Metabolic Alterations Produced in the Liver by Chronic Ethanol Administration

COMPARISON BETWEEN THE EFFECTS PRODUCED BY ETHANOL AND BY THYROID HORMONES

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(Received 18 December 1972)

1. Liver slices from rats treated with thyroxine show an increased rate of O_2 consumption. The extra consumption, but not the basal respiration, can be abolished by ouabain. 2. Dinitrophenol is not effective in increasing the rate of O_2 consumption of liver slices from thyroxine-treated animals but its effectiveness can be recovered in the presence of ouabain. 3. $(Na^+ + K^+)$ -stimulated adenosine triphosphatase activity of liver was increased by administration of thyroxine *in vivo*. No changes were found in total Mg^{2+} -stimulated adenosine triphosphatase activity. 4. Mitochondrial α -glycerophosphate dehydrogenase and microsomal NADPH oxidase activity were increased by both thyroxine and chronic ethanol treatment. 5. Liver slices from animals chronically treated with ethanol synthesize urea at an increased rate. 6. Mitochondrial size (sectional area) is markedly increased in the liver of animals chronically treated with ethanol. 7. Acute administration of ethanol in doses of 4 and 6 g/kg significantly increases the uptake of ^{131}I -labelled thyroxine by the liver. 8. Work reported here, along with results from other investigators, indicates marked similarities between the effects produced in the liver by chronic administration of ethanol and by thyroid hormones.

Work reported in the two preceding papers (Videla *et al.*, 1973; Bernstein *et al.*, 1973) shows that chronic administration of ethanol results in a decrease in the phosphorylation potential ($[ATP]/[ADP][P_i]$) and a concomitant increase in the respiratory rate in liver tissue. The extra respiration was blocked by ouabain, an inhibitor of the $(Na^+ + K^+)$ -stimulated adenosine triphosphatase $[(Na^+ + K^+)-ATPase^*]$. In the livers of animals chronically treated with ethanol the active transport of ^{86}Rb and the activity of $(Na^+ + K^+)-ATPase$ were also increased. Similar changes have been recently reported for animals rendered hyperthyroid by administration of thyroid hormones. In the livers of these animals O_2 consumption and $(Na^+ + K^+)-ATPase$ activity are increased, and the extra respiration elicited by thyroid hormones could be abolished by ouabain (Ismail-Beigi & Edelman, 1970; 1971). Further, the results of Hassinen *et al.* (1971) suggest that thyroid hormone administration also leads to a decrease in phosphorylation potential in the liver cell.

The similarities described above suggested that the changes in the liver of the ethanol-treated animal might be produced by an increased availability or effectiveness of thyroid hormones in this organ. We have therefore compared several relevant variables in the livers of ethanol-treated and thyroxine-treated animals. Apart from confirming the observations of

Ismail-Beigi & Edelman (1970, 1971) in animals treated with thyroid hormones, we have found striking similarities between the changes produced by ethanol and by thyroxine which suggest that ethanol produced a functional hyperthyroid state in the liver.

Materials and Methods

Complete details regarding the chronic treatment with ethanol, type of diet used, pairing schedule and time of decapitation have been described by Videla *et al.* (1973). Basically, adult male Wistar rats were given liquid diets containing 35% of the total energy (cal) as ethanol (experimental group) for 21–27 days; for the pair-fed controls, ethanol was replaced by an amount of sucrose of equivalent energy. The average daily ethanol intake of the animals in the experimental group was 14.3 ± 0.2 g/day per kg. Other groups of rats, of 220–260 g body weight and fed Purina chow *ad libitum*, were given a single intraperitoneal dose of either L-thyroxine (6 mg/kg, freshly dissolved in 1 ml of 10 mM-NaOH in 0.9% NaCl) or the same amount of vehicle alone. The animals were killed 24–30 h after the administration of the hormone or the vehicle.

