Differential Effect of L-Thyroxine on Phospholipid Biosynthesis in Mitochondria and Microsomal Fraction

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1. The action of L-thyroxine on the incorporation of radioactive choline or CDP-choline into phosphatidylcholine *in vitro* was explored in liver and brain microsomal fraction and mitochondria obtained from young adult rats. 2. In liver mitochondria isolated from animals treated with L-thyroxine (40 mg/kg body wt. during 6 days), the incorporation of both radioactive precursors into phosphatidylcholine was significantly decreased compared with normal controls, whereas in the total homogenate and in the microsomal fraction the incorporation was similar in the experimental and control groups. In subcellular fractions isolated from brain, the incorporation of precursors was similar in L-thyroxine-treated and normal animals. 3. Liver mitochondria isolated from normal animals incubated *in vitro* with CDP-choline, in the presence of different concentrations of L-thyroxine, showed also a marked decrease in the incorporation of label into phosphatidylcholine, whereas no significant changes were found in the total homogenate and in the microsomal fraction compared with control experiments. 4. The differential effect of L-thyroxine on the incorporation of radioactive precursors into phosphatidylcholine of isolated liver subcellular fractions gives further support to the hypothesis that liver mitochondria can independently synthesize part of their own phospholipids. 5. Possible mechanisms of the action of the hormone at the mitochondrial level are discussed.

Work from this laboratory (Soto *et al.*, 1972; Krawiec *et al.*, 1975) as well as results presented by Bossman & Case (1969) and by Van Schijndel *et al.* (1973), at variance with results obtained by other investigators (McMurray & Dawson, 1969; Jungalwala & Dawson, 1970; McMurray, 1974), has indicated that both liver mitochondria and microsomal fraction are able to independently synthesize phosphatidylcholine.

Nelson & Cornatzer (1965) showed that the administration of L-thyroxine for 6 days to young adult rats resulted in an increased incorporation of ^{32}P into different phospholipids of liver mitochondria. On the other hand, studying the effects of thyroidectomy on the turnover rate of protein of rat liver mitochondria, Satav *et al.* (1976) concluded that the lack of hormone produced an increase in the half-lives of mitochondrial proteins.

These results, as well as data published by Sterling *et al.* (1977) that indicate a possible direct effect of thyroxine on mitochondria, prompted us to explore the action of thyroid hormone on the incorporation of labelled precursors into phosphatidylcholine of liver and brain mitochondria and microsomal frac-

tion to obtain further support for the hypothesis of an independent synthesis of phosphatidylcholine by mitochondria.

Experimental

Adult Wistar rats of either sex (40–50 days old, 100–120 g body wt.) were used in all studies. To explore the effect of thyroxine *in vivo*, animals were made hyperthyroid as described by Nelson & Cornatzer (1965). L-Thyroxine (sodium salt; kindly supplied by Glaxo Laboratories, Buenos Aires), dissolved in 5 mM-NaOH at a concentration of 4 mg/ml and adjusted to pH 8.1 with HCl, was injected intraperitoneally into each animal once a day (40 mg/kg body wt. per day) for 6 days. Controls were similarly injected with 5 mM-NaOH. On the seventh day, animals were killed by decapitation and brains and livers were rapidly removed. Serum concentrations of L-thyroxine were determined in controls and in experimental animals by radioimmunoassay. All subsequent operations were performed at 0–4°C. Brain and livers were homogenized at 10% (w/v) in ice-cold 0.32 M-sucrose, pH 7.4, by using a Potter-

Elvehjem-type homogenizer. A portion of the total homogenate was saved for subsequent analysis and the remainder was used for the separation of subcellular fractions as previously described (Soto *et al.*, 1972; Krawiec *et al.*, 1975). Isolated liver and brain mitochondria and microsomal fraction were employed to explore the incorporation of radioactive precursors *in vitro* as described below.

Cross contamination of subcellular fractions

For the detection of microsomal contamination of the mitochondrial fraction we used NADPH-cytochrome *c* reductase, cytochrome *P*-450 and a method that was devised in our laboratories as previously described (Krawiec *et al.*, 1975).

