosides inhibit the transport cycle presumably by binding at an external membrane site (67,184). However, by binding covalently cardiac glycoside to large protein molecules it was suggested recently, that the receptors for the drug may lie deep within the plasma membrane (160). It was found that specific ligands had to be present to stimulate binding of the labeled glycoside (1,107,177). At least two ligand conditions were found to yield maximal glycoside binding:

a) ATP plus magnesium plus sodium and b) magnesium plus inorganic phosphate. These two conditions produce a phosphorylated enzyme system which chromatographically seems to be a single chemical species (154). The maximal amount of tritiated cardiac glycoside bound to NaK-ATPase correlated with the enzyme activity (1,62,148) and with the degree of enzyme inhibition (6,18). The relationship between glycoside binding and glycoside-induced inhibition of NaK-ATPase was further defined by experiments indicating that the apparent affinity of various glycosides for binding to the enzyme evaluated by a competitive displacement method, correlated with their ability to inhibit NaK-ATPase activity (107).

Based on the findings that the same conditions that favor glycoside binding also produce a phosphorylated enzyme system, it was thought that the glycoside was bound and stabilized the phosphorylated enzyme state

(94,77). Formation of the phosphorylated intermediate, however, is not required for specific binding of the glycosides (148). Nevertheless, the evidence available indicates that glycoside-induced inhibition of NaK-ATPase activity is a consequence of glycoside binding to a site that stabilizes the conformation of the phosphorylated enzyme (148). Thus, the quantity of glycoside bound to the enzyme preparation under appropriate conditions will reflect the number of active Na^+-pump sites. The forthcoming section will deal mainly with the methodology involved in using ^{3}H-ouabain to measure the number of active NaK-ATPase sites on a plasma membrane preparation from rat jejunum and the results obtained. A formidable difficulty in these experiments was the fact that all the experiments were done on rat tissue. With the exception of the brain, rat tissues are relatively insensitive to cardiac glycosides. The complex formed between rat NaK-ATPase and ouabain is far less stable than that formed in dog or beef NaK-ATPase preparations (3,7,176).

B. __Materials and Methods.__

1. __Plasma membrane preparation.__

Thyroidectomized and euthyroid rats weighing 200-240 g were used for these experiments. Thyroidectomized rats were injected with T_3, 50 µg/100g body weight according to the protocol described in Methods of Chapter II. The animals were sacrificed 16-20 hours after the last injection and had access to food and water __ad libitum__. The intestine was prepared as described in Methods in Chapter III, with one difference: starting from the ligament of Treitz, four 10 cm segments of intestine were used, rather than two segments. The mucosa was scraped from two segments and combined in a Petri dish. Preparations from paired rats, either control or T_3-treated were processed simultaneously. The final pellets of

the 10,000xg spin were pooled together; thus the crude jejunal plasma membrane fractions from two rats were combined into a single pool, consisting of four segments of intestine taken out from each animal. The pools were prepared from two T_3-treated or two control rats in the morning and from the remaining two rats in the afternoon. The preparations were kept overnight in the refrigerator at 1-3°c, and the ^{3}H-ouabain binding assay performed the following day, in the order of the preparation sequence. NaK-ATPase assays were done on the day of preparation of the fraction and repeated the following day at the same time that ^{3}H-ouabain binding was measured. The NaK-ATPase specific activity levels summarized in the Tables and Figures are those recorded at the same time that the binding assay was performed.

2. ^{3}H-ouabain binding assay.

Uniformly labelled G-strophanthin was obtained from New England Nuclear Corp. ^{3}H-ouabain (G), lot Numbers 184-196 and 747-009, 13 Ci/m-mole and 12.7 Ci/mmole specific activity respectively. The lot #747-009 was used for all the experiments in which thyroidectomized animals were injected with T_3. To achieve high concentrations of ouabain the specific activity was diluted with unlabelled ouabain (Sigma analytical grade) the dilution did not exceed a ratio of 1:50. The incubation medium contained 100 mM-Na$^+$, 5 mM-Mg^{2+}, 5 mM-Tris-ATP, and 50 mM-Tris HCl buffer, pH 7.4 in a final volume of 1 ml. Various concentrations of plasma membrane protein (to be described below) were added to the tubes, the mixture was warmed to 37°c in a water bath for 3 minutes and the reaction started by the addition of Tris-ATP. The incubation was carried on for 1 to 15 minutes in the time-course experiment and for 5 minutes in all other experiments. To differentiate between specific and non-specific binding two

tubes with the same enzyme content were incubated in parallel. In pre-
liminary experiments the nonspecific binding was measured by two differ-
ent methods: a) By adding a 10^3 - 10^6 excess of unlabelled ouabain, which
would dilute out specific ^{3}H-ouabain binding assuming that the nonspecific
binding was unsaturable in these range of ^{3}H-ouabain concentration (see
Figure 4-IV) and b) By eliminating Na^+, Mg^{2+}, and ATP from the media. As
the two methods yielded the same results, only the second i.e. suing an
incubation medium without electrolytes and ATP, was employed for subsequent
experiments. In the first few experiments we tried to estimate ^{3}H-ouabain
binding by the method of Matsui and Schwartz (107). The binding reaction
was terminated by transferring the sample tubes into ice, and centrifuged
for 5 minutes in a refrigerated ultra centrifuge at 105,000xg. The super-
natant was discarded and the pellet was dissolved in 0.3 ml of 0.2 N NaOH.
However, no specific binding to rat jejunal plasma membrane fractions could
be demonstrated by this method; similiar negative results were mentioned in
the literature (176). A rapid method which will retain the enzyme-glycoside
complex and will enable washings of the complex was desirable. The tech-
nique that seemed to be most suited for this purpose, was a filtration
method. Several filters were tested for this purpose, the most suitable
one was found to be a Millipore 0.45 μ filter (Millipore, Bedford, Mass.).
The reaction media and time of incubation were as described before. Five
minutes after the reaction was started 200 μl in quadruplicates were as-
sayed as will be described.

Millipore filters were soaked before use in ice cold 2.5 mM Tris-
EDTA (pH-7.4), filters that were not wetted uniformally within two minutes
were discarded. The filter discs were placed on a 10-piece filter mani-
fold (Hoeffer Scientific, San Francisco, Calif.) and kept wet and cold by

addition of 1 ml of ice-cold 2.5 mM Tris-EDTA. One minute before the

samples were applied to the filters, the filters were freed of excess

moisture by application of the vacuum. The vacuum was broken, and 200

μl samples in quadruplicates from each tube were uniformly applied to

each filter. The application time was 30-40 seconds for each tube. One

minute after the last sample was put on the filter, the vacuum was applied

and the filters were washed with three 1.0 ml portions of ice cold 2.5 mM

Tris-EDTA (Cornwall syringe). The damp filters were dissolved in 10 ml

of a modified Bray's solution [xylene - 429ml, p-Dioxan 1284 ml, Ethylene

glycol monoethyl ether 1284 ml, PPO (2,5 - Dephenyloxozole) 30 g, POPOP

(Dimethyl-1,4-bis-2-(4-methyl-5-phenyloxazole) 1.5 g and Naphtalene-240g].

(All reagent were analytical grade or spectroquality). Quadruplicate 25

μl aliquots of the incubation mixture were added directly without filtra-

tion to vials containing 10 ml of the counting solution. The vials were

counted in a Mark I Nuclear Chicago Liquid Scintillation Counter (Chicago,

Ill.). Counting efficiencies (25% with a filter and 27% without) were

monitored by the external-standard, channel ratio method. The average of

the quadruplicates was used for calculations. The results of the repli-

cates were usually in close agreement. The concentration of ^{3}H-ouabain in

each incubation mixture was calculated from the actual radioactivity

measured and the activity of the stock solution. The concentration of the

free ^{3}H-ouabain in the filtrate, was calculated as the difference between

^{3}H-ouabain concentration in the incubation medium and the total amount of

^{3}H-ouabain bound to the filters. ^{3}H-ouabain bound specifically to the en-

zyme system was calculated by subtracting the amount of ^{3}H-ouabain retained

nonspecifically as defined above, from the amount of ^{3}H-ouabain bound to

the filter when Na^+, Mg^{2+} and ATP were included in the incubation medium.

C. Results and Discussion

1. Effect of filter washing on nonspecific ^{3}H-ouabain retention.

Figure 1-IV shows that when ^{3}H-ouabain in an incubation solution, without plasma membranes, is applied to Millipore filters, about 15% of the radioactivity is retained on the filter. One washing of the filter, by 1 ml 2.5 mM Tris-EDTA reduces the amount retained to 1% and two additional washings further reduces it to about 0.1 to 0.2% of the amount applied to the filters. Exactly the same washing curve was obtained when ^{3}H-ouabain was incubated with Serum Bovine Albumin only (protein concentrations were comparable to those used with plasma membrane fractions). These results indicate that the filter per se retains only small amounts of ^{3}H-ouabain even in the presence of a non-specific protein such as Albumin.

2. Protein leakage from the filters: Effect of protein load and number of washes.

To measure protein leakage through the filters, under experimental conditions, the filters were loaded with various concentrations of plasma membrane fractions. The filters were washed 3 times, 1 ml each, with 2.5 mM Tris-EDTA, the filtrates were collected and assayed for protein content. The results are shown in Figure 2-IV. When more than 500 μg of plasma membrane protein were applied per filter, an appreciable protein leakage, ranging from 2.5% after one wash to about 5% of the amount applied, after three consecutive washes was observed. At the same time, difficulties in filtration were encountered as well. When the protein load was reduced to 250-300 μg per filter, the leakage ranged between 0 to 0.8% from the first to the third washings and the filtration was rapid and smooth. When 200 μg of jejunal plasma membrane protein were applied to each filter, the amount of protein in the filtrate was nil or negligible even
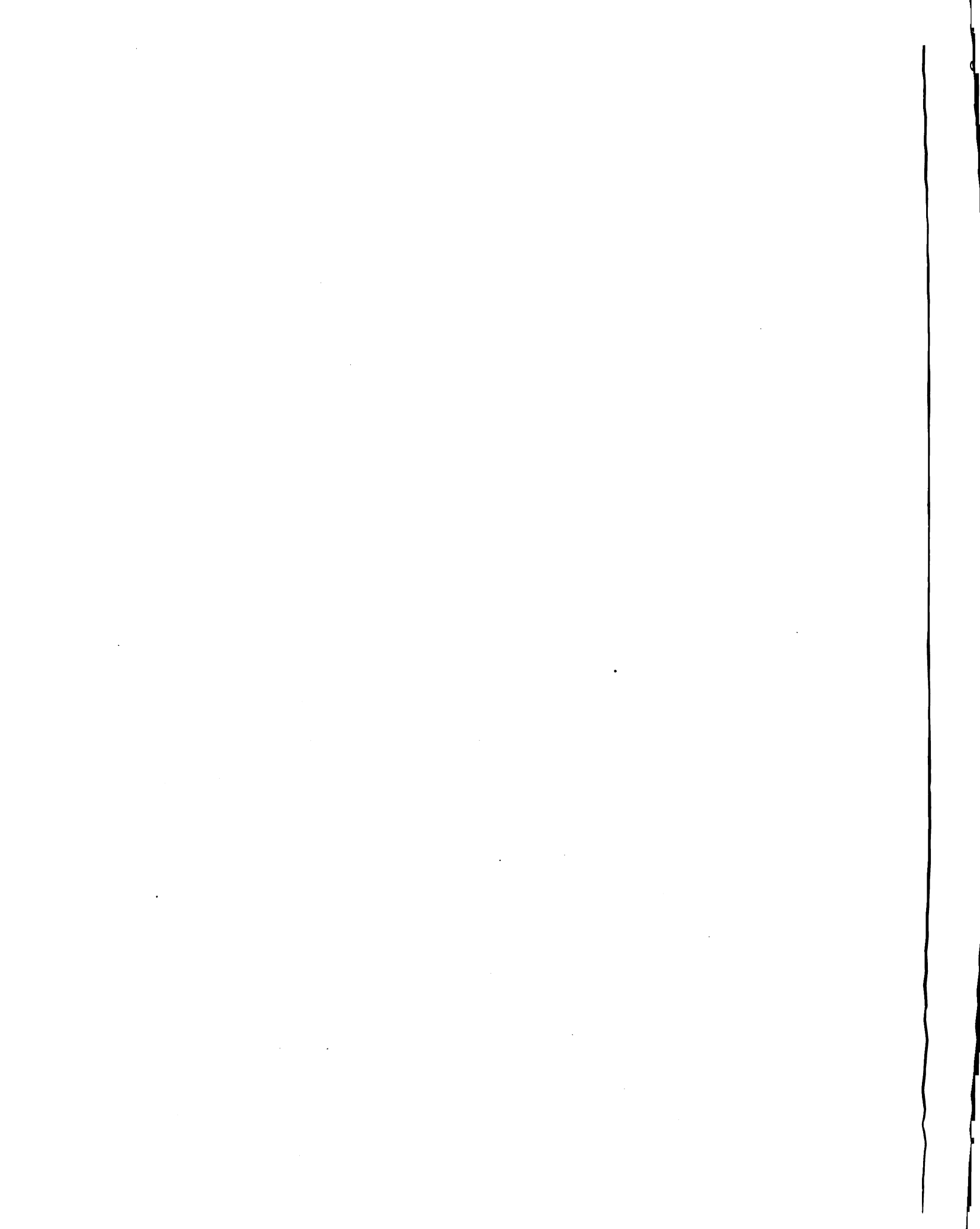
% RADIOACTIVITY
RETAINED

NUMBER OF 1 ml WASHES

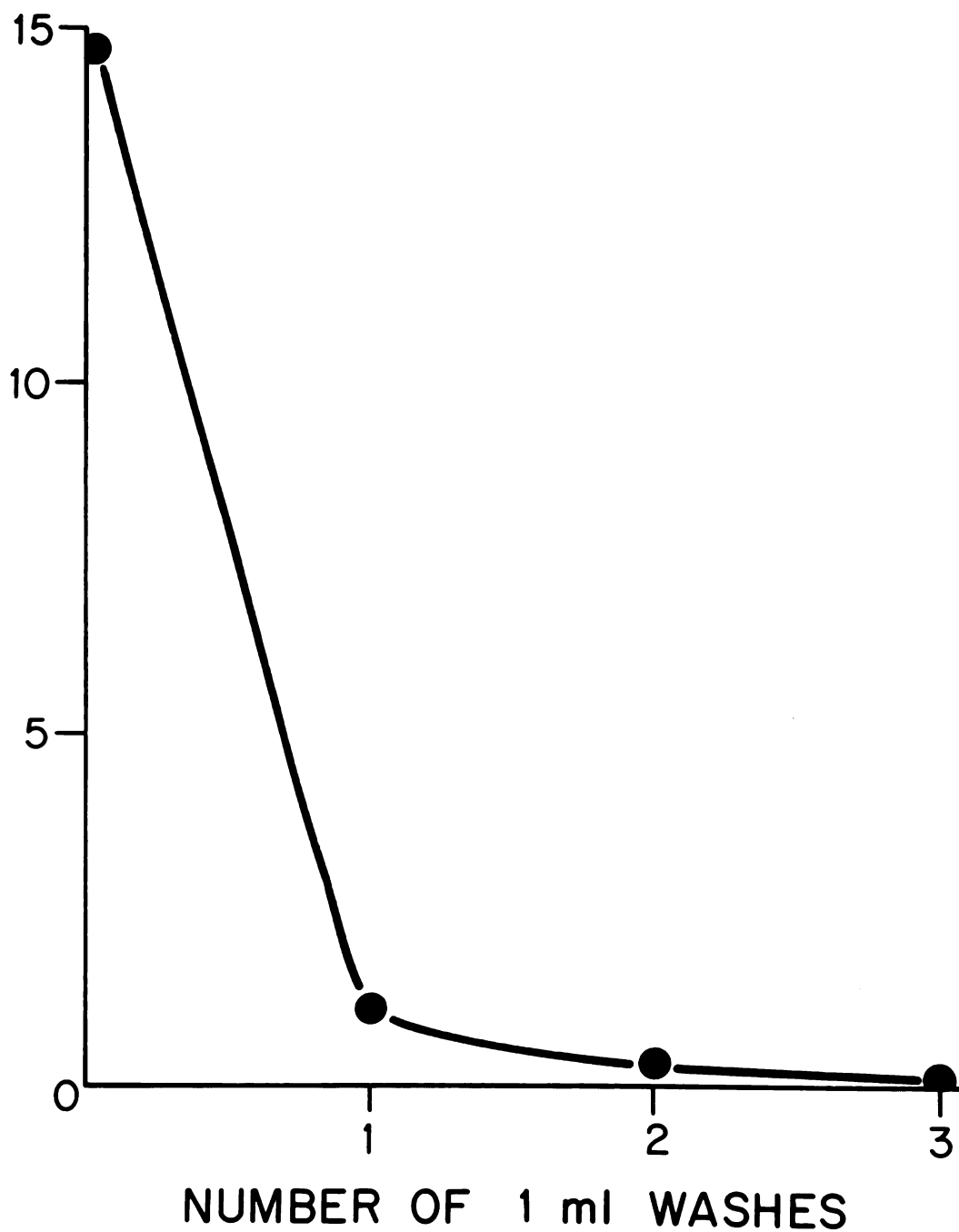Figure 1-IV. EFFECT OF WASHINGS WITH 2.5 mM TRIS-EDTA ON THE

RELATIVE AMOUNT OF [^{3}H] OUABAIN RETAINED ON

MILLIPORE FILTERS. (n = 4).

