allowed. This demonstrates that the Pb-containing precipitates near to the plasmalemma are formed from soluble inorganic phosphate which is lost into the medium if a glutaraldehyde prefixation is used. The similarity in location of the two precipitates thus seems fortuitous, which is borne out by the fact that the amount of pyroantimonate precipitate was increased by an increase in glucose concentration (*7–9*), whereas the lead phosphate precipitate disappeared.

As far as we are aware, this is the first demonstration by any electron microscope technique of a specific localization of phosphate at the plasmalemma. The localization of orthophosphate in the nucleolus was previously described in maize root-tip cells (*4, 5*). The early autoradiographic experiments of Causey and Harris (*10*) suggested that ^{32}P injected into the frog was selectively concentrated near to the cell surface of the muscle cells, but there was no proof that this existed as inorganic P. These results on muscle can also be contrasted with more recent experiments which show no preferential localization of ^{32}P near the plasmalemma of the hepatocyte both by light (*11*) or electron microscope (*12*) autoradiography. The fact that the plasmalemma precipitate is found only on the B cells and not on A, D, or exocrine cells under the present experimental conditions, coupled with the disappearance of the precipitate under conditions which are known to deplete the B cell phosphate and cause insulin secretion (*13*), complement the evidence already obtained that the two processes are connected in some way. Although the form in which the phosphate is kept near to the plasmalemma is not apparent, it is obvious that it would be strategically placed to support rapid extracellular migration and passage to the perfusion fluid on stimulation, as is observed in practice.

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Mitochondrial Thyroid Hormone Receptor: Localization and Physiological Significance

Abstract. *Binding studies of thyroid hormone to submitochondrial fractions from rat liver suggest that the component responsible for high-affinity, low-capacity (saturable) binding of hormones arises from the inner mitochondrial membrane. The partially purified component, approximately 150,000 daltons, appears to be half protein and half lipid, largely phospholipids, tentatively identified as lecithin, phosphatidyl ethanolamine, and cardiolipin. A similar hormone-binding macromolecule was found in mitochondria from rabbit kidney, from human liver and kidney, and from rat kidney, myocardium, skeletal muscle, intestinal mucosa, whole small intestine, adipose tissue, and lung. It was absent from mitochondria of adult rat brain, spleen, and testis, organs calorigenically unresponsive to thyroid hormones injected in vivo, but was present in mitochondria from brains of rats 12 days old and younger. The organ distribution of the hormone-binding protein and its presence in neonatal brain mitochondria supports the biological relevance of the mitochondrial component as a thyroid hormone receptor.*

We have described a membrane component of rat liver mitochondria (*1, 2*) which binds triiodothyronine (T_3) with an extremely high association constant ($K_A > 10^{11} M^{-1}$). This binding exhibits high-affinity, low-capacity (saturable) characteristics; we infer that binding by mitochondria may be of importance in thyroid hormone action. This view is supported by the demonstration of binding of thyroid hormone analogs in proportion to the potency of the analog (*2, 3*).

In view of data indicating a direct effect of T_3 on mitochondrial oxidative phosphorylation (*3*), we investigated the intramitochondrial localization of the presumed receptor. We now report that the receptor appears to be part of the inner mitochondrial membrane and provide data on its nature and organ distribution.

Materials and methods. Euthyroid male Sprague-Dawley rats (Charles River, North Wilmington, Massachusetts), 400 to 700 g, were used after an overnight fast. Pregnant female rats were purchased from Camm Research, Wayne, New Jersey. Mitochondria were prepared as described (*1, 3*). A highly purified preparation of phospholipase A_2 from cobra venom was provided by M. Rapport, Columbia University.

Two methods of mitochondrial fractionation were used (Fig. 1).