Incubation conditions

Two radioactive precursors (both obtained from New England Nuclear Corp., Boston, MA, U.S.A.) were used for our studies: [*Me*-³H]choline (sp. radioactivity 69.5 Ci/mmol) and CDP-[*Me*-¹⁴C]choline (sp. radioactivity 49 mCi/mmol). To study the incorporation of choline, the incubation was done in a medium containing 20 mM-KH₂PO₄/K₂HPO₄ buffer (pH 7.4)/20 mM-sodium pyruvate/1.3 mM-sodium malate/5 mM-MgCl₂/1.7 mM-ATP/0.25 mM-CTP/0.1 mM-CoA / 1.7 mM-CMP/[*Me*-³H]choline (5 μmol, 0.5 μCi) (McMurray & Dawson, 1969). The final volume was 2 ml. The amount of fraction added to the incubation medium was equivalent to 0.5 g of fresh tissue. For the study of the incorporation of CDP-choline, the medium contained 37.5 mM-Tris/HCl buffer, pH 7.4/10 mM-cysteine hydrochloride (adjusted to pH 7.4 with 0.1 M-NaOH)/20 mM-MgCl₂/0.17 M-sucrose/CDP-choline (0.10 μCi, 0.8 μmol) (final volume 2 ml) as described by McMurray & Dawson (1969). The amount of fraction obtained from liver added to the incubation medium was equivalent to 50 mg of fresh-tissue total homogenate or microsomal fraction and to 100 mg of fresh-tissue mitochondria per tube as stated in a previous paper (Krawiec *et al.*, 1975). For the incubation of brain subcellular fractions we used the equivalent of 100 mg of fresh tissue for all fractions.

In the experiments in which we explored the incorporation into liver and brain subcellular fractions obtained from L-thyroxine-treated and control animals, the reaction was started by adding the labelled precursor to the medium containing the fraction and incubation was carried out for 30 min at 37°C with continuous shaking. In the experiments in which we explored the effect of L-thyroxine added *in vitro* on the incorporation of CDP-[*Me*-¹⁴C]choline, we used liver and brain total homogenate, mitochondria and microsomal fraction obtained from normal animals. To facilitate the entrance of the hormone, the fractions were first preincubated for 15 min in the presence of three different concentrations of added L-thyroxine: 1 mM, 10 μM and 0.1 μM.

The reaction was started by addition of the complete medium and incubation was carried out for 30 min at 37°C with continuous shaking. Appropriate normal controls without L-thyroxine were processed similarly. Incorporation was stopped in all cases by addition of chloroform/methanol (1 : 1, v/v).

Lipid extraction and radioactive counting

Extraction of lipids from the various samples and washing of the extracts was done by the method of McMurray & Dawson (1969). To check that the radioactivity was incorporated into phosphatidylcholine, lipids were separated by t.l.c. as previously described (Seminario *et al.*, 1965) and areas of silica gel corresponding to the various lipids were scraped off the plate and placed in counting vials containing 10 ml of scintillation solution (see below) and 0.7 g of Cab-O-Sil. Of the total radioactivity found in the plate, 90–95% was present in the spot corresponding to phosphatidylcholine. To measure the incorporation of the radioactive precursor, portions of the total lipid extract were evaporated to dryness in counting vials and re-dissolved in 10 ml of a scintillation solution containing 4 g of 2,5-diphenyloxazole and 0.1 g of 1,4-bis(5-phenyloxazol-2-yl)benzene/litre of toluene.

Chemical and enzymic determinations

ATPase (Mg²⁺-dependent) activity was measured as described by Richards *et al.* (1977). Proteins were determined by the method of Lowry *et al.* (1951) and lipid phosphorus as described by Chen *et al.* (1956).

The amount of radioactive CDP-choline present in the medium was assessed by extraction of the nucleotide derivative from samples incubated for 30 min with and without subcellular fractions, using the method described by Mandeles & Kammen (1966). Adequate zero-time controls were processed similarly for the purpose of comparison. Briefly, samples were precipitated with an equal volume of cold 20% trichloroacetic acid. After separation of the supernatant, the precipitate was washed once with 10% trichloroacetic acid. Absorbance of the combined supernatants was measured at 260 nm and acid-washed charcoal (Norit) was added in amounts corresponding to 5 mg/unit of $A_{260} \cdot \text{cm}^2$ {1 unit of $A_{260} \cdot \text{cm}^2 = [A_{260} \cdot \text{volume (cm}^3)]/\text{path length (cm)}$ }.

