constructive piece of information resulting from our discrepancies is that we are actually looking at two different things by optical rotation and ultraviolet difference spectra. The tyrosine side chains seem to be so situated that they will become exposed to water with either transition, depending on a delicate balance of factors. It would be interesting to observe the ultracentrifugal behavior of enolase under the conditions used by Rosenberg and Lumry, but charge effects in the lower ionic strength solutions might complicate the interpretation of such data.

It is interesting that no change in rotatory dispersion or ultraviolet spectrum occurs around *p*H 2.7, where the aggregated molecules dissociate. One might expect a change in tryptophyl absorption, for example, but perhaps intermolecular association of hydrophobic groups is replaced quantitatively by intramolecular association.

I would like to express appreciation for the help of Mr. G. McLain who prepared most of the enolase used in these studies, and of Mr. K.-Y. Wong who helped with the ultracentrifugation.

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Quantitative Study of the Effects of Thyroxine on Components of the Electron-Transfer System*

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The purpose of this work was to examine the effects of thyroxine on several components of the electron-transport system of rat liver. Doses of 0.005, 0.05, 0.1, 0.5, 1.0, and 2.0 mg of L-thyroxine per day were injected into normal white rats for 2 weeks. The highest dose was shown to increase succinoxidase activities of liver homogenates to 165% of the untreated control, succinic-ferricyanide reductase to 177%, succinic–cytochrome c reductase to 230%, cytochrome oxidase to 273%, and coenzyme Q to 279%. NADH–cytochrome c–reductase activity was decreased to 55% of the control value and this decrease is shown to be due to the antimycin A–insensitive microsomal system. These results are in agreement with an effect of thyroxine on protein synthesis which precedes and is responsible for the effect on oxidative metabolism as suggested independently by Tata and by Sokoloff in 1963.

Since the discovery of coenzyme Q (Co Q)[1] by Crane *et al.* (1957), and its involvement in electron transport (Hatefi *et al.*, 1959), there have been several proposals (Hatefi, 1959a, b; Clark and Todd, 1961) suggesting that Co Q might be an intermediate in one of the phosphorylation reactions. Definite proof of a role for Co Q in phosphorylation has thus far been elusive. Recently it has been shown that desiccated thyroid, when fed to rats, could cause dramatic increases in levels of Co Q in the liver (Aiyar and Sreenivasan, 1962; Beyer *et al.*, 1962) as well as smaller increases in other tissues (Beyer *et al.*, 1962). Others have found, however, that at physiological levels of thyroxine no increase could be observed (Pederson *et al.*, 1963).

Our interest in this area stemmed from these observations as well as the report (Leonhauser *et al.*, 1962) that there is a small amount of Co Q in the microsomal fraction of rat liver. The object of this work was to establish a quantitative relationship between the level of thyroxine administered and resulting changes in Co Q level, and to observe any change in the intracellular distribution of Co Q which might result.

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[1] Abbreviations used in this work: Co Q, coenzyme Q; ETP, electron-transfer particle; TSH, thyroid-stimulating hormone.

Experimental

Experimental Material.—Normal white male rats of the Harvard-Wistar strain were placed on experiment

at 15 weeks of age and were supplied standard diet of "Wayne Lab Blox" (Allied Mills, Chicago) and water *ad libitum*. Doses of aqueous L-thyroxine were injected daily in a volume of 0.2 ml at *p*H 11.0. At the end of the treatment period, animals were sacrificed by decapitation and the organs to be studied were removed and chilled in the homogenizing medium, 0.25 M sucrose.

Tissues were homogenized in a Potter-Elvehjem homogenizer fitted with a Teflon pestle, and filtered through a double layer of cheesecloth. Assays were performed on the whole homogenate and, in those experiments involving differential centrifugation, on each resulting fraction.

Chemicals.—NADH, cytochrome c, and L-thyroxine (sodium salt) were obtained from Sigma Chemical Co. Inorganic reagents were supplied by Fisher Scientific Co.

