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EFFECT OF THYROIDECTOMY ON THE PROTEIN CONTENT OF RAT ADRENAL CORTEX MITOCHONDRIA AND MICROSOMES

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SUMMARY

The effects of thyroidectomy on the protein content and properties of adrenorcortical mitochondria and microsomes were investigated in the rat. Thyroidectomy caused a drop in the cytochrome P450 concentration in both particulate fractions. Polyacrylamide gel electrophoresis of mitochondrial and microsomal proteins showed a reduction in the protein constituent with a molecular weight of 52,000 daltons. This constituent corresponded to the cytochrome P450 subunit. Isolated adrenal cortex mitochondria from thyroidectomized rats incorporated 30% less leucine than normal mitochondria. Electrophoresis of labelled proteins showed no change in radioactivity distribution. In microsomes, the rate of 21-hydroxylase activity dropped by 30% after thyroidectomy.

Although these effects were similar to those observed after dexamethasone treatment, there was no doubt that they were caused by the absence of thyroid hormones, since no reduction was found in the plasma ACTH concentration in thyroidectomized animals. The drop in the content of cytochrome P450 in adrenal cortex mitochondria and microsomes could explain the diminished hydroxylating activity of these organelles.

INTRODUCTION

The thyroid state affects the functions of the adrenal cortex and corticoid metabolism. The problem has been studied in the course of many clinical trials on Man and the resulting data have often been contradictory [1–3]. However investigations on animals have been relatively few. In the rat, hypothyroidism was shown to reduce the weight of the adrenals and slow down cholesterol metabolism [4]. Biosynthesis of corticosterone, the chief glucocorticoid in the rat, brings localized enzyme complexes into play in microsomes and mitochondria. In addition to a respiratory chain, adrenal cortex mitochondria have a special chain responsible for 20, 22, 11β and 18 hydroxylations [5, 6]. The absence of thyroid hormones does not change either oxidative phosphorylation mechanisms or the activities of the various multienzyme systems of the inner membrane involved in electron transport [7]. It does, however, affect the hydroxylation chain as shown by the slowing down of corticosterone biosynthesis from deoxycorticosterone [7]. The effect of thyroidectomy is probably not located at NADPH energy sources since the transhydrogenases or the malic enzyme are either unchanged or increased [7, 8]. Thyroid hormone absence might make itself felt in one or more constituents of the hydroxylating chain: cytochrome P450 is the terminal oxygenase in this chain and is responsible for electron transport from the NADPH to the oxygen [9–11]. Accurate data are not yet available on this subject. Labelled aminoacid incorporation in the thyroidectomized rat is lower in isolated liver [12] and muscle [13] mitochondria. The reduced weight of the adrenals in the thyroidectomized animals might thus be caused by a drop in protein synthesis.

We therefore set out to study the effects of thyroidectomy in the rat, firstly on the concentration of cytochrome P450 and secondly on the protein constituents electrophoretic patterns of adrenal cortex mitochondria and microsomes. We also measured labelled leucine incorporation in mitochondria and determined the 21-hydroxylation kinetics of 17α-hydroxyprogesterone in adrenal cortex microsomes from normal and thyroidectomized animals.

MATERIALS AND METHODS

Experiments were performed on normal male Wistar rats weighing about 200 g and on rats weighing 50 g at the time of the thyroidectomy by surgery and which were used 8 weeks afterwards. Hypothyroidism was verified by observing changes in weight and using radioimmunology to ascertain iodothyronines content in plasma. Some of the animals were given dexameth-

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asone* at the rate of 200 μg per 100 g body weight in two injections separated by a 12 h interval. These animals were killed 24 h after the first injection.

Preparation of mitochondria and microsomes

Mitochondria. Each assay required a total of 40 normal or thyroidectomized rats. They were killed by carotid cutting. The adrenals were quickly removed and carefully dissected to eliminate the medulla. The amount of adrenal cortex tissue thus obtained was 0.5–1 g. Mitochondria were isolated in 0.33 M sucrose in accordance with Nakamura and Tamaoki's technique [14]. The homogenate was centrifuged twice for 15 min, once at 600 *g* and once at 5000 *g*. The pelleted mitochondria were washed twice with the 0.33 M sucrose and centrifuged for 15 min at 5000 *g*.