O_2 consumption by liver slices was determined by a polarographic technique as described by Videla *et al.* (1973). ATPase activity in liver homogenates was determined essentially as described by Bernstein

* Abbreviation: ATPase, adenosine triphosphatase.

et al. (1973), except that AT^{32}P (γ -labelled, New England Nuclear Corp., Boston, Mass., U.S.A.) was used as substrate at a final concentration of 2 mM ($50\text{ nCi}/\mu\text{mol}$). $^{32}\text{P}_i$ released was measured in a liquid-scintillation counter.

The activity of liver mitochondrial α -glycerophosphate dehydrogenase was determined polarographically (Estabrook, 1967) by measuring the change in the rate of O_2 utilization of mitochondrial preparations (Wainio, 1970) after addition of $30\mu\text{mol}$ of α -glycerophosphate to 3 ml of incubation medium (Hagihara, 1961) containing $1\mu\text{mol}$ of ADP at 37°C . Under these conditions the activity was linear with respect to the amount (2–4 mg) of mitochondrial protein added. For the determination of liver mitochondrial size, mitochondrial profiles in electron micrographs were cut and weighed. Liver samples were fixed in 3% (v/v) glutaraldehyde in cacodylate buffer, pH 7.1. Areas of liver were photographed at random by an independent observer and all mitochondria in each field were measured in this fashion. Microsomal NADPH oxidase activity was determined spectrophotometrically as previously described (Videla *et al.*, 1973).

Urea synthesis was measured by incubating 50–80 mg of liver slices at 37°C in 3 ml of Ringer bicarbonate buffer at pH 7.4 (Umbreit *et al.*, 1959) containing 10 mM-L-alanine. The slices were pre-incubated for 20 min in this medium saturated with $\text{O}_2 + \text{CO}_2$ (95:5) and were then transferred to fresh medium saturated with $\text{O}_2 + \text{N}_2 + \text{CO}_2$ (18:77:5) and shaken at 80–90 strokes/min. After 1 h 0.2 ml of 4% HClO_4 was added to the flasks and a portion of the medium was neutralized with 5 M- K_2CO_3 by using Methyl Orange as indicator. The sample was centrifuged, and urea in the supernatant was measured by the urease method of Bernt & Bergmeyer (1965).

For the study of the acute effects of ethanol on the uptake of thyroxine by the liver *in vivo*, male Wistar rats (160–180 g) were injected with $0.5\mu\text{Ci}$ of ^{131}I -labelled thyroxine (Abbott Laboratories, North Chicago, Ill., U.S.A.; sp. radioactivity $0.5\mu\text{Ci}/\text{ng}$) and $1\mu\text{Ci}$ of ^{125}I -labelled albumin (Abbott Laboratories; sp. radioactivity $1\mu\text{Ci}/\text{mg}/100\text{ g body wt.}$). These two substances were dissolved together in 0.9% NaCl, the pH was adjusted to 9–9.5 with Tris base, and the solution was injected into the tail vein in a final volume of 1.25 ml/kg. Since protein-bound iodine in the rat is about $3\mu\text{g}/\text{ml}$ of serum (Mendoza *et al.*, 1966), it can be calculated that the amount of ^{131}I -labelled thyroxine injected corresponds to less than 0.01% of the total amount of circulating thyroxine. After a 4 h period of equilibration the animals were injected intraperitoneally with 20 ml of iso-osmotic saline (0.9% NaCl)/kg containing ethanol in concentrations such that the dose of ethanol was 0, 2, 4 or 6 g/kg. All animals were killed

1 h after the last injection and radioactivity of ^{131}I and ^{125}I was determined in heparinized plasma, whole blood and liver in a two-channel Autogamma counter (Nuclear-Chicago, Des Plaines, Ill., U.S.A.). The amount of ^{131}I -labelled thyroxine in the liver was calculated after correction (from the ^{125}I -labelled albumin results) for the amount bound in the blood that remains trapped in the tissue (Oppenheimer *et al.*, 1968). Although this correction decreased the sample-to-sample variation, it did not essentially modify the interpretation of the results. It has been reported previously (Oppenheimer *et al.*, 1968) that even 48 h after the injection of the radioactive hormone more than 85% of the radioactivity in plasma or liver corresponds to unchanged thyroxine. It is reasonable to assume therefore that under the conditions of our experiment, in which the animals were killed 5 h after the administration of the hormone, virtually all the ^{131}I radioactivity in plasma and liver corresponds to the unchanged hormone.