1) Digitonin fractionation was performed as described by Greenawalt (*4*). A mitochondrial suspension (100 mg of mitochondrial protein per milliliter) was treated with digitonin (0.12 mg per milligram of mitochondrial protein) by adding an equal volume of digitonin solution (12 mg/ml) and stirring for 15 minutes at 0°C. After centrifugation twice at 9250 rev/min (1000*g*), the resultant "mitoplast" fraction was resuspended in medium at a protein concentration of 30 mg/ml. Lubrol WX (19 mg/ml) was then added (final concentration, 0.16 mg per milligram of mitoplast protein). After 15 minutes the suspension, diluted 1 : 2 with isolation medium, was centrifuged at 144,000*g* for 60 minutes. The sediment (inner mitochondrial membrane) was resuspended in tris buffer, *p*H 7.2, containing 0.01*M* EDTA. The supernatant from this spin represents matrix protein solution (*4*). Outer mitochondrial membranes were obtained by collecting the sediment after centrifugation (144,000*g*) of the combined supernatants from the mitoplast preparation. The supernatant from this spin is intermembrane protein solution (*4*).

2) Hypotonic lysis was done by the method of Caplan and Greenawalt (*5*). Rat liver mitochondria were prepared in 0.25*M* sucrose and washed twice. The pellet was then treated for 10 minutes with distilled water at 4°C (1 ml per gram of liver equivalent). The suspension was centrifuged at 105,000*g* for 30 minutes

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and the procedure was repeated. The sediment (ghosts) consists of outer and inner membranes (*5*) while the combined supernatants from the two water washes contain matrix and intermembrane proteins (*5*).

Monoamine oxidase (MAO) was measured with benzylamine as substrate (*6*, *7*). Malate dehydrogenase was determined by the method of Ochoa (*8*) and cytochrome oxidase by the polarographic method of Schnaitman *et al.* (*9*).

All mitochondrial fractions were subjected to Sephadex G-200 gel filtration as described (*1*). The major peak of absorbance at 280 nm, immediately after the void volume and close to the immunoglobulin G peak (determined by prior calibration of the column with human serum), was designated the A peak; a much later emerging peak of absorbance, presumed to be a small polypeptide (*1*, *2*), was called the B peak. The latter peak, apparently a fragment of the principal macromolecule, contained more nonspecific binding material. In any fraction, if an A peak was obtained by gel filtration, a Scatchard analysis (Fig. 2) of the binding of $[^{125}I]T_3$ to the protein was performed as described (*1–3*). The mass, ~ 150,000 daltons, was inferred from the position of emergence of the A peak.

In enzyme incubation studies, 1.2 ml of A peak solution (~1.2 mg of protein per milliliter) was incubated with 60 μl of $[^{125}I]T_3$ solution (2.25×10^{-6} μM) and 0.1 ml of enzyme solution was added. Enzymes studied were pepsin, phospholipase A_2, Pronase, ribonuclease A, and deoxyribonuclease. After 24 hours, 0.6 ml of solution of dextran-coated charcoal (1 g of Norit A and 1 g of dextran in 100 ml of tris buffer, *p*H 7.2) was added. Tubes were incubated for 10 minutes and then spun at 9250 rev/min (1000*g* for 10 minutes) to separate bound $[^{125}I]T_3$ from free hormone (*3*). Total radioactivity was determined by counting 1 minute before incubation. Experiments were done in duplicate with appropriate control tubes lacking enzyme, as well as control tubes with enzymes but no mitochondrial protein.

Lipids were extracted from A peak material with chloroform-methanol (2:1, by volume) (*10*). Nitrogen was bubbled through the solvent mixture before use. After fractions were extracted for 1 to 2 hours in solvent mixture, the extract was washed with 0.2 volume of 0.05*M* KCl. The chloroform phase was reduced to dryness in a rotary ("flash") evaporator at 30°C. The lipid material was dissolved in chloroform, transferred to a tared vessel, and dried to constant weight under vacuum before weighing. Lipid samples awaiting further analysis were stored in chloroform (which had been bubbled with nitrogen) at −12°C.

Table 1. Binding of T_3 to mitochondrial fractions. All values are the mean of two or more determinations. The value for inner membrane is the mean ± standard deviation of six determinations. "Nonspecific" indicates virtually unsaturable binding sites, as discussed.

