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Thyroid Control over Biomembranes

Liver-Microsomal Cytochrome b_5 in Hypothyroidism

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Hypothyroid rats were prepared by thyroidectomy and maintenance on a low-iodine diet (group A); Group B was additionally pretreated with 0.5 mCi of ^{131}I as NaI, given intraperitoneally. Liver microsomes obtained from hypothyroid and normal rats were compared. After fasting and refeeding on 20% sucrose solution, high levels of microsomal fatty-acyl-CoA Δ^9-desaturase (as measured spectrophotometrically by the rate constants for cytochrome b_5 reoxidation) were induced in all the normal animals, half of the group A hypothyroid rats, and none of the group B hypothyroid rats. Hypothyroidism did not change desaturase Arrhenius profiles or V and K_m for NADH–cytochrome c reductase, but increased content of cytochrome b_5. The inability of adequately hypothyroid rats to induce the Δ^9-desaturase seems to be specific, in that injection of methylcholanthrene successfully induced microsomal benzpyrene monooxygenase activity and increased cytochrome b_5 contents in hypothyroid animals. The defects in overall fatty acyl desaturation reported in hypothyroid animals [Landriscina, C., Gnoni, G. V. & Quagliariello, E. (1976) *Eur. J. Biochem. 71*, 135–143] are suggested to be due to deficiencies in the specific desaturase(s).

The level of unsaturation in the phospholipid fatty acyl chains in mitochondrial and nuclear membranes obtained from the livers of hypothyroid rats is abnormally low and returns to normal within 3 days after a single injection of thyroid hormone [1–5]. Since 18:2 is the only source of (*n*-6) fatty acids in these laboratory animals, (where *n*-6 denotes the position of the double bond at the carbon atom numbered 6 from the non-carboxyl end) the accumulation of 18:2, 18:3(*n*-6) (γ-linoleic) and 20:3(*n*-6) and the deficiency of 20:4(*n*-6) fatty acyl chains in membranes during hypothyroidism suggests that microsomal Δ^5-desaturase activity [which converts 20:3(*n*-6)-CoA to 20:4(*n*-6)-CoA] depends on adequate levels of thyroid hormone. Such a dependence might explain the depression of overall desaturation activity in hypothyroid rats that has been deduced from measurements of aerobic and anaerobic fatty acid synthesis from acetate, acetyl-CoA or malonyl-CoA [6]. Furthermore, rates of overall fatty acid desaturation and of microsomal Δ^9-desaturation are above control levels in hyperthyroid rats [7].

Since there is evidence that at least the specific Δ^6 and Δ^9 desaturases share a common microsomal electron transport system, namely NADH–cytochrome b_5 reductase and cytochrome b_5 [8–11], a defect in one of these proteins or in their interactions might account for the findings in hypothyroidism, even though these components are said to be not normally rate-limiting [12,13]. Reported effects of thyroid state on liver microsomal NADH–cytochrome b_5 reductase and cytochrome b_5 are inconsistent. For instance, the activity of the system with cytochrome c as acceptor is said by some investigators to be similar in control and hypothyroid rats [14,15] and by others to rise to more than twice control levels abruptly about 2 weeks after thyroidectomy [16]. On the other hand, decreased flavoprotein cytochrome-b_5-reductase activity is reported to depress fatty acyl desaturation in riboflavin-deficient rats [17], and flavoproteins are not synthesized adequately from dietary riboflavin by hypothyroid rats [18], which might lead one to expect decreased cytochrome-b_5-reductase activity. Microsomal cytochrome b_5 content is found to be either normal [15] or above normal

This is fifth in a series of investigations on thyroid control over biomembranes.

Enzymes. Acyl-CoA Δ^9-desaturase (EC 1.14.99.5); NADH:ferricytochrome *b* oxidoreductase or NADH-cytochrome b_5 reductase (EC 1.6.2.2); NADH:(acceptor) oxidoreductase or NADH-cytochrome *c* reductase.

[16] in hypothyroid rats, but is also said to be elevated in hyperthyroid rats [19].

