The Effects of Acute Ethanol Feeding and of Chronic Benfluorex Administration on the Activities of some Enzymes of Glycerolipid Synthesis in Rat Liver and Adipose Tissue

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Rats were treated for 5 days with benfluorex {1-(3-trifluoromethylphenyl)-2-[N-(2-benzoyloxyethyl)amino]propane} or with suspending medium (controls). They were then intubated with an acute intoxicating dose of ethanol or with glucose of equivalent energy content. Treatment of the control rats with ethanol specifically increases the hepatic activity of the soluble phosphatidate phosphohydrolase by about 5-fold in 6 h. The equivalent increase for the benfluorex-treated rats were about 2-fold. The results are discussed in relation to the effects of ethanol and benfluorex on glycerolipid synthesis.

The ingestion of ethanol stimulates the incorporation of fatty acids into triacylglycerol by mammalian livers (Hawkins & Kalant, 1972; Lieber, 1974). By contrast, benfluorex {1-(3-trifluoromethylphenyl)-2-[N-(2-benzoyloxyethyl)amino]propane or S-780} decreases the rate of triacylglycerol synthesis in a number of tissues (for references see Pritchard & Brindley, 1977). Administration of benfluorex to rats for 5 days significantly decreases the ethanol-induced synthesis and accumulation of triacylglycerol in rat liver (Pritchard & Brindley, 1977). The earlier experiments were extended to investigate the effects of ethanol and benfluorex on the activities of some of the enzymes of glycerolipid synthesis in rat liver and adipose tissue.

Materials and Methods

Materials were prepared or purchased as described previously (Pritchard & Brindley, 1977; Whiting *et al.*, 1977).

Treatment of animals

Male Wistar rats (160–170 g) were kept in a room provided with artificial light for 12 h/day, commencing at midnight. They were allowed free access to Pilsbury's 41B diet (Brindley *et al.*, 1976) and to water, and were given 14 days in which to acclimatize before the experiments. They were then placed individually into Jencons Metabowls so that their food and water consumptions could be measured. After 3 days, the rats were treated on each of 5 successive days with 50 mg of benfluorex/

kg body wt. (Pritchard & Brindley, 1977). This treatment is known to produce a marked hypotriglyceridaemic effect (Duhault *et al.*, 1976) and it was continued long enough for the acute effects of benfluorex on lipid metabolism to be seen (Brindley *et al.*, 1976). Control rats were given gum tragacanth suspending medium (Brindley *et al.*, 1976) and all intubations were performed within 1 h of the start of the 'daylight' period. At 2 h after receiving the benfluorex or suspending medium on the fifth day, the rats were intubated with 5 g of ethanol/kg body wt. or with glucose of equivalent energy content (Pritchard & Brindley, 1977). They were then deprived of food but not of water.

Tissue preparation

At 6 h after the treatment with ethanol or glucose, the rats were killed by cervical dislocation and their livers perfused with ice-cold 0.15 M-NaCl. Microsomal and particle-free supernatant fractions were prepared from liver homogenates as described previously (Mangiapanne *et al.*, 1973; Whiting *et al.*, 1977). Fat-free homogenates were prepared from epididymal fat-pads (Dodds *et al.*, 1976), and these were dialysed twice for 90 min at 4°C against 0.25 M-sucrose containing 6 mg of fatty acid-poor bovine serum albumin/ml and 1 mM-Hepes* buffer, adjusted to pH 7.4 with KOH.

Determination of enzyme activities

Enzyme activities in liver were measured and

* Abbreviation: Hepes, 4-(2-hydroxyethyl)-1-piperazine-ethanesulphonic acid.

expressed relative to DNA P by using arylesterase (EC 3.1.1.2) as a marker for the recovery of endoplasmic-reticulum membranes (Whiting *et al.*, 1977).

