Biochemical Pharmacology, Vol. 37, No. 4, pp. 695–705, 1988.
Printed in Great Britain.

0006-2952/88 $3.00 + 0.00
Pergamon Journals Ltd.

EFFECTS OF CHRONIC ADMINISTRATION OF BENFLUOREX TO RATS ON THE METABOLISM OF CORTICOSTERONE, GLUCOSE, TRIACYLGLYCEROLS, GLYCEROL AND FATTY ACID

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(*Received* 29 *June* 1987; *accepted* 21 *August* 1987)

Abstract—(1) Rats were fed on diets enriched with sucrose, beef tallow or corn oil and treated for 11–16 days with 50 mg of benfluorex per kg of body weight. By these times the growth rate and food intake were not significantly different from those of control rats. (2) Benfluorex approximately halved the concentration of circulating triacylglycerol in rats fed the beef tallow or sucrose diets. (3) It did not significantly alter the total lipoprotein lipase activity in diaphragm, heart and adipose tissue. (4) The clearance of triacylglycerols from chylomicrons exhibited two $t_{1/2}$ values of about 0.6 and 6.9 min in rats fed the beef tallow diet. Benfluorex did not significantly alter these values. (5) Benfluorex did not significantly alter the rate of appearance of triacylglycerol in the blood of rats injected with Triton WR 1339 to block triacylglycerol uptake. It did, however, decrease the rise in circulating glucose which presumably resulted from the stress of the procedure. (6) Benfluorex decreased the extent and duration of the rise in serum corticosterone when rats maintained on the corn oil diet were fed acutely with fructose. It also decreased the circulating concentrations of glycerol, triacylglycerol and glucose after fructose feeding. (7) Rats fed on the corn oil diet and then treated with benfluorex had lower concentrations of circulating glucose, triacylglycerol, glycerol and fatty acids after being injected with 2-deoxyglucose. (8) It is proposed that some of the long-term hypoglycaemic and hypotriglyceridaemic effects of benfluorex could be mediated indirectly through changes in endocrine balance, perhaps via the serotonergic system and in particular, by decreasing the effects of stress hormones relative to insulin. The implications of these findings are discussed in relation to controlling metabolism in stress conditions and for the management of obesity, diabetes and atherosclerosis.

Insulin-insensitivity and hyperglycaemia often accompany obesity, diabetes and atherosclerosis. Metabolism is therefore controlled to a greater extent by the stress hormones (e.g. glucocorticoids, corticotropin, catecholamines, glucagon etc.) and less by insulin. Obese animal models exhibit high concentrations of circulating glucocorticoids and the obesity can often be partially reversed by adrenalectomy [1]. Most human obesity is not associated with obvious hypercortisolism but post-obese women showed an exaggerated cortisol output and an impaired release of growth hormone and prolactin during insulin-induced hypoglycaemia. This suggested an altered hypothalamic control [2].

Stress is a major risk factor in atherosclerosis, and high concentrations of glucocorticoids are associated with it [3–6]. Glucocorticoids could also contribute to the ageing process in mammals and to neuronal loss [7, 8]. Stress hormones antagonize the actions of insulin on the breakdown of protein, glycogen and triacylglycerol. The amino acids and glycerol that are released can be used for gluconeogenesis and together with glucose formed from glycogen breakdown this could cause hyperglycaemia. Similarly, the fatty acids that are released from adipose tissue are converted by the liver to ketones and triacylglycerol. The stimulation of triacylglycerol synthesis in the liver can be facilitated by the stress hormones which increase the activity of phosphatidate phosphohydrolase [9, 10] and promote its stability [11]. The expression of the high phosphatidate phosphohydrolase activity depends upon an increased net availability of fatty acid in the liver resulting from lipolysis in adipose tissue [12]. Triacylglycerol synthesis protects the liver against the toxic accumulation of fatty acids and their CoA esters but it can result in a fatty liver. Alternatively, the triacylglycerols can be secreted as VLDL and this process is stimulated by glucocorticoids and antagonized by insulin [13]. In stress and diabetes, VLDL are preferentially metabolized by heart and skeletal muscle rather than by adipose tissue [14]. The hyperglycaemia and hypertriglyceridaemia that can occur in these conditions results from increased hepatic secretion and decreased removal from the blood.