The charcoal-treated material was filtered through a 0.025 mm Millipore filter and the adsorbed nucleotide was eluted by repeated washings with water. Radioactivity present in the water eluate (which corresponds to the CDP-bound radioactive choline) was measured by liquid scintillation by using 10 ml of scintillation solution (Bray, 1960) per 2 ml of the water eluate. Recovery was better than 70% in three different control experiments. Statistical analysis of the results was carried out by the Student's *t* test. A probability level of 0.05 or less was considered a significant difference.

Results

Hormone serum concentrations in treated animals showed values six to seven times higher than those present in normal controls, indicating that L-thyroxine treatment was effective in producing hyperthyroidism. Cross contamination of isolated mitochondria by microsomal fraction, assessed by the different methods mentioned above, was similar in fractions obtained from normal or thyroxine-treated animals and varied between 3 and 7%, depending on the method used (cytochrome *P*-450 gave negative values in most cases).

Table 1 shows that the administration of L-thyroxine to young adult rats during six days produced a 10–20% decrease in body weight and a 10% decrease in liver weight compared with control animals. In L-thyroxine-treated animals there was a statistically significant decrease in the total proteins of liver mitochondria, whereas total protein content in the liver total homogenate and microsomal fraction was not significantly affected in treated animals compared with normal controls. Phospholipid phosphorus on the other hand showed similar results in all fractions in both experimental and control rats.

In animals injected with L-thyroxine the incorporation of radioactive choline into phosphatidylcholine either expressed as nmol/mg of total protein or as nmol/ μ g of lipid P showed a marked decrease in liver mitochondria in comparison with data obtained in normal animals (Table 2). In the total homogenate and in the microsomal fraction on the

contrary, the incorporation was similar in both experimental conditions. Since Nelson & Cornatzer (1965) found that the activity of liver ATPase was significantly increased in animals treated with thyroxine, we decided to investigate the activity of this enzyme in our fractions. A high activity of ATPase could invalidate the results mentioned above, since a decreased availability of ATP in the incubation medium could unspecifically interfere with the incorporation of choline into phosphatidylcholine. Our results (Table 2) shows an increase in the activity of the enzyme of 28% in liver total homogenate, 50% in mitochondria and 41% in microsomal fraction obtained from thyroxine-treated animals compared with controls. Although apparently the decreased incorporation of choline into mitochondria was not due to an increase in the activity of ATPase (the activity of the enzyme was also increased in the total homogenate and specially in microsomal fraction, with no apparent effect on the incorporation of the labelled precursor), we decided to use CDP-choline as precursor in our further experiments, since ATP is not necessary at this stage of the biosynthetic pathway of phosphatidylcholine.

In animals treated with thyroxine, the incorporation of CDP-choline into phosphatidylcholine was significantly decreased in liver mitochondria compared with normal controls. In the total homogenate and in the microsomal fraction, however, the incorporation was similar in experimental and normal animals (Table 2). To ascertain that in hyperthyroid rats there was no increased degradation of CDP-choline by enzymes present in the tissue fractions during incubation, decreasing the availability of CDP-choline in the medium and thus diminishing the synthesis of phosphatidylcholine, radioactive CDP-choline was estimated in control experiments as described in the Experimental section. Results showed that the total nucleotide-bound radioactivity remaining after incubation was quite similar in tubes containing added tissue fraction obtained from hyperthyroid rats and in control tubes containing tissue obtained from normal animals, indicating that the decreased incorporation found in mitochondria was not due to a decreased availability of the labelled precursor, but to other reasons. Results obtained with brain total homogenate, microsomal fraction and mitochondria indicated that the administration of thyroid hormone produced no significant effect on the nervous tissue in adult rats. Brain weight, total protein and phospholipid phosphorus, as well as incorporation of CDP-choline, were similar in both experimental and control animals (Table 3).

In the experiments in which the isolated subcellular fractions were incubated in the presence of thyroxine added to the medium, we also found a decrease in the incorporation of CDP-choline into

Table 1. *Effect of L-thyroxine on total protein and phospholipid content in liver subcellular fractions and on body and liver weight*

Rats (40–50 days old) were injected with L-thyroxine (40mg/kg body wt.) during 6 days. Liver subcellular fractions were obtained and total protein and phospholipid P were determined as described in the text. Results are means $\pm$ S.E.M. for six different experiments. Statistical analysis was carried out by Student's *t* test. A probability level of 0.05 or less was considered a significant difference. Body weight refers to increase in weight during the experimental period (6 days).