Results

As shown in Table 1 thyroxine treatment 24–30 h before the rats were killed produced a 36% ($P < 0.01$) increase in the respiratory rate of liver slices *in vitro*. This is slightly less than the 50% increase reported by Ismail-Beigi & Edelman (1971) in the livers of rats pretreated for 6 days with tri-iodothyronine. Ouabain completely abolished the extra O_2 consumption in the livers of the thyroxine-treated animals but did not significantly alter the respiration in the livers of control animals. On the other hand, dinitrophenol increased by 50% ($P < 0.001$) the rate of O_2 consumption by the livers of control animals but had no effect on those from thyroxine-treated animals. In the livers of treated animals, the stimulatory effect of dinitrophenol on O_2 consumption was recovered in the presence of ouabain. Thyroxine treatment increased the ($\text{Na}^+ + \text{K}^+$)-ATPase activity by 70%, but did not alter the Mg^{2+} -ATPase activity (Table 2). These results are similar to those obtained in ethanol-treated animals by the same methods of investigation (Bernstein *et al.*, 1973).

The effect of ethanol and thyroxine treatments on mitochondrial α -glycerophosphate dehydrogenase activity is shown in Table 3. Ethanol and thyroxine treatments produced very similar increases in α -glycerophosphate dehydrogenase activity (190% and 180% respectively). Both treatments increased the activity of microsomal NADPH oxidase. This activity $\pm$ S.E.M. (numbers of animals) increased from 4.5 ± 0.7 (6) nmol/min per mg of protein in saline controls to 6.8 ± 0.8 (6) ($P < 0.05$) in thyroxine-treated animals. A similar increase was also found in animals chronically treated with ethanol (Videla *et al.*, 1973).

Table 1. *Effect of dinitrophenol and ouabain on the respiration of liver slices after the administration of thyroxine*

For details see the text. The values represent the means $\pm$ S.E.M. with the numbers of animals in parentheses. Significance studies were done by Student's *t* test for unpaired results: (a) versus (b), $P < 0.001$; (a) versus (d), $P < 0.001$; (b) versus (c), $P < 0.001$; (d) versus (e), $P < 0.001$; (g) versus (h), $P < 0.001$; (e) versus (g), $P < 0.02$; (e) versus (h), $P < 0.05$; (a) versus (e), $P < 0.01$; (a) versus (c), (a) versus (g), (b) versus (e), (e) versus (f), (b) versus (f), (c) versus (g), (d) versus (h), (b) versus (d) and (f) versus (h) were not statistically significantly different. Dinitrophenol (0.1 mM) and ouabain (1 mM) were used.

		Additions		O ₂ consumption (μ mol of O ₂ /min per g of liver)
		Dinitrophenol	Ouabain	
Saline control	(a)	—	—	0.88 $\pm$ 0.04 (8)
	(b)	+	—	1.32 $\pm$ 0.09 (8)
	(c)	—	+	0.94 $\pm$ 0.07 (6)
	(d)	+	+	1.47 $\pm$ 0.05 (6)
Thyroxine-treated	(e)	—	—	1.19 $\pm$ 0.07 (8)
	(f)	+	—	1.29 $\pm$ 0.08 (8)
	(g)	—	+	0.93 $\pm$ 0.03 (6)
	(h)	+	+	1.44 $\pm$ 0.07 (6)

Table 2. *ATPase activity in liver homogenates of rats treated with thyroxine*

For details see the text. The values represent the means $\pm$ S.E.M. with the numbers of animals used in parentheses. *P* values were calculated by analysis of variance. N.S., not significant.