VLDL are converted to LDL and the accumulation of these particles in the blood is associated with an increased atherosclerotic risk. Insulin increases

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whereas glucocorticoids decrease the activity for LDL receptors and the degradation of LDL in hepatocytes [15]. This, together with the increased secretion of VLDL [13] could account for part of the increased risk of premature atherosclerosis which is associated with stress and diabetes.

Glucocorticoids are not always antagonistic to insulin since they can further enhance the synthesis of glycogen [16] and fatty acids [17, 18], and the activity of lipoprotein lipase in adipose tissue [19] provided that insulin is also available. Cortisol and insulin concentrations in the blood of human beings rise during the consumption of meals [20–22] thus promoting energy storage. High concentrations of glucocorticoids can also decrease proton conduction in brown adipose tissue [23] and this could decrease the loss of energy stores. Genetically obese (OB/OB) mice are hypersensitive to the glucocorticoid-induced stimulation of feeding, but they are resistant to the glucocorticoid-induced weight loss [24].

It therefore seems to be important in the management of obesity, diabetes and atherosclerosis to improve insulin-sensitivity and to decrease the effectiveness of the stress hormones in regulating metabolism. Two drugs that act partly through the serotonergic system could exert some of their actions by decreasing an excessive release of glucocorticoids. These drugs are benfluorex which is an anti-hyperglycaemic and a hypolipdaemic agent [25–27] and D-fenfluramine which is used to treat obesity [28, 29]. The present work was designed to investigate further the mechanisms by which benfluorex might produce its hypoglycaemic and hypotriglyceridaemic effects.

MATERIALS AND METHODS

Unless stated to the contrary the source of the materials, animals and pelleted diets have been presented previously [30–32]. These references also describe the effects of dietary modification on growth rates of rats, body composition, the concentrations of some metabolites and hormones in blood, and some enzyme activities in liver, heart and adipose tissue. The methods of measurement were the same as were employed in this previous work [30–32], except that in Fig. 3 and Table 3 the concentration of corticosterone was measured by using HPLC and an internal standard of 11-deoxy-17-hydroxycorticosterone [33]. Benfluorex (1-(*m*-trifluoromethylphenyl)-2-(β-benzoyloxyethyl)-aminopropane hydrochloride; 780SE) was provided by Les Laboratoires Servier, Neuilly, France.

Treatment of rats. Male Wistar rats weighing 150–170 g were placed in grid-bottomed cages (3–5 rats per cage) and were maintained on a 41B diet [30] for 7 days. The food was then changed to the sucrose, beef tallow or corn oil diet [30]. After a further 7 days some of the rats were fed daily by stomach tube at 8.00–12.00 hr with 50 mg of benfluorex/kg of body wt. The benfluorex suspension was prepared fresh each day at 10 mg/ml in 0.5% (w/v) gum tragacanth. Control rats were fed the equivalent volume of suspending medium for the time period indicated. All rats were kept in a room at 22° that was lit from 08.00 hr to 20.00 hr. Further treatment of the rats is indicated in figures and tables.

Measurement of the clearance of [^{3}H]triacylglycerol from chylomicrons in the circulation of rats. The method is based on that of Harris and Felts [34, 35]. In order to prepare chylomicrons, about 1.5 mCi [^{3}H]palmitate was dissolved in 0.6 ml of water by warming to 60° and adding a slight molar excess of KOH. Six ml of evaporated milk was added and the mixture was shaken. Rats were dosed by stomach tube with 1 ml of the mixture and after about 30 min they were anaesthetized and the thoracic duct was cannulated but without the use of heparin [36]. Chylomicrons were collected over the next 4 hr and kept at room temperature. The clot was removed by centrifugation and the lipids were extracted from a 50 μl sample [37]. The concentration of lipid was determined by the method of Bragdon [38] with palmitic acid as a standard and by using a twentieth of the recommended volumes. Analysis of the radioactive lipids [39] by TLC on plates of silica gel G and development with hexane–diethylether–acetic acid (60:40:1; by vol.) showed that about 90% of the ^{3}H was in triacylglycerol. The chylomicron preparation was diluted with sterile 0.9% NaCl. In experiment 1, the diluted sample contained 9.4×10^6 dpm and 4.1 mg of lipid per ml. In experiment 2 the equivalent values were 12.9×10^6 dpm and 5.4 mg/ml.