Phosphatidate phosphohydrolase activity (EC 3.1.3.4) in adipose-tissue homogenates was determined as described for liver (Bowley *et al.*, 1977), except that the pH of the reaction was 6.8 and the concentration of phosphatidate was 1 mM. The incubation time was 30 min at 37°C and each assay contained 50–500 µg of homogenate protein. The rates of acylation of glycerol phosphate and of 2-hexadecylglycerol, and protein determination, in adipose tissue were measured as described by Dodds *et al.* (1976). The second activity was measured by using 2 mM-[U-³H]hexadecylglycerol (2 µCi/µmol) and [1-¹⁴C]palmitate (0.5 µCi/µmol) as substrates. The incorporation of both radioisotopes into acylalkylglycerol was determined after chromatography of the products on columns of alumina and t.l.c. on silica gel (Dodds *et al.*, 1976). EGTA (1 mM) was added to the assays to minimize phospholipase activities of type A, which might cause the acylation of 2-hexadecylglycerol by an acyl-exchange reaction (Waite & Sisson, 1976). Parallel incubations were also performed in the absence of ATP and CoA to distinguish true monoacylglycerol acyltransferase activity (EC 2.3.1.22) from a reversal of lipase activity. The results in Table 1 show the rates of incorporation of 2-hexadecylglycerol. Those for palmitate were similar, except that the proportion of the total activity that was dependent on ATP and CoA was slightly greater (59–67%), compared with 40–56% for hexadecylglycerol. 2-Hexadecylglycerol was used as an analogue for 2-monoacylglycerol, whose esterification may contribute to the synthesis of acylglycerols in adipose tissue (Dodds *et al.*, 1976). This rate of esterification may help to control that of glycerol phosphate in this tissue, since monoacylglycerols are inhibitors of glycerol phosphate acyltransferase (Schultz *et al.*, 1971).

Results and Discussion

Treatment of rats with benfluorex caused a severe decrease in food consumption and a loss in body weight over the first day, as shown previously (Brindley *et al.*, 1976). The consumption of water in the present experiments essentially paralleled the eating pattern. The rats soon became more tolerant to benfluorex with respect to these parameters; in the 24 h period just before treatment with ethanol, the 14 benfluorex-treated rats ate 18±3 (S.D.) g of food, which was significantly less ($P<0.001$) by Student's *t* test than the 15 control rats (22±2 g). They drank 22±7 (S.D.) ml of water and

gained 2±4 (S.D.) g of body wt., but this was not significantly different from control rats (25±4 ml and 1±3 g respectively).

Feeding of an acute dose of ethanol to the control rats produced approximately a 4.7-fold increase after 6 h in the activity of the soluble phosphatidate phosphohydrolase in the liver (Table 1). The other enzymes of glycerolipid synthesis that were measured in liver and adipose tissue did not change significantly. The administration of ethanol to the benfluorex-treated rats also increased the activity of the soluble phosphohydrolase, but only by about 2-fold. The microsomal activities of phosphatidate phosphohydrolase and of choline phosphotransferase (EC 2.7.8.2) were also increased in these rats (Table 1).

Ethanol treatment has been shown to increase the accumulation of intravenously injected [¹⁴C]palmitate in hepatic triacylglycerol in rats prepared in a similar way to those described in Table 1. This accumulation probably resulted from the increased relative rate of hepatic triacylglycerol synthesis that was measured *in vivo* (Pritchard & Brindley, 1977). In these rats, the relative rates of phosphatidate conversion into diacylglycerol were increased by ethanol treatment, and the accumulations of [³H]glycerol and [¹⁴C]palmitate in phosphatidate were decreased. Pretreatment of the rats with benfluorex significantly decreased the ethanol-induced increase in triacylglycerol synthesis and accumulation (Pritchard & Brindley, 1977). These results can probably be explained at the enzymic level by changes in the activity of the soluble phosphatidate phosphohydrolase (Table 1).