Treatment and fraction	K_A (M^{-1})
Digitonin-treated	
Outer membrane*	Nonspecific
Intermembrane protein	1.6×10^{11}
Mitoplast	Nonspecific
Matrix	$\sim 2 \times 10^{11}$
Inner membrane	1.97×10^{11} $\pm 0.81 \times 10^{11}$
Water-washed	
Ghosts	1.0×10^{11}
Matrix + intermembrane protein*	Nonspecific

*No A peak on Sephadex G-200 gel filtration.

Total lipids were separated into neutral lipids and phospholipids by silicic acid column chromatography (*11*). Neutral lipids and phospholipids were further separated on plates coated with silica gel. The solvent system for neutral lipids was hexane–ethyl ether–acetic acid (90:10:1), and for phospholipids, chloroform-methanol-water (75:26:4.5). On the phospholipid plates, lipid spots were detected with I_2 vapor and scraped off the plate into digestion tubes for determination of lipid phosphorous (*12*). The various spots on the thin-layer plates were identified by the following markers: cholesterol (free and esterified), lecithin, lysolecithin, phosphatidyl ethanolamine, and triglycerides.

For studies of neonatal brain, 2-, 5-, 8-, 10-, 12-, 14-, and 17-day-old litters were used. The neonates were killed by decapitation, brain mitochondria were prepared in 0.25*M* sucrose as described for liver (*1*), and A peak material was subjected to Scatchard analysis.

Specific saturable binding of $[^{125}I]T_3$ to mitochondrial membrane preparations of liver, kidney, myocardium (atrial and ventricular), skeletal muscle, small intestinal mucosa, and whole small intestine, lung, and adipose tissue from the rat was also sought. These tissues were treated as described for rat liver mitochondria.

The studies were extended to species other than the rat. Kidneys from a New Zealand white rabbit, and human renal and hepatic tissues were handled as were rat tissues. Human tissues were obtained from six autopsies within 3 to 18 hours of death from cardiovascular diseases.

Results. A typical Scatchard plot showing "specific" or high-affinity, limited-capacity binding, with $K_A \sim 10^{11} M^{-1}$ is shown in Fig. 2. The "nonspecific" binding obtained by adding a millionfold excess of unlabeled T_3 has been subtracted from each experimental point. Addition of amounts of T_3 greater than those illustrated usually revealed a flattening of the curve, ascribed to secondary or higher order classes of binding sites, on either the major binding macromolecule or other contaminating proteins. In preparations lacking the primary class of binding sites with $K_A \sim 10^{11} M^{-1}$, the plot was essentially horizontal despite definite binding of T_3. This signified low-affinity, high-capacity, or virtually unsaturable binding sites, termed "nonspecific." The data of Fig. 2, for rabbit renal mitochondria, are similar to those for rat hepatic and renal mitochondria and also to those for human liver and kidneys.

The mean K_A value reported previously for T_3 binding to mitochondrial

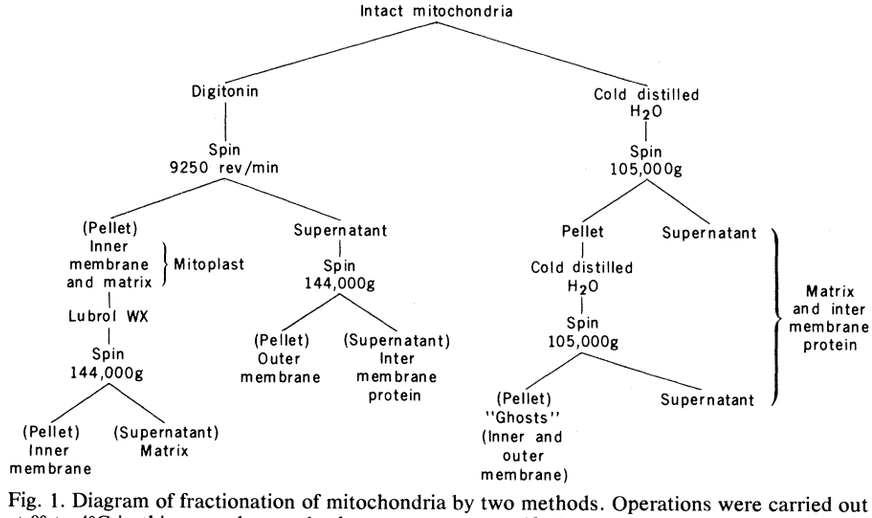

Fig. 1. Diagram of fractionation of mitochondria by two methods. Operations were carried out at 0° to 4°C in this procedure and others, except as specified.