Rats that had been maintained on the beef tallow diet and treated with 11 consecutive daily doses of benfluorex or suspending medium were anaesthetized with Brietal (sodium methohexitone) on the eleventh day of treatment between 06.00 hr and 08.00 hr. There were three benfluorex-treated and three control rats in both experiments 1 and 2. Catheters containing citrated-saline were implanted in the jugular vein and the carotid artery. The rats were allowed to regain consciousness and they were then given their final dose of benfluorex, or suspending medium at 08.15–12.00 hr. At about 270 min later the rats were infused through the venous catheter at a rate of 50 μl of the diluted chylomicron preparation/min. The infusion was stopped at 20 min. Blood samples (0.25 ml) were collected at intervals between 2 and 28 min through the arterial cannula, and fluid was replaced with 0.9% NaCl. No heparin was used during any of these procedures. Samples of the serum were extracted [37], and the radioactivity in triacylglycerol was determined [39].

RESULTS

Effects of benfluorex on the food intake and growth rates of rats

Diets enriched with sucrose, beef tallow or corn oil were used in this paper to produce the required metabolic condition under which to study the effect of benfluorex treatment. The effects of benfluorex on growth rates and food intakes were similar with all three diets, and the results for rats fed the beef tallow diet are shown in Fig. 1 as an example.

There was a marked fall ($P < 0.05$) in body weight in the benfluorex-treated rats on the first day of treatment (Fig. 1A). After about 5 days the growth of these rats was re-established such that the rate paralleled that of the control rats. The change in the

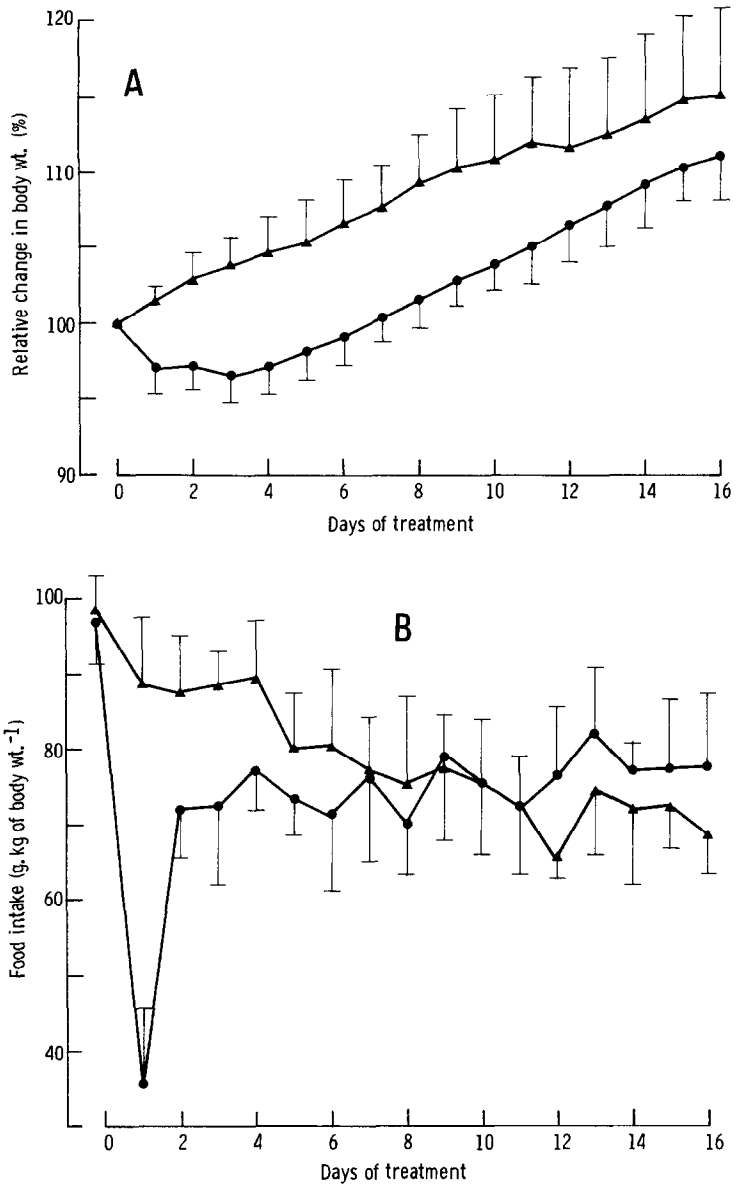

Fig. 1. Effect of benfluorex on the growth rate and food intake of rats fed the beef tallow diet. Rats were given daily doses of 50 mg of benfluorex·kg of body wt^{-1} (●), or suspending medium (▲) (Materials and Methods section). The changes in body weight are expressed relative to that at the beginning of the treatment (A). There were 27 control rats and 32 benfluorex-treated rats up to day 11, and thereafter 13 control rats and 15 benfluorex-treated rats. Food intake is shown per cage of rats relative to the weight of rats in the cage. There were 7 cages of control rats and 8 cages of benfluorex-treated rats up to day 11, and thereafter 3 cages of control and 4 cages of benfluorex-treated rats. Results are given as means ± SD.