The ethanol-induced changes in lipid synthesis may result partly from a change in the availability of substrates. An increased concentration of glycerol phosphate that accompanies the change in the redox state may be one of these (Nikkilä & Ojala, 1963; Hawkins & Kalant, 1972; Lieber, 1974). However, glycerol phosphate concentrations alone cannot account for the ethanol-induced stimulation of triacylglycerol synthesis (Kalant *et al.*, 1972; Fellenius *et al.*, 1973; Estler, 1974; Abrams & Cooper, 1976a). The increased uptake of fatty acids from the circulation appears to be far more important (Bode & Goebell, 1971; Johnson *et al.*, 1975; Abrams & Cooper, 1976a,b). It has been suggested that ethanol inhibits the β -oxidation of these fatty acids, thus making them more available for esterification (Lieber & Schmid, 1961; Lieber *et al.*, 1967). However, the net rate of conversion of [U-¹⁴C]palmitate into ¹⁴CO₂ by the rats treated with ethanol was higher than that produced by the glucose-treated rats (Pritchard & Brindley, 1977). These experiments do not exclude the possibility that the rate of palmitate oxidation in the livers of ethanol-treated rats was decreased.

Table 1. *Effects of treating rats with ethanol and benfluorex on the activities of some enzymes of glycerolipid synthesis in liver and adipose tissue*

Enzyme activities in liver were measured in the microsomal fraction, except for the soluble phosphatidate phosphohydrolase, which was determined in the particle-free supernatant. The activities are expressed as nmol of substrate converted/min per μ mol of DNA P. Enzyme activities from adipose tissue were measured in the fat-free homogenate and are shown as nmol of substrate converted/min per mg of protein. All activities are expressed as means $\pm$ S.E.M. Significant differences between the treatments, which are described in the Materials and Methods section, are indicated as follows: * $P < 0.05$; † $P < 0.005$; the numbers of rats in each group are shown in parentheses.

Tissue	Enzyme activity	Specific activity			
		(I) Control + glucose (7)	(II) Control + ethanol (8)	(III) Benfluorex + ethanol (7)	(IV) Benfluorex + glucose (7)
Liver	Glycerol phosphate acyltransferase	47 $\pm$ 5	54 $\pm$ 5	46 $\pm$ 3	49 $\pm$ 5
	Microsomal phosphatidate phosphohydrolase	115 $\pm$ 22	158 $\pm$ 14	159 $\pm$ 20 III versus IV*	108 $\pm$ 11
	Soluble phosphatidate phosphohydrolase	15 $\pm$ 2 I versus II†	71 $\pm$ 15 II versus III*	32 $\pm$ 4 III versus IV†	14 $\pm$ 2
	Phosphatidate cytidyltransferase	8 $\pm$ 0.4	8 $\pm$ 0.4	7 $\pm$ 0.4	7 $\pm$ 0.4
	Choline phosphotransferase	59 $\pm$ 5	62 $\pm$ 5	62 $\pm$ 5 III versus IV*	47 $\pm$ 3
	Diacylglycerol acyltransferase	41 $\pm$ 8	42 $\pm$ 6	51 $\pm$ 6	45 $\pm$ 4
	Glycerol phosphate acyltransferase	9 $\pm$ 1	11 $\pm$ 1	10 $\pm$ 1	10 $\pm$ 1
Adipose tissue	Total phosphatidate phosphohydrolase	5 $\pm$ 0.6	6 $\pm$ 0.6	4 $\pm$ 0.7	5 $\pm$ 0.6
	ATP-+CoA-dependent synthesis of acylalkylglycerol from hexadecylglycerol	0.20 $\pm$ 0.05	0.28 $\pm$ 0.03	0.30 $\pm$ 0.04	0.35 $\pm$ 0.02
	ATP-+CoA-independent synthesis of acylalkylglycerol from hexadecylglycerol	0.30 $\pm$ 0.06	0.25 $\pm$ 0.02	0.27 $\pm$ 0.03	0.28 $\pm$ 0.03

There is evidence that ethanol directly stimulates hepatic triacylglycerol synthesis (Beaugé *et al.*, 1972; Estler, 1974; Abrams & Cooper, 1976a). Liver homogenates obtained from rats treated 16h previously with ethanol are known to exhibit enhanced triacylglycerol synthesis (Scheig & Isselbacher, 1965). The enhanced synthesis can now be ascribed to a selective increase in the activity of phosphatidate phosphohydrolase (Table 1; Lamb & Fallon, 1977). Rats have to be treated for 10 days with ethanol before the activity of glycerol phosphate acyltransferase is increased (Joly *et al.*, 1973).