	Enzyme activity (nmol/h per mg of protein)	
	(Na ⁺ + K ⁺)-ATPase	Mg ²⁺ -ATPase
Saline control	289 $\pm$ 39.6 (15) ($P < 0.01$)	3502 $\pm$ 310 (15) (N.S.)
Thyroxine-treated	435 $\pm$ 45.0 (15)	3474 $\pm$ 256 (15)

Table 3. *Effect of thyroxine administration and of chronic ethanol treatment on liver mitochondrial glycerophosphate dehydrogenase activity*

For details see the text. The values represent the means $\pm$ S.E.M. with the numbers of animals in parentheses. *P* values were calculated by Student's *t* test for unpaired results.

		α -Glycerophosphate dehydrogenase activity (nmol of O ₂ /min per mg of protein)
Expt. A.	Sucrose control	4.0 $\pm$ 0.4 (7) ($P < 0.001$)
	Alcohol-treated	11.8 $\pm$ 1.4 (7)
Expt. B.	Saline control	3.1 $\pm$ 0.8 (5) ($P < 0.02$)
	Thyroxine-treated	8.7 $\pm$ 1.7 (5)

Urea synthesis $\pm$ S.E.M. (numbers of animals) by liver slices was increased from 12.7 $\pm$ 2.6 (6) μ mol/h per g in controls to 19.3 $\pm$ 3.5 (6) in animals chronically treated with ethanol ($P < 0.01$). Mitochondrial

size (sectional area in electron micrographs) was measured in a total of 96 mitochondria in six control livers and 107 in livers from seven alcohol-treated animals. Mean areas/mitochondrion in the two groups

Fig. 1. Effect of acute ethanol administration on the uptake of ^{131}I -labelled thyroxine by liver *in vivo*

^{131}I -labelled thyroxine was given 5 h and ethanol or 0.9% NaCl 1 h before the animal was killed. Distribution of radioactivity was then determined in liver and plasma. Values presented for liver are corrected for the amount of hormone contained in blood present in the hepatic vascular bed (see the Materials and Methods section). The bars represent $\pm$ S.E.M. and the numbers in parentheses represent the numbers of animals for each point. *P* values refer to the differences between values obtained with no ethanol and those at the other points shown. (a) Uptake of ^{131}I -labelled thyroxine in liver; (b) radioactivity in plasma; (c) liver/plasma ratio of ^{131}I -labelled thyroxine.

were $0.59 \pm 0.06 \mu\text{m}^2$ and $0.98 \pm 0.07 \mu\text{m}^2$ respectively, indicating an increase of about 80% in the ethanol-treated group ($P < 0.01$).

Ethanol in doses of 4 and 6 g/kg significantly increased by 30% and 55% respectively the liver/plasma distribution ratio of injected ^{131}I -labelled thyroxine (Fig. 1). The actual net accumulation of the hormone in liver was also increased by ethanol, but owing to the larger variations inherent in this technique and to the decrease of the circulating hormone the increase was statistically significant only with the larger dose. Total amount (bound + free) of circulating hormone was significantly decreased by the doses of 4 and 6 g/kg.

Discussion

Results presented here and in the two preceding papers (Videla *et al.*, 1973; Bernstein *et al.*, 1973) indicate many resemblances between the hepatic biochemical effects of chronic treatment with ethanol and with thyroxine. Both treatments result in increased O_2 consumption by liver slices and this increase can be blocked by addition of ouabain *in vitro*. Also both treatments result in loss of activation of O_2 consumption by dinitrophenol, but the activation is recovered on addition of ouabain in both cases. This could be interpreted as an indication that in both ethanol-treated and thyroxine-treated groups

the extra O_2 consumption by liver slices depends on an increased rate of hydrolysis of ATP by the $(\text{Na}^+ + \text{K}^+)\text{-ATPase}$ system.

This conclusion is strengthened by the finding that the $(\text{Na}^+ + \text{K}^+)\text{-ATPase}$ activity was increased in both groups (Table 2, and Bernstein *et al.*, 1973). The increase in the thyroxine-treated group is in agreement with that reported by Ismail-Beigi & Edelman (1971). Other findings by these authors (Ismail-Beigi & Edelman, 1970) suggest that thyroxine increases the activity of the sodium pump and we have observed similar increases in ethanol-treated animals (Bernstein *et al.*, 1973). Increased utilization of ATP by the $(\text{Na}^+ + \text{K}^+)\text{-ATPase}$ system in the liver of animals chronically treated with thyroid hormones or ethanol might explain the decrease in cellular ATP amounts that also occur in both (Fletcher & Myant, 1961; French, 1966; Ammon & Estler, 1967; Walker & Gordon, 1970; Lindros & Hillbom, 1971; Bernstein *et al.*, 1973).