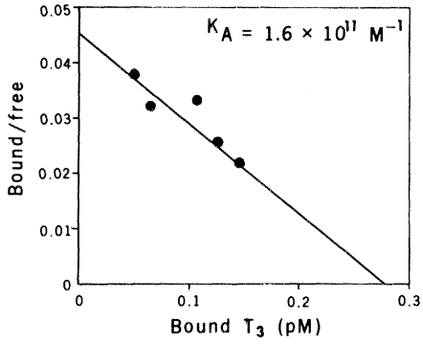

Fig. 2. Scatchard plot showing interaction between triiodothyronine (T_3) and rabbit renal mitochondrial protein. The medium contained 0.05*M* tris-HCl, *p*H 7.0, and 0.01*M* EDTA.

protein from rat liver was $1.9 \pm 0.2 \times 10^{11} M^{-1}$ (*3*) in 31 studies. Values for mitochondrial subfractions, based on fewer determinations, may be cruder approximations indicative only of order of magnitude (Table 1).

Purity of membrane preparations was confirmed by the enzyme marker assays. More than 95 percent of MAO activity was in outer membrane preparations, and more than 90 percent of cytochrome oxidase activity was in inner membrane preparations. The ghosts from hypotonic lysis contained both enzymes, which indicates the presence of inner and outer membranes (*5*). Malate dehydrogenase was present predominantly in the matrix solution from the digitonin-treated mitochondria and in the supernatant after hypotonic lysis.

Crude mitochondrial fractions showed no specific binding because of the high nonspecific binding encountered. After gel filtration, specific binding was observed in the intermembrane, inner membrane, and matrix fractions from the digitonin and Lubrol WX–treated mitochondria (Table 1). No A peak was observed in the water lysis supernatant, but the ghost fraction showed specific binding. The data suggested that specific binding sites were located on the inner mitochondrial membrane.

Binding of T_3 by the partially purified (A peak) material is unaffected by treatment with ribonuclease and deoxyribonuclease (Fig. 3). The proteolytic enzymes pepsin and Pronase significantly reduced the binding, whereas treatment with phospholipase A_2 increased it by about 60 percent. The results are compatible with a lipoprotein component.

Lipid analysis showed that the partially purified membrane component was approximately one-half lipid, largely phospholipids tentatively identified as lecithin, phosphatidyl ethanolamine, and cardiolipin. Small amounts of free and esterified cholesterol and free fatty acids were found. Relative thermostability was inferred from essentially unaltered binding of T_3 by crude mitochondrial membrane protein despite freezing and thawing several times. Purer materials, however, exhibited some precipitation after thawing.

The K_A values (Table 2) for the interaction between [^{125}I]T_3 and A peak from various rat tissues were essentially the same as those reported for rat liver and kidney ($\sim 2 \times 10^{11} M^{-1}$). Specific saturable binding sites were found in brains from animals up to 12 days old. The specific binding protein was not present in brains of older animals (14 and 17 days) or in adult brain; the Scatchard plot for adult brain yielded a nearly horizontal line, which signifies absence of saturable binding sites and presence of only nonspecific or virtually unsaturable sites.