PROTEIN RECOVERED
IN FILTRATE AS % OF
PROTEIN APPLIED

● 500 - 750 μg protein per filter

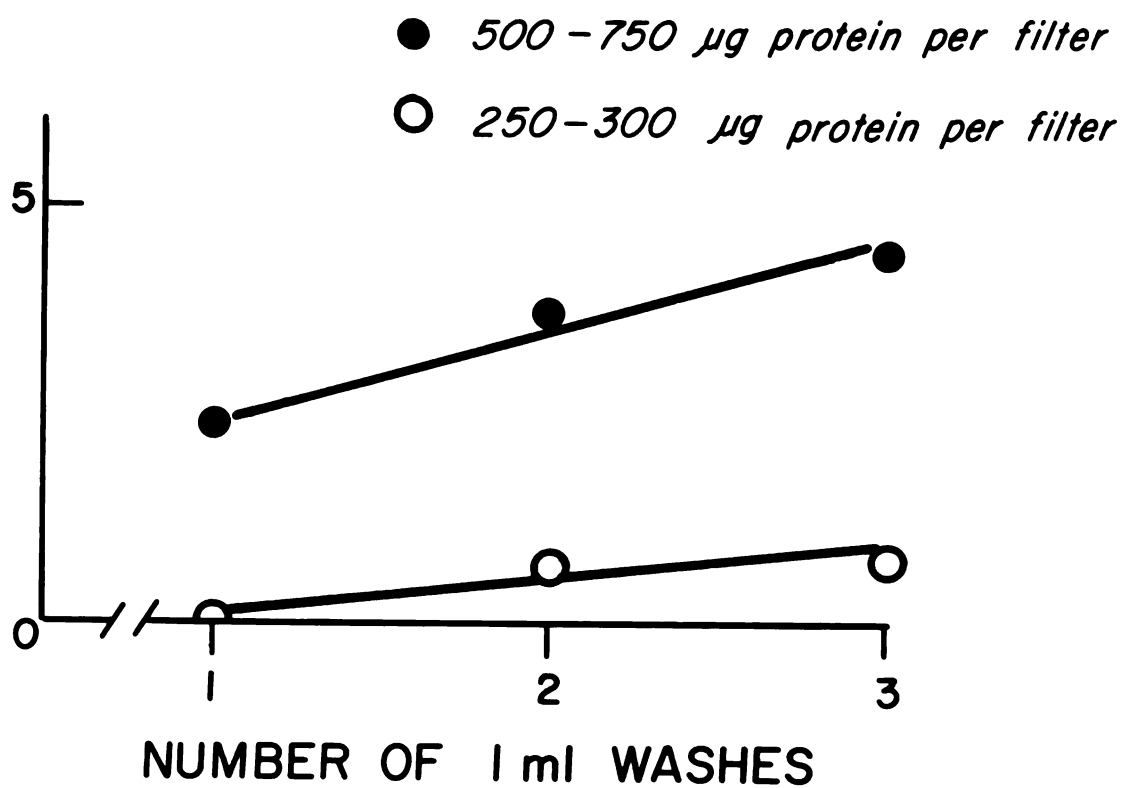○ 250 - 300 μg protein per filter

NUMBER OF 1 ml WASHES

Figure 2.-IV. LEAKAGE OF PROTEIN FROM FILTERS AS A FUNCTION

OF THE CONCENTRATION APPLIED AND THE NUMBER

OF 2.5 mM TRIS-EDTA WASHINGS.

after three washings of the filters and no NaK-ATPase activity was de-
tected in the filtrate. Based on the control experiments mentioned in
the last two sections, the concentration of plasma membrane applied to
each filter was less than 200 µg and usually in the range between 120 to
180 µg, and the filters were washed 3 times with 1 ml of 2.5 mM Tris-
EDTA. With this technique all of the protein was retained on the filters
and most of the non-specifically bound ^{3}H-ouabain was eluted.

3. Effect of incubation time on ^{3}H-ouabain specific binding.

The binding procedure was as detailed above, but the reaction at 37°c
was continued for various periods of time.As shown in Figure 3-IV the
binding of ^{3}H-ouabain to the enzyme-system is complete after one minute of
incubation, and there was no further specific binding of the glycoside
during the next 15 minutes of incubation. Since the time involved in ap-
plying the samples to the filters and subsequent washing, the actual min-
imum incubation time is about 2 minutes. Based on these time-course ob-
servations, I elected to use 5 minutes of incubation at 37°c as the stan-
dard time for the binding reaction. My results are in agreement with
time-course studies of ouabain binding and/or inhibition of NaK-ATPase
activity observed in lamb brain (18) and in   enzyme preparations
from guinea pig and dog (176), calf heart (107,148) calf brain (4) and
human erythrocytes (97).

4. Specific and nonspecific ^{3}H-ouabain binding.

Nonspecific binding was determined by two different methods: In the
first technique, 10^3-10^6 excess of nonradioactive ouabain was used as a
competitor for specific ouabain binding (94). In the second method, Na$^+$
Mg^{2+} and ATP were omitted from the incubation media. The plasma membrane
fraction was assumed to be free of these cations, as during the prepara-
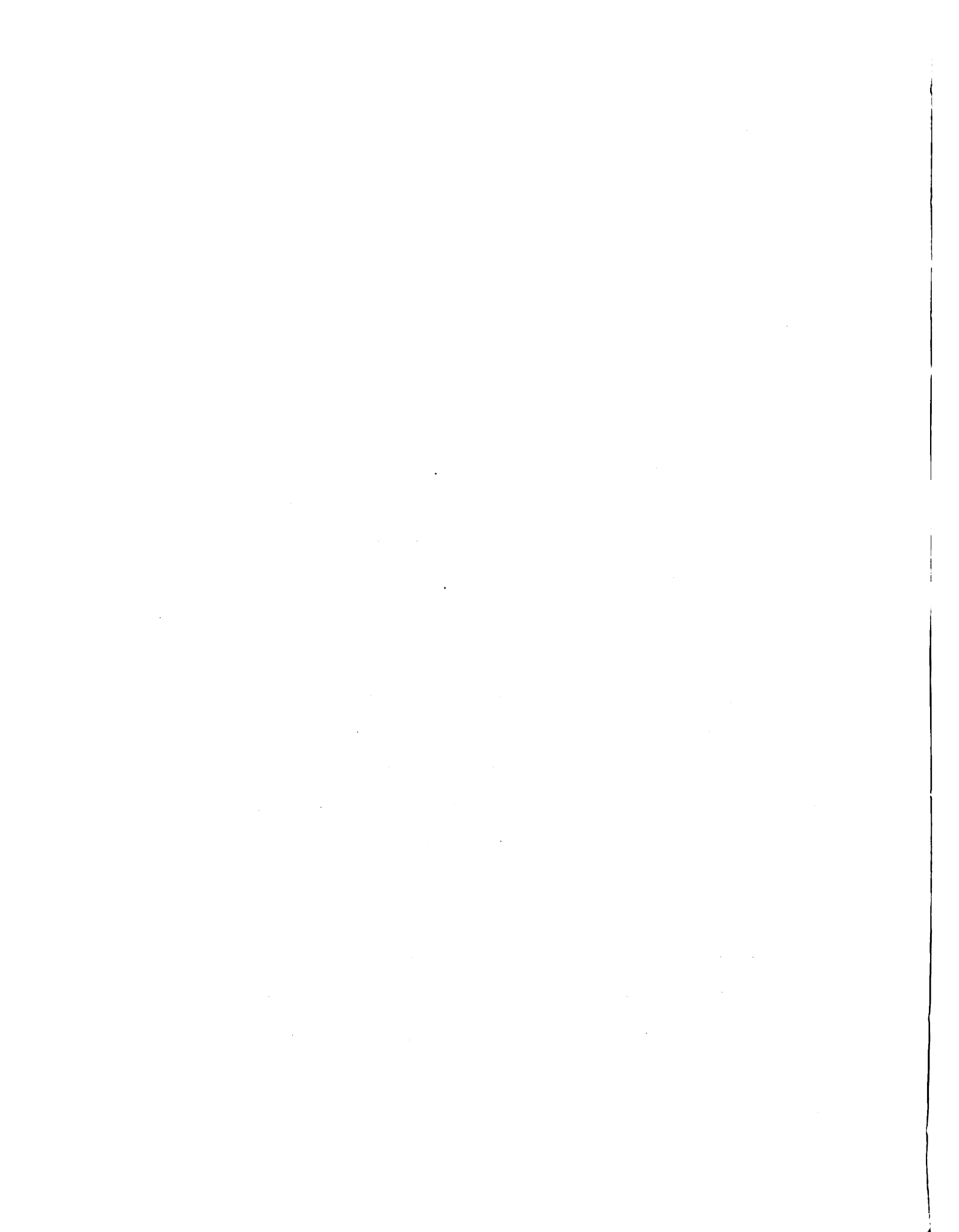
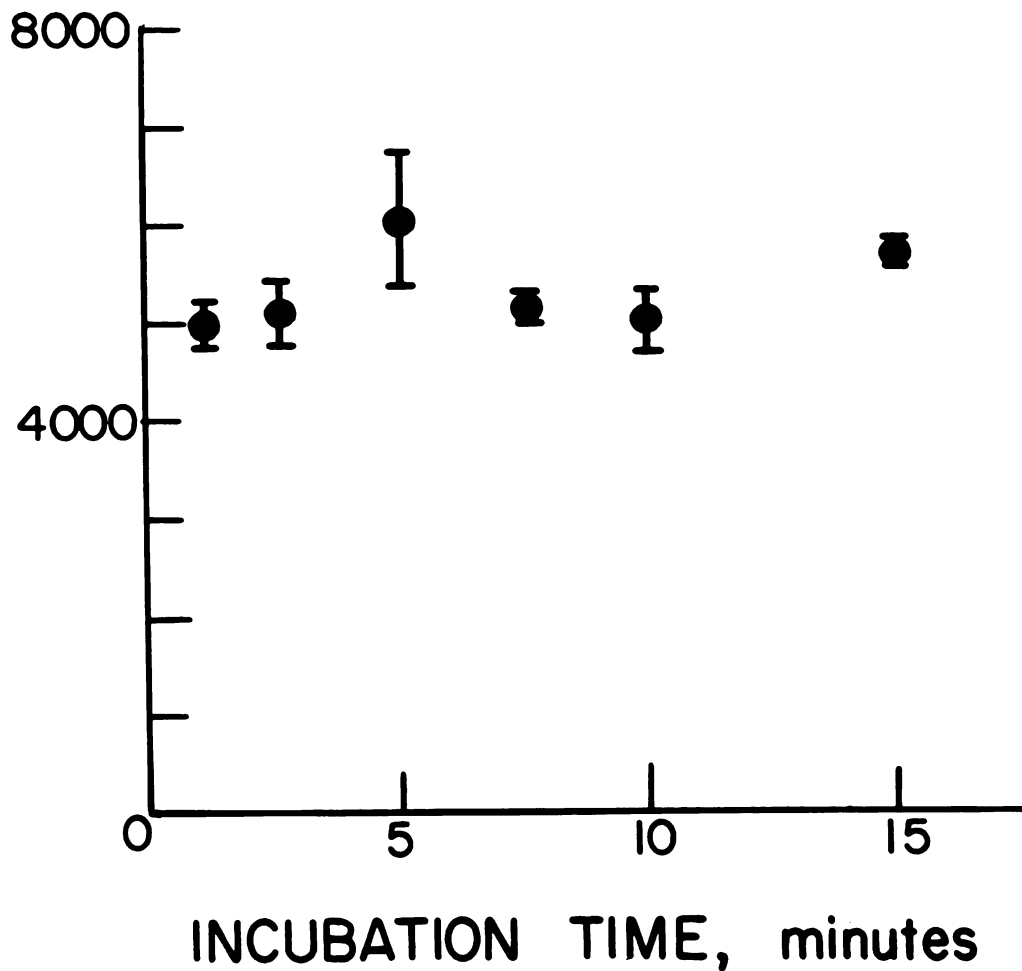Figure 3-IV. SPECIFIC BINDING OF [^{3}H] OUABAIN TO A JEJUNAL CRUDE PLASMA MEMBRANE FRACTION AS A FUNCTION OF INCUBATION TIME IN 37°C. (n = 6).

tion procedure it was washed and finally resuspended in 2.5 mM Tris-EDTA.
For subsequent experiments the non-specific binding was measured by the
second method as: a) There was no difference in the results obtained
when the two methods were employed simultaneously with the same prepara-
tion and b) When higher concentration of ^{3}H-ouabain, in the range of 10-5
M were used, it was impossible to prepare an aqueous solution that would
contain 10^{-2}-10^{-1} M cold ouabain.

In Figure 4-IV a representative experiment indicates the amount of
^{3}H-ouabain bound specifically and nonspecifically to a jejunal plasma
membrane fraction, as a function of free ^{3}H-ouabain concentration. Spe-
cific binding of the glycoside shows typical saturation kinetics but non-
specific binding is linearly correlated to the free ^{3}H-ouabain concentra-
tion in the range measured. This observation further supports the assump-
tion that when Na$^+$, Mg^{2+} and ATP are eliminated from the reaction medium,
only nonspecific binding components, which are present in large excess
over the specific sites, are measured. It is of importance to note that
the ratio of specifically bound over nonspecifically bound is inversely
proportional to the free ^{3}H-ouabain concentration. This observation im-
plies that binding measures performed at high concentrations of ^{3}H-ouabain
may be less accurate than at low concentrations of the glycoside.

5. ^{3}H-ouabain specific binding as a function of protein content and
   NaK-ATPase specific activity.

Five sets of experiments were performed. In each set, 4-6 samples,
of the same jejunal plasma membrane fraction were added to a final volume
of 1 ml reaction medium, ^{3}H-ouabain in a final concentration of 2.59×10^{-6}M
and from the same stock solution was used for all these experiments. The
binding assay procedure was followed as detailed in the Methods section.
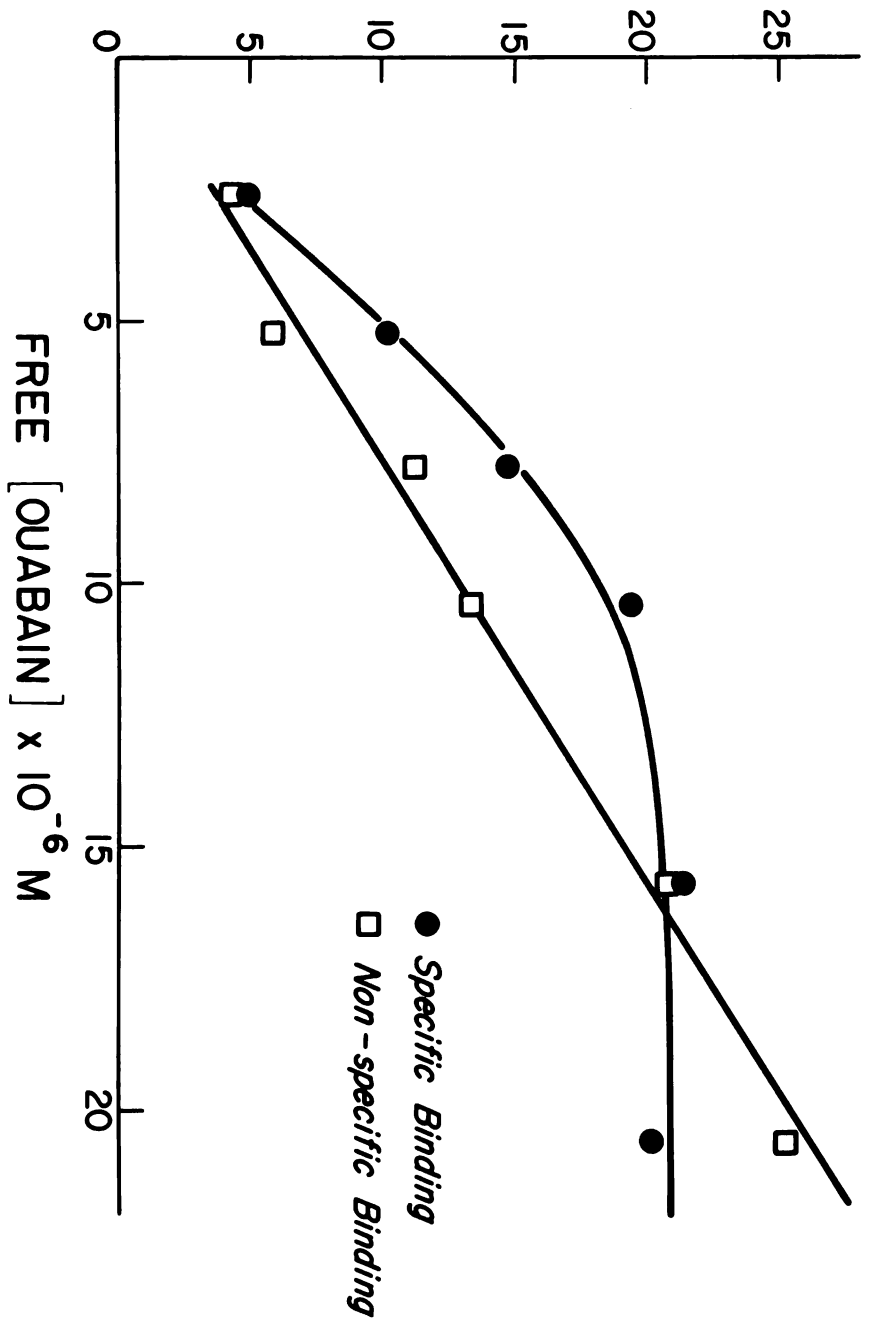
Figure 4-IV. SPECIFIC AND NON-SPECIFIC BINDING OF [³H] OUABAIN TO JEJINAL CRUDE PLASMA MEMBRANE FRACTION, AS A FUNCTION OF FREE [³H] OUABAIN CONCENTRATION.

The amount of plasma membrane protein applied ranged from 40-240 µg per filter. Two of the jejunal plasma membrane fractions were prepared from pools of hypothyroid rats, and three from euthyroid rats. The amount of ^{3}H-ouabain bound specifically, as a function of protein applied per filter is shown in Figure 5-IV. A close, linear correlation with a regression coefficient of 0.85 was obtained, implying a ratio of 1:1 between specific ^{3}H-ouabain binding and plasma membrane protein concentration. Moreover, the results from the hypothyroid group tend to be below the regression line, reflecting lesser specific glycoside binding to these preparations. When specific ^{3}H-ouabain binding is expressed as a function of NaK-ATPase activity applied to each filter (Figure 6-IV), the linear correlation is even closer, with a correlation coefficient of 0.91; indicating a 1:1 ratio between ^{3}H-ouabain binding and NaK-ATPase activity. In this figure the hypothyroid and euthyroid values are spread equally along the regression line, with a tendency of the hypothyroid data to concentrate in the lower left quarter of the figure, reflecting again the lower NaK-ATPase activity of these preparations and the associated decreased binding of glycoside.