Assays—NADH- and succinic–cytochrome c–reductase activities were determined by modifications of assays described previously (Green *et al.*, 1955; Mackler and Green, 1956). The former contained 40 μmoles of phosphate, *p*H 7.4, 1.5 μmoles of cytochrome c, 0.03 μmole of KCN, 0.5 μmole of NADH, and 0.1–0.4 mg of protein. The latter contained 300 μmoles of phosphate, *p*H 7.0, 1.5 μmoles of cytochrome c, 0.02 μmole of KCN, 60 μmoles of succinate, *p*H 7.0, and 0.1–0.4 mg of protein. Final volumes were 3.0 ml and absorbancy changes were followed at 550 mμ in a Beckman DK-2 spectrophotometer.

Cytochrome-oxidase activity was measured spectrophotometrically as described (Wharton and Griffiths, 1962) except that reduced cytochrome c was prepared by reduction with KBH_4 followed by addition of acetic acid; 0.1–0.4 mg of protein was used. Previously described assays were used for succinic-oxidase activity (Mackler and Green, 1956) and succinic-ferricyanide-reductase activity (Green *et al.*, 1955). All assays were performed at 38°. Co Q was determined by the Craven test (Crane and Dilley, 1963) and protein by the biuret reaction (Gornall *et al.*, 1949).

Results

The data on body weight (Fig. 1) show that on a gross physiological level there was a quantitative graded response to thyroxine. In this graph the average body weight of the animals in each group (8–12 rats) on the first day was taken to be 100% and the per cent charge was plotted against time. The control values show that the animals were still growing during the sixteenth and seventeenth weeks of life, the 2 weeks of experimental treatment; and that while the two lower dose levels of thyroxine only retarded growth, doses of 0.1 mg per day and greater actually induced a weight loss.

In initial experiments it was observed that livers from rats treated with thyroxine weighed less than those from normal animals. However, since body weight also decreased, the liver weight per 100 g of body weight remained the same (Table I). In later experiments, in which rats were starved for 24 hours before sacrificing, there was no change in actual liver weight and the liver weight per 100 g of body weight actually increased with increasing thyroxine dose (Table I).

The succinoxidase, succinic-ferricyanide-reductase, cytochrome c–oxidase, and succinic- and NADH–cytochrome c–reductase activities obtained in a typical experiment (Table II) show that while all components of the succinoxidase system from succinic dehydrogenase to cytochrome c oxidase and coenzyme Q levels are doubled; NADH–cytochrome c–reductase activity

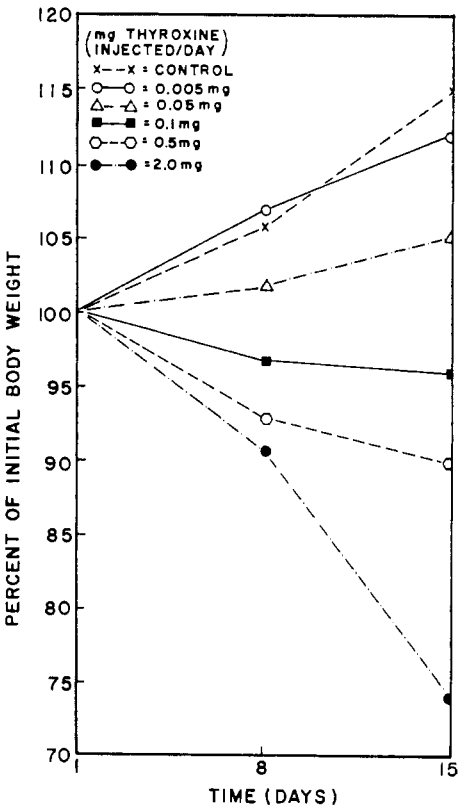

FIG. 1.—Effect on body weight of daily injections of varying doses of L-thyroxine. The injection series was begun on the first day and continued for 14 days. Weights on the first day were assigned a value of 100%.