Animals chronically treated with ethanol show an increased capacity to metabolize ethanol both *in vivo* and *in vitro* (Hawkins *et al.*, 1966; Videla & Israel, 1970; Lieber & de Carli, 1970b; Tobon & Mezey, 1971; Mezey, 1972; Rawat & Kuriyama, 1972; Videla *et al.*, 1973). Thyroid hormone treatment has been reported by some investigators to increase the rate of ethanol metabolism (Goldberg *et al.*, 1960; Ylikahri & Mäenpää, 1968; Hillbom, 1971), whereas

others have found no change or a slight decrease (Newman & Smith, 1959; Kalant *et al.*, 1962; Smith *et al.*, 1963; Rawat & Lundquist, 1968; Hillbom & Pikkarainen, 1970; Stokes, 1971). It must be considered that apart from increasing the tissue oxidative capacity, thyroid hormones are effective reversible inhibitors of alcohol dehydrogenase (McCarthy *et al.*, 1968; Gilleland & Shore, 1969). Ylikahri & Mäenpää (1968) and Hillbom & Pikkarainen (1970) have shown that with the doses of thyroid hormones which they employed, the activity of alcohol dehydrogenase was inhibited at least 30–70%. Thus the effect on the rate of ethanol metabolism will depend not only on the oxidative capacity of the tissue but also on the residual amount of hormone present in the liver at the time of the experiment. Kahonen *et al.* (1971) produced mild liver hyperthyroidism by chronic administration of clofibrate (ethyl- α -*p*-chlorophenoxyisobutyrate). This drug is known to release thyroxine from circulating carrier proteins, to increase the uptake of this hormone by liver and to produce thyroxine-like effects in the liver (Westerfeld *et al.*, 1968; Ruegamer *et al.*, 1969). Treatment with clofibrate increased the rate of O₂ consumption by liver *in vitro* and increased the rate of ethanol metabolism *in vivo*, even when calculated per g of liver, without altering alcohol dehydrogenase activity.

Mitochondrial α -glycerophosphate dehydrogenase activity is believed to be a good indicator of thyroid hormone activity in the liver (Lee & Lardy, 1965; Ruegamer *et al.*, 1965). We found this activity markedly increased in the livers of both chronically ethanol-treated and thyroxine-treated animals. The increase in liver mitochondrial α -glycerophosphate dehydrogenase activity after chronic ethanol treatment is in agreement with findings by several workers (Kiessling, 1968; Marciniak *et al.*, 1968; Sardesai & Walt, 1969; Rawat & Kuriyama, 1972) but at variance with the report by Rubin *et al.*, (1972).

Liver mitochondrial size is known to be increased in animals treated with thyroid hormones (Paget & Thorp, 1963; O'Brien & Klitgaard, 1965; Shamoto, 1968). We have obtained similar results in animals chronically treated with ethanol in which mitochondria were photographed in random orientations. This is in agreement with previous findings by Porta (1969). Concomitant with the changes in liver mitochondrial size observed in hyperthyroid animals, marked increases in the rate of swelling and in the permeability to several substrates, co-factors and enzymes are known to occur in isolated mitochondria from these animals (see Tapley, 1964 for general references). Similar increases in swelling and in permeability also occur in liver mitochondria from animals chronically treated with ethanol (French, 1971; Rubin *et al.*, 1972; Rawat & Kuriyama, 1972).