These data serve as another control for the binding assay, showing that the amount of specifically-bound glycoside retained on the filters is linearly related to the protein, applied up to about 250 µg per filter. Because of protein leakage as mentioned before, no higher protein concentrations were used. But even more important, these observations imply that changes in NaK-ATPase specific activity, measured under V_{max} conditions reflects changes in the number of active Na^+-pump sites and that the increase in NaK-ATPase activity observed in jejunal plasma membrane fractions after administration of T_3 to hypothyroid and euthyroid rats, is a

Figure 5-IV. SPECIFIC BINDING OF [³H] OUABAIN (2.59 X 10⁻⁶ M) TO JEJUNAL CRUDE PLASMA MEMBRANE PREPARATION FROM EUTHYROID AND THYROIDECTOMIZED RATS, AS A FUNCTION OF THE AMOUNT OF PROTEIN APPLIED TO EACH FILTER. THE INNER LINES REPRESENT 95% CONFIDENCE INTERVAL FOR THE LINE, THE OUTER LINES THE 95% CONFIDENCE INTERVAL FOR EACH POINT.
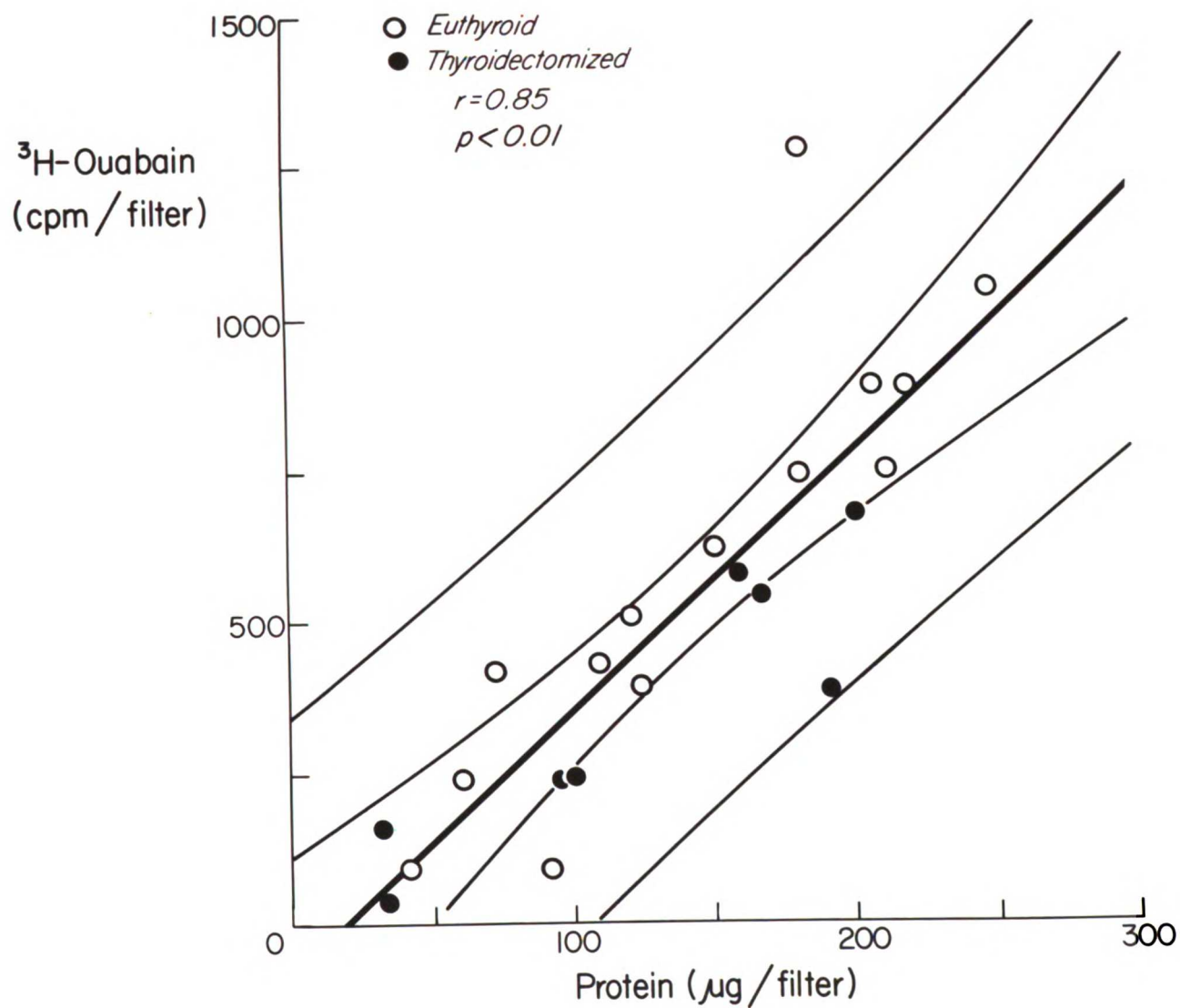
Figure 6-IV. SPECIFIC BINDING OF [³H] OUABAIN TO JEJUNAL CRUDE PLASMA MEMBRANE PREPARATION FROM EUTHYROID AND THYROIDECTOMIZED RATS, AS A FUNCTION OF NaK-ATPASE ACTIVITY APPLIED TO EACH FILTER. THE INNER LINES REPRESENT 95% CONFIDENCE INTERVAL FOR THE LINE, THE OUTER LINES, THE 95% CONFIDENCE INTERVAL FOR EACH POINT.

result of an increase in the number of active Na^+-pump sites. To obtain more direct estimates of the number of active enzyme sites, the following experiments were performed.

6. ^{3}H-ouabain binding to plasma membrane fractions from thyroidecto-mized rats $\pm$ T_3.

In preliminary experiments I found an increase in specific binding of ^{3}H-ouabain to jejunal plasma membrane fractions from hypothyroid rats injected with T_3 to the uninjected (diluent only) thyroidectomized group, at ^{3}H-ouabain concentrations of 3.5×10^{-8}M to 1.9×10^{-5}M. To estimate the maximal number of binding sites and the dissociation constant for ouabain, there was a need to measure the binding at or near saturation levels. Therefore, the effect of thyroid status on glycoside binding was tested at ^{3}H-ouabain concentrations in the range of 2.4×10^{-6}M to 1.9×10^{-5}M.

A total of 36 thyroidectomized rats were used; 18 of the rats were injected with T_3 (50 μg/100 g body wt, q.48 hrs x 3) and the other 18 served as controls. As the small intestinal plasma membrane fractions were pooled from 2 rats, the number of fractions examined from each group was 9.

Specific binding of ^{3}H-ouabain as a function of the free glycoside concentration is depicted in Figure 7-IV. Average specific binding at each concentration of free glycoside of the fractions from the T_3-treated rats was compared to the corresponding binding of the controls by a non-paired t-test. Thyroid hormone administration to hypothyroid rats caused a statistically significant increase in ^{3}H-ouabain specific binding at each level of glycoside concentration. The 'P' value was less than 0.01 at all concentrations of free ^{3}H-ouabain. The increase in specific gly-
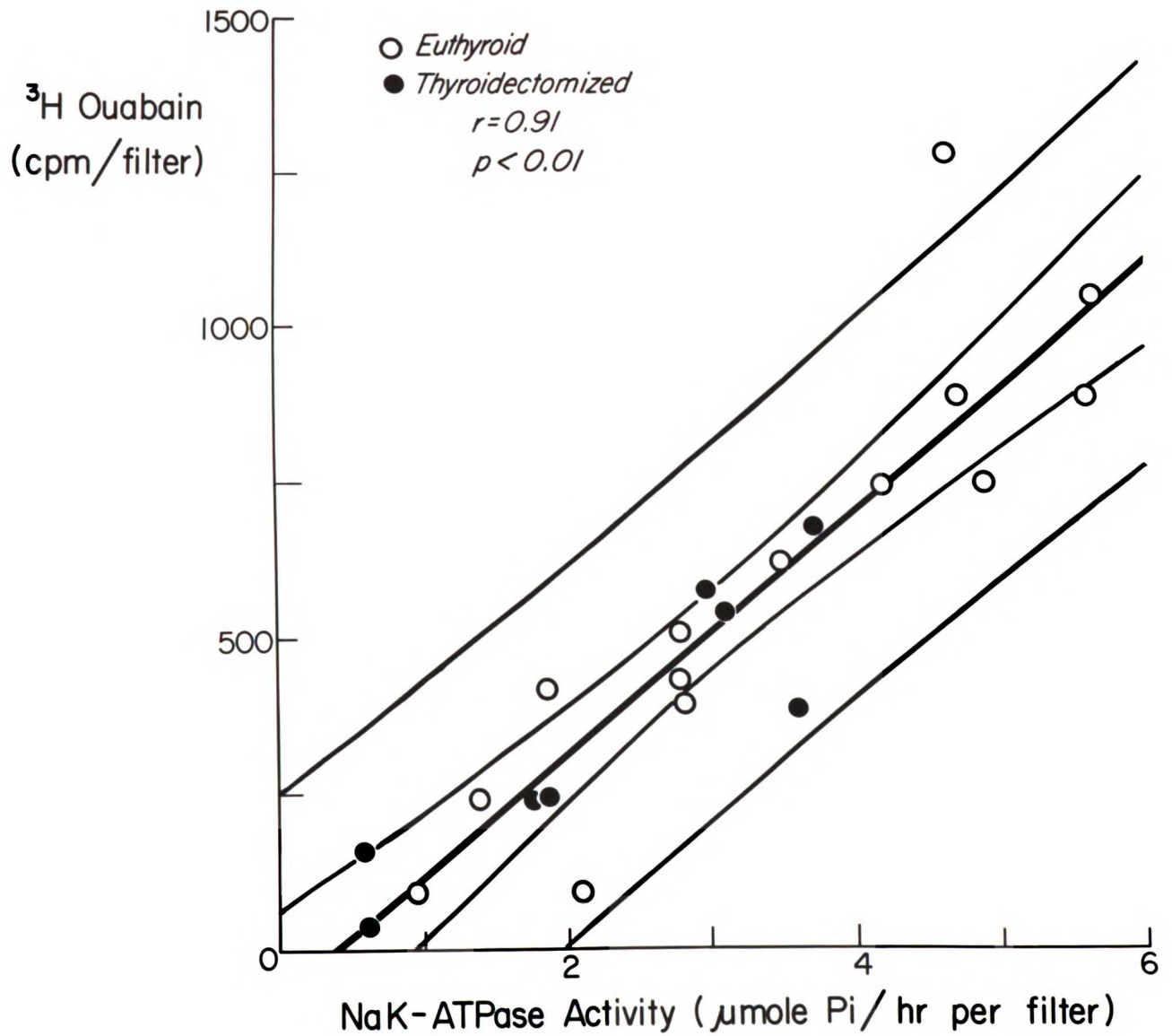
Figure 7-IV. SPECIFIC BINDING OF [³H] OUABAIN TO JEJUNAL CRUDE PLASMA MEMBRANE PREPARATION FROM THYROIDECTOMIZED RATS ± T₃ (50 µg/100 g BODY WT.).

coside binding of the fractions from T_3-treated rats, ranged from 23% to 40%. Through the range of $2x10^{-6}M$ to $9x10^{-6}M$ free ^3H-ouabain, specific binding of the glycoside to preparations from T_3-treated animals and controls, followed first order kinetics (Figure 7-IV). NaK-ATPase activity was directly proportional to specific ^3H-ouabain binding. These results point out that thyroid hormone does not affect the linear correlation between enzyme activity and glycoside binding, I demonstrated in a previous section of this chapter, and as reported previously for preparations from normal animals and a variety of species (1,62,148).

Figure 7-IV shows that specific binding to the crude plasma membrane preparation conforms to first-order saturation kinetics. By employing the Linewaver-Burk technique, a plot of the reciprocal values of ^3H-ouabain bound specifically as a function of free glycoside concentration, N_{max} values were calculated from the intercept on the Y-axis and the apparent K_d from the slope. N_{max} and K_d values were calculated separately for each experiment. Regression lines were obtained by the least mean square method with the aid of a digital PDP-12 Computer (Digital Equipment Corporation, Mywnard, Mass.) and the Hewlett Packard 9100B Calculator. Regression coefficients calculated for each line ranged between 0.97 to 0.997 with one exception in which r = 0.94. The close linear correlation observed confirms that specific binding of ^3H-ouabain was a saturable process, and that only one class of binding sites for the glycodide was detected. Two examples of Lineweaver-Burck plots of ^3H-ouabain binding of T_3 treated and control preparations are shown in Figure 8-IV.

The estimates of N_{max} were calculated for the individual fractions (pool of fractions from rats); the mean value of the estimates for the T_3-treated and control groups are presented in Table 1-IV, as well as the
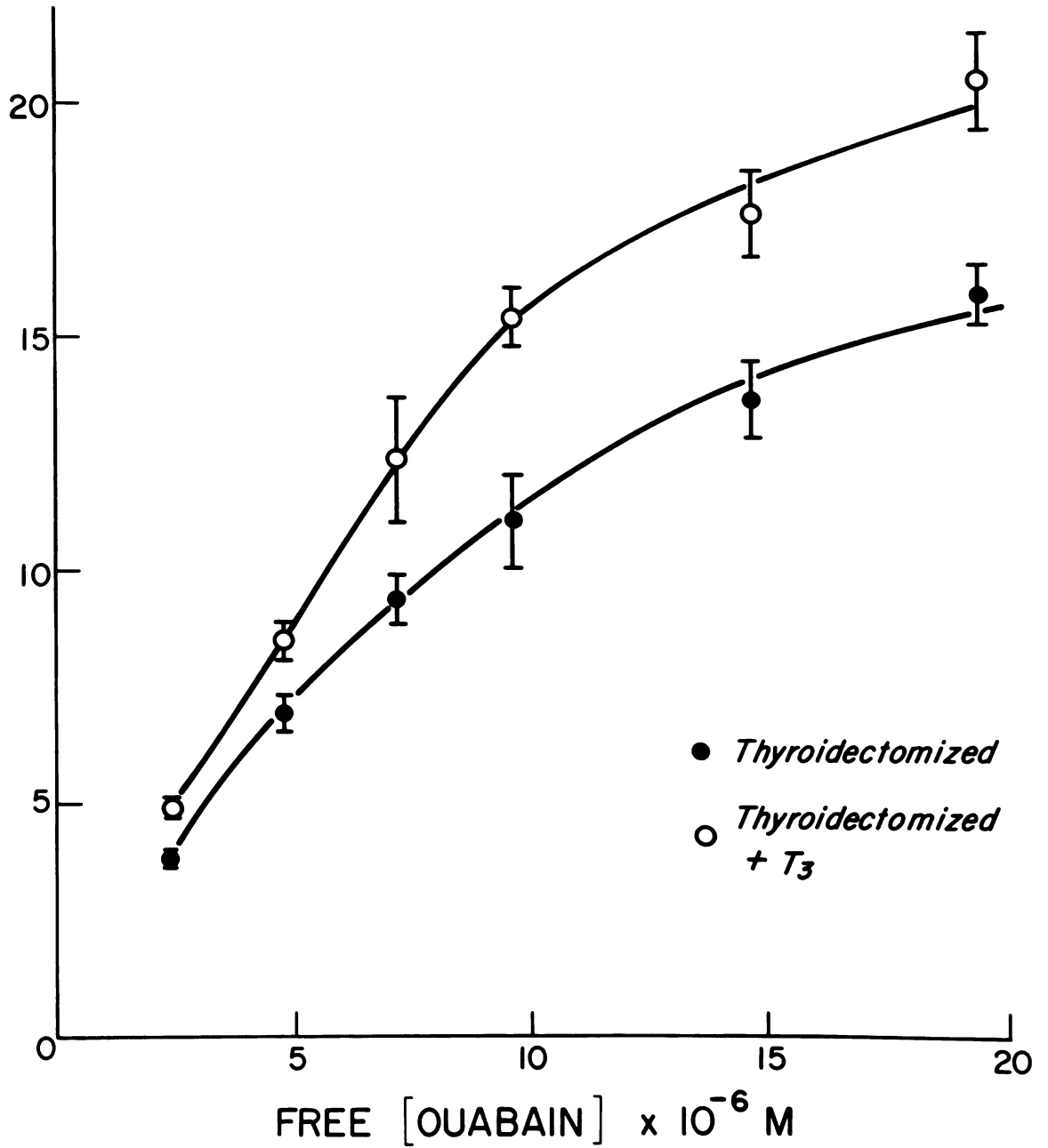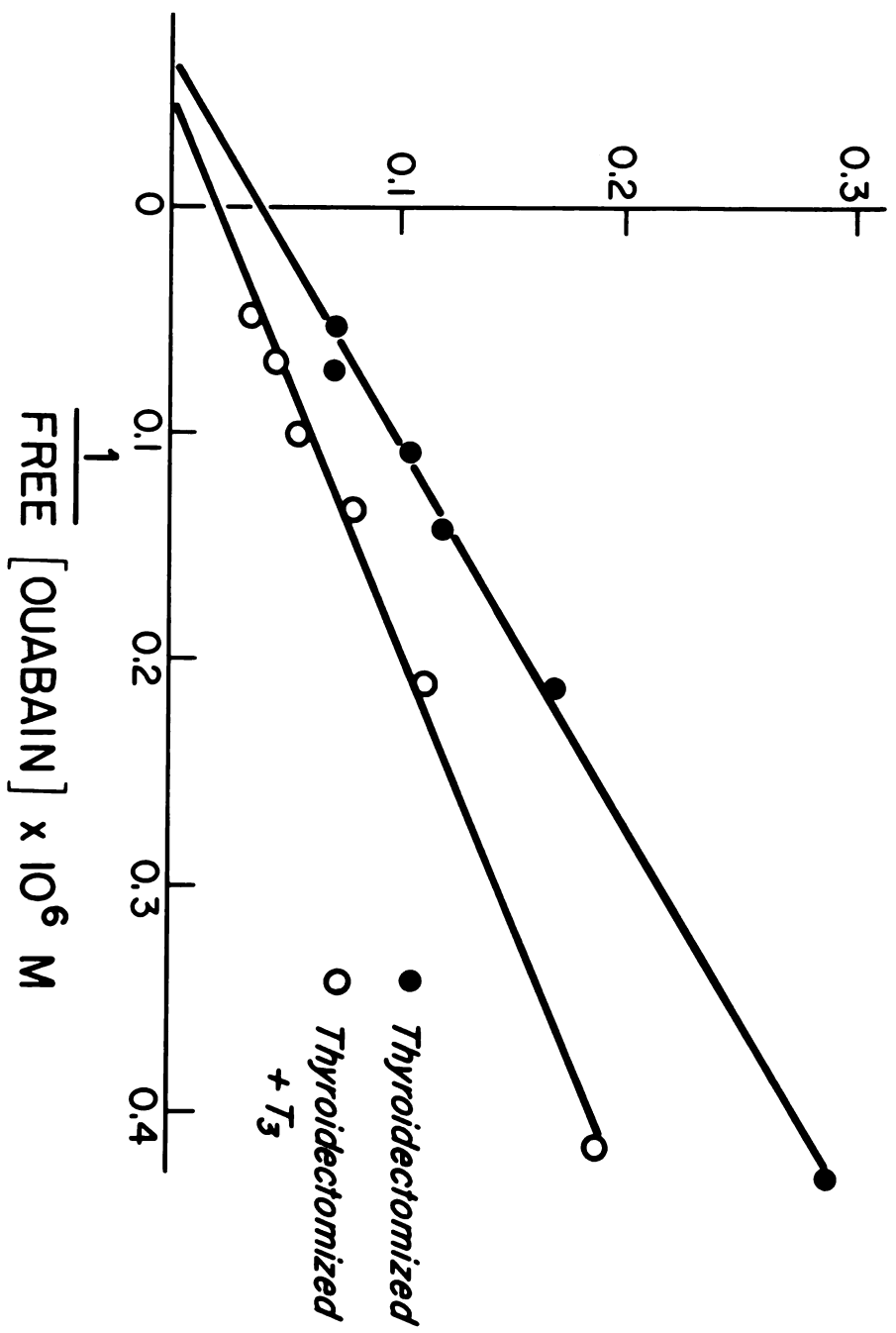
Figure 8-IV. LINEWEAVER-BURK PLOT OF THE SPECIFIC BINDING OF [^{3}H] OUABAIN TO JEJUNAL CRUDE PLASMA MEMBRANE PREPARATION FROM THYROIDECTOMIZED RATS ± T$_3$ (50 μg/100 g BODY WT.), AS A FUNCTION OF FREE [^{3}H] OUABAIN CONCENTRATION.