	Control	Experimental
Body weight (g)	+17.7 $\pm$ 2.0	+7.5 $\pm$ 1.9
Liver weight (g)	11.0 $\pm$ 0.3	10.0 $\pm$ 0.6
Total protein (mg/g of fresh tissue)		
Total homogenate	194.8 $\pm$ 17.9	223.8 $\pm$ 18.0
Mitochondria	31.6 $\pm$ 3.0	23.0 $\pm$ 1.6*
Microsomal fraction	27.0 $\pm$ 2.5	28.2 $\pm$ 2.2
Phospholipid phosphorus (μ g/g of fresh tissue)		
Total homogenate	1007.4 $\pm$ 62.3	1095.8 $\pm$ 91.0
Mitochondria	154.4 $\pm$ 14.1	132.0 $\pm$ 8.3
Microsomal fraction	240.4 $\pm$ 18.1	212.6 $\pm$ 12.2

* $P < 0.025$.

Table 2. *Effect of L-thyroxine on the incorporation of radioactive choline and CDP-choline into phosphatidylcholine and on ATPase activity of liver subcellular fractions*

Rats (40–50 days old) were injected with L-thyroxine (40 mg/kg body wt.) during 6 days. Liver subcellular fractions were obtained and incubated for 30 min in a medium containing the radioactive precursor as described in the Experimental section. Results are means $\pm$ S.E.M. for six different experiments. Data of incorporation into mitochondria are those obtained after subtraction of the possible incorporation due to contaminating microsomal fraction, which was estimated as described in the Experimental section. Statistical analysis was carried out by the Student's *t* test. A probability level of 0.05 or less was considered a significant difference. †, $P < 0.05$; ‡, $P < 0.01$; **, $P < 0.02$; *, $P < 0.01$.

	Choline incorporation			
	Control		Experimental	
	(nmol/ μ g of P)	(nmol/mg of protein)	(nmol/ μ g of P)	(nmol/mg of protein)
Total homogenate	0.24 $\pm$ 0.05	1.32 $\pm$ 0.22	0.21 $\pm$ 0.04	1.45 $\pm$ 0.28
Mitochondria	0.23 $\pm$ 0.05	1.51 $\pm$ 0.13	0.13 $\pm$ 0.02†	0.85 $\pm$ 0.13‡
Microsomal fraction	0.09 $\pm$ 0.02	0.96 $\pm$ 0.07	0.11 $\pm$ 0.03	1.02 $\pm$ 0.08
	CDP-choline incorporation			
	Control		Experimental	
	(nmol/ μ g of P)	(nmol/mg of protein)	(nmol/ μ g of P)	(nmol/mg of protein)
Total homogenate	1.10 $\pm$ 0.04	4.83 $\pm$ 0.30	0.95 $\pm$ 0.08	3.85 $\pm$ 0.17
Mitochondria	0.28 $\pm$ 0.03	1.32 $\pm$ 0.27	0.15 $\pm$ 0.02**	0.82 $\pm$ 0.09†
Microsomal fraction	1.69 $\pm$ 0.10	12.23 $\pm$ 1.28	1.69 $\pm$ 0.08	10.91 $\pm$ 0.89
	ATPase activity (nmol of P _i released/min per mg of protein)			
	Control		Experimental	
	(nmol/min per mg of protein)	(nmol/min per mg of protein)	(nmol/min per mg of protein)	(nmol/min per mg of protein)
Total homogenate	105.7 $\pm$ 9.6	135.2 $\pm$ 12.3*		
Mitochondria	114.9 $\pm$ 11.4	172.3 $\pm$ 22.3*		
Microsomal fraction	70.9 $\pm$ 9.9	100.3 $\pm$ 11.6*		

Table 3. *Effect of L-thyroxine on the incorporation of radioactive CDP-choline into phosphatidylcholine of brain subcellular fractions*

Rats (40–50 days old) were injected with L-thyroxine (40 mg/kg body wt.) during 6 days. Brain subcellular fractions were obtained and incubated in an appropriate medium containing the radioactive precursor as described in the Experimental section. Results are means for six different experiments $\pm$ S.E.M.