TABLE I
EFFECT OF STARVATION ON WEIGHTS OF RAT LIVERS[a]

Treatment Prior to Killing	Thyroxine Dose (mg/day)	Liver Weight (g)	Liver Weight (g/100 g body wt)
Not starved	0.0	14.1	3.5
	0.005	12.3	3.4
	0.05	12.0	3.3
	0.1	10.6	3.9
	0.5	9.5	3.2
	2.0	10.2	3.7
Starved 24 hours	0.0	9.1	2.7
	0.005	8.2	2.5
	0.05	9.5	2.8
	0.1	9.8	3.2
	0.5	8.5	3.4
	1.0	9.3	3.4

[a] The effect of 24 hours' starvation on the weights of livers from rats injected with varying doses of L-thyroxine daily for 15 days.

is halved. These data indicate that there is no specific effect of thyroxine on Co Q but rather a general effect on the entire mitochondrial electron-transport system. Figure 2 shows that the decrease in NADH–cytochrome c–reductase activity is due to the antimycin A–insensitive microsomal-enzyme system. The activity of this enzyme in the 200 × *g* and 1000 × *g* fractions is about 60% inhibited by antimycin A while the activity in the 78,000 × *g* fraction is 100% insensitive.

Since it has been reported (Leonhauser *et al.*, 1962) that rat liver microsomes contain Co Q, the intracellular distribution of Co Q in various thyroid states was studied, using succinic-ferricyanide- and succinic–cytochrome c–reductase activities to characterize the

TABLE II
EFFECTS OF L-THYROXINE ON ENZYME ACTIVITIES OF RAT LIVER[a]

Thyroxine Dose (mg/day for 2 weeks)	Cytochrome Oxidase	Succinic Oxidase	Succinic–Cytochrome c Reductase	Succinic-Ferricyanide Reductase	NADH–Cytochrome c Reductase	Coenzyme Q (μg/g liver)
Control (0.0)	1.5	0.17	19.2	34.3	84.6	131
0.005	1.4	0.22	15.6	43.4	70.3	142
0.05	3.5	0.24	40.7	55.0	64.8	139
0.1	2.4	0.25	35.7	41.0	40.4	215
0.5	3.7	0.51	41.8	59.0	47.7	224
2.0	4.1	0.28	44.1	60.8	46.5	365

[a] Effects of L-thyroxine on the activities of several enzymes of the electron-transport system of rat liver. All assays were performed on whole homogenates of livers pooled from a pair of animals and activities are expressed as μmoles of substrate or μatoms of oxygen reduced/min per g of liver (wet wt). Succinoxidase and succinic-ferricyanide reductase activities were measured manometrically and all other assays were spectrophotometric.

TABLE III
EFFECTS OF L-THYROXINE ON FRACTIONS FROM RAT LIVER[a]

Thyroxine Dose (mg/day for 2 weeks)	Fraction (× g)	Succinic–Cytochrome c Reductase	Succinic-Ferricyanide Reductase	Co Q (μg/mg protein)
0.00	800	0.189 (89.2)	0.304 (83.7)	0.83 (66.0)
	8,700	0.069 (10.8)	0.154 (14.1)	0.44 (12.9)
	78,000	0.0 (0.0)	0.020 (1.1)	0.38 (6.1)
	Super	0.0 (0.0)	0.022 (1.1)	0.42 (15.0)
0.005	800	0.125 (92.6)	0.213 (90.4)	1.33 (85.3)
	8,700	0.062 (6.4)	0.115 (6.8)	0.82 (7.3)
	78,000	0.008 (1.0)	0.019 (1.4)	0.39 (4.3)
	Super	0.0 (0.0)	0.006 (1.4)	0.09 (3.1)
0.05	800	0.219 (77.2)	0.295 (78.8)	0.36 (36.3)
	8,700	0.259 (22.8)	0.292 (19.5)	1.00 (25.0)
	78,000	0.004 (tr.)	0.051 (1.2)	0.40 (33.7)
	Super	0.0 (0.0)	0.008 (0.5)	0.19 (5.0)
0.1	800	0.251 (97.3)	0.289 (92.7)	0.97 (83.3)
	8,700	0.050 (2.7)	0.110 (4.6)	1.03 (11.7)
	78,000	0.004 (tr.)	0.070 (1.9)	0.46 (3.3)
	Super	0.0 (0.0)	0.009 (0.8)	0.07 (1.7)
0.5	800	0.257 (95.0)	0.326 (93.3)	1.10 (62.6)
	8,700	0.079 (4.8)	0.108 (5.1)	3.48 (32.4)
	78,000	0.006 (0.2)	0.086 (0.2)	0.64 (2.8)
	Super	0.0 (0.0)	0.014 (1.4)	0.11 (2.2)
2.0	800	0.242 (97.5)	0.376 (94.7)	1.35 (71.6)
	8,700	0.056 (2.5)	0.081 (2.4)	3.20 (20.6)
	78,000	0.003 (tr.)	0.076 (2.5)	0.81 (5.6)
	Super	0.0 (0.0)	0.008 (0.4)	0.13 (2.2)