Table 1-IV NaK-ATPase Activity and Turnover Number, and Estimated Maximal Number of Binding Sites

(N_{max}) of ^{3}H-Ouabain and Apparent Dissociation Constant (K_{diss}) of a Crude Plasma Membrane

Preparation from Jejunal Mucosa from Thyroidectomized Rats $\pm$ T_3 (50 μg per 100 g Body Weight).*

	NaK-ATPase μmoles Pi/hr per mg protein	N_{max} p-moles ouabain bound per mg protein	Turnover number Pi released/min per enzyme site	K_{diss} 10^{-5}M
Thyroidectomized	11.54 ± 0.88	33.30 ± 3.85	6429 ± 965	1.94 ± 0.32
Thyroidectomized $+ T_3$	15.64 ± 0.83	44.53 ± 5.40	6376 ± 739	2.00 ± 0.27
Δ	4.10 (36%)	11.23 (34%)	-53 (-1%)	0.06 (3%)
P	< 0.005	NS	NS	NS

*Mean $\pm$ SE. n=9

mean NaK-ATPase activity of the same preparations. Thyroid hormone treatment caused a mean increase of 34% in the estimated maximal number of ^{3}H-ouabain binding sites that correlates very well with a mean increase of 36% in NaK-ATPase activity. However, the difference between the estimated N_{max} of the treated and untreated groups fell short of statistical significance, p=0.1.

In Figure 9-IV, estimated N_{max} values calculated for enzymes prepared from hypothyroid $\pm$ T_3 and euthyroid $\pm$ T_3 rats are plotted as a function of NaK-ATPase specific activity values measured in the same crude plasma membrane fractions. A statistically significant linear correlation between the two parameters exists. This observation emphasizes again that an increase in NaK-ATPase activity measured under N_{max} conditions reflects an increase in the number of active enzyme sites, as measured by ^{3}H-ouabain binding.

Apparent K_d values were estimated from the slope of the Lineweaver-Burk plot. If it is assumed that a) The enzyme (E) ouabain (G) interaction can be described as a one step binding reaction in which the enzyme binds ouabain to form a complex (EG) which dissociates reversibly, the reaction can be formulated as:

$$E + G \underset{K_2}{\overset{K_1}{\rightleftharpoons}} EG$$
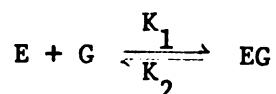
then the apparent K_d values may be taken as representing the equilibrium dissociation constant (K_{diss}). The calculated K_{diss} values for ouabain are presented in Table 1-IV. It is obvious that no change in K_{diss} was observed after T_3-treatment of hypothyroid rats. My results agree with those of Allen and Schwartz (7) on NaK-ATPase from rat heart and kidney, employing a ^{3}H-ouabain binding assay.

ESTIMATED Nmax
pmoles Ouabain bound / mg protein

120

80

40

0

$r = 0.51$
$p < 0.01$

● Thyroidectomized
O Thyroidectomized + T_3
▲ Euthyroid
△ Euthyroid + T_3

NaK-ATPase, μmoles Pi / mg protein / hr

5   10   15   20   25
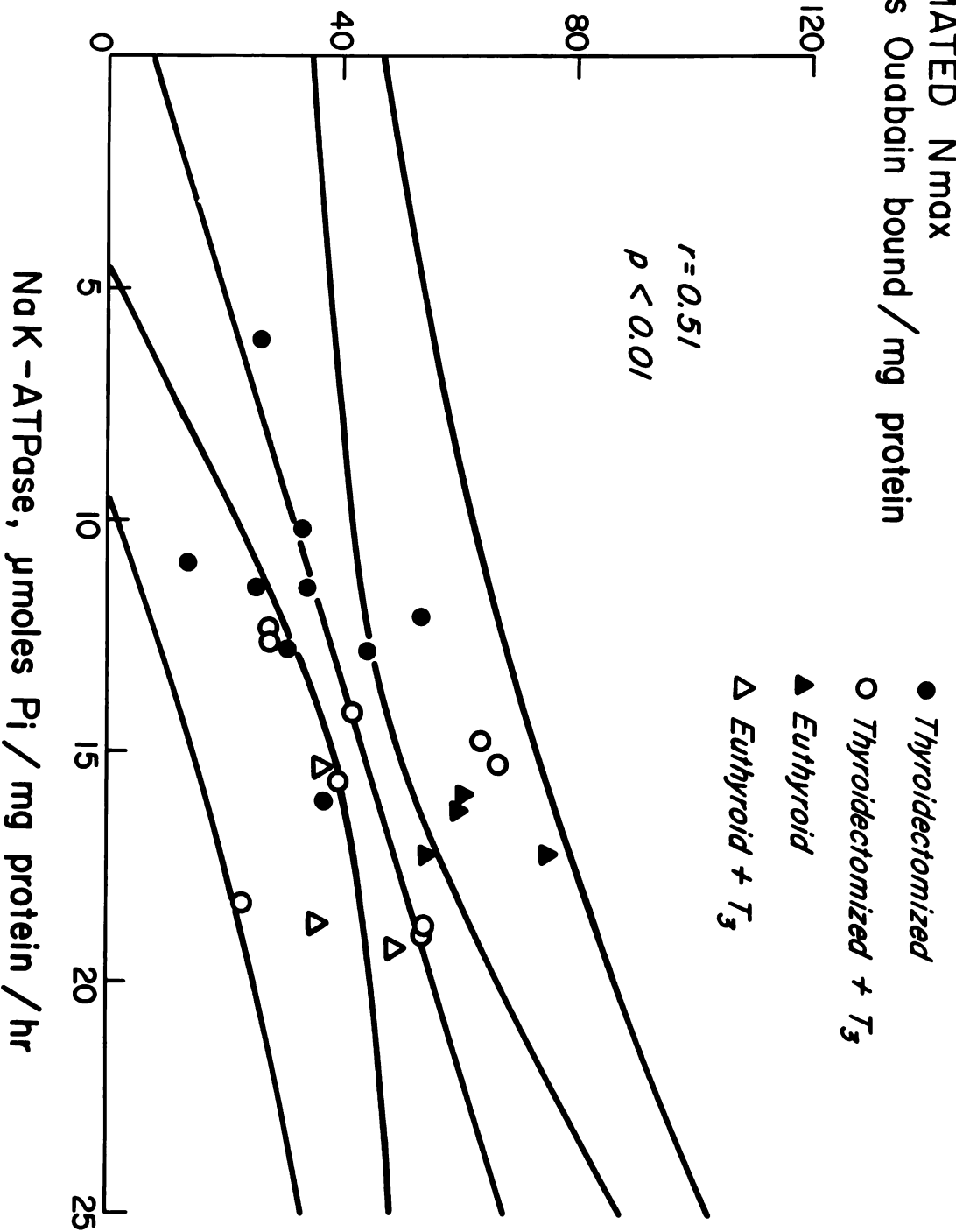
Figure 9-IV.   ESTIMATED MAXIMAL NUMBER OF [^{3}H] OUABAIN BINDING SITES (N$_{max}$), TO JEJUNAL CRUDE PLASMA MEMBRANE PREPARATION FROM THYROIDECTOMIZED AND EUTHYROID RATS ± T_3 (50 μg/100 g BODY WT.), AS A FUNCTION OF NaK-ATPASE SPECIFIC ACTIVITY.   THE INNER LINES REPRESENT 95% CONFIDENCE INTERVAL FOR THE LINE, THE OUTER LINES, THE 95% CONFIDENCE INTERVAL FOR EACH POINT.

Barnett (18) and Wilson et al (189) inferred that one molecule of

ouabain binds to one transport enzyme. Assuming such a 1:1 relationship,

a turnover number for the NaK-ATPase enzyme system in our preparation can

be calculated from the specific activity of the enzyme and the estimated

N_{max} of the same preparation. The mean turnover number for NaK-ATPase

from T_3 treated and control rats is presented in Table 1-IV. No differ-

ence between the turnover numbers calculated for each group was docu-

mented. From the similarity between the K_d values and calculated turn-

over numbers, it may be concluded that the NaK-ATPase obtained from ani-

mals treated with T_3 is similiar if not identical to the enzyme in the

control group; thyroid hormone does not appear to induce qualitative

changes in the NaK-ATPase enzyme system. Bader et al. (13) measured the

phosphorylated intermediate of NaK-ATPase, and estimated the turnover num-

ber of the enzyme in 6 tissues from 11 species. The range in specific ac-

tivity of the enzyme was more than 400-fold, yet the turnover number

varied 2-fold; from 8,100 to 16,700 moles of Pi released per site per

minute. These investigators, however, used the phosphorylated interme-

diate measured at 0°c as the base for their calculations. I found only

one report in the literature (7), in which ouabain binding to rat NaK-

ATPase other than the brain was measured, and I calculated the turnover

number to be about 8000 moles of Pi released per minute per site. In the

above mentioned work, the separation of the bound ^{3}H-ouabain from free

was performed by centrifugation and the pellet was not washed. Our fig-

ures for the turnover number when compared to the studies are about 20%

lower than the figures obtained by centrifugation, perhaps because of

differences in efficiency of the methods.

The efficiency of the filter assay is determined by probability that

the complex will survive the filtration and washing procedures and be detected when the filter is assayed (192,193). The efficiency may be measured by titration of a large excess of enzyme so that the maximal possible amount of ligand is bound. This procedure could not be performed in our assay system because of the very high K_d of the enzyme-ouabain complex. However, the lower values for turnover number obtained by us in comparison to those cited above will not affect the validity of the comparison between NaK-ATPase activities or of the amounts of ^{3}H-ouabain bound (i.e. N_{max}) in thr fractions from the two groups of rats, T_3-treated and untreated.

While these experiments were in progress, Dr. C.S. Lo in our laboratory developed a method of measuring the quantity of phosphorylated intermediate formed in kidney membrane preparations from ATP-γ-^{32}P (98). In collaboration, with Dr. Lo the same procedure was carried out on jejunal crude plasma membrane fractions from thyroidectomized rats $\pm$ T_3. NaK-ATPase activity was measured simultaneously in each preparation. The results are summarized in Table 2-IV. The increase of about 35% in enzyme specific activity of T_3 treated rats was accompanied by a 54% increase in the amount of the phosphorylated intermediate. The direct proportionality between NaK-ATPase activity in the intestinal membrane fractions from the treated and untreated animals and the phosphorylated intermediate is shown in Figure 10-IV. These observations lead to the conclusion that: a) as described previously (13,126), the ^{32}P-intermediate is a measure of the number of Na$^+$-transport units and b) The increase in transport enzyme activity after thyroid hormone treatment, represents an increase in the number of active Na$^+$ pump sites.

Recently Tata and his associates (171,175) observed that thyroid

Table 2-IV NaK-ATPase Activity and Phosphorylated Intermediate of a
Partially Purified Membrane Fraction from Small Intestinal
Mucosal Cells. Thyroidectomized Rats $\pm$ T_3 (50 μg/100 g Body
Wt.)[*]

	NaK-ATPase	^{32}P-Intermediate
	μmoles Pi/hr/ mg protein	pmoles ^{32}P bound/ mg protein
$- T_3$[+]	13.89 $\pm$ 1.26	44.50 $\pm$ 4.57
$+ T_3$[‡][+]	18.68 $\pm$ 1.04	68.42 $\pm$ 3.97
Δ	5.79 (34.5%)	23.92 (54%)
p	< 0.025	< 0.005

[*]In collaboration with Dr. C. S. Lo.

[+]Mean $\pm$ SE (n = 6). [‡]Mean $\pm$ SE (n = 5).

³²P INTERMEDIATE
pmoles ³²P bound /mg protein

NaK - ATPase, μmoles Pi/mg protein/hr

r = 0.71
p < 0.05

● Thyroidectomized
○ Thyroidectomized
+ T₃
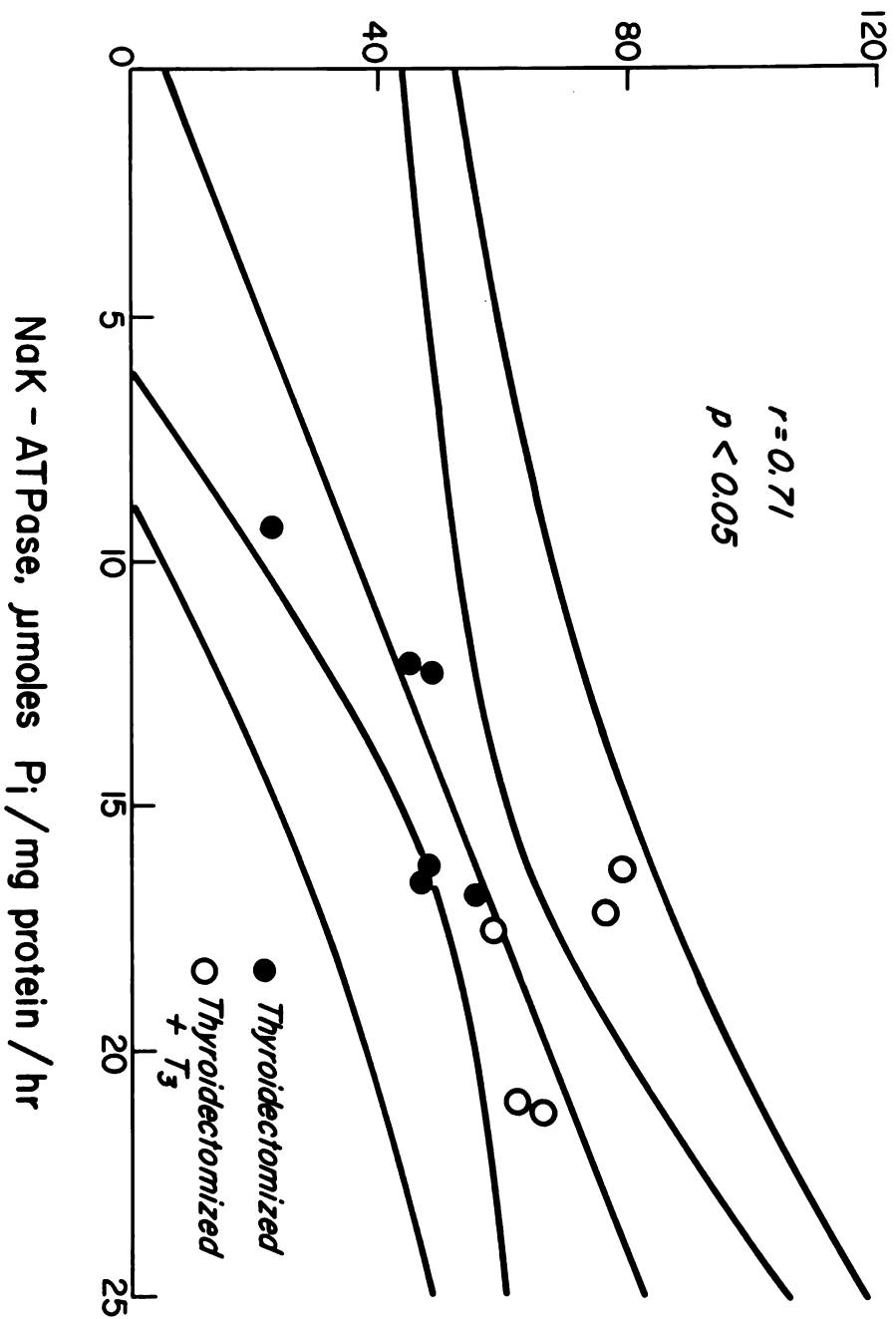
Figure 10-IV. THE FORMATION OF NaK-ATPASE PHOSPHORYLATED INTERMEDIATE AS A FUNCTION OF NaK-ATPASE SPECIFIC
ACTIVITY. MEASUREMENTS WERE MADE ON JEJUNAL CRUDE PLASMA MEMBRANE PREPARATION FROM
THYROIDECTOMIZED RATS ± T₃ (50 μg/100 g BODY WT.). THE INNER LINES REPRESENT 95% CONFIDENCE
INTERVAL FOR THE LINE, THE OUTER LINES, THE 95% CONFIDENCE INTERVAL FOR EACH POINT.

hormone augments RNA synthesis in vivo and RNA polymerase activity of isolated nuclei. Increased rates of ribosmol RNA synthesis were most obvious during the early phase of hormone action (175). In addition, increased rates of cytoplasmic protein synthesis followed the increase in RNA synthesis. The rise in amino acid incorporation into cytoplasmic ribosomes was accompanied by the appearance of an additional amount of newly formed ribosomes (175). Furthermore it was observed (170-173) that the labeling of phospholipid of all cellular membranes was stimulated by thyroid hormone; the most impressive net membrane accumulation took place in the microsomal fraction (172,173). Thus it is possible that thyroid hormone affects NaK-ATPase by synthesizing newly formed Na^+-pumps, that are then inserted into the membrane. Alternatively thyroid hormone may induce the synthesis of a protein or a lipoprotein that activates pre-existing pump sites in the plasma membrane.