	Specific radioactivity			
	Control		Experimental	
	(d.p.m./ μ g of P)	(d.p.m./mg of protein)	(d.p.m./ μ g of P)	(d.p.m./mg of protein)
Total homogenate	21.6 $\pm$ 3.6	249 $\pm$ 12.8	18.8 $\pm$ 2.1	215 $\pm$ 18.7
Mitochondria	89.3 $\pm$ 7.5	716 $\pm$ 54.5	81.6 $\pm$ 7.6	654 $\pm$ 55.7
Microsomal fraction	22.9 $\pm$ 2.8	291 $\pm$ 25.2	22.5 $\pm$ 3.5	287 $\pm$ 30.7
Myelin	34.1 $\pm$ 2.9	941 $\pm$ 87.9	40.8 $\pm$ 5.2	946 $\pm$ 61.5
Nerve endings	28.4 $\pm$ 4.2	286 $\pm$ 31.2	25.8 $\pm$ 3.5	278 $\pm$ 19.8

phosphatidylcholine of liver mitochondria at all hormone concentrations, whereas in total homogenate and microsomal fraction there were no differences in specific radioactivity compared with control (Table 4). Total protein, lipid phosphorus and ATPase activity in fractions incubated with the hormone showed no changes relative to controls.

Discussion

Three points are relevant in the discussion of our results: (a) the differential effect of thyroxine (in

both experimental conditions) on protein content and on incorporation of radioactive CDP-choline, into phosphatidylcholine of isolated liver mitochondria, total homogenate and microsomal fraction; (b) the lack of effect of thyroxine on the various parameters analysed in adult (mature) brains; (c) the possible mechanisms through which the hormone exerts its action on phosphatidylcholine synthesis at the mitochondrial level.

Enzymes involved in the biosynthesis of phospholipids have been found in the endoplasmic reticulum by Wilgram & Kennedy (1963), but work by McCaman & Cook (1966) in brain and Stoffel &

Table 4. *Effect of added L-thyroxine on the incorporation of radioactive CDP-choline into phosphatidylcholine of liver mitochondria and microsomal fraction*

Liver mitochondria and microsomal fraction were obtained from normal 40–50 day-old rats. The isolated fractions were pre-incubated for 15 min in the presence of different concentrations (1 mM–0.1 μ M) of L-thyroxine and further incubated for 30 min in the complete medium containing radioactive CDP-choline. For details see the Experimental section. Results are means $\pm$ S.E.M. for six different experiments. Data of incorporation into mitochondria are those obtained after subtraction of the possible incorporation due to contaminating microsomal fraction, which was estimated as described in the Experimental section. Statistical analysis was carried out by Student's *t* test. A probability level of 0.05 or less was considered a significant difference. Abbreviation: N.S., not significant.

	Incorporation into phosphatidylcholine			
	Mitochondria		Microsomal fraction	
	(nmol/ μ g of P)	(nmol/mg of protein)	(nmol/ μ g of P)	(nmol/mg of protein)
Control	0.27 $\pm$ 0.03	1.13 $\pm$ 0.12	1.60 $\pm$ 0.12	15.38 $\pm$ 1.06
1 mM-Thyroxine	0.15 $\pm$ 0.03	0.62 $\pm$ 0.12	1.69 $\pm$ 0.16	14.99 $\pm$ 1.25
	<i>P</i> < 0.02	<i>P</i> < 0.02	N.S.	N.S.
10 μ M-Thyroxine	0.15 $\pm$ 0.01	0.67 $\pm$ 0.10	1.72 $\pm$ 0.14	16.06 $\pm$ 1.20
	<i>P</i> < 0.02	<i>P</i> < 0.02	N.S.	N.S.
0.1 μ M-Thyroxine	0.17 $\pm$ 0.01	0.67 $\pm$ 0.10	1.67 $\pm$ 0.16	16.75 $\pm$ 1.07
	<i>P</i> < 0.05	<i>P</i> < 0.05	N.S.	N.S.