[a] Effects of injections of varying doses of L-thyroxine on the intracellular distribution of succinic–cytochrome c and succinic-ferricyanide-reductase activities and the coenzyme Q levels of fractions from rat liver. Livers from groups of 8–12 animals were pooled and centrifuged as shown. Values given as μmoles of substrate reduced/min per mg protein. Numbers in parentheses represent per cent of total activity in each fraction.

various fractions. The results of a typical experiment (Table III) show that most of the mitochondrial activity is sedimented in the 800 × *g* fraction. It can also be seen that while all of the succinic–cytochrome c-reductase activity is in the 800 × *g* and 8700 × *g* fractions, there is a greater proportion of succinic-ferricyanide-reductase activity and Co Q in the 78,000 × *g* fraction and supernatant.

DISCUSSION

Despite the great number of effects of thyroxine which have been observed, there is no general agreement as to which, if any, of these effects constitutes the basic physiological mechanism by which thyroxine acts. The thyroxine-induced uncoupling phenomenon observed by Lardy and Feldott (1951) and linked to mitochondrial swelling by Lehninger (1961) has been accepted by many as the true biochemical function of thyroxine. These phenomena are observed only at high levels of thyroxine and swelling can be induced by a great variety of nonspecific agents (Ernster, 1956; Ernster and Low, 1955; Lehninger, 1960a; Lehninger and Schneider, 1958; Slater and Cleland, 1953). It has also been shown that thyroxine can stimulate the ATP-induced contraction of mitochondria (Lehninger, 1960b).

Recent work indicates that thyroxine-induced hypermetabolism may be secondary to an effect of thyroxine on protein synthesis. It has been shown that puromycin (Weiss and Sokoloff, 1963) and actinomycin D (Tata, 1963), two inhibitors of protein synthesis, cause a complete reversal of the effects of thyroxine on oxygen consumption and that thyroxine causes an increased incorporation of amino acids into protein (Michels *et al.*, 1963).

Our work shows that in thyrotoxicosis there are increases in all components of the succinoxidase system of rat liver. It is felt that, in these experiments, doses of 0.1 mg of thyroxine per day or greater produced a thy-

rotoxic state as shown by weight gain (Fig. 1). This is consistent with a stimulation of protein synthesis by thyroxine. If the role of thyroxine were to uncouple one rate-limiting phosphorylation as proposed (Lardy and Feldott, 1961), one would expect an acceleration of only that segment of the chain to which the phosphorylation was coupled. That is, either the succinic–cytochrome c–reductase or the cytochrome c–oxidase rate would be increased, but not both. A primary effect on protein synthesis also explains the time lag that has been observed between thyroxine treatment and response (Pitt-Rivers and Tata, 1960).

The anomalous decrease in succinic–cytochrome c–reductase activity (Tables II and III) is consistent and reproducible and may be explained by postulating that the exogenous thyroxine level is great enough to partially inhibit TSH production and thereby decrease thyroid output but not high enough to replace the lost thyroid function.