Preparations were checked for quality by polarography which was used to measure mitochondrial respiration and phosphorylation; the purity of the preparations was verified by electron microscopy [15]. Protein analysis was carried out using the technique described by Lowry *et al.* [16] and Gornall *et al.* [17]. Six to 8 mg mitochondrial protein were obtained from each preparation.

Microsomes. The mitochondrial supernatant was centrifuged at 27,000 *g* for 15 min to eliminate light mitochondria and chromaffine particles. Microsomes were sedimented for 1 h at 105,000 *g* and resuspended with 0.25 M sucrose. Proteins were measured by established methods [16, 17]. 8–10 mg microsomal protein were obtained from each preparation.

Determination of cytochrome P450 by spectrophotometry

Cytochrome P450 was determined by differential spectra, according to Omura and Sato's method [18]. Mitochondria or microsomal proteins (about 2 mg) were suspended in 1 ml 0.1 M phosphate buffer, pH 7.0. A few crystals of sodium dithionite ($Na_2S_2O_4$) were added, to reduce possible blood pigment traces. The contents of one cuvette were then gassed with CO which forms a P450-CO complex ($CO\text{-}Fe^{2+}$). The concentration of P450 can be calculated by using the extinction coefficient difference between 450 (peak) and 490 nm which is $91 \cdot cm^{-1} \cdot mM^{-1}$.

Polyacrylamide gel electrophoresis

Electrophoresis was performed in polyacrylamide gel (8.5% total acrylamide concentration, 0.22% methylene bisacrylamide to acrylamide, 0.075% ammonium peroxodisulfate and 0.15% tetramethylene diamine) in 0.01 M sodium phosphate (pH 7.0) with 0.1% sodium dodecyl sulphate and 0.1% mercaptoethanol [19]. Samples (50–100 μg protein) dissolved in the buffer used for electrophoresis were submitted to a constant current of 8 mA/tube (90 × 6 mm) for 6 h. Gels were stained overnight with 0.04% Coomassie blue (w/v), 45% methanol (v/v), 9% acetic acid (v/v) and destained with 5% methanol and 7% acetic acid. Photometric tracings of the stained gels were performed in a Gilford gel spectrophotometer. Molecular weights were determined by using pure marker proteins of commercial origin as follows (20): β-galactosidase (130,000 daltons), phosphorylase (94,000 daltons), bovine serum albumin (68,000 daltons), creatine kinase (40,000 daltons), trypsin (23,300 daltons) and cytochrome *c* (11,700 daltons).

[^3H]-leucine incorporation into mitochondrial proteins

Adrenal cortex mitochondria (2 mg) were incubated *in vitro* for 20 min in a metabolic shaker at 30°C in 2 ml of medium containing 50 mM Bicine buffer (N,N-Bis(2-hydroxyethyl-glycine), pH 7.6, 10 mM $MgCl_2$, 5 mM KH_2PO_4, pH 7.6, 90 mM KCl, 22.5 μg/ml of a complete aminoacid mixture minus leucine and 100 μCi of L-[4,5-^{3}H]-leucine (S.A. 60 Ci/mmol, Amersham) with an artificial ATP generating system formed by 2 mM ATP, 5 mM phosphoenol pyruvate and 20 μg pyruvate kinase [21]. The reaction was stopped by adding 0.25 M cold sucrose containing an excess of unlabelled leucine. Mitochondria were again isolated at 9000 *g* for 10 min and washed twice with 0.25 M sucrose. Mitochondrial proteins were precipitated with 10% trichloracetic acid and afterwards dissolved for electrophoretic analysis. Electrophoresis of each sample was performed in duplicate, one being stained and the other cut into 1.0 mm slices using a Gilford model gel slicer. The slices were burned in a Tricarb oxidizer (Packard 306 model). Radioactivity was counted in a Packard liquid scintillation counter.

Enzyme activity: 21 hydroxylase

The activity of this enzyme was determined by measuring the cortexolone formed during microsome incubation at 25°C in the presence of 17α-hydroxyprogesterone [22, 23]. The reaction medium included 0.12 M NaCl containing 2% bovine serum albumin (pH 7.4), 22 mM glycylglycine buffer (pH 7.4), 0.88 mM $MgCl_2$, 4.8 mM KCL, 6–8 mg microsomal proteins, 100 μM 17α-hydroxyprogesterone in ethanol and 35 μM NADPH. The final vol. was 25 ml. Aliquots were taken at various intervals and the reaction was stopped by adding 1 ml 0.5% $HgCl_2$. The cortexolone which formed was extracted with 10 ml dichloromethane [24] and measured by Porter and Silber's technique [25].