We have compared the cytochrome b_5 and the cytochrome-b_5-reductase segments in liver microsomes from hypothyroid and normal rats, using spectrophotometric methods and stearoyl-CoA as substrate for the Δ^9-desaturase. Rates of NADH-reduced cytochrome b_5 reoxidation were measured over a wide temperature range in order to assess possible alterations connected with the general increase in saturation of the fatty acyl chains of phospholipids in liver membranes observed in hypothyroidism [1–6, 20]. Because the Δ^9-desaturase is usually at low levels in animals fed routinely and can be induced by feeding sugars after a fast [13, 14, 21–23], we tested its inducibility in hypothyroid rats. The inducibility of other microsomal enzymes by phenobarbital or methylcholanthrene [24] was also examined.

MATERIALS AND METHODS

Two groups of male rats were used. Group A were Sprague Dawley rats (Anglia Laboratory Animals, Huntingdon Research Center, Cambs, England) weighing about 100 g, of which half were thyroidectomized and litter-mates were sham-operated as controls. Group B were Sprague Dawley rats (Spartan Animal Research, Haslett, MI, USA) of which half were thyroidectomized and in addition injected intraperitoneally with 0.5 mCi of $^{131}I^-$ as NaI. The thyroidectomized rats in both groups received 0.5% calcium lactate in their drinking water and the controls received 0.0005% KI in their drinking water. All the animals were fed a low-iodine, vitamin-enriched diet, obtained either from Astra Ewos AB (Stockholm, Sweden) or Nutritional Biochemicals (Cleveland, OH, USA) to avoid the thyroactive ingredients of the usual laboratory chows [25]. The rats were maintained on this regimen for at least 3 weeks, and were fed *ad libitum* up to the time of killing, unless otherwise stated. For the dietary induction of the Δ^9-desaturase, the animals were first starved for 24 or 48 h and then refed by being given access to a 20% (w/v) solution of carbohydrate in 0.45% sodium chloride for 24 h before killing [26]. To test the induction of liver microsomal cytochrome *P*-450 systems [24] the animals were given intraperitoneal injections of methylcholanthrene (20 mg/kg body weight) in corn oil, 5 days, 3 days and 1 day before sacrifice. Control animals received equal volumes of corn oil or isotonic NaCl solution. In these experiments the rats were starved overnight before preparation of liver microsomes to allow comparison with previous results.

Animals were killed by decapitation, and the livers were minced with scissors and homogenized in Teflon-glass to give a 20% (w/v) suspension in cold 0.25 M sucrose. Microsomes were prepared as described elsewhere [27]. Using a Teflon-glass homogenizer, the pellet was resuspended gently in 0.15 M Tris acetate pH 8.1, leaving behind most of the glycogen (when present), and the microsomes were resedimented at $105000 \times g$ for 45 min. The final pellet was suspended in 0.25 M sucrose and the protein concentration was adjusted to 20 mg/ml. Protein was measured by a rapid biuret reaction [28] in the presence of sodium deoxycholate; decolorization with KCN revealed no residual turbidity with microsome samples. The standard for protein measurements was bovine serum albumin.

The activity of the microsomal Δ^9-desaturase system was estimated spectrophotometrically by measuring the rate of cytochrome b_5 reoxidation in a dual wavelength spectrophotometer at 424–409 nm ($\varepsilon = 185\,mM^{-1}\,cm^{-1}$) [29]. The 2.0-ml reaction mixture contained 15 mM Tris-acetate pH 7.1, 10 µM stearoyl-CoA (Sigma, St. Louis, MO, USA), and 0.1 ml of a microsomal suspension (20 mg protein/ml). The concentration of cytochrome b_5/mg microsomal protein was estimated from the absorption difference between full reduction, obtained by adding 1.7 µmol of β-NADH (Sigma), and after reoxidation. Reduction of cytochrome b_5 with dithionite gave identical results, indicating that NADH addition reduced the cytochrome completely. The rate of cytochrome b_5 reoxidation followed first-order kinetics, at least initially, as reported by Oshino et al. [12, 13]; first-order rate constants were calculated from the recorded absorption changes [13]. The temperature of the reaction mixture was controlled by a cell-holder designed by B. Höijer, using Peltier elements to heat or cool. Arrhenius plots of the cytochrome b_5 reoxidation rate constants were obtained over the temperature range 7 °C to 44 °C (F. L. Hoch and B. Höijer, unpublished data).