Ethanol has been reported to decrease triacylglycerol synthesis in adipose tissue (Fan *et al.*, 1974). However, treatment of our rats with ethanol did not significantly alter the specific activities of the enzymes of glycerolipid synthesis that were measured in homogenates of the epididymal fat-pad (Table 1). This treatment may stimulate adipose-tissue lipolysis (Kessler & Yalovsky-Mishkin, 1966; Guidicelli *et al.*, 1972; Hawkins & Kalant, 1972), although no change was observed by Poggi & DiLuzio (1964).

Benfluorex and its metabolites may decrease the

ethanol-induced stimulation of triacylglycerol synthesis by a direct inhibition of phosphatidate phosphohydrolase (Brindley & Bowley, 1975; Pritchard & Brindley, 1977). However, the effects shown in Table 1 are probably not caused by this. Benfluorex and its metabolites would have been separated from the microsomal and supernatant fractions during their preparations, and the excess of phosphatidate used in the assays would have overcome the effects of any residual drug (Bowley *et al.*, 1977). The mechanism by which benfluorex alters the long-term control of phosphatidate phosphohydrolase activity is not known. Some of the differences in the response to ethanol between the control and benfluorex-treated rats may have been caused by differences in food intake. However, the benfluorex-treated rats consumed only about 20% less food than the controls in the 24h preceding treatment with ethanol, and their weight gains were not significantly different during this period. Furthermore food restriction is known to increase rather than decrease the hepatic activity of phosphatidate phosphohydrolase (Vavrečka *et al.*, 1969; Mangiapane *et al.*, 1973) and to enhance the ethanol-induced stimu-

lation of hepatic triacylglycerol synthesis (Johnson, 1974). Benfluorex may also reverse some of the effects of ethanol on glycerolipid synthesis by decreasing the ethanol-induced increase in hepatic blood flow and the resulting increase in the uptake of fatty acids (Abrams & Cooper, 1976a,b).

The soluble phosphatidate phosphohydrolase of rat liver is thought to be the major phosphohydrolase activity involved in the synthesis of triacylglycerol *de novo* (Hübscher *et al.*, 1967). The microsomal activity of the phosphohydrolase (assayed with a phosphatidate emulsion) appears to be up to 8 times greater than the soluble activity (Table 1; Whiting *et al.*, 1977). However, the composition of the lipids synthesized during the assay of the microsomal glycerol phosphate acyltransferase in Table 1 of the present work was determined and less than 10% of the total glycerol phosphate incorporated was converted into diacylglycerol and triacylglycerol. A similar result is reported in Table 2 of Whiting *et al.* (1977), who used identical techniques. The inability of the microsomal phosphatidate phosphohydrolase to act on microsomally bound phosphatidate has been reviewed by Hübscher *et al.* (1967). They demonstrated that the soluble fraction, and particularly the soluble phosphohydrolase, is required in order to obtain maximum rates of conversion of microsomally synthesized phosphatidate into neutral lipid. Of all the enzymes of triacylglycerol synthesis examined, it is only the activity of the soluble phosphatidate phosphohydrolase that changes dramatically and rapidly (Table 1) to parallel the observed rates of triacylglycerol synthesis (Pritchard & Brindley, 1977) in the livers of rats treated with ethanol and benfluorex. These results are compatible with the hypothesis that changes in the activity of the soluble phosphatidate phosphohydrolase contribute to the control of the metabolism of fatty acids and their incorporation into hepatic triacylglycerols (Mangiapane *et al.*, 1973; Lamb & Fallon, 1974; Fallon *et al.*, 1977; Whiting *et al.*, 1977).

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