Radioimmunological measurements

ACTH. Plasma ACTH was measured by radioimmunology (CEA Kit). The blood of 7 batches, comprising 3–4 normal or thyroidectomized animals per batch, was collected at 0°C in the presence of EDTA (1 mg/ml blood). Plasma from each batch was centrifuged at 2°C and ACTH concentrations were measured in duplicate.

* Dexamethasone 21-phosphate. Merck, Sharp and Dohme.

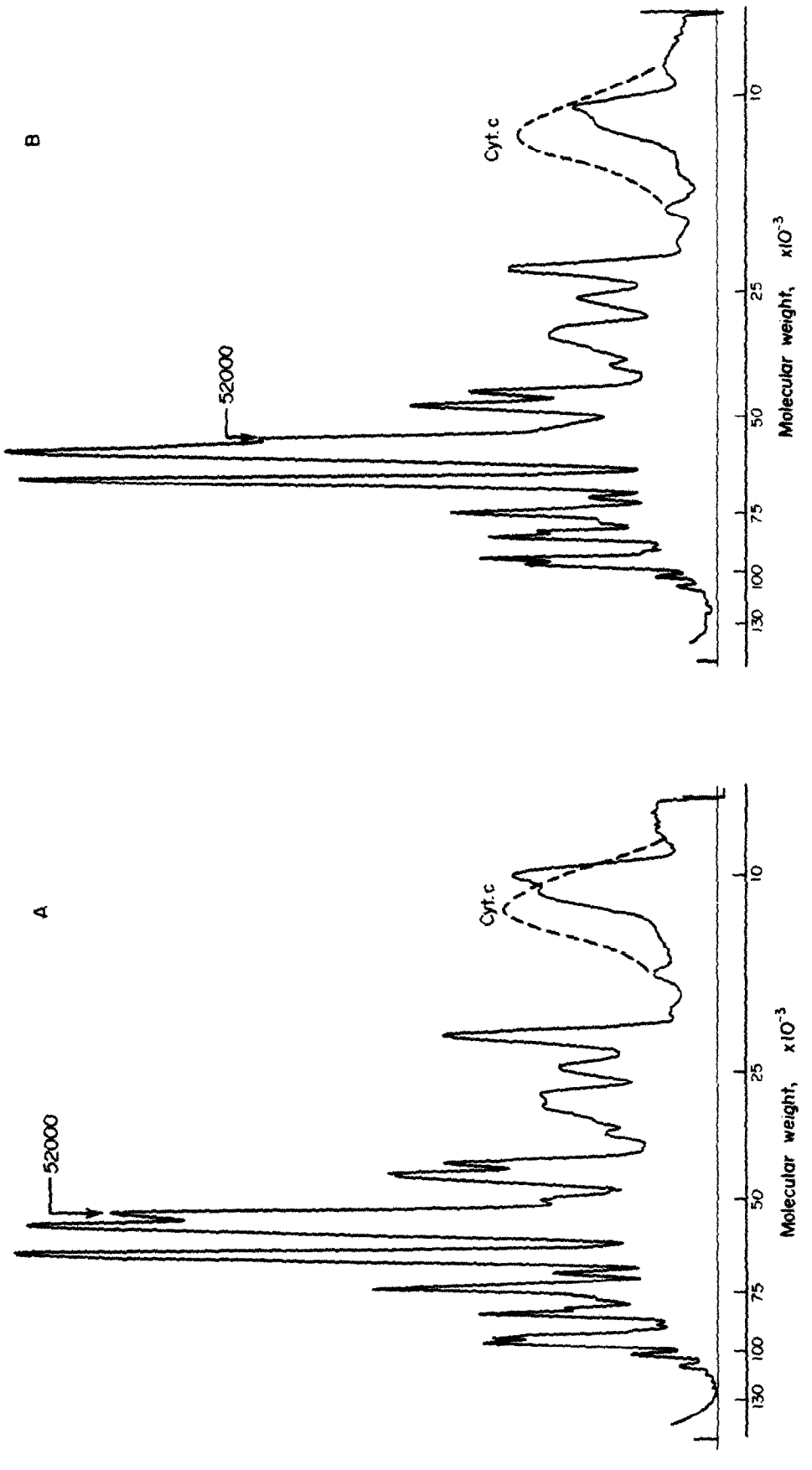

Fig. 1. Densitometric traces of polyacrylamide gel electrophoresis of adrenal cortex *mitochondria* proteins. A: Normal. B: Thyroidectomized. Cytochrome *c*, represented in *broken lines*, was used as a marker.