7. Appraisal of the binding assay for ^{3}H-ouabain to rat tissue.

As mentioned in the introduction to this chapter, rat tissues with the exception of the brain are relatively insensitive to cardiac glycoside (3,7,132,133). The concentration of ouabain that inhibits 75% of cardiac NaK-ATPase activity from rats is about 100 to 200 fold higher than the dose employed to obtain the same relative inhibition in dog, pig, sheep and guinea pig cardiac NaK-ATPase (3). I found only one study in the literature (7) in which ^{3}H-ouabain binding to rat kidney and heart NaK-ATPase was demonstrated. The binding was measured at 4 different ^{3}H-ouabain concentrations, in the range of 10^{-6} to 5×10^{-4}M. Separation between bound and unbound glycoside was by centrifugation, and the radioactivity in the unwashed pellet was measured. On resuspension of the pellet the ^{3}H-ouabain was completely released from the complex. In my

method, specific binding of ^{3}H-ouabain is detectable at concentrations of

free ouabain as low as 1.5×10^{-9}M. In Table 3-IV, specific binding of

ouabain to jejunal crude plasma membrane from euthyroid rats, as a func-

tion of free glycoside concentrations is presented. Specific binding of

9.8×10^{-15} moles of glycoside per mg protein was observed when the prep-

aration is incubated with 1.5×10^{-9}M ^{3}H-ouabain, the amount bound is

very low but the measurements are reproducible. An increase of ^{3}H-ouabain

concentration in 10 fold steps yielded a linear increase in specific

binding (Table IV-3).

^{3}H-ouabain specific binding to kid-

ney plasma membrane preparation obtained from thyroidectomized rats $\pm$ T$_3$
   also

was measured. Only one concentration of ^{3}H-ouabain 9.5×10^{-6} was used.
   ^

It was observed that thyroid hormone treatment caused a statistically sig-

nificant increase of 65% in the specific binding of the glycoside. NaK-

ATPase activity of the preparations measured at the same time revealed

that T$_3$ injection caused a 71% increase in enzyme specific activity.

These observations demonstrated that: a) The increase in the kidney

plasma membrane NaK-ATPase specific activity after thyroid hormone admin-

istration represents an increase in the number of active enzyme sites,

and confirms the results obtained with the jejunal preparation, and b)

My ^{3}H-ouabain binding assay is applicable to other tissues.

It was suggested by several authors that the species differences in

the sensitivity to cardiac glycoside are principally related to the dif-

ferent dissociation rates of the drug-enzyme complex (5,176) my results

with the rat jejunal NaK-ATPase support this view. The rates of associa-

tion of ouabain with the different enzyme preparations did not differ

appreciably (176). However, these comparative studies were not performed

Table 3-IV Specific ^{3}H-Ouabain Binding as a Function of Free ^{3}H-Ouabain

Concentration of a Jejunal Crude Plasma Membrane Preparation

from Euthyroid Rats.*

Free ^{3}H-ouabain 10^{-6} M	Bound ^{3}H-Ouabain 10^{-12} moles/mg protein
0.0015	0.0098 ± 0.000
0.015	0.116 ± 0.008
0.153	0.873 ± 0.217
1.53	8.064 ± 0.344

*Mean ± SE (n=6)

with enzymes prepared from rat tissues, since the enzyme-ouabain complex is almost totally dissociated under the assay conditions (176), no specific binding was demonstrated (5,176). It was observed that while glycoside induced inhibition of the NaK-ATPase system in sensitive species was time and temperature dependent (1,147) rat heart and kidney NaK-ATPase did not manifest these relations (7). It was suggested therefore that the enzyme isolated from rat tissues may contain a different kind of receptor (7). Studies on the effect of ouabain on Na^+ dependent transport of L-methyl-D-glucoside in rabbit kidney slices, and the binding of the drug to the slices and to rat kidney cortex slices, showed that the maximum number of binding sites to the tissue was one order of magnitude lower in the rat than in the rabbit (132). Thus the nature of the differences in NaK-ATPase from rat tissue as compared to other species is still obscure.

The sensitivity of the binding technique used by me, may provide a tool for further insight into the nature of the glycoside-enzyme interaction and some qualitative aspects of the NaK-ATPase system, by comparing glycoside sensitive and insensitive preparations.

V. EFFECT OF T_3 ON TRANSEPITHELIAL POTENTIAL DIFFERENCE (PD) AND SHORT

CIRCUIT CURRENT (I_{sc})

A. Introduction

The small intestinal epithelium is a mosaic structure, characterized by in parallel, as well as in series, inhomogenieties. Solute transport across small intestinal epithelium takes place, in general, through two routes: 1) A transcellular route that involves movements across at least two membranes in series, e.g. the brush border and plasma membranes and 2) An extracellular route that includes the tight junctions and the underlying lateral intercellular spaces and is referred as the shunt pathway (143). There is good evidence that the shunt pathway plays a major role in the movements of ions, small nonelectrolytes and water across small intestine, as well as across "leaky" epithelia in general. Total ionic conductance of the shunt pathway in rabbit ileum, accounts for at least 85% of the total tissue conductance (54). This implies that in response to the electrochemical gradients, i.e. the driving force for passive ion flows, transepithelial movements through the shunt pathway will exceed flows through the transcellular route by a factor of about 6.

The mammalian jejunum has a high capacity to absorb Na^+, Cl^- and HCO_3^- but few studies have been carried out on possible underlying mechanisms. Though differences in function between jejunum and ileum exist, the basic cellular mechanism involved in transcellular Na^+ transport are probably common to both parts of the small intestine. Recent studies on mammalian ileum in vitro disclosed similarities with ileal function in vivo, and in addition provided some insight into specific transport processes. The following discussion on transcellular transport in the gut will be based therefore, on some jejunal studies but mainly on studies

with the ileum.

The small intestinal epithelium consists of various cell types (178) and each cell is bounded by two membranes; the mucosal (brush border) and basolateral plasma membranes. Active transepithelial transport is a result of the unique transport properties of each of these membranes. The following model was proposed by Schultz and is based for the most part on work performed by his group (51-54, 87,116,117,138,142-144). Na^+ entery into the cell across the brush border is driven by a steep electrochemical potential difference. In the absence of actively transported sugars or amino acids Na^+ may cross the brush border by two routes: 1) By a neutral NaCl influx carrier mechanism, that is inhibited by intracellular cyclic adenosine mono-phosphate (cAMP) and acetazolamide. This process is apparently reversible and may mediate the net efflux of NaCl and/or $NaHCO_3$ out of the cells. 2) A Cl^- independent mechanism the nature of which is yet unclear. Both mechanisms are not inhibited by ouabain or metabolic inhibitors such as 2,4-dimitrophenol and sodium fluoroacetate. Net Cl^- movement into the cell across the brush border is against an electrochemical potential difference. Since Cl^- transport does not seem to be directly linked to metabolic energy it is probably driven by the Na^+ gradient. The extrusion of Na^+ across the basolateral plasma membrane is an active transport process that is dependent on a source of metabolic energy, and is inhibited by ouabain in the serosal solution. These findings suggest that this process is mediated by a NaK-ATPase (this subject was discussed at some length in chapter III. Recent studies of subcellular fractions of small intestinal epithelium (55,128,129) as well as autoradiographic studies (164) indicate that most of the Na-K stimulated ouabain-sensitive ATPase, is localized in the basolateral membranes. That transmembrane Na^+ transport

is current-generating (or electrogenic) is implied by the finding that the rate of active Na^+ extrusion exceeds the rate of coupled K^+ uptake (54,138). In the light of these observations: The results cited above of an increase in QO_2 coupled to active Na^+ transport (i.e. $QO_2(t)$) in the small intestinal mucosa, following thyroid hormone treatment as well as the demonstration of an increase in NaK-ATPase activity raised the question of whether thyroid hormone would induce measurable changes in transepithelial PD and short circuit current (I_{sc}) across the jejunal epithelium.

B. Materials and Methods

Male Sprague-Dawley rats, weighing 330-400g, were thyroidectomized and the experiments were done 3-4 weeks later. The rats were injected with T_3, 50 μg/100 g body weight at 48 hour intervals for 3 doses. The injections were given in divided doses, ½ of each into two subcutaneous sites because of the large volumes required. For each set of experiments two rats, T_3 treated and control, were used. The rats were sacrificed by decapitation. The small intestine was rapidly exposed by a midline abdominal incision. The first 25 cm of intestine distal to the pylorus was discarded. A 10-20 cm section was excised and the lumen was flushed with Krebs-Henseleit bicarbonate buffer, pH-8 (88) at room temperature. The jejunal segment was pulled onto a wet glass rod and placed in chilled buffer solution. The serosa and muscularis externa were stripped as described in Methods in Chapter II. Two successive 2 cm sections, from each rat, not containing any Peyer's patches, were mounted on two lucite half-

chambers. (Research and Development Laboratory, S.F. Medical Center).

Each half chamber was clamped together with its corresponding half and

attached to a water-jacketed gas-life, circulating system (144), main-

tained at 37.5°c. Both the mucosal and serosal surfaces of the tissue

(exposed area = $1.12cm^2$) were separately bathed with 10ml of the buffer.

D-glucose at a 25 mM concentration was present in the solutions. The gas

used was a water-saturated 95% O_2 - 5% CO_2 mixture. About 15 minutes

elapsed from the time the animal was sacrificed until the first electrical

measurements were done. The preparations from two animals for each ex-

periment were performed in succession, thus the second preparation was

mounted on the chambers about 15-20 minutes after the first one.

The flux chamber and associated apparatus for measuring transmural

electrical potential difference (PD) and short-circuit current (I_{sc}) were

similar to those previously described by Schultz and Zalusky (144) and

Field, Fromm and McColl (46). The electrodes were connected to the cham-

bers through salt-agar bridges containing the same physiological solution

as the fluid bathing the tissue. For PD measurements the tips of the

bridges were placed within 1 mm of each surface of the membrane at the

center, and the potential difference between these bridges was measured

by a pair of matched calomel electrodes. The external current was applied

to the system employing immersed Ag-AgCl electrodes connected to a varia-

ble voltage source. Short-circuiting was achieved with a specially de-

signed automatic voltage clamp (2). The determination of the short-circuit

current was complicated by the low resistance of the intestinal wall. The

resistance of the solution between the potential electrodes and the surface

of the gut contributes an appreciable proportion of the total resistance of

the system. Therefore to abolish the potential difference across the in-

testinal wall a correction was made for the resistance between the electrodes and the tissue (46). The current - voltage relationship over the range of PD's encountered in our studies was measured in the absence of the intestine, and again when the tissue was mounted in the apparatus. The value of the true short circuit current and the potential at which the volt meter must be set in order to eliminate the potential across the gut wall were calculated from the two sets of current-voltage data, analyzed graphically.

The tissues were kept under short circuited conditions and the I_{sc} was opened briefly and the PD recorded after about a minute. One hundred to one hundred and forty minutes from the time that the animals were sacrificed, while PD and I_{sc} readings were relatively stable, ouabain (Aldrich Chemical Co) in a final concentration of 10^{-3} M was added to the serosal side of the tissue. PD and I_{sc} were monitored in 5 minutes intervals and the experiment discontinued when the electrical measurements reached a new stabilized level. The changes in PD and I_{sc} due to ouabain action were taken as the difference between the plateau levels. The results were expressed in mV for PD and μA for I_{sc}.

C. Results

1. Effect of T_3 on I_{sc} and transepithelial PD.

Figure 1-V illustrates electrical measurements recorded from one representative jejunal segment. I_{sc} and PD became relatively stable 20-30 minutes after the intestinal sheets were mounted in the chambers. Addition of ouabain caused an abrupt decrease, of about 70-80%, in I_{sc} and PD, that became relatively stable at the new lower level, after about 30 minutes.

The experiments on intestine from thyroidectomized rats $\pm$ T_3 were
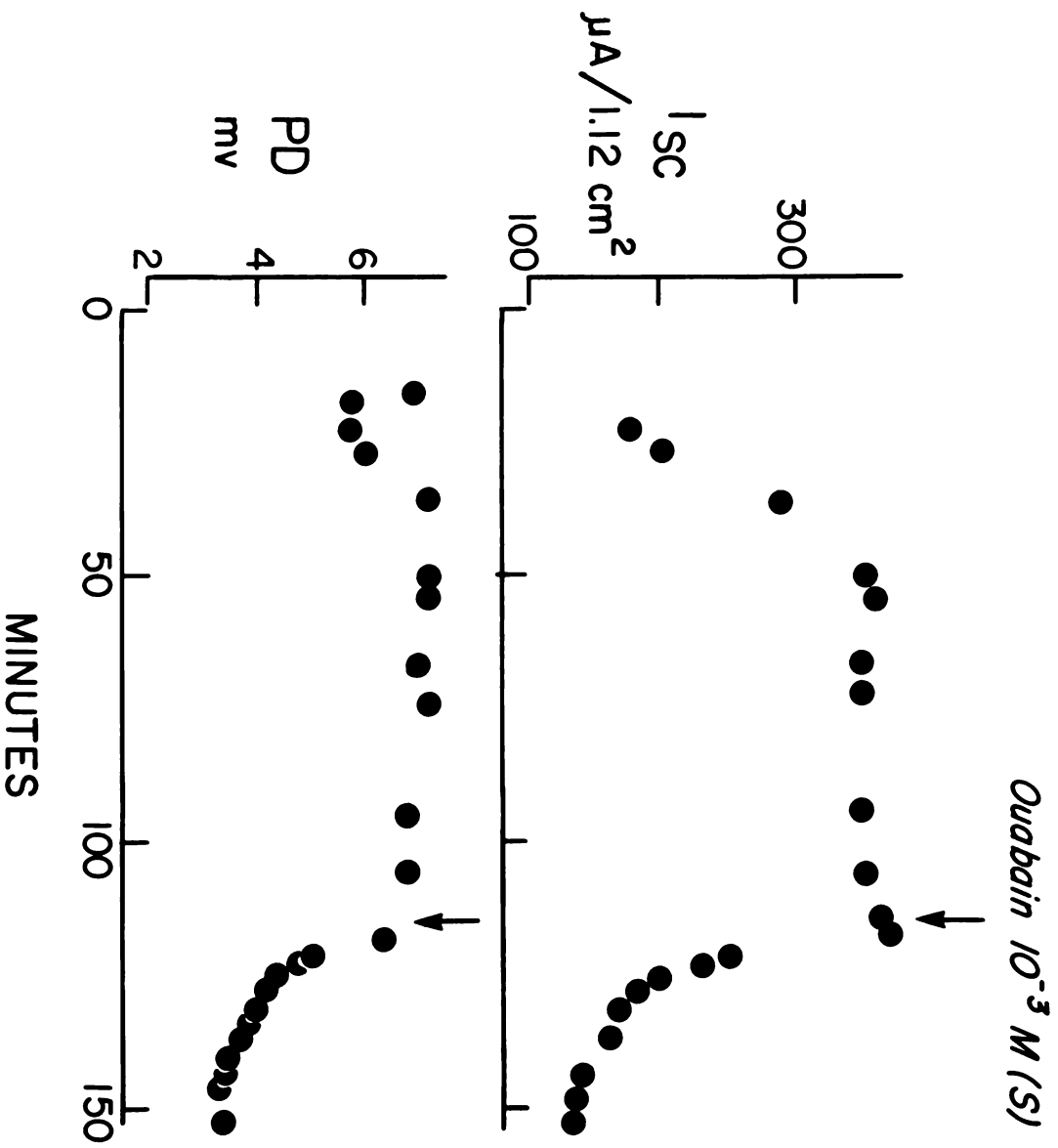
Figure 1-V. TRANSEPITHELIAL POTENTIAL DIFFERENCE (PD) AND SHORT-CIRCUIT CURRENT (I$_{sc}$) MEASUREMENT IN A STRIPPED JEJUNAL EPITHELIUM. OUABAIN IN A FINAL CONCENTRATION OF 10^{-3} M WAS ADDED AT THE TIME INDICATED

performed on separate dates, a few months apart. In the first set of ex-
periments 5 pair of rats (control and T_3 treated) were used, and in the
second set 8 pairs. No differences were found in the results obtained
from the two sets of experiments and therefore the experimental data were
pooled and treated as one population. In Table 1-V the measured I_{sc}'s
and PD's are presented. The changes in I_{sc} and PD after the addition of
ouabain are designated as ΔI_{sc} and ΔPD respectively and are expressed
in absolute units as well as a relative change. Administration of T_3 had
no effect on average I_{sc} or PD.

2. Effect of T_3 on jejunal crude plasma membrane preparation.

The rats used for the experiments cited in Chapters 2-4, as well as
in previous studies from this laboratory (11,42,72,73) were relatively
young, and weighed 120 to 250 g. To obtain adequate current/voltage read-
ings the lucite chambers were constructed to accomodate intestinal seg-
ments from rats that weighed 350 to 400 g. It is possible that the older
(∿ 400 g body wt) rats may not respond to thyroid hormone administration
in the same way that younger (100-250g) rats would. To test this possi-
bility, measurements were made of NaK-ATPase activity of crude plasma
membrane fractions prepared from older thyroidectomized rats, some of
whom were given T_3. Twenty-four rats with an average body weight of 400 g
were used. The protocol for T_3 administration was as mentioned in
Material and Methods of this Chapter. Jejunal crude plasma membrane
fractions were prepared and assayed for ATPase activity as described in the
Method section of Chapter III. Four fractions, two controls and two T_3-
treated were prepared simultaneously, and ATPase assays were completed on
the same day. The results are presented in Table 2-V. An increase of 57%
in NaK-ATPase specific activity, which is statistically highly significant,

Table 1-V   Potential Differences (PD) and Short Circuit Current (I_{sc}) Measurements of Stripped Jejunal Mucosa from Thyroidectomized Rats $\pm$ T_3 (50 µg per 100 g Body Weight).[*]

| | I_{sc} | ΔI_{sc} | | PD | ΔPD | |
	µA	µA	%	mV	mV	%
Thyroidectomized	245±17	186±14	77±2	4.3±0.2	3.2±0.2	77±3
Thyroidectomized + T_3	231±18	185±14	81±2	4.1±0.3	3.3±0.2	80±2
P	NS	NS	NS	NS	NS	NS

[*]Mean $\pm$ SE (n=13)

ΔI_{sc} and ΔPD represent the decrease of I_{sc} and PD respectively after the addition of ouabain.

Table 2-V ATPase Activity of Jejunal Crude Plasma Membrane Fractions

from Thyroidectomized Rats $\pm$ T_3 (50 µg per 100 g Body Weight)[*]

	Mg-ATPase	NaK-ATPase
	µmole Pi/hr per mg protein	
Hypothyroid	39.32 $\pm$ 1.04	7.59 $\pm$ 0.44
Hypothyroid + T_3	39.59 $\pm$ 0.92	11.94 $\pm$ 0.59
Δ	0.27 (1%)	4.35 (57%)
P	NS	< 0.001

[*]Mean $\pm$ SE (n=12)

was observed, and no changes in Mg-ATPase activity were demonstrated. These results are in the same range of changes observed in jejunal plasma membrane fractions obtained from younger rats, after thyroid hormone treatment (as discussed in chapter III).