Cytochrome *P*-450 was measured as the reduced carbon monoxide complex according to Omura and Sato [30]. Benzpyrene monooxygenase activity was assayed using radioactive substrate and by separation of products from remaining substrate with a simple extraction procedure [31]. Microsomal cytochrome *c* reductase activity was measured as described by [32], using 0.02 mg of microsomal protein and varying the NADH concentration to determine V and K_m.

RESULTS

Cytochrome b_5

Table 1 shows cytochrome b_5 contents, the rate constants for NADH-reduced cytochrome b_5 reoxidation, cytochrome *c* reductase activity, and the effects thereon of dietary induction. Microsomal cytochrome b_5 content is elevated above control levels in routinely fed hypothyroid rats, in agreement with [16]. After they undergo a fasting-refeeding cycle, the hypothy-

Table 1. *Effects of dietary induction on cytochrome* b_5 *levels and rates of reoxidation (fatty-acyl-CoA,* Δ^9*-desaturase rate constants), and NADH-cytochrome* c *reductase activity in liver microsomes from control and hypothyroid rats*
Experimental details are described under Materials and Methods. Two groups of rats were used: (A) the hypothyroids were thyroidectomized and maintained on a low-iodine diet; (B) the hypothyroids received in addition 0.5 mCi of $^{131}I^-$ intraperitoneally as NaI just after thyroidectomy. Animals were fed *ad libitum* to the time of killing. The rate constants for the reoxidation of NADH-reduced cytochrome b_5 were measured with added stearoyl-CoA (k) and without stearoyl-CoA (k^-); the rate constant for Δ^9-desaturation, $k^+ = k - k^-$. Dietary induction comprised a 24–48-h fast followed by access to a 20% sucrose/0.25 M NaCl solution overnight. The results are shown as means ± S.E., and P is calculated from group comparisons by Student's t-test

Animals (n)	Dietary Induction	Cytochrome b_5	Cytochrome b_5 reoxidation rate constants			NADH-cytochrome c reductase	
			k	k^-	$\frac{(k-k^-)}{k^+}$	V	K_m
		nmol/mg	min^{-1}			nmol · min^{-1} · mg^{-1}	μM
A. Controls (7)	0	0.23 ± 0.03	3.14 ± 0.61	1.19 ± 0.01	1.95 ± 0.60		
Hypothyroids (6)	0	0.34[e] ± 0.04	2.03 ± 0.10	1.28 ± 0.04	0.75 ± 0.09		
Controls (6)	+	0.27 ± 0.01	10.8[d] ± 0.64	1.29 ± 0.09	9.5[d] ± 0.64		
Hypothyroids (10)	+	0.34[f] ± 0.02	5.13[a,g] ± 1.39	1.33 ± 0.08	3.80[g] ± 1.49		
B. Controls (11)	0	0.16 ± 0.01	3.05 ± 0.55	1.88 ± 0.31	1.17 ± 0.33	1.42 ± 0.37	25.6 ± 10.3
Hypothyroids (5)	0	0.16 ± 0.01	3.61 ± 0.91	2.86 ± 0.71	0.75 ± 0.24	1.85 ± 0.48	6.3 ± 3.4
Controls (7)	+	0.10[d] ± 0.01	9.13[b] ± 1.79	1.66 ± 0.16	7.47[c] ± 1.86	0.72 ± 0.21	18.3 ± 6.6
Hypothyroids (5)	+	0.17[f] ± 0.02	2.69[f] ± 0.45	1.63 ± 0.22	1.06[f] ± 0.28	0.96 ± 0.39	19.2 ± 16.0

[a–d] Induced versus uninduced. [e–g] Hypothyroids versus controls. [a,e] $P < 0.05$. [b,f] $P < 0.01$. [c,g] $P < 0.005$. [d] $P < 0.001$.