Table 1. Content of cytochrome P450 in adrenal cortex mitochondria and microsomes of normal, thyroidectomized and dexamethasone treated rats

	Normal	Thyroidectomized	Dexamethasone
Mitochondria	1.62 ± 0.31	1.19 ± 0.20*	1.38 ± 0.31*
Microsomes	0.97 ± 0.22	0.61 ± 0.05**	—

Concentrations are expressed in nmol × mg^{-1} mitochondrial or microsomal protein. Values given are the mean of 6 experiments ± S.E.M. For each experiment 30–50 rats were used.
* $P < 0.02$; ** $P < 0.01$.

3,5,3'-triiodo-L-thyronine (T_3), *3,3',5'-triiodo-L-thyronine* (rT_3) *and L-thyroxine* (T_4). Thyroid hormone concentrations in serum were measured by radioimmunology in the case of T_3 (Seralute Ames Kit) and rT_3 (Biodata Hypolab Kit) and for T_4 by competition (Tetralute Ames Kit). The serum came from both normal and dexamethasone-treated animals. Measurements were made on 7–8 batches of serum, each batch coming from 3 to 4 rats.

RESULTS

Mitochondria

Table 1 shows cytochrome P450 concentrations in adrenal cortex mitochondria of normal and thyroidectomized rats. In normal animals, the concentration found was 1.62 nmol × mg^{-1} protein. In organelles from thyroidectomized animals, this value dropped by more than 25%.

Polyacrylamide gel electrophoresis revealed the presence of more than 20 protein constituents in rat adrenal cortex mitochondria, as shown by the densitometric tracing in Fig. 1. Their molecular weight ranged from about 10,000 to 130,000 daltons. Clearly defined relative migrations were established by using cytochrome *c* as an internal standard. Under the electrophoretic conditions chosen, migration chiefly depends on the size of protein constituents [20], which means it is possible to determine their molecular weight in relation to reference proteins. Section B of Fig. 1 reproduces the densitometric tracing for mitochondrial proteins from thyroidectomized animals. The number of bands is identical to that

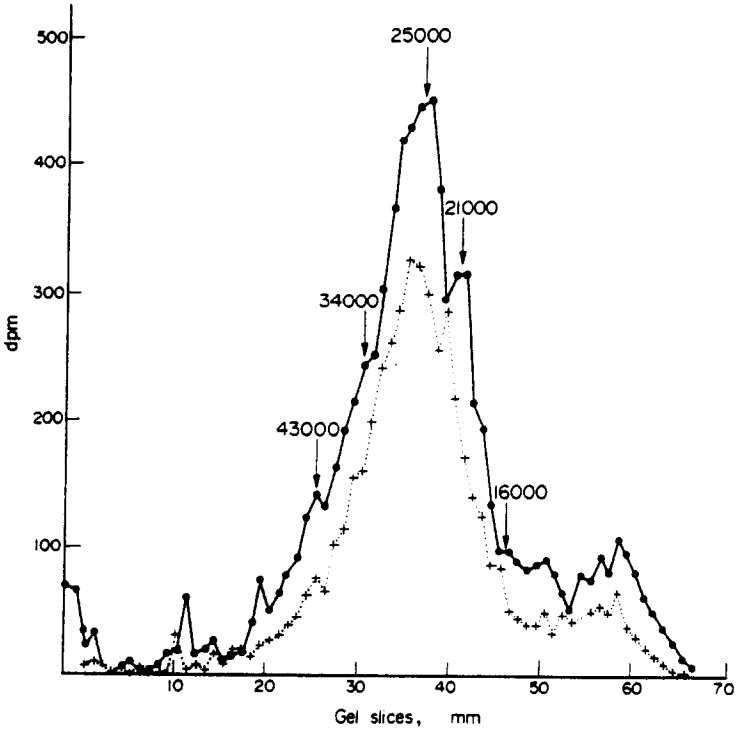

Fig. 2. Polyacrylamide gel electrophoresis of the mitochondrial proteins from rat adrenal cortex, labelled *in vitro*. The molecular weights of the peaks were calculated according to marker proteins. ●——● Normal. +---+ Thyroidectomized.