Schiefer (1968), Kaiser & Bygrave (1968) and Kaiser (1969) in rat liver suggested that mitochondria can synthesize phospholipids through the CTP-mediated pathway. Work by Jungalwala & Dawson (1970), Miller & Dawson (1972) and McMurray (1974), however, indicated that mitochondria were unable to synthesize their own phospholipids and postulated that the low synthesizing activity found in these organelles was due to microsomal contamination. In previous work we have dealt extensively with this subject and have come to the conclusion that mitochondria have a certain degree of autonomy for the synthesis of phospholipids (Soto *et al.*, 1977). Clear evidence supporting this point of view was presented by Van Schijndel *et al.* (1973).

Although the results obtained with radioactive choline agree with those obtained using CDP-choline, for the reasons mentioned above (see the Results section) we shall only deal in the present discussion with the latter ones. Whatever the final mechanism of action of thyroxine on the synthesis of phosphatidylcholine might be, it is clear from our results that the hormone affects the CTP-mediated pathway in mitochondria, but not in microsomal fraction. The observed phenomenon cannot be due to differences in recovery of mitochondrial and endoplasmic-reticulum membranes in their respective fractions produced by thyroxine treatment, since microsomal contamination of mitochondria, assessed by different methods, is similar in fractions obtained from normal controls and thyroxine-treated animals. Besides, the fact that the differential effect on microsomal fraction and mitochondria is also seen in fractions obtained from normal (untreated)

animals, when they are incubated in the presence of different concentrations of thyroxine added to the medium, indicates that possible variations in the purity of the fractions are not the explanation for our observations.

If the synthesis of the phospholipid in mitochondria is due only to microsomal contamination (as proposed by Dawson and his co-workers), one would expect to find a similar effect of the hormone on microsomal fraction and mitochondria. The fact that it produces a marked decrease in the incorporation of labelled precursor into mitochondria in the two experimental conditions used in the present work, having no significant effect on the activity of microsomal fraction, seems to indicate that the two synthesizing systems are different and to a certain extent independent. Thus the results presented here give further support to our previous findings and seem to prove definitely that liver mitochondria have the machinery for the synthesis of phosphatidylcholine, irrespective of the presence of endoplasmic reticulum.

Our data in rats injected with thyroxine agree with those of Nelson & Cornatzer (1965) regarding the gross effect of the hormone on body and liver weight. Decrease in protein content in mitochondria was not reported by these investigators. Regarding the effects of the hormone on phospholipid metabolism and content in liver subcellular fractions, our results differ completely from those reported by these investigators. The data referring to incorporation of labelled precursor could be at variance because (a) the precursor used was different and (b) we measured the incorporation *in vitro* in the isolated fractions after treatment of the animal with

thyroxine, whereas Nelson & Cornatzer (1965) injected the precursor *in vivo* in the treated animals. The problem nevertheless is that we have been unable to confirm their finding of a decreased content of phospholipids in thyroxine-treated liver mitochondria. Unfortunately we have no valid explanation for this discrepancy. Kaiser & Bygrave (1969) explored the incorporation of choline into phosphatidylcholine of liver mitochondria isolated from animals treated with tri-iodothyronine and liver mitochondria isolated from normal animals but incubated with various concentrations of hormone. The results of the first group of experiments are opposite to those obtained by us, since they found an increase in the incorporation of the precursor by mitochondria instead of a decrease. However, in the second group of experiments the results obtained are coincident with our own. Unfortunately Kaiser & Bygrave (1969) do not make any comments on the discrepancies in the results of their two sets of experiments. There are several differences in the experimental design used by these investigators and by us which might explain the discrepancies mentioned above. The low doses of tri-iodothyronine used by these investigators to treat animals compared with very high doses used by us could be an explanation, since it is apparent from the results of their second group of experiments, that when high doses of tri-iodothyronine are present in the medium, the incorporation of the precursors into phosphatidylcholine is completely inhibited.

Regarding the second point, a large number of reports from different laboratories have shown that the effects of thyroxine on brain are only exerted during maturation (Krawiec *et al.*, 1969). Neonatal hyperthyroidism affects the lipid composition of rat cerebral cortex and cerebellum (Faryna de Raveglia *et al.*, 1972) and treatment of newborn rats with thyroxine markedly decreases all lipid classes in the same tissues (Faryna de Raveglia *et al.*, 1973). There are no reports in the literature, to our knowledge, with reference to the effects of thyroxine and thyroid deprivation on the biosynthesis and turnover of phospholipids in brain subcellular fractions. Although we expected to obtain negative results in the present group of experiments with brain tissue, we are presently studying the action of neonatal hyper- and hypo-thyroidism on phospholipid metabolism in the nervous tissue, which, based on the results presented in this paper, appears to be a promising and interesting line of research.