roids do not alter cytochrome b_5 content whereas the group B controls decrease it markedly, as was reported by Jansson and Schenkman [33]. The diet affects cytochrome b_5 contents significantly, the Swedish diet producing a higher level in both controls and hypothyroids than the American diet. The rate constant for the reoxidation (autoxidation) of reduced cytochrome b_5 in the absence of stearoyl-CoA, k^-, is similar in all groups and is not affected by thyroid state or diet. The rate constants for Δ^9-desaturation, k^+, are at similarly low levels in uninduced hypothyroids and normals. Refeeding sucrose to fasted normal animals results in a fivefold to sixfold increase in the rate constant, as expected [13, 21]. An increased rate constant for cytochrome b_5 reoxidation occurs in the refed hypothyroid rats of group A, although the value is still significantly below the induced levels in control animals. The hypothyroid rats of group B fail to induce the Δ^9-desaturase, despite their having consumed 20.5 mg of the offered sucrose/g body weight as compared with 17.6 mg/g for the control animals.

Certain membrane-dependent processes in tissues from hypothyroid rats are slower than in normal rats only over a narrow temperature range, because of abnormal velocity-temperature relationships [1–5]. Fig. 1 shows representative Arrhenius profiles for the observed k in normals and hypothyroids, either routinely fed (uninduced) or induced by sucrose refeeding. In all cases the profile is linear from about 7 °C to at least 38 °C (confirming data in [34] on activities between 28 °C and 40 °C), with evidence of inhibition of rates at higher temperatures. In rats fed routinely,

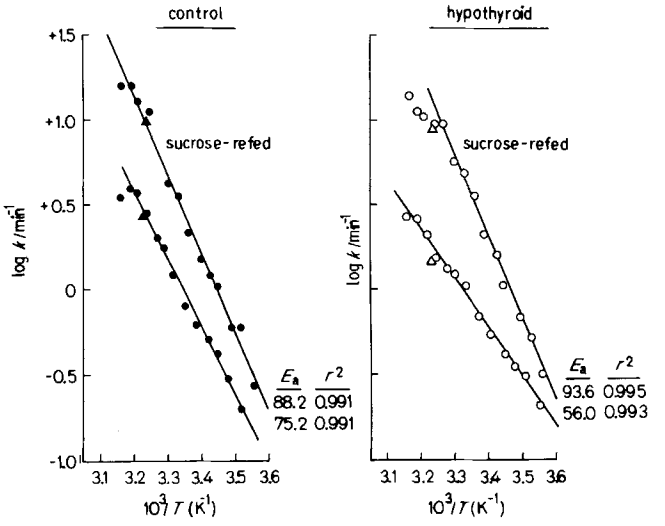

Fig. 1. *Arrhenius plots of observed rate constants,* k, *in sucrose-fed and routinely fed rats (group A).* The lines are calculated by a least mean square method, with the regression coefficient, E_a, in kJ/mol; the correlation coefficient, r^2, is also shown, Each line is one experiment, and rate measurements all start at the highest temperature; the triangular symbols are activities obtained at the end of each experiment (3–4 h)

the apparent activation energy, E_a, is greater in normals than in hypothyroids: in six controls E_a = 77.8 ± 3.93 (S.E.) kJ/mol, and in four hypothyroids E_a = 58.5 ± 2.13 kJ/mol, with $P < 0.005$. E_a is raised to higher levels in rats that have successfully induced desaturase activity after sucrose refeeding. Only a small difference is then observed between the E_a

Table 2. *The induction of liver microsomal enzymes by methylcholanthrene in control and hypothyroid rats*
Animals were prepared as in Table 1, except that they were fasted overnight before killing. 'Induced' rats received methylcholanthrene in oil [31]; uninduced rats were injected with oil