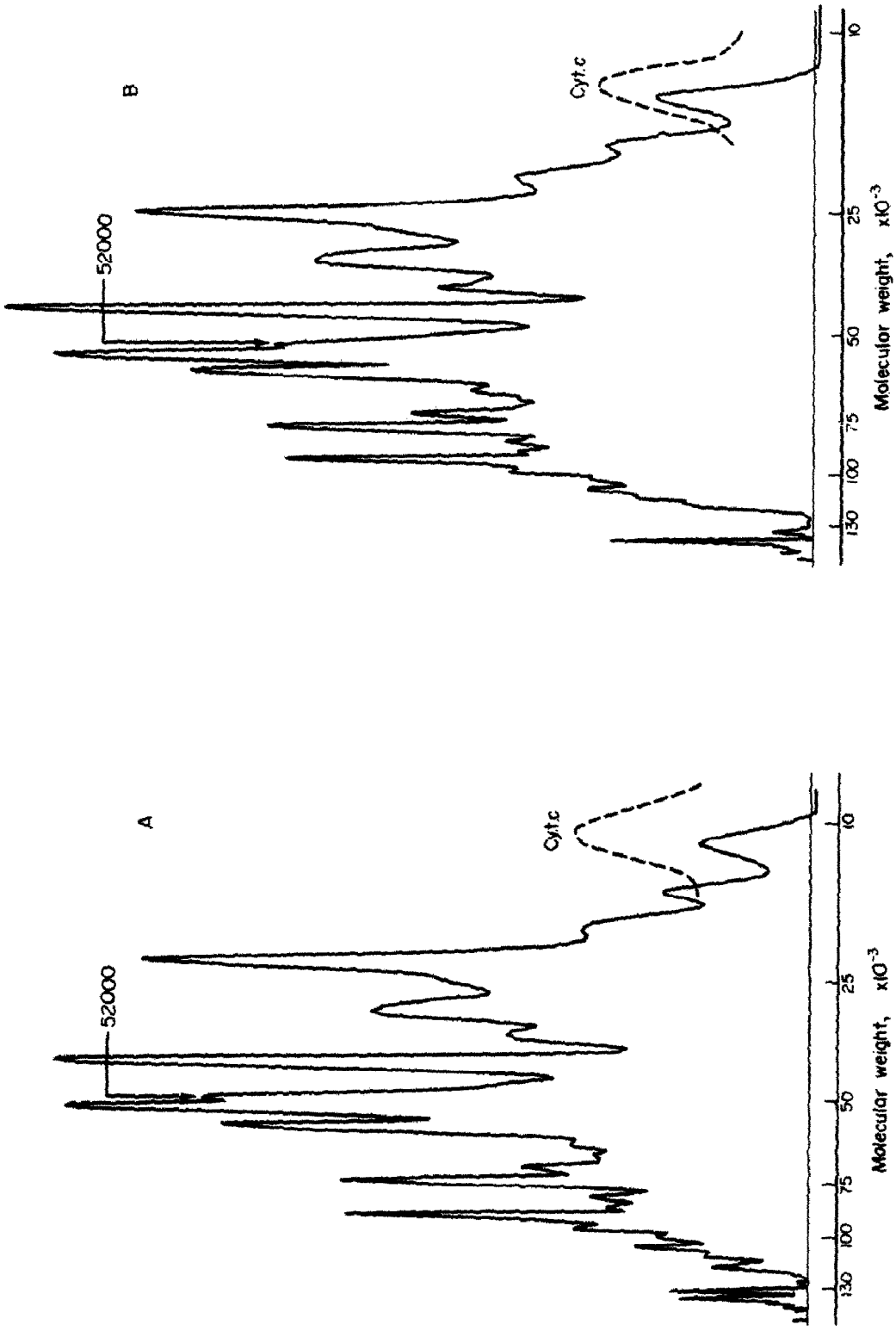

Fig. 3. Densitometric traces of polyacrylamide gel electrophoresis of adrenal cortex microsomes. A: Normal. B: Thyroidectomized.