Discussion. The results of the binding studies in mitochondrial fractions prepared by digitonin and Lubrol WX excluded the outer membrane as a source of the binding protein but did not establish that binding sites were localized in the inner membrane, since specific binding was also observed in the matrix and the intermembrane protein. However, treatment with digitonin may detach various protein moieties from either the outer or inner membrane (*6*). Therefore, hypotonic lysis was also used since it avoids any chemical additive and yields different fractions (*5*). After hypotonic lysis, specific binding was found in the pellet fraction containing inner and outer membranes, but not in the supernatant fraction containing both the intermembrane and matrix proteins. These data suggest that binding in the intermembrane and matrix fractions prepared with digitonin resulted from detachment from the inner membrane. Taken together, these results strongly suggest that the specific binding protein for thyroid hormone arises from the inner membrane. It is not known whether it is a part of the membrane or attached to the surface.

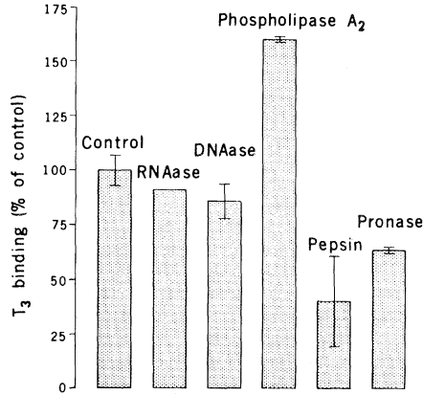

Fig. 3. Effect of enzymatic digestion on specific mitochondrial receptor binding of T_3. Incubation time was 24 hours; similar results were obtained with 1-hour incubations. Enzyme concentrations, *p*H, and temperatures were as follows: pepsin, 1 mg/ml, *p*H 2, 37°C; Pronase, 2 mg/ml, *p*H 7.5, 37°C; phospholipase A_2, 120 μg/ml, *p*H 7.5, 37°C; ribonuclease A, 5 mg/ml, *p*H 7.2, 25°C; and deoxyribonuclease, 1 mg/ml, *p*H 5.0, 25°C.

Table 2. Distribution of mitochondrial T_3 receptor in rat tissues. Association constants are for high-affinity, low-capacity (saturable) binding. No specific binding was found in adult brain, spleen, or testis.

Tissue	K_A (M^{-1})
Liver	1.9×10^{11}
Kidney	2.0×10^{11}
Myocardium (ventricular)	2.0×10^{11}
Myocardium (atrial)	1.6×10^{11}
Skeletal muscle	3.7×10^{11}
Neonatal brain	2.8×10^{11}
Lung	1.8×10^{11}
Small intestinal wall with mucosa	2.3×10^{11}
Small intestinal mucosa	3.6×10^{11}
Adipose tissue	2.4×10^{11}

Dry weight determinations of lipid extracts suggest that the partially purified membrane component (A peak) is approximately half lipid. However, the lipid moiety is partially oxidized during extraction, which precludes a quantitative analysis. Although a cardiolipin marker was not used, the chromatographic mobility of one lipid component was consistent with this mitochondrial lipid in the solvent system used (*13*). The other lipid components tentatively identified are also constituents of mitochondria. Consistent with the lipid profile is the fact that treatment with phospholipase A_2 significantly increased the binding of T_3 to the receptor. Since the major substrate of this enzyme is lecithin, this treatment may expose an underlying protein binding site. Current work indicates that lipid is present after further purification. The results of the other enzyme treatments were as expected: the nucleases were without effect, and the proteolytic enzymes partially destroyed binding capacity.

The physiological relevance of the mitochondrial binding protein is strengthened by its presence in mitochondria isolated from brains of neonatal rats up to 12 days old. Several biochemical functions are believed to be dependent on the presence of thyroid hormones until approximately this age. The data are consistent with the absence of calorigenic response in adult brain (*14*), where no specific binding was found.

Previous data supported the sugges-

tion that an early action of thyroid hormones is stimulation of oxidative phosphorylation (*3*). Since this process occurs on the inner mitochondrial membrane, the present evidence suggests a direct action of thyroid hormones on mitochondria independent of nuclear involvement. The model of direct thyroid hormone action on mitochondria (*15*) in no way negates the concept of other more sustained if somewhat delayed effects mediated by the nucleus (*16*); such effects might include increased synthesis of messenger RNA directing the synthesis of inducible proteins. This pathway would presumably account for thyroid hormone stimulation of adenosine triphosphatase, which apparently entails formation of additional "sodium pump" units in the plasma membrane (*17*).