Animals (n)	Methylcholanthrene induction	Cytochrome P-450	Benzpyrene metabolized	Cytochrome b_5	Cytochrome b_5 reoxidation k^+
		nmol/mg	pmol · s^{-1} · mg^{-1}	nmol/mg	min^{-1}
A. Control	0	0.78	4.17	0.27	0.63
Hypothyroid	0	0.62	1.17	0.43	0.83
Controls (2)	+	1.53	28.9	0.56	0.28
Hypothyroids (3)	+	1.84 ± 0.08	16.9 ± 1.1	0.65 ± 0.02	0.39 ± 0.03
B. Controls (5–6)	0	0.50 ± 0.08	1.75 ± 1.00		
Hypothyroids (5–6)	0	0.42 ± 0.02	1.98 ± 0.83		
Controls (3–5)	+	0.82[a] ± 0.13	30.9[c] ± 2.0		
Hypothyroids (5)	+	0.75[b] ± 0.12	18.6[d] ± 2.0		

[a–c] Induced vs uninduced. [a] $P < 0.01$. [b] $P < 0.025$. [c] $P < 0.001$. [d] Hypothyroids vs controls: $P < 0.005$.

values in hypothyroids and controls; the means of four experiments are 86.9 and 95.7 kJ/mol, respectively (cf. 91.1 kJ/mol in [34]). The Arrhenius profiles for k^- (not shown in Fig. 1) are also linear between 7 °C and 38 °C and show little or no difference with diet change or altered thyroid state. The $E_a = 51.8 \pm 3.59$ kJ/mol in four experiments, a value which is significantly lower than the E_a for uninduced controls ($P < 0.001$) but is not lower than that for uninduced hypothyroids ($P > 0.1$).

Induction of Cytochrome P-*450 Systems*

Liver protein synthesis being slower in hypothyroid animals than in normals [35–37] may explain the failure of dietary induction of stearoyl-CoA desaturase, which normally involves protein synthesis [21,38]. This hypothesis was tested with injected inducing agents that are known to stimulate the synthesis of microsomal cytochrome P-450 systems in normal animals. A group of three hypothyroid rats was injected intraperitoneally with phenobarbital at a dose that normally induces microsomal cytochrome P-450 [24]. All three rats died with 3 days but the controls lived. Hypothyroid rats injected intraperitoneally with methylcholanthrene [24] survived as well as control animals; Table 2 compares measurements of microsomal enzymes after this treatment. Methylcholanthrene injection produces the following changes: a 7–18-fold increase in the rate of benzpyrene monooxygenase activity in control rats and a 9–14-fold increase in hypothyroids, although the rates in the induced hypothyroids are significantly lower than those in the induced control rats; a shift in the absorption maximum of the complex between the reduced cytochrome P-450 and carbon monoxide to 448 nm; a significant increase in the level of this cytochrome in liver microsomes from hypothyroids as well as in control rats; and an increase in microsomal cytochrome b_5 content in both control and hypothyroid rats, in the latter to levels even higher than the elevated ones seen in untreated hypothyroids (cf. Table 1), which is consistent with a report [39] that cytochrome b_5 is a component of the induced cytochrome P-450 system. The Δ^9-desaturase activity remains very low, however, both in hypothyroids and in controls; the low activities in the controls (cf. Table 1) may be connected with the overnight fasting of animals in the standard procedure for testing cytochrome P-450 induction (see Materials and Methods).

DISCUSSION

In normal animals liver microsomal fatty-acyl-CoA Δ^9-desaturase is induced within 24 h by feeding sugars after a fasting period [13,21,23]. In group B rats that are thyroidectomized surgically and chemically, sucrose refeeding does not induce the Δ^9-desaturase, although it can induce as high levels of Δ^9-desaturase activity as in normal rats in about half the animal not treated with $^{131}I^-$ (group A). It seems that group B must be more hypothyroid than group A, although none of the criteria used substantiate this conclusion. Both groups huddled for warmth, had eyelid retraction and scaly tails, stopped gaining weight beyond 150–200 g, had abnormally low liver :body weight ratios, and had low serum levels of thyroxine (<25% of controls) but not as low concentrations of triiodothyronine (group A 50% and group B 70% of controls). Although the iodine contents of the Swedish and the United States low-iodine diets were not measured, the feeding of the latter diet to rats prepared as were group A produces mitochondrial lipid changes that are less striking than in rats treated with $^{131}I^-$ (Hoch, F. L., Subramanian, C., Dhopeshwarkar, G. A., and Mead, J. F., unpublished

results). We therefore include radio-thyroidectomy in our preparation of hypothyroid rats.