Table 2. Plasma concentrations of ACTH in normal and thyroidectomized rats

Nornal	Thyroidectomized
13.7 ± 5.9	22.7 ± 4.4*

Concentrations are expressed in pg/ml plasma. Values given are the mean of 6 experiments ± S.E.M.
* $P < 0.02$.

obtained for mitochondria from normal animals. There was, however, a drop of about 30% in the 52,000 daltons molecular weight band. The corresponding constituent has the same relative mobility as the protein fraction containing the P450 subunit in bovine adrenal cortex mitochondria. These results were confirmed in the course of 12 experiments. Bovine adrenocortical mitochondria were also submitted to electrophoresis. A 52,000 daltons peak analogous to the peak of rat adrenocortical mitochondria was demonstrated.

In the presence of an artificial ATP generating system, isolated adrenal cortex mitochondria from normal animals incorporated [^{3}H]-L-leucine into their proteins. Under the experimental conditions chosen, the incorporation rate was 141/200 d.p.m. × mg^{-1} protein for 20 min, for an average of 7 experiments. It did not change in the presence of 1.5 mM cycloheximide but dropped by about 70% with 0.5 mM chloramphenicol.

Polyacrylamide gel electrophoresis showed that radioactivity was chiefly localized in proteins whose molecular weight ranged from 17,000 to 32,000 daltons (Fig. 2). Smaller radioactive peaks were recorded in the 43,000–47,000 and 9000–10,000 daltons molecular weight ranges. In the thyroidectomized rat, radioactivity incorporation dropped by an average of 30% (98,000 d.p.m. × mg^{-1} protein) but the radioactivity distribution did not change after electrophoresis.

Microsomes

The cytochrome P450 concentration in microsomes is indicated in Table 1; in normal rats, it was 0.97 nmol/mg protein. After thyroidectomy, it dropped by about 37%.

Figure 3 gives densitometric tracings for microsomal proteins in both normal and thyroidectomized rats. Proteins are divided into more than 20 constituents, their molecular weight ranging from 18,000 to 160,000 daltons. Thyroidectomy reduced the protein constituent with a molecular weight of 52,000 daltons. The amount of this constituent doubled in liver microsomes of phenobarbital-treated rats.

Figure 4 shows the kinetics for 21 hydroxylation of 17α-hydroxyprogesterone by isolated adrenal cortex microsomes. Thyroidectomy slowed down hydroxylation by about 30%.

Radioimmunological ACTH measurements

Table 2 indicates plasma ACTH concentrations: in normal rats, the concentration is 13.7 pg/ml whereas in thyroidectomized animals it is 22.7 pg/ml. The difference is statistically significant.

Rats treated with dexamethasone

In the rat, mitochondrial cytochrome P450 content dropped by 15%, 24 h after dexamethasone treatment (Table 1).

Electrophoresis of mitochondrial proteins shows a reduction of the protein constituent whose molecular weight is 52,000 daltons.

Concentration of thyroid hormones in plasma are given in Table 3. Dexamethasone reduced the proportion of T_3 and T_4 by about 10% but the differences observed were not statistically significant. rT_3, on the other hand, rose by 34%, a difference which was highly significant.

Table 3. Plasma concentrations of T_4, T_3 and rT_3 in normal and dexamethasone treated rats

	Normal	Dexamethasone
T_4	50.3 ± 3.2 (14)	43.9 ± 5.8 (13)
T_3	1.14 ± 0.10 (15)	1.02 ± 0.12 (14)
rT_3	0.14 ± 0.03 (7)	0.21 ± 0.03 (7)*

Concentrations are expressed in ng/ml plasma. Values are mean ± S.E. The number of experiments are given in parenthesis.
* $P < 0.01$.