The results with succinic-ferricyanide reductase are less consistent but a decrease is generally seen with this assay as well.

The difference in the distribution of succinic–cytochrome c–reductase and succinic-ferricyanide-reductase activities may be explained by the presence of a particle (Moury and Crane, 1964) which sediments with the microsomes at 78,000 × *g* and is similar in all respects to the ETP isolated from beef heart by Crane *et al.* (1956). This particle may be more "open" to ferricyanide than to exogenous cytochrome c.

Tata *et al.* (1963) have presented data which are in agreement with observations made in our laboratory (Moury, 1964) showing that mitochondrial effects reach a maximum 45–60 hours after a single injection and further showed that amino acid incorporation reaches a maximum in 40–50 hours.

If the effect of thyroxine is to be explained on the basis of increased protein synthesis, it must be postuated that it is not a simple stimulation of total protein synthesis, but rather a stimulation of those proteins involved directly and indirectly in electron transport. This stipulation is necessary since the protein concentration of liver is actually decreased by thyroxine treatment (Table IV). This indicates that the protein involved in electron transport is synthesized at the expense of other proteins.

TABLE IV
CHANGES IN PROTEIN CONTENT OF RAT LIVER WITH THYROXINE TREATMENT[a]

Expt	Thyroxine Treatment (mg/day for 2 weeks)	Total Liver Weight (g)	Total Protein Weight (g)	Protein (mg/g liver)	Control (%)
7	0.0	96.3	19.4	202	
7	1.0	89.4	15.0	168	83.1
8	0.0	105.7	29.4	278	
8	1.0	82.9	18.3	221	79.5

[a] Values represent pooled livers of 9 or 10 animals. Protein determined by the biuret reaction.

The data in Table I are in agreement with the report (Kuriyama, 1917, 1918) that the thyroid hormone can cause a depletion of liver glycogen and points up the necessity of fasting rats prior to sacrificing in order to eliminate the weight variability due to stored glycogen.

It is also of interest to note that in those experiments involving fractionation of homogenates, most of the mitochondrial activity is sedimented in the heaviest fraction (800 × *g* or 200 × *g*). This phenomenon was unaffected by variation of *p*H from 6.5 to 7.8 and may have been due to incomplete cell disruption and/or trapping of mitochondria by the cell debris.

Most, if not all, of the increase in Co Q is mitochondrial and any increases in the microsomal fraction may be due to contamination by mitochondrial-derived ETP (Moury and Crane, 1964). Also, there has been no attempt in these experiments to obtain a physiological state. Experiments will soon be undertaken to examine this facet and to correlate these changes with a direct measurement of protein synthesis.

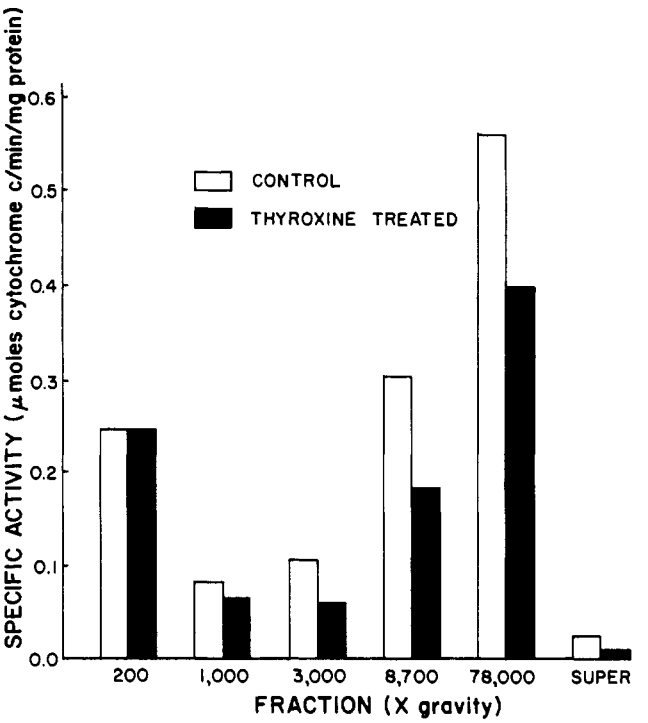

FIG. 2.—Effect of injection of 1 mg/day of L-thyroxine for 2 weeks on the specific activity of NADH–cytochrome c reductase in fractions from rat liver.