Although hypothyroidism prevents the dietary induction of the fatty-acyl-CoA Δ^9-desaturase, injection of methylcholanthrene stimulates the production of benzpyrene-metabolizing enzymes and cytochrome b_5 in the hepatic endoplasmic reticulum in hypothyroid animals as well as in normal ones (Table 2). Similarly, intraperitoneal injection of coumarin, phenobarbitone or butylated hydroxytoluene successfully induces the synthesis of specific hepatic microsomal proteins in hypothyroid rats [40]. Although overall protein synthesis is slowed in the livers of hypothyroid animals [35–37], the existing ribosomal apparatus and RNA complement are obviously sufficient to respond to chemical inducers, since the induction by methylcholanthrene involves protein synthesis [38].

The failure of fasted and refed hypothyroid animals to increase the rate constant for electron-transfer between cytochrome b_5 and the Δ^9-desaturase thus appears to be a specific defect in the induction of the Δ^9-desaturase protein. As noted, dietary induction in normal animals involves the synthesis of this molecule [21]. The slowed rate constant can not be attributed to a defect in cytochrome b_5, which is indeed increased in content above control levels in one group of hypothyroid rats that are routinely fed (Table 1). Nor does hypothyroidism involve any change in the electron-transport capacity of the flavoprotein component of the fatty-acyl-CoA desaturase system, as shown by cytochrome *c* reductase activities (Table 1). Just how the inability to induce the Δ^9-desaturase would contribute to the observed decrease in overall desaturation rates in hypothyroidism [6] or to the depressed membrane contents of polyunsaturated fatty acyls [1–5] is not clear, however. The only Δ^9-desaturation product normally found in quantity in biomembranes is oleic acid as 18:1 fatty acyl, which accounts for up to 20% of the total fatty acyl content of phospholipids [41] and only about 10% of the normal unsaturation index. Further, 18:1 acyl content does not change with thyroid state [1–5] perhaps because it is also a common dietary component—39.3% of the total fatty acids in the low-iodine diet (Nutritional Biochemical)—and so its content does not necessarily reflect endogenous fatty acid metabolism. In view of the intactness of the desaturase electron-transport system proximal to the specific desaturase molecule, the desaturation defect in hypothyroidism is probably caused by deficiencies or defects in the various fatty-acyl-specific desaturases.

The rate constant for the autoxidation of cytochrome b_5, k^-, is independent of diet and thyroid state (Table 1), which indicates that reduced cytochrome b_5 is reoxidized by a separate and concurrent Δ^9-desaturation mechanism in the presence of stearoyl-CoA. If this is the case, the observed $k = k^+ + k^-$, and the E_a for k would be the sum of the E_a values for k^+ and k^- [42]. As the contribution of the Δ^9-desaturase as an acceptor increases as a result of dietary induction, E_a values would increase. In this way the observation that the E_a is higher in microsomes from routinely fed normal rats than in microsomes from hypothyroid rats (Fig. 1) can be attributed to the higher levels of Δ^9-desaturation activity in the normals (Table 1).

Arrhenius profiles that lack the transition points normally observed occur in some mitochondrial processes in hypothyroid tissues [1,2,4]. No transition appears in the temperature-dependence of the liver microsomal Δ^9-desaturase system of either control or hypothyroid rats (Fig. 1). The microsomal fatty acyl composition is, like that of mitochondria [2,4] and nuclei [3], depleted in arachidonoyl moieties in the hepatocytes of hypothyroid rats, but in addition the microsomes have an abnormally low cholesterol content [43]. The constancy of membrane-dependency of the microsomal fatty-acyl-CoA desaturase system even when the lipid composition is abnormal is consistent either with the known organization of this complex that allows the approximation of the active sites in the aqueous phase [9] and with the successful reconstitution of the complex by combining the protein components with relatively nonspecific phospholipids [44], or with the compensatory changes in fatty acyl unsaturation and cholesterol content as concerns membrane fluidity.

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