It is well known that treatment with thyroid

hormones leads to a marked decrease in the amount of glycogen stored in the liver (Coggeshall & Greene, 1933; MacLagan & Rundle, 1940; Chilson & Sacks, 1959). Similar results have been reported in animals chronically treated with ethanol and examined in either the fed or the starved state (Lefèvre *et al.*, 1970; Walker & Gordon, 1970). In the absence of glycogen circulating glucose in the starved state should be produced by gluconeogenesis, which in most cases occurs with concomitant production of urea. Therefore both thyroxine and ethanol treatments should result in an increased rate of urea synthesis and an increased urinary nitrogen excretion. This is known to occur in hyperthyroidism (Rawson, 1964) and our findings, together with those of Klatskin (1962) and Rodrigo *et al.* (1971), suggest that it also occurs in animals chronically treated with ethanol.

Apart from the similarities in a number of alterations in mitochondria, cell membrane and cytosol, thyroxine and chronic ethanol treatment also cause increased activity of microsomal NADPH oxidase. For ethanol, this observation confirms those of Lieber & de Carli (1970a) and Carter & Isselbacher (1971). Thyroid hormones have also been reported to increase the microsomal NADPH-cytochrome *c* reductase activity (Tata *et al.*, 1962), which appears to form part of the NADPH oxidase system (Gillette, 1971).

Acute ethanol administration increased the uptake of thyroxine by the liver and decreased the total amount of hormone (free + bound) in plasma. These changes may be a reflexion of an increased binding or decreased excretion of thyroxine by the liver, or of an increase in the concentration of the free hormone available to the liver. Although direct changes in the liver cannot be discarded, the third explanation seems likely for two reasons. First, ethanol is known to produce metabolic acidosis, probably by production of lactate and acetate in the liver, which may be compounded by respiratory acidosis (Beard & Knott, 1971). A very small decrease in blood pH within the physiological range is known to release thyroxine from its bound form and to markedly increase the uptake of the hormone by the liver (Gordon & Coutsoftides, 1971). Secondly, intoxicating doses of ethanol are known to increase the plasma concentration of non-esterified fatty acids (Brodie *et al.*, 1961; Lieber *et al.*, 1966), which also increase the concentration of circulating free thyroxine (Radomski *et al.*, 1968).

Although ethanol intoxication equivalent to that caused by doses of about 4g/kg or more in rats is known to occur in human alcoholics, somewhat lower levels are likely to occur in rats consuming ethanol in liquid diets. It may be, however, that only a small increase in the availability of thyroxine to the liver

is necessary to cause the hepatic changes when the treatment is carried for a long time, as in our experiments (21–27 days). Kinetic studies (L. Videla, J. Bernstein & Y. Israel, unpublished work) have shown that with the ethanol-containing liquid diets used here a maximum increase in mitochondrial α -glycerophosphate dehydrogenase activity occurs only after 19–21 days of treatment.

Liver necrosis and cirrhosis occurred in 50–60% of patients, who died of severe thyrotoxicosis before the advent of modern therapy (Weller, 1933; Beaver & Pemberton, 1933). Although only minor histological deviations are detected in liver biopsies of hyperthyroid patients during life (Piper & Poulsen, 1947), it is clear that when the disease is severe enough it may produce (or predispose to) liver pathological changes resembling those found in chronic alcoholics.

Despite the large number of similarities between the effects produced in liver by thyroxine treatment and by chronic ethanol administration, one difference stands out: thyroid hormones are known to decrease the amount of circulating cholesterol (Boyd & Oliver, 1960; Young, 1968), whereas chronic treatment with ethanol increases it (Lieber *et al.*, 1963; De Carli & Lieber, 1967; Lefèvre *et al.*, 1972). However, in this respect, animals treated with thyroid hormones (but without ethanol simultaneously) might not be strictly comparable with animals chronically treated with ethanol.

In conclusion, a number of similarities between the effects produced in liver by chronic ethanol ingestion and by thyroxine treatment have been pointed out. The hypothesis that chronic ethanol treatment results in a functional liver hyperthyroid state should be readily testable in the laboratory and the clinic.

We thank Dr. Harold Kalant for suggesting the thyroxine-uptake experiments and for his critical review of this manuscript. The support of the Alcoholism and Drug Addiction Research Foundation (Ontario, Canada) is gratefully acknowledged.

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