DISCUSSION AND CONCLUSION

Our results make it clear that thyroidectomy reduces the amount of cytochrome P450 in rat adrenal cortex mitochondria. Spectrophotometric measurements do not reveal the heterogeneous nature of cytochrome P450 [26] but give overall values. In thyroidectomized animals, there is a reduction in

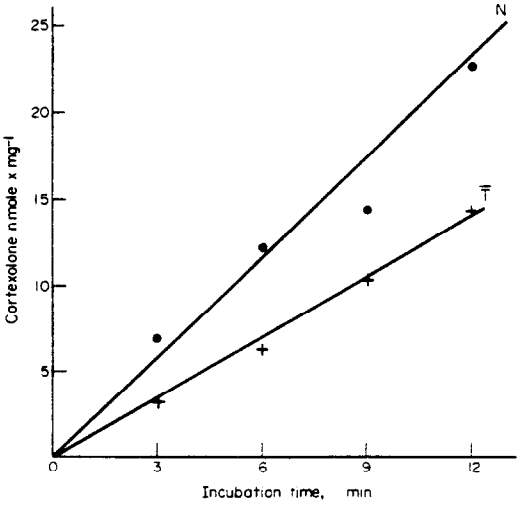

Fig. 4. Hydroxylation kinetics of 17α-hydroxyprogesterone in the presence of microsomes. N: Normal. T: Thyroidectomized.

the activity of the system that hydroxylates deoxycorticosterone into corticosterone [4]. This reduction may therefore conceivably be caused, at least in part, by the drop in cytochrome P450 since as we have previously found, the phosphorylating oxidation mechanisms are not affected in mitochondria [7]. Our results showed that cortexolone formation also decreases in the absence of thyroid hormone. The drop in 21-hydroxylase activity in adrenal cortex microsomes is not due to decreased NADPH biosynthesis, since this nucleotide is added to the reaction medium, but may be partly attributable to the reduced cytochrome P450 content which we observed in microsomes of thyroidectomized animals.

Polyacrylamide gel electrophoresis showed that about 15% of the mitochondrial proteins migrate into the 40,000–60,000 daltons ranges. Two molecular weight peaks 56,000 and 52,000 daltons are well individualized. The 52,000 dalton peak corresponds to the protein that contains cytochrome P450 subunit in bovines [27]. After thyroidectomy, this protein fraction was reduced by 30% which is in correlation with the decrease in the cytochrome P450 concentration. This constituent corresponds to the cytochrome P450 because it has, in our electrophoresis conditions, the same relative migration as the cytochrome P450 of liver microsomes. This fact is supported when we observe liver microsomes coming from rats which have been treated with phenobarbital, a well known agent for strongly increasing this cytochrome [28, 29].

It may be asked whether the decrease of cytochrome P450 concentration which is observed in rat adrenal cortex mitochondria and microsomes is due to lack of thyroid hormones or to a decrease of ACTH pituitary secretion. We observed that cytochrome P450 concentration is lower in adrenal cortex mitochondria from rats treated with dexamethasone which is a synthetic steroid well known for causing a drop in plasma ACTH concentration [30]. This drop is only due to the decrease of ACTH because we have not observed modifications in T_3 and T_4 hormone concentration in these animals, contrary to that which is observed in man [31–33]. The hormone concentration is increased only for rT_3. Corticotrope hormone concentration in the thyroidectomized rat, not only failed to diminish but in fact, increased. This rise might possibly be due to the slowing down of protein catabolism during hypothyroidism. Thus the adrenal cortex changes, observed in the thyroidectomized rat, are definitely the result of direct thyroid hormonal action.

Isolated rat adrenal cortex mitochondria incorporate labelled amino acids into their proteins. Protein electrophoresis showed that radioactivity was localized in the same areas as in the liver [34, 35]. Our results show that the incorporation which drops after thyroidectomy in liver and muscle mitochondria [12, 13], also diminishes in adrenal cortex particles. The lack of thyroid hormones does not seem to affect any particular protein, since the reduction in the incorporation is evenly distributed. In liver, nuclear and microsomal RNA were shown to drop after thyroidectomy [36], which also reduced RNA polymerase activity in mitochondria [37] and slowed down the replacement rate of mitochondrial proteins [38]. It is conceivable that the absence of thyroid hormones may have the same effect on the systems responsible for protein synthesis in the adrenal cortex.

Thus, the lack of thyroid secretion, which reduces protein synthesis in isolated adrenal cortex mitochondria, also causes a drop in the cytochrome P450 concentration in mitochondria and microsomes. This may explain the slowing down of the 11β and 21-hydroxylation rates observed in thyroidectomized rats.

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