Previous reports (*18*) have implicated the mitochondria as a target of thyroid hormone action. While some reported effects might entail enhanced protein synthesis mediated by either nuclear or mitochondrial DNA, the effects we have observed (*3*) would appear to be too rapid for such a mechanism. Future studies may indicate the mechanism whereby interaction of the hormone with the postulated inner membrane receptor may activate oxidative phosphorylation.

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The Fanconi Syndrome in Basenji Dogs: A New Model for Renal Transport Defects

Abstract. *The renal defects resulting in a Fanconi syndrome were seen in eight Basenji dogs by measuring renal clearance and in vitro amino acid and sugar uptake and performing histopathologic evaluations. Renal tubular handling of glucose, phosphate, sodium, potassium, uric acid, and amino acids was abnormal, and in vitro uptake of labeled lysine, glycine, and α-methyl-D-glucoside by renal cortical slices was impaired. Histopathology was normal except for enlarged nuclei in some renal tubule cells. These Basenji dogs, which may be genetically affected, represent a likely model for idiopathic Fanconi syndrome in humans.*

The Fanconi syndrome in humans is a constellation of abnormalities associated with renal defects of tubular reabsorption; it is manifested by excessive urinary loss of glucose, amino acids, phosphate, bicarbonate, sodium, potassium, and water (*1–3*). The etiology may be unknown or idiopathic, but frequently the disorder is associated with a variety of inherited diseases (such as cystinosis, tyrosinemia, Lowes syndrome, and hereditary fructose intolerance in children) or with acquired conditions (such as multiple myeloma, amyloidosis, drug intoxication in adults). Such widespread derangement of renal tubular cell transport function could result from disordered membrane structure or abnormal cell metabolism, but as yet, the underlying mechanisms are unknown.

An animal model exists for Fanconi syndrome in which maleic acid is injected into dogs (*4–6*) and rats (*7–9*). We have characterized a spontaneous renal tubular disorder in eight dogs of the Basenji breed; this disorder resembles human idiopathic Fanconi syndrome. Our studies, which include physiological assessment of renal function, in vitro uptake of a sugar and amino acids by kidney cortex slices, and pathological evaluation of the kidney, indicate this disorder is a new model of deranged active renal transport.

Clinical signs first appear in adult Basenjis of both sexes and progress to renal failure after months or years. These signs, similar to those in humans with Fanconi syndrome, include polydipsia, polyuria, dehydration, weight loss, and weakness. Profound glycosuria and hypotonic urine are present in the absence of diabetes mellitus. Plasma electrolytes are normal, but arterial blood gas values in affected dogs suggest a moderate metabolic acidosis.

Renal clearance studies were performed on seven affected and three nor-

Table 1. Renal handling of solutes by normal and affected Basenji dogs. One week prior to clearance studies, all dogs were fed a standard diet. None of the dogs were volume depleted or debilitated by routine clinical evaluation. Glucose, phosphate, and sodium values are expressed as the fractional reabsorption of the filtered load ± standard error (S.E.). The potassium value for normal animals is the mean ratio of urinary excretion to filtered load. Potassium values for affected dogs and the uric acid values are expressed as the mean ± S.E. ratio of urinary excretion to filtered load. Numbers in parentheses indicate the number of animals in each group.

Solute	Normal	Affected	*P*
Glucose	99.6 ± 0.2 (3)	71.7 ± 8.1 (7)	<.01
Phosphate	91.6 ± 4.2 (3)	61.3 ± 6.2 (7)	<.01
Sodium	97.4 ± 0.6 (3)	90.0 ± 3.1 (7)	<.05
Potassium	0.2 (2)	2.53 ± 0.77 (7)	
Uric acid	0.34 ± 0.08 (3)	0.95 ± 0.11 (4)	<.01

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