D. Discussion

Administration of T_3 to thyroidectomized rats resulted in a profound increase in NaK-ATPase activity of jejunal plasma membrane fractions, and had no effect on I_{sc} or PD of rats of comparable sex and weight. One straight forward inference is that there is no association between NaK-ATPase activity and the transmembrane electrical measurements. An impressive body of evidence, however, contradicts this view: Na^+ extrusion from the cell into the serosal solution is an active process, inhibited by ouabain in the serosal fluid (103,144) as well as by metabolic inhibitors such as 2,4-diritrophenol and sodium fluoroacetate (19). Impressive evidence is also been adduced that the membrane bound $Na^+ + K^+$ stimulated, ouabain sensitive ATPase is the biochemical expression of the Na^+-pump (24,40,125,156,159). It is highly unlikely, therefore, that the increase in jejunal NaK-ATPase activity demonstrated after T_3 treatment does not reflect changes in Na^+ pump activity. A probable explanation for the dissociation between the effects on I_{sc}, PD and NaK-ATPase activity may be inferred from the anatomy of the intestinal epithelium. The measurements of transmural PD and I_{sc} treat the intestinal epithelium as a "black box" in which a single membrane separates two solutions. The small intestinal epithelium, however, is a mosaic structure, characterized by parallel inhomogenieties due to the presence of more than one type of cell and the shunt pathway, as well as by series inhomogenieties in that each cell is bounded by two different membranes, mucosal and basolateral. Transmural

PD values represent the integral difference in the mucosal and basolateral transmembrane potential differences of all of the cells in the area measured (19,103).

In the absence of an actively transported sugar or amino acids, the transepithelial PD in jejunum and ileum are very low, in the range of 1-3 mV (19,103,138). Thus even an increase of 50% in the PD will not be detectable. Furthermore, it should be stressed that in the presence of a high conductance shunt pathway, which accounts for approximately 90% of the total tissue conductance, a change in the electromotive force across the basolateral membranes, due to electrogenic active Na^+ extrusion will be considerably attenuated and fall out of the range of detectable measurements. In the absence of glucose in the media Munck has observed (111) that short circuited preparations of rat jejunum actively secrete Cl^- and absorb Na^+; the former accounts for approximately two-thirds of the total short-circuit current and the latter accounts for the remainder. Thus, if T_3 stimulated both Cl^- and Na^+ transport little change in I_{sc} or PD would result. In my studies, however, the measurements were made in the presence of 25mM D-glucose in the bathing media.

Actively transported sugars and amino acids stimulate Na^+ absorption across the epithelium of the small intestines of a variety of species both in vivo and in vitro (19,103,138,141). The presence of the actively transported sugars or amino acids in the mucosal solution apparently enhances the entry of Na^+ into the epithelial cells across the brush border and the Na^+ is then extruded from the cell by the ouabain-sensitive active transport mechanism that operates in the absence of nonelectrolytes (143). By application of intracellular recording techniques it has been shown that the 5-10 fold increase in transmural PD in the presence of an actively

transported sugar, in the rat jejunum, is due to an increase in the sero-
sal potential (19,103) and in the rabbit ileum to changes in mucosal po-
tential as well (138). In autoradiographic studies (84) in which everted
sacs of hamster jejunum were incubated in vitro with ^{14}C or ^{3}H labelled
sugars and amino/acids, it was observed that columnar absorptive cells at
tips of villi accumulated the radioactive material. These observations
suggest that only some of the epithelial cells are primary responsible
for sugar and amino acids transport. Thus thyroid hormone may affect
only one type of intestinal epithelial cells, such as the crypt and in-
tervillus cells (see Discussion, Chapter II). In the presence of glucose
in the bathing solution (as is the case in our experimental procedure)
the increase in transepithelial PD and I_{sc}, due to sugar absorption and
the associated enhancement of Na^{+} transport by the villus cells would
mask small changes in electrical parameters due to the effects of thyroid
hormone on crypt cells. An alternative possibility is that mucosal cells
sensitive to the action of thyroid hormone are not involved in transepith-
elial Na^{+} transport, and that the hormone acts only on the steady state
Na^{+}/K^{+} gradients of these cells by virtue of an effect on the Na^{+}-pump.
The magnitude of the observed changes in NaK-ATPase, after T_3 is such,
that an appreciable part of the epithelial cell population would have to
be excluded from involvement in transepithelial transport, which seems un-
likely.

An alternative possibility is that the hormone affects either directly
or indirectly more than one transport system. Munck (110) found that
transport of lysine across the rat jejunum is active and Na^{+} dependent,
and that the failure to observe a substantial increment in short circuit
current is due to a lysine-induced net Cl^{-} flux from mucosa to serosa.

If thyroid hormone simultaneously induces changes in Na^+ transport and other ion fluxes a dissociation between the effect on Na^+ flux and/or on PD and I_{sc} may occur.

The main part of the above discussion was dedicated to speculations in order to explain an experimental observation. There is no way to resolve the problem other than by additional experiments. Measurements of bidirectional Na^+ flux across the stripped jejunal mucosa from T_3 treated and untreated rats may point to one of the mechanisms postulated. These studies will be performed in the future.

VI. EFFECT OF T_3 ON SERUM ELECTROLYTES

A. Introduction

Changes in the extracellular concentration of electrolytes especially Na^+ and K^+ may affect transepithelial transport, both through the shunt pathway as well as the transcellular route, by affecting intracellular concentration and membrane potentials. Of special interest are the divalent cations Ca^{2+} and Mg^{2+}, alterations of which may lead to changes in the activity of the sodium pump. Ca^{2+} and Mg^{2+} compete with Na^+ for the sodium-activation site (80,95,123). Calcium alters potassium activation curves of NaK-ATPase so that the K_m for K^+ is decreased. It was of interest therefore, to find out if various thyroid states are accompanied by changes in extracellular electrolyte composition, and in particular Ca^{2+} and Mg^{2+} concentrations.

B. Materials and Methods

These experiments were carried out in collaboration with Dr. Y. Asano.

Euthyroid and thyroidectomized animals were used. The rats were injected with T_3, 50 µg/100 g body weight at 48 hours interval for 3 doses. Thyroid gland ablation by I^{131} was achieved as described by Asano et al (11). Twenty-four to 48 hours after the third injection of T_3 or diluent the animals were anesthetized with inactin (see Methods II). Blood was collected in dry test tubes by percutaneous or open cardiac puncture. The blood was allowed to clot at room temperature and the supernatant serum was analyzed for Na^+, K^+, Ca^{2+}, PO_4^{3-}, total protein and albumin with the Technicon Auto-Analyzer, and for Cl^- by the method of Zall et al. (194).

C. Results and Discussion

Serum Na^+, K^+, and Cl^- concentrations were determined in rats of varying thyroid status and the results are given in Table 1-VI. Injection

Table 1-VI   Effect of T_3 on Serum Electrolytes Concentrations of

Thyroidectomized and Euthyroid Rats.[*]

	$[Na^+]$	$[K^+]$	$[Cl^-]$
		mEg/liter	
Thyroidectomized	145 ± 5 (14)	4.4 ± 0.3 (15)	104 ± 2 (13)
Thyroidectomized + T_3	147 ± 5 (16)	4.3 ± 0.5 (12)	103 ± 3 (14)
P	NS	NS	NS
Euthyroid	144 ± 3 (21)	4.6 ± 0.3 (11)	100 ± 3 (18)
Euthyroid + T_3	145 ± 3 (16)	4.7 ± 0.4 (10)	102 ± 2 (13)
P	NS	NS	NS

[*]Mean ± SD (n in brackets).

of T_3 had no significant effects on serum levels of these electrolytes.
In addition, no difference was observed between the average concentrations
of Na^+, K^+ and Cl^- of serum of euthyroid and thyroidectomized rats. Serum
Ca and P levels in euthyroid and two groups of hypothyroid rats with and
without T_3 treatment are presented in Table 2-VI. In athyroid animals
serum Ca was about 8% less than in euthyroid rats; these differences were
statistically significant. During surgical thyroidectomy only two para-
thyroid glands were left in place and these probably may have been trau-
matized resulting in a partial parathyroid insufficiency. The lower serum
P observed in hypothyroid animals does not support this hypothesis how-
ever. In hypoparathyroidism, while serum Ca is low serum P is above the
normal range. However, the finding that serum P is as low in the I^{131}
treated group as well (in which hypoparathyroidism is not apparent), may
point to some other mechanism accounting for the decrease in serum P in
the hypothyroid state. Injection of T_3 had no effect on serum Ca in
euthyroid rats. In contrast, a marked decrease, of about 20%, in serum
calcium was observed when T_3 was injected into thyroidectomized rats.
Several explanations for this effect may be considered. Thyroid hormone
inhibits calcium absorption from the gastrointestinal tract (121), in-
creases urinary excretion of calcium and accelerates bone turnover. The
sum of these effects will tend to lower serum calcium and to impose a
stimulus for parathyroid hormone (PTH) secretion. If a partial insuffi-
ciency of the parathyroid glands exists, serum Ca will remain below the
normal level. The elevation in serum P observed in the same animals sup-
ports this explanation. It has to be noted however, that a similar in-
crease, of about 100%, in serum P was documented in the I^{131} treated
group, as well as in the euthyroid animals though to a lesser extent in

Table 2-VI Serum Calcium and Phosphorus Levels in Euthyroid and Hypothyroid Rats $\pm$ T$_3$ (50 μg/100 g Body Wt)[#]

	Ca	P	No. of Animals
	mg/100 ml		
Euthyroid	10.0 ± 0.2	8.7 ± 0.4	(11)
Euthyroid + T$_3$	9.9 ± 0.2	9.9 ± 0.4	(11)
Δ	-0.1	+1.3	
p	NS	< 0.05	
Hypothyroid (Surgical)	9.2 ± 0.2*	6.4 ± 0.2	(21)
Hypothyroid (Surgical) + T$_3$	7.4 ± 0.4	12.9 ± 0.7	(21)
Δ	-1.8	+6.5	
p	< 0.001	< 0.001	
Hypothyroid (I^{131}-Treated)	9.4 ± 0.3+	6.5 ± 0.3	(11)
Hypothyroid (I^{131}-Treated) + T$_3$	8.8 ± 0.3	12.2 ± 0.8	(11)
Δ	-0.6	+5.7	
p	NS	< 0.001	

[#]Mean ± SE (n in brackets)
*Significant difference from euthyroid control mean (p 0.05). + Not significant difference from euthyroid control.

the latter group. These findings suggest that parathyroid insufficiency is not the only mechanism that elevates serum P after thyroid hormone administration. Another factor that contributes to the change in serum Ca after administration of T_3 to thyroidectomized rats is the decrease of about 30%, in serum albumin concentration (Table 3-VI). It is well established that about 50% of serum Ca is bound to albumin. Ionized calcium accounts for about 40% of the total serum calcium, but is the physiologically important species of Ca and the signal for the feed-back loop that controls serum Ca levels (130). Any change in serum albumin will affect total serum Ca but ionized serum calcium levels should remain unchanged. Thus, a part of the fall in serum calcium in the thyroidectomized rats is attributable to changes in serum protein concentrations and probably all of the relatively small change in total serum calcium observed in the ^{131}I treated rats (Table 2-VI) results from the effect of T_3 on serum albumin.

There is no good explanation for the increased serum P observed after administration of T_3 to either euthyroid or hypothyroid rats (Table 2-VI). The finding that 24 hours of starvation had no effect on the change in serum P after thyroid hormone treatment, doesn't support the idea of enhanced gastrointestinal absorption of phosphate but does not exclude this explanation completely, because of the availability of phosphate from the drinking water and gastrointestinal secretions. However, Noble and Matty (121) observed a lower rate of phosphate absorption from the gut after thyroid hormone administration.

These observations imply that thyroidectomy is attended by damage to the remaining parathyroid glands and produces changes in Ca metabolism that are aggravated by T_3 injection.

Table 3-VI   Serum Protein Levels in Thyroidectomized Rats $\pm$ T_3

(50 μg per 100 g Body Weight).*

	Total Protein	Albumin	Globulin
		g/100 ml	
Thyroidectomized	7.4 $\pm$ 0.2	4.2 $\pm$ 0.2	3.2 $\pm$ 0.2
Thyroidectomized + T_3	5.6 $\pm$ 0.1	2.9 $\pm$ 0.1	2.7 $\pm$ 0.2
Δ	1.8	1.3	0.5
p	< 0.001	< 0.01	NS

*Mean $\pm$ SE. (n = 5 and 6 for thyroidectomized and thyroidectomized + T_3 respectively).

Asano and I assessed the possibility that the changes in QO_2, $QO_2(t)$ and NaK-ATPase activity observed after T_3 administration to thyroidectomized rats are secondary to changes in Ca metabolism. We used skeletal muscle as a model target organ in these studies. Athyroid animals were prepared by ^{131}I injection, as described above and produced almost no changes in serum Ca levels. The effect of T_3 on QO_2, $QO_2(t)$ and NaK-ATPase activity of plasma membrane fractions from skeeltal muscle were measured. The results were identical to those obtained in thyroidectomized rats $\pm$ T_3. Thus, the changes observed in QO_2, $QO_2(t)$ and NaK-ATPase activity after thyroid hormone administration to thyroidectomized rats, are not attributable to changes in serum Ca concentrations. Furthermore, thyroid hormone elicits the same effects (qualitatively) in euthyroid rats, in which no changes in Ca metabolism were demonstrated. The problem of changes in phosphate metabolism and its relationship to thyroid hormone action is yet unresolved and will have to be clarified by additional experiments.

REFERENCES

1. Albers, R.W., G.J. Koval, and G.J. Siegel. Studies on the interaction and other cardioactive steroides with sodium-potassium-activated adenosine triphosphatase. Mol. Pharmacol. 4:324-336, 1968.

2. Adair, S.F., R. Vreeland, and L.Z. Benet. To be published.

3. Akera, T., F.S. Larsen, and T.M. Brody. The effect of ouabain on sodium and potassium activated adenosine triphosphatase from the hearts of several mammalian species. J. Pharmacol. Exp. Ther. 170:17-26, 1969.

4. Allen, J.C., R.A. Harris, and A. Schwartz. The nature of the transport ATPase-digitalis complex. I. Formation and reversibility in the presence and absence of a phosphorylated enzyme. Biochem. Biophys. Res. Commun. 42:366-370, 1971.

5. Allen, J.C., R.A. Harris, and A. Schwartz. The nature of the transport ATPase-digitalis complex. II. Some species differences and ouabain "exchange" characteristics. J. Mol. Cell. Cardiol. 3:297-300, 1971.

6. Allen, J.C., G.E. Lindenmayer, and A. Schwartz. An allosteric explanation for ouabain induced time-dependent inhibition of sodium, potassium-adenosine triphosphatase. Arch. Biochem. Biophys. 141:322-328, 1970.

7. Allen, J.C., and A. Schwartz. A possible biochemical explanation for the insensitivity of the rat to cardiac glycosides. J. Pharmacol. Exp. Ther. 168:42-46, 1969.

8. Almendares, J.A., and A. Kleinzeller. The ouabain inhibition of sugar transport in kidney cortex cells. Arch. Biochem. Biophys. 145:511-519, 1971.

9. Althausen, T.L. Hormonal and vitamin factors in intestinal absorption. Gastroenterology 12:467-480, 1949.

10. Anderson, J.W., and D. Zakim. The influence of alloxan-diabetes and fasting on glycolytic and gluconeogenic enzyme activities of rat intestinal mucosa and liver. Biochem. Biophys. Acta. 201:236-241, 1970.

11. Asano, Y., U.A. Liberman and I.S. Edelman. In preparation.

12. Babior, B.M., S. Creagan, S.H. Ingbar, and R.S. Kipnes. Stimulation of mitochondrial adenosine diphosphate uptake by thyroid hormones. Proc. Natl. Acad. Sci. U.S.A. 70:98-102, 1973.

13. Bader, H., R.L. Post, and G.H. Bond. Comparison of sources of a phosphorylated intermediate in transport ATPase. Biochim. Biophys. Acta 150:41-46, 1968.

14. Bader, H., and A.K. Sen. (K^+)-dependent acyl phosphatase as a part of the $(Na^+ + K^+)$ dependent ATPase of cell membranes. Biochim. Biophys. Acta 118:116-123, 1966.

15. Bamford, D.R., and J.E. Holmes. Regional variation in the aerobic metabolism of intestinal mucous membrane. Comp. Biochem. Physiol. 38A:603-610, 1971.

16. Barker, S.B. Effect of triiodothyromine on oxygen consumption of tissues not responsive to thyroxine. Proc. Soc. Exp. Biol. Med. 90:109-111, 1955.

17. Barker, S.B. Physiological activity of thyroid hormones and analogues. In: The Thyroid Gland. Ed. R. Pitt-Rivers and W.R. Trotter. Vol. I:199-236. Butterworths, Washington 1964.

18. Barnett, R.E. Effect of monovalent cations on the ouabain inhibition of the sodium and potassium ion activated adenosine triphosphatase. Biochem. 9:4644-4648, 1970.

19. Barry, R.J.C., and J. Eggenton. Membrane potentials of epithelial cells in rat small intestine. J. Physiol. 227:201-216, 1972.

20. Barry, R.J.C., D.H. Smyth, and E.M. Wright. Short circuit current and solute transfer by rat jejunum. J. Physiol. (Lond.) 181: 410-431, 1965.

21. Baumann, E. Uber das thyrojodin. Munch. Med. Wschr. 43:309-312, 1896.

22. Berg, G.G., and Chapman, B. The sodium and potassium activated ATPase of intestinal epithelium. 1. Location of enzymatic activity in the cell. J. Cell. Comp. Physiol. 65:361-372, 1965.

23. Blond, D.M., and R. Whittam. The regulation of kidney respiration by sodium and potassium ions. Biochem. J. 92:158-167, 1964.

24. Bonting, S.L., L.L. Caravaggio, and N.M. Hawkins. Studies on sodium-potassium-activated adenosinetriphosphatase. IV. Correlation with cation transport sensitive to cardiac glycoside. Arch. Biochem. Biophys. 98:413-419, 1962.

25. Booth, C.C. Effect of location along the small intestine on absorption of nutrients. Handbook of Physiology, Section 6: Alimentary Canal (Edited by Heidel W.) III, 1513-1529, 1968.

26. Boyd, C.A.R., D.S. Parsons, and A.V. Thomas. The presence of K^+-dependent phosphatase in intestinal epithelial cell brush borders isolated by a new method. Biochim. Biophys. Acta 150:723-726, 1968.