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Activated Hydrogens in Compounds Related to Thiamine. II*

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Two model compounds were studied in order to determine whether the rapid exchange at the two position of thiamine was because of the aromaticity of the thiamine molecule or because of its cyclic structure. The unstable *N*-methyl-2-thiazolinium methyl sulfate was found to hydrolyze in tritium-enriched water with subsequent methylation to give the product *S*-methyl-*N*,*N*-dimethyl-*N*-formyl-β-aminoethanol. This product contained nonexchangeable tritium, which demonstrates that the thiazolinium salt must have very rapidly exchanged protons with solvent prior to hydrolyses. However, the hydrogens on the open-chain analog *S*-methyl-*N*,*N*-dimethylthioformimidinium iodide have an unmeasurably high half-life for exchange with protons of solvent. These results show that the rapid exchange of the proton at the two position in thiamine is dependent upon ring formation but not on aromaticity. This result can be explained by carbene character to the intermediate carbanion. The aromatic character in the thiazolium ring of thiamine is not necessarily for carbanion formation but is necessary for other further reactions of the thiamine.

Carbanion formation in the thiazolium ring of thiamine is the first step in all mechanisms (Breslow, 1958; White and Ingraham, 1962) that may be written for reactions catalyzed by thiamine in biological systems. A carbanion is surprisingly easy to form in thiazolium salts. Breslow found that the half-life of the hydrogen at the 2- position of 3,4-dimethylthiazolium iodide was on the order of minutes in neutral D_2O. This paper gives a second report of studies designed to learn the reason for this unexpected high acidity in thiazolium ions. The previous report (Hafferl *et al.*, 1963) on the H-exchange rate in thiazolium, oxazolium, and imidazolium salts, and on the lack of exchange in noncyclic quaternized Schiff bases showed that neither sulfur-shell expansion nor sp^2 hybridization of a carbon adjacent to a charged nitrogen causes this high rate of H exchange.

The present study was undertaken to determine whether the exchange might be a result of the aromaticity or cyclic structure of all models which were found to exchange earlier. Experiments with a partially hydrogenated thiazole and its open-chain analog were performed to study the influence of both aromaticity and ring structure.

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EXPERIMENTAL

Preparations

2-Thiazoline (I).—This compound was synthesized by the method of Wenker (1935). Some decomposition of the crude base was caused by the potassium hydroxide drying agent. The purified compound was stable to metallic sodium so that the last traces of water were removed by high-vacuum distillation over sodium. It boiled at 38–42° (13 mm Hg) and at 139° (760 mm Hg). The infrared absorption is listed in Table I.

N-Methyl-2-thiazolinium Methyl Sulfate.—Three g of thiazoline (freshly distilled in high vacuum over sodium) was diluted with 15 ml benzene (dried over sodium). The solution was kept at 0° as 4.4 g of dimethyl sulfate (twice-distilled) was added. Soon a crystalline precipitate formed, which was allowed to stand 0.5 hour at 0°. The reaction product was very unstable in all polar solvents and even in benzene at room temperature. It was used immediately without further purification or identification.

S-Methyl-N,N'-dimethyl-N-formyl-(^3H)-β-aminoethanol (III).—The methylated thiazoline (II) was added to a solution of 12.5 g of dibasic potassium phosphate and 10 g monobasic potassium phosphate in 20 ml of tritiated water (1 mc/ml). The solution was shaken and almost immediately droplets of an oil appeared. The *p*H dropped from 9.4 to 7.3 during the few seconds of shak-