With reference to the possible mechanism of action of thyroxine on phospholipid metabolism at the mitochondrial level, it must be borne in mind that in spite of the large amount of work devoted to this fundamental subject, the effects of thyroxine at the molecular level are far from being known. Sterling *et al.* (1977) have reviewed the literature on this topic

and have also demonstrated the presence of thyroxine-binding sites in mitochondrial inner and outer membranes, supporting previous findings of other investigators that indicate that one of the target elements of thyroxine could be the mitochondrion. A long list of enzyme activities has been shown to be affected (decreased or increased) in liver and in other tissues by thyroxine administration (Wolff & Wolff, 1964). The list of enzymes is so diverse that no common mechanism of action of thyroxine can be recognized.

The decreased incorporation of CDP-choline into phosphatidylcholine in liver mitochondria isolated from hormone-treated animals could be explained by a decrease in the activity of CDP-choline-1,2-diacylglycerol cholinephosphotransferase as a consequence of a reduction in the protein content found in these mitochondria. It would be another of the various enzymes adversely affected by thyroxine. Roncari & Murthy (1975) have shown changes in the activity of liver enzymes related to lipid metabolism in rats treated with thyroxine, which were proved to be due to an altered content of enzyme protein and not to variations in the catalytic activity or to the presence of activators or inhibitors. If the activity of CDP-choline-1,2-diacylglycerol cholinephosphotransferase were similarly decreased by the action of thyroid hormone, this could be a possible explanation for our findings. However, the fact that similar results were obtained in the experiments in which the action of the hormone on normal liver mitochondria was explored *in vitro* tends to preclude this hypothesis, since no decrease in protein content was found in this experimental condition.

Since the activity of diacylglycerol acyltransferase is increased in the liver of animals treated with thyroxine and a greater proportion of diacylglycerol is diverted to the synthesis of triacylglycerol (Young & Lynen, 1969), a decrease in the availability of diacylglycerol (see Glenny & Brindley, 1978) could contribute to the diminished synthesis of phosphatidylcholine. However, the same objections mentioned above apply to this interpretation. It has been reported that thyroxine forms a highly insoluble complex with Mg^{2+} (Wolff & Wolff, 1964). CDP-choline-1,2-diacylglycerol cholinephosphotransferase is a Mg^{2+} -dependent enzyme and the decreased incorporation of CDP-choline into mitochondria found in our experiments could be due to the lack of this bivalent cation. We have carried out a few experiments to explore this possibility and have found that the decreased incorporation into mitochondria was not corrected by the addition of Mg^{2+} to the incubation medium. In fact, the incorporation was further decreased when the Mg^{2+} concentration in the medium was higher than 150 mM. Since Ca^{2+} is a potent inhibitor of phosphatidylcholine syn-

thesis, a change in partition of this cation between mitochondria and medium produced by thyroxine could explain our results. We have performed a set of experiments in which 1 mM-EGTA (final concentration) was present in the solution used for the isolation of subcellular fractions and in the incubation medium and have obtained similar results, ruling out this possibility. Coates *et al.* (1978) showed that the activity of an acid lipase present in liver increases with thyroid treatment. An uneven distribution of this enzyme in the microsomal and mitochondrial fractions due to the action of the hormone might perhaps explain our results. There are several reasons that make this hypothesis unlikely: optimum pH of this enzyme is acidic, whereas our assays are performed at neutral pH; triacylglycerols and cholesteryl esters (not diacylglycerols) are considered to be their substrates; the enzyme is dependent on Ca^{2+} , which is deliberately omitted from the incubation medium. The main argument against this possibility is again that the differential effect of thyroxine is observed with fractions treated *in vitro* with thyroxine, in which no possible uneven distribution could be expected to occur, since they are obtained from normal animals.

Further studies are required to elucidate the possible mechanisms of action of L-thyroxine on phospholipid metabolism in mitochondria.

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