27. Braverman, L.E., S.H. Ingbar, and K. Sterling. Conversion of thyroxine (T_4) to triiodothyronine in athyreotic human subjects. J. Clin. Invest. 49:855-864, 1970.

28. Bronk, J.R., and D.S. Parsons. The polarographic determination of
    the respiration of the small intestine of the rat. Biochim.
    Biophys. Acta 107:397-404, 1965.

29. Bronk, J.R., and D.S. Parsons. Influence of the thyroid gland on
    the accumulation of sugars in rat intestinal mucosa during
    absorption. J. Physiol. 184:942-949, 1965.

30. Burton, K. A study of the conditions and mechanism of the diphenyl-
    amine reaction for the calorimetric estimation of DNA. Biochem.
    J. 62:315-323, 1956.

31. Clarkson, T.W., and A. Rothstein. Transport of monovalent cations
    by the isolated small intestine of the rat. Am. J. Physiol.
    199:898-906, 1960.

32. Clarkson, T.W., and S.R. Toole. Measurement of short-circuit current
    and ion transport across the ileum. Amer. J. Physiol. 206:
    658-668, 1964.

33. Creamer, B. The turnover of the epithelium of the small intestine.
    Brit. Med. Bull. 23:226-230, 1967.

34. Csaky, T.Z. A possible link between active transport of electrolytes
    and nonelectrolytes. Fed. Proc. 22:3-7, 1963.

35. Curling, T.B. Two cases of absence of the thyroid body and symmet-
    rical swellings of fat tissue at the sides of the neck, connected
    with defective cerebral development. Trans. Med. Chir. London
    33:303-306, 1850.

36. Dickens, F., and G. Weil-Malherbe. Metabolism of normal and tumor
    tissue. The metabolism of intestinal mucos membrane. Biochem.
    J. 36:7-16, 1941.

37. Doluisio, J.T., G.H. Tan, N.F. Billups, and L. Diamond. Drug absorp-
    tion II. Effect of fasting on intestinal drug absorption. J.
    Pharm. Sci. 58:1200-1202, 1969.

38. Douglas, J.E. Thyroxine induced alterations in the fine structure
    of rat liver. John Hopkins Hosp. Bull. 114:253-265, 1964.

39. Dunham, P.B., and J.F. Hoffman. Partial purification of the ouabain-
    binding component and of Na, K-ATPase from human red cell mem-
    branes. Proc. Nat. Acad. Sci. 66:936-943, 1970.

40. Dunham, E.T., and I.M. Glynn. Adenosinetriphosphatase activity and
    the active movements of alkali metal ions. J. Physiol. 156:
    274-293, 1961.

41. Edelman, I.S. Thyroid thermogenesis. New Eng. J. Med. 290:1303-
    1308, 1974.

42. Edelman, I.S. and F. Ismail-Beigi. Role of ion transport in thyroid
    calorigenesis. In: Proceedings of the International Symposium
    on Environmental Physiology. Bioenergetics. Dublin. Ireland.
    July 1971. FASEB 1972. Robert E. Smith, J.P. Hannor, J.L.
    Shields and B.A. Horvvitz, eds. pp 67-70.

43. Emmelot, P., C.J. Bos, E.L. Benedetti, and Ph. Rumke. Studies on
    plasma membranes. I. Chemical composition and enzyme content of
    plasma membranes isolated from rat liver. Biochim. Biophys.
    Acta 90:126-145, 1964.

44. Fahns, S., G.J. Koval, and R.W. Albers. Sodium-potassium-activated
    adenosine triphosphatase of electrophorus electric organ. I.
    An associated sodium-activated trans-phosphorylation. J. Biol.
    Chem. 243:1993-2002, 1968.

45. Fairhurst, T.A.S., J.C. Roberts, and R.E. Smith. Effect of physio-
    logical levels of thyroxine in vivo on respiration and phosphory-
    lation in rat liver fractions. Amer. J. Physiol. 197:370-376,
    1959.

46. Field, M., D. Fromm, and I. McColl. Ion transport in rabbit ileal
    mucosa. I. Na and Cl fluxes and short-circuit current. Am. J.
    Physiol. 220:1388-1396, 1971.

47. Fiske, C.R., and Y. Subbarow. The colorimetric determination of
    phosphorous. J. Biol. Chem. 66:375-400, 1925.

48. Fletcher, K., and N.B. Myant. Partial reversal of the effects of
    thyroxine on lipid synthesis in rat liver by the addition of
    cofactors in vitro. J. Physiol. 157:542-564, 1961.

49. Fletcher, K., N.B. Myant, and D.D. Tyler. The influence of thyroid
    hormone upon the metabolism of adenosine triphosphate in rat
    liver. J. Physiol. 162:345-357, 1962.

50. Forstner, G.G., S.M. Sabesin, and K. J. Isselbacher. Rat intestinal
    microvillus membranes. Purification and biochemical characteri-
    zation. Biochem. J. 106:381-390, 1968.

51. Frizzell, R.A., L. Markscheid-Kaspi, and S.G. Schultz. Oxidative
    metabolism of rabbit ileal mucosa. Am. J. Physiol. 226:1142-
    1148, 1974.

52. Frizzell, R.A., N.H. Nellans, R.C. Rose, L. Markscheid-Kaspi, and
    S.G. Schultz. Intracellular Cl concentrations and influxes
    across the brush border of rabbit ileum. Am. J. Physiol. 224:
    328-337, 1973.

53. Frizzell, R.A., and S.G. Schultz. Ionic conductances of extracel-
    lular shunt pathway in rabbit ileum: Influence of shunt on
    transmural sodium transport and electrical potential differences.
    J. Gen. Physiol. 59:318-346, 1972.

54. Frizzell, R.A., and S.G. Schultz. Ionic conductances of extracellular shunt pathway in rabbit ileum: Influence of shunt on transmural sodium transport and electrical potential differences. J. Gen. Physiol. 59:318-346, 1972.

55. Fujita, M., H. Ohata, K. Kawai, H. Matsui, and M. Nakao. Differential isolation of microvillus and basolateral plasma membranes from intestinal mucosa: Mutually exclusive distribution of digestive enzymes and ouabain-sensitive ATPase. Biochim. Biophys. Acta 274:336-347, 1972.

56. Glynn, I.M. The action of cardiac glycosides on sodium and potassium movements in human red cells. J. Physiol. 136:148-173, 1957.

57. Gross, J., and R. Pitt-Rivers. Unidentified iodine compounds in human plasma in addition to thyroxine and iodide. Lancet 2:766-767, 1951.

58. Gross, J., and R. Pitt-Rivers. The identification of 3,5,3'-L-triiodothyronine in human plasma. Lancet 1:439-441, 1952.

59. Gross, J., and R. Pitt-Rivers. 3,5,3'-triiodothyronine. Isolation from thyroid gland and synthesis. Biochem. J. 53:645-650, 1953.

60. Gull, W.W. On a cretinoid state supervening in adult life in women. Trans. Clin. Soc. London. 7:180-185, 1874.

61. Gustafsson, R., J.R. Tata, O. Lindberg, and L. Ernster. The relationship between the structure and activity of rat skeletal muscle mitochondria after thyroidectomy and thyroid hormone treatment. J. Cell Biol. 26:555-578, 1965.

62. Hansen, O. The relationship between G-Strophantin-binding capacity and ATPase activity in plasma-membrane fragments from ox brain. Biochim. Biophys. Acta 233:122-132, 1971.

63. Harington, C.R., and G. Barger. Chemistry of thyroxine. III. Constitution and synthesis of thyroxine. Biochem. J. 21:169-183, 1927.

64. Hepner, G.W., and A.F. Hofmann. Different effects of free and conjugated bile acids and their keto derivatives on (Na^+, K^+)-stimulated and Mg^{2+}-ATPase of rat intestinal mucosa. Biochim. Biophys. Acta 291:237-245, 1973.

65. Hindmarsh, J.T., D. Kilby, B. Ross, and G. Wiseman. Further studies on intestinal active transport during semistarvation. J. Physiol. 188:207-218, 1967.

66. Hoch, F.L., and F. Lipmann. The uncoupling of respiration and phosphorylation by thyroid hormones. Proc. Natl. Acad. Sci. 40:909-921, 1954.

67. Hoffman, J.F. The red cell membrane and the transport of sodium and
    potassium. Amer. J. Med. 41:666-680, 1966.

68. Hafkenscheid, J.C.M. Occurence and properties of a (Na^+-K^+)-activated
    ATPase in the mucosa of the rat intestine. Pflugers Arch.
    338:289-294, 1973.

69. Hokin, L.E., P.S. Sastry, P.R. Galsworthy, and A. Yoda. Evidence
    that a phosphorylated intermediate in a brain transport adeno-
    sine triphosphatase is on a cyl phosphate. Proc. Nat. Acad.
    Sci. U.S. 54:177-184, 1965.

70. Hopper, A.F., R.W. McGovern, and R.W. Wannemacker. Cell population
    changes in the intestinal epithelium of the rat following star-
    vation and protein depletion. Proc. Soc. Exptl. Biol. Med.
    128:695-698, 1968.

71. Ingbar, S.H., and K.A. Woeber. The Thyroid Gland In: Text Book of
    Endocrinology. Ed. R.H. Williams. W.B. Saunders Company.
    Philadelphia. London. Toronto. p. 105-286. 1968.

72. Ismail-Beigi, F., and I.S. Edelman. Mechanism of thyroid calori-
    genesis: role of active sodium transport. Proc. Nat. Acad.
    Sci. USA 67:1071-1078, 1970.

73. Ismail-Beigi, F., and I.S. Edelman. The mechanism of the calori-
    genic action of thyroid hormone. J. Gen. Physiol. 57:710-722,
    1971.

74. Ismail-Beigi, F., and I.S. Edelman. Effects of thyroid status on
    electrolyte distribution in rat tissues. Am. J. Physiol.
    225:1172-1177, 1973.

75. Ismail-Beigi, F., and I.S. Edelman. Time Course of the effects of
    thyroid hormone on respiration and $Na^+ + K^+$-ATPase activity in
    rat liver. Proc. Soc. Exptl. Biol. & Med. 146:983-988, 1974.

76. Ismail-Beigi, F., A. Salibian, E. Kirsten, and I.S. Edelman. Effects
    of thyroid hormone on adenine nucleotide content of rat liver.
    Pro. Soc. Exp. Biol. Med. 144:471-474, 1973.

77. Jasper, D.K., and J.R. Bronk. Studies on the physiological and
    structural characteristics of rat intestinal mucosa.
    Mitochondrial structural changes during amino acid absorption.
    J. Cell Biol. 38:277-291, 1968.

78. Jones, J.K., F. Ismail-Beigi, and I.S. Edelman. Rat liver adenyl
    cyclase activity in various thyroid states. J. Clin. Invest.
    51:2498-2501, 1972.

79. Jorgensen, P.L., and J.C. Skou. Purification and characterization of $(Na^+ + K^+)$-ATPase. I. The influence of detergents on the activity of $(Na^+ + K^+)$-ATPase in preparation from the outer medulla of the rabbit kidney. Biochim. Biophys. Acta 233: 366-380, 1971.

80. Judah, J.D., and K. Ahmed. The biochemistry of sodium transport. Biol. Rev. 39:160-193, 1964.

81. Kendall, E.C. The isolation in crystalline form of the compound containing iodine which occurs in the thyroid. Its chemical nature and physiological activity. Trans. A. Am. Physicians. 30:420-447, 1915.

82. Kershaw, T.G., K.D. Neame, and G. Wiseman. The effect of semistarvation on absorption by the rat small intestine in vitro and in vivo. J. Physiol. 153:182-190, 1960.

83. Kim, K.H., and P.P. Cohen. Actinomycin D inhibition of thyroxine-induced synthesis of carbamyl phosphate synthetase. Biochem. Biophys. Acta 166:574-577, 1968.

84. Kinter, W.B., and T.H. Wilson. Autoradiographic study of sugar and amino acid absorption by everted sacs of hamster intestine. J. Cell Biol. 25:19-39, 1965.

85. Klingenberg, M. The respiratory chain. In: Biological Oxidation. Ed. T.P. Singer. p. 3. Interscience Publishers. 1968.

86. Kocher, T. Ueber kropfextirpation und thihre folgen. Arch. Klin. Chir. 29:254-337, 1883.

87. Koopman, W., and S.G. Schultz. The effect of sugars and amino acids on mucosal Na and K concentrations in rabbit ileum. Biochim. Biophys. Acta 173:338-340, 1969.

88. Krebs, H.A., and K. Henseleit. Untersuchungen uber die Harnstoffbildung im Tierkorper. Hoppe-Seyler's Z. Physiol. Chem. 210:33-36, 1932.

89. Kyte, J. Purification of the sodium- and potassium-dependent adenosine triphosphatase from canine renal medulla. J. Biol. Chem. 246:4157-4165, 1971.

90. Langer, P. History of goitre. In: Endemic Goitre. Geneva, World Health Organization. 1960.

91. Lardy, H.A., and G. Feldott. Metabolic effects of thyroxine in vitro. Ann. N.Y. Acad. Sci. 54:636-648, 1951.

92. Lehninger, A.L. Bioenergetics, the molecular basis of biological energy transformations. W.A. Benjamin, Inc. New York. 1965.

93. Levin, R.J. The effects of hormones on the absorbtive, metabolic and digestive functions of the small intestine. J. Endocr. 45:315-348, 1969.

94. Levin, R.J., and D.H. Smyth. The effect of the thyroid gland on intestinal absorption of hexoses. J. Physiol. 169:755-769, 1963.

95. Lindenmayer, G.E., and A. Schwartz. Sodium-calcium competition on molecular level: Function in glycoside-induced inotropism. Circulation 41-42, Suppl. III, 112, 1970.

96. Lindenmayer, G.E., and A. Schwartz. Conformational changes induced in Na^+ K^+-ATPase by ouabain through a K^+-sensitive reaction: Kinetic and spectroscopic studies. Arch. Biochem. Biophys. 140:371-378, 1970.

97. Lishko, V.K., M.K. Malysheva, and T.I. Grevizirskaya. The interaction of the (Na^+, K^+)-ATPase of erythrocyte ghosts with ouabain. Biochim. Biophys. 288:103-106, 1972.

98. Lo, C.S., and I.S. Edelman. In preparation.

99. Loehry, C.A., D.N. Croft, A.K. Singh and B. Creamer. Cell turnover in the rat small intestinal mucosa: An appraisal of cell loss. Gut. 10:13-18, 1969.

100. Loomis, W.F., and F. Lipmann. Reversible inhibition of the uncoupling between phosphorylation and oxidation. J. Biol. Chem. 173:807-808, 1948.

101. Lowry, O.H., and J.A. Lopez. The determination of inorganic phosphate in the presence of labile phosphate esters. J. Biol. Chem. 162:421-428, 1946.

102. Lowry, O.H., N.J. Rosebrough, A.L. Farr, and R.J. Randall. Protein measurements with folin phenol reagent. J. Biol. Chem. 193: 267-275, 1951.

103. Lyon, I., and H.E. Sheerin. Studies on transmural potentials in vitro in relation to intestinal absorption. IV. The effect of sugars on electrical potential profiles in jejunum and ileum. Biochim. Biophys. Acta 249:1-14, 1971.

104. Magnus-Levy, A. Ueber den respiratorischen gaswechsel unter dem einfluss der thyroidea sowie unter verschiedenen pathologische zustanden. Berl. Klin. Wochenschr. 32:650-652, 1895.

105. Major, R.H. Classic Descriptions of Disease. 3rd Edition. Oxford. Blackwell. 1945.

106. Marcus, G.J., and J.F. Manery. Potassium stimulation of oxidation and phosphorylation in pigeon-muscle mitochondria. Can. J. Biochem. 44:1127-1144, 1966.

107. Matsui, H., and A. Schwartz. Mechanism of cardiac glycoside inhibition of the $(Na^+ + K^+)$-dependent ATPase from cardiac tissue. Biochim. Biophys. Acta. 151:655-663, 1968.

108. Medzihradsky, F., M.H. Kline, and L.E. Hokin. Studies on the characterization of the sodium-potassium transport adenosinetriphosphatase. 1. Solubilization, stabilization and estimation of apparent molecular weight. Arch. Biochem. Biophys. 121:311-316, 1967.

109. McManus, J.P.A., and K.J. Isselbacher. Effect of fasting versus feeding on the rat small intestine. Gastroenterology. 59:214-221, 1970.

110. Munck, B.G. Interactions between lysine, Na^+ and Cl^- transport in rat jejunum. Biochim. Biophys. Acta 203:424-433, 1970.

111. Munck, B.G. Effects of sugar and amino acid transport on transepithelial fluxes of sodium and chloride of short circuited rat jejunum. J. Physiol. 223:699-717, 1972.

112. Murphy, B.E.P., and C. Jachan. Determination of thyroxine by competitive protein binding analysis employing an anion-exchange resin and radiothyroxine. J. Lab. Clin. Med. 66:161-167, 1965.

113. Murphy, B.E.P., and C.J. Pattee. Determination of thyroxine utilizing the property of protein binding. J. Clin. Endocr. Metabol. 24:187-196, 1964.

114. Murray, G.R. Note on the treatment of myxoedema by hypodermic injections of an extract of the thyroid gland of a sheep. Brit. Med. J. 2:796-797, 1891.

115. Martius, C., and B. Hess. The mode of action of thyroxine. Arch. Biochem. Biophys. 33:486-487, 1951.

116. Nellans, N.H., R.A. Frizzell, and S.G. Schultz. Coupled sodium-chloride influx across the brush border of rabbit ileum. Am. J. Physiol. 225:467-475, 1973.

117. Nellans, H.N., R.A. Frizzell, and S.G. Schultz. Brush border processes and transepithelial Na and Cl transport by rabbit ileum. Am. J. Physiol. 226:1131-1141, 1974.

118. Newey, H., P.A. Sanford, and D.H. Smyth. Effects of fasting on intestinal transfer of sugars and amino acids in vitro. J. Physiol. 208:705-724, 1970.

119. Niemeyer, H., R.K. Crane, P. Kennedy, and F. Lipmann. Action of thyroids on oxygen consumption and phosphorylation by mito-

chondria isolated from rat liver. Santiago, Chile. Bol. Soc. Biol. 10:54-58, 1953.

120. Nishikawara, M.T. Hexokinase and phosphatase activity of intestinal mucosa following thyroidectomy and thyroid administration. Endocrinology. 68:850-854, 1961.

121. Noble, H.M., and A.J. Matty. The effect of thyroxine on the movement of calcium and inorganic phosphate through the small intestine of the rat. J. Endocr. 37:111-117, 1967.

122. Ord, W.M. On myxoedema, a term proposed to be applied to an essential condition in the "cretinoid" affection occasionally observed in middle-aged women. Trans. Med. Chir. London. 61: 57- , 1878.

123. Portius, H.J., and K.R.H. Repke. Eigenschaften und funktion des $Na^+ + K^+$-aktivierten, Mg^{++}-abhangigem adenosintriphosphat phosphohydrolase-systems. Acta Biol. Med. Ger. 19:907-938, 1967.

124. Post, R.L., S. Kume, T. Tobin, B. Orcutt, and A.K. Sen. Flexibility of an active center in sodium plus potassium adenosine triphosphatase. J. Gen. Physiol. 54:306-326, 1969.

125. Post, R.L., C.R. Merrit, C.R. Kinsolving, and C.D. Albright. Membrane adenosine triphosphatase as a participant in the active transport of sodium and potassium in the human erythrocyte. J. Biol. Chem. 235:1796-1802, 1960.

126. Post, R.L., A.K. Sen, and A.S. Rosenthal. A phosphorylated intermediate in adenosine triphosphate-dependent sodium and potassium transport across kidney membranes. J. Biol. Chem. 240:1437-1445, 1965.

127. Quigley, J.P., and G.S. Gotterer. Properties of a high specific activity, $(Na^+ - K^+)$ stimulated ATPase from rat intestinal mucosa. Biochim. Biophys. Acta 173:469-476, 1969.

128. Quigley, J.P., and G.S. Gotterer. Distribution of $(Na^+ - K^+)$-stimulated ATPase activity in rat intestinal mucosa. Biochim. Biophys. Acta. 173:456-468, 1969.

129. Quigley, J.P., and G.S. Gotterer. A comparison of the $(Na^+ - K^+)$-ATPase activities found in isolated brush border and plasma membrane of the rat intestinal mucosa. Biochim. Biophys. Acta 255:107-113, 1972.

130. Rasmussen, H. The parathyroids. In: Text Book of Endocrinology. Ed. R.H. Williams. W.B. Saunders Company. Philadelphia. London. Toronto. p. 847-965, 1968.

131. Reiss, J.M., and A. Wyatt. Action of thyroid hormone on brain metabolism of newborn rats. Proc. Soc. Exp. Biol. Med. (N.Y.) 93:19-22, 1956.

132. Repke, K. Effects of digitalis on membrane adenosine triphospha-
     tase of cardiac muscle. In Proceeding of the Second Interna-
     tional Pharmacological Meeting, Vol. 4, Drugs and Enzymes, ed.
     by B.B. Brodie and J.R. Gillette, p. 65-88. Pergamon Press,
     London, 1965.

133. Repke, K., M. Est, and H.J. Portius. Ueber die ursache der
     speciesunterschiede in der digitalis empfindlichkeit.
     Biochem. Pharmacol. 14:1785-1802, 1965.

134. Reverdin, J.L. Communication sur les accidents consecutifs a
     l'ablation totale du goitre. Rev. Med. Suisse Rom. 2:530-540,
     1882.

135. Richardson, S.H. The ion translocase system from rabbit intestinal
     mucosa preparation and properties of the (Na^+-K^+)-activated
     ATPase. Biochim. Biophys. Acta 150:572-577, 1968.

136. Robinson, J.W.L. The difference in sensitivity to cardiac steroides
     of (Na^++K^+)-stimulated ATPase and amino acid transport in the
     intestinal mucosa of the rat and other species. J. Physiol.
     206:41-60, 1970.

137. Rohrer, A. Vergleich des sauerstoffverbrauchs uber lebenden
     saugtierorgane in normalen zustande und nach futtering mit
     schieddrusenhormon. Biochem. Zeitschmift. 145:154-167, 1924.

138. Rose, R.S., and S.G. Schultz. Studies on the electrical potential
     profile across rabbit ileum: Effects of sugars and amino acids
     on transmural and transmucosal electrical potential differences.
     J. Gen. Physiol. 57:639-663, 1971.

139. Sachs, G., J.D. Rose and B.I. Hirschowitz. Acetyl phosphatase in
     brain microsomes: A partial reaction of $Na^+ + K^+$ ATPase. Arch.
     Biochem. Biophys. 119:277-281, 1967.

140. Schiff, M. Resume d'une s'erie d'experiences sur les effects de
     l'ablation des corps thyroides. Rev. Med. Suisse Rom. 4:65-
     75, 1884.

141. Schultz, S.G., and P.F. Curran. Coupled transport of sodium and
     organic solutes. Physiol. Rev. 50:637-718, 1970.

142. Schultz, S.G., R.E. Fuisz, and P.F. Curran. Amino acid and sugar
     transport in rabbit ileum. J. Gen. Physiol. 49:849-866, 1966.

143. Schultz, S.G., R.A. Frizzell, and H.N. Nellans. Ion transport by
     mammalian small intestine. Ann. Rev. Physiol. 36:51-91, 1974.

144. Schultz, S.G., and R. Zolusky. Ion transport in isolated rabbit
     ileum. I. Short-circuit current and Na fluxes. J. Gen. Physiol.
     47:567-584, 1964.

145. Schultz, S.G., and R. Zalusky. Ion transport in isolated rabbit
     ileum. II. The interaction between active sodium and active
     sugar transport. J. Gen. Physiol. 47:1043-1059, 1964.

146. Schwartz, A. A sodium and potassium-stimulated adenosine triphos-
     phatase from cardiac tissues. I. Preparation and properties.
     Biochem. & Biophys. Res. Commun. 9:301-306, 1962.

147. Schwartz, A., J.C. Allen, and S. Harigaya. Possible involvement
     of cardiac Na^+, K^+-adenosine triphosphatase in the mechanism
     of action of cardiac glycosides. J. Pharmacol. Exp. Ther.
     168:31-41, 1969.

148. Schwartz, A., H. Matsui, and A.H. Laughter. Tritiated digoxin
     binding to (Na^++ K^+)-activated adenosine triphosphatase:
     possible allosteric site. Science. 159:323-325, 1968.

149. Schwartz, H.L., M.I. Surks, and J.H. Oppenheimer. Quantitation of
     extrathyroidal conversion of L-thyroxine to 3,5,3'-triiodo-
     thyronine in the rat. J. Clin. Invest. 50:1124-1130, 1971.

150. Seshardi, B. Action of thyroxine on the intestinal tissue of rat.
     Indian J. Exp. Biol. 3:97-99, 1965.

151. Shakespeare, P., L.M. Srivastava, and G. Hubscher. Glucose metab-
     olism in the mucosa of the small intestine. The effect of
     glucose on hexokinase activity. Biochem. J. 111:63-67, 1969.

152. Sensenbach, W., L. Madison, S. Eisenberg, and L. Ochs. The cere-
     bral circulation and metabolism in hyperthyroidism and my-
     xedema. J. Clin. Invest. 33:1434-1440, 1954.

153. Shirachi, D.Y., A.A. Allard, and A.J. Trevor. Partial purification
     and ouabain sensitivity of Lubrol-extracted sodium-potassium
     transport adenosine triphosphatase from brain and cardiac
     tissue. Biochem. Pharmacol. 19:2893-2906, 1970.

154. Siegel, G.J., G.J. Koval, and R.W. Albers. Sodium-potassium-
     activated adenosine triphosphatase. VI. Characterization of the
     phosphoprotein formed from orthophosphate in the presence of
     ouabain. J. Biol. Chem. 244:3264-3269, 1969.

155. Siekevitz, P., H. Low, L. Ernster, and O. Lindberg. On a possible
     mechanism of the adenosinetriphosphatase of liver mitochondria.
     Biochim. Biophys. Acta. 29:378-3-1, 1958.

156. Skou, J.C. The influence of some cations on an adenosine triphos-
     phatase from peripheral nerves. Biochim. Biophys. Acta. 23:
     394-401, 1957.

157. Skou, J.C. Further investigation on a Mg^{++} + Na^+-activated adeno-
     sinetriphosphatase, possible related to the active, linked
     transport of Na^+ and K^+ across the nerve membrane. Biochim.
     Biophys. Acta 42:6-23, 1960.
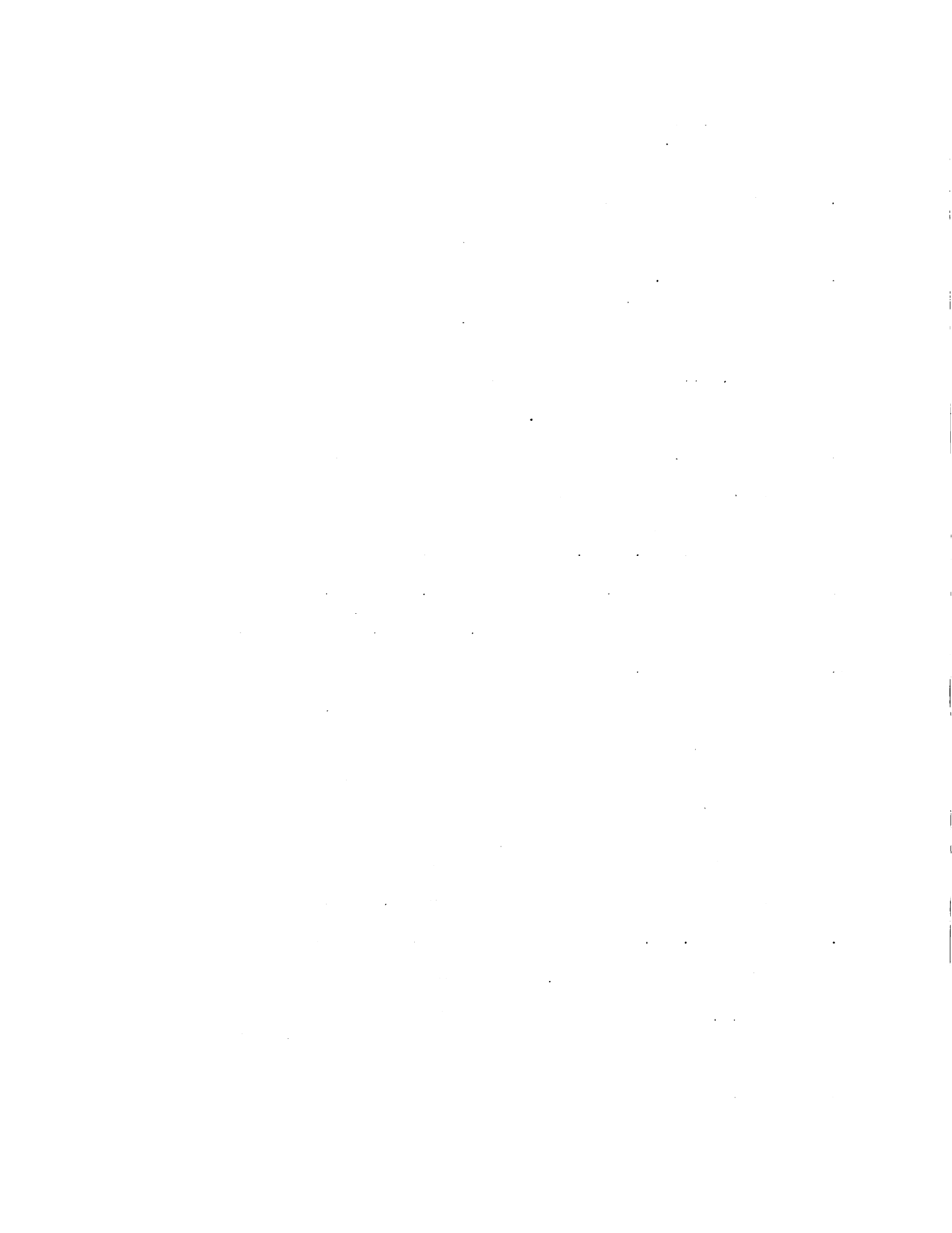
158. Skou, J.C. Preparation from mammalian brain and kidney of the enzyme system involved in active transport of Na^+ and K^+. Biochim. Biophys. Acta 58:314-325, 1962.

159. Skou, J.C. Enzymatic basis for active transport of Na^+ and K^+ across cell membrane. Physiol. Rev. 45:546-617, 1965.

160. Smith, T.W., H. Wagner Jr., J.E. Markis, and M. Young. Studies on the localization of the cardiac glycoside receptor. J. Clin. Invest. 51:1777-1789, 1972.

161. Snedecor, G.W., and W.G. Cochran. Statistical Methods. Sixth Edition. The Iowa State University Press, Ames, Iowa, U.S.A. 1967.

162. Srivastara, L.M., P. Shakespeare, and G. Hubscher. Glucose metabolism in the mucosa of the small intestine. A study of hexokinase activity. Biochem. J. 109:35-42, 1968.

163. Stifel, F.B., R.H. Herman, and N.S. Rosensweig. Dietary regulation of glycolytic enzymes. III. Adaptive changes in rat jejunal pyruvate kirase, glycerol-3-phosphate dehydrogenase. Biochim. Biophys. Acta. 184:29-34, 1969.

164. Stirling, C.E. Radioautographic localization of sodium pump sites in rabbit intestine. J. Cell Biol. 53:704-714, 1972.

165. Tapley, D.F. The effect of thyroxine and other substances on the swelling of isolated rat liver mitochondria. J. Biol. Chem. 222:325-339, 1956.

166. Tapley, D.F., and C. Cooper. The effect of thyroxine and related compounds on oxidative phosphorylation. J. Biol. Chem. 222:341-349, 1956.

167. Tata, J.R., L. Ernster, and O. Lindberg. Control of basal metabolic rate by thyroid hormones and cellular function. Nature (Lond.) 193:1058-1060, 1962.

168. Tata, J.R. Biological action of thyroid hormones at the cellular and molecular levels. In: Actions of Hormones on Molecular Processes, ed. by G. Litwack and D. Kritchevsky. New York: Wiley, 1964, p. 58-131.

169. Tata, J.R. The regulation of mitochondrial structure and function by thyroid hormones under physiological conditions. In: Regulation of metabolic processes in mitochondria. Eds. J.M. Tager, S. Papa, E. Quagliariello, and E.C. Slater. Biochim. Biophys. Acta Library 17:489-505, Elsevier Publ. Co. Amsterdam, 1966.

170. Tata, J.R. The formation and distribution of ribosomes during hormone-induced growth and development. Biochem. J. 104: 1-16, 1967.

171. Tata, J.R. The formation, distribution and function of ribosomes and microsomal membranes during induced amphibian metamorphosis. Biochem. J. 105:783-801, 1967.

172. Tata, J.R. Membrane phospholipid synthesis and the action of hormones. Nature 213:566-569, 1967.

173. Tata, J.R. Coordination between membrane phospholipid synthesis and accelerated biosynthesis of cytoplasmic, ribonucleic acid and protein. Biochem. J. 116:617-630, 1970.

174. Tata, J.R., L. Ernster, O. Lindberg, E. Arrhenius, S. Pedersen, and R. Hedman. The action of thyroid hormone at the cell level. Biochem. J. 86:408-428, 1963.

175. Tata, J.R., and C.C. Windell. Ribonucleic acid synthesis during the early action of thyroid hormones. Biochem. J. 98:604-620, 1966.

176. Tobin, T., R. Henderson, and A.K. Sen. Species and tissue differences in the rate of dissociation of ouabain from $(Na^+ + K^+)$-ATPase. Biochim. Biophys. Acta 274:551-555, 1972.

177. Tobin, T., and A.K. Sen. Stability and ligand sensitivity of ^3H-ouabain binding to $(Na^+ + K^+)$-ATPase. Biochim. Biophys. Acta. 198:120-131, 1970.

178. Trier, J.S., and C.E. Rubin. Electronmicroscopy of the small intestine: A review. Gastroenterology 49:574-603, 1965.

179. Tyler, D.B., and A. van Harreveld. The respiration of the developing brain. Amer. J. Physiol. 136:600-603, 1942.

180. Tyrrell, J.B., and J.W. Anderson. Glycolytic and pentose phosphate pathway enzymes in jejunal mucosa, adaptive responses to alloxan-diabetes and fasting in the rat. Endocrinol. 89:1178-1185, 1971.

181. Ulrich, F. The inhibition of mitochondrial adenosine triphosphatase. The effects of alkali-metal ions. Biochem. J. 88:193-206, 1963.

182. Umbeit, W.W., R.H. Burris, and J.F. Stauffer. Manometric techniques. Burgess Publishing Co., Minneapolis, Minn. 1957.

183. Valcana, T., and P.S. Timiras. Effect of hypothyroidism on ionic metabolism and NaK-activated ATP phosphorylase activity in the developing rat brain. J. Neurochem. 16:935-943, 1969.

184. Whittam, R. The asymmetrical stimulation of a membrane adenosine triphosphatase in relation to active cation transport. Biochem. J. 84:110-118, 1962.

185. Whittam, R. The cellular functions of membrane transport. ed. J.F. Hoffman. Prentice-Hall, New York, N.Y. 1964.

186. Whittam, R. The interdependence of metabolism and active transport. In: The cellular function of membrane transport. Ed. J.F. Hoffman. p. 139. Prentice-Hall Inc. 1964.

187. Whittam, R., and D.M. Blond. Respiratory control by an adenosine triphosphatase involved in active transport in brain cortex. Biochem. J. 92:147-158, 1964.

188. Whittam, R. and J.S. Willis. Ion movements and oxygen consumption in kidney cortex slices. J. Physiol. 168:158-177, 1963.

189. Wilson, W.E., W.I. Sivitz, and L.T. Hanna. Inhibition of calf brain membranal sodium- and potassium-dependent adenosine triphosphatase by cardioactive sterols. A binding site model. Mol. Pharmacol. 6:449-459, 1970.

190. Wilson, T.H., and G. Wiseman. Metabolic activity of the small intestine of the rat and golden hamster. (Mesocricetus auratus). J. Physiol. 123:126-130, 1954.

191. Wolfe, D.L., S.C. Forland, and L.Z. Benet. Drug transfer across intact rat intestinal mucosa following surgical removal of serosa and muscularis externa. J. Pharm. Sci. 62:200-205, 1973.

192. Yarus, M., and P. Berg. Recognition of tRNA by aminoacyl tRNA synthetase. J. Mol. Biol. 28:479-490, 1967.

193. Yarus, M., and P. Berg. On the properties and utility of a membrane filter assay in the study of isoleucyl-tRNA synthetase. Analyt. Biochem. 35:450-465, 1970.

194. Zall, D.M., D. Fisher, and M.Q. Garner. Photometric determination of chlorides in water. Anal. Chem. 28:1665-1668, 1956.