slope of the curve for the control rats after 11 days results from the change in the numbers of animals and not from a convergence of the growth rate curves. The initial loss in body weight in the benfluorex-treated group was associated with a severe decrease in food intake on the first day (Fig. 1B). By about 5 days of treatment the food intake of the treated rats was not significantly different from that of the controls. It was therefore decided to treat the rats for at least 11 days (i.e. 12 doses) in other experiments. This provides a stable situation in which the metabolic effects of benfluorex can be investigated and in which the growth rates and food intakes of the control and treated rats are essentially the same.

Effects of benfluorex treatment on the concentrations of some metabolites and hormones in the serum of rats

Rats were fed on the beef tallow and sucrose diets, since these are known to increase the concentrations of circulating triacylglycerol relative to rats fed on the corresponding starch diet [30]. Treatment of

Table 1. Effects of benfluorex treatment on the concentrations of some metabolites and hormones in the blood serum of rats

	Beef tallow diet		Sucrose diet	
Concentration	Control	Benfluorex treated	Control	Benfluorex treated
Glucose (mM)	8.2 ± 0.3 (13)	7.9 ± 0.2 (15)	8.4 ± 0.3 (8)	8.9 ± 0.4 (7)
Fatty acid (mM)	0.27 ± 0.02 (11)	0.29 ± 0.02 (13)	Not measured	
Glycerol (mM)	0.13 ± 0.009 (12)	0.09 ± 0.008 (12)	0.20 ± 0.02 (8)	0.13 ± 0.03 (7)
	$P < 0.005$		$P < 0.01$	
Triacylglycerol (mM)	3.63 ± 0.04 (13)	1.95 ± 0.19 (15)	4.05 ± 0.60 (8)	2.04 ± 0.12 (7)
	$P < 0.001$		$P < 0.005$	
Total cholesterol (mM)	2.38 ± 0.1 (13)	2.40 ± 0.07 (15)	Not measured	
HDL cholesterol (mM)	0.87 ± 0.05 (8)	0.80 ± 0.13 (6)	Not measured	
Corticosterone (nM)	208 ± 96 (5)	231 ± 87 (9)	Not measured	
Insulin (mU/litre)	81 ± 19 (6)	72 ± 7 (10)	43 ± 5 (8)	30 ± 6 (7)

The rats on the sucrose diet were treated with 12 consecutive daily doses of benfluorex or suspending medium, and those on the beef tallow diet were given 17 consecutive doses (Materials and Methods section). They were then killed 3 hr after the last dose. Concentrations are given as means ± SEM with the numbers of rats shown in parentheses. P values were calculated by an unpaired *t* test.

the rats fed the beef tallow diet with benfluorex significantly decreased the concentration of circulating glycerol and triacylglycerol. Benfluorex did not significantly change the basal concentrations of circulating glucose, fatty acid, total HDL–cholesterol, corticosterone and insulin. Decreases in the concentrations of glycerol and triacylglycerol were also seen after benfluorex treatment of the rats fed the sucrose diet (Table 1).

Effect of benfluorex treatment on tissue lipoprotein lipase activities

These experiments were performed to see whether the hypotriglyceridaemic action of benfluorex (Table 1) could be attributed to increases in lipoprotein lipase activity particularly in adipose tissue. The beef tallow diet used in these experiments decreased lipoprotein lipase activity in adipose tissue and increased that in heart compared to rats fed on a diet rich in starch [32]. The sucrose diet had an intermediate effect between the starch and tallow diets [32]. These changes were paralleled by increases in the concentration of triacylglycerol in the serum of rats fed on the sucrose and beef tallow diets [30]. A direct comparison between the lipase activities with the two diets is not possible for the results in Table 2 since the measurements were performed with different batches of rats at different times.

Treatment with benfluorex did not significantly change the total lipoprotein lipase activity of heart and diaphragm in either of the dietary groups (Table 2). The activity in adipose tissue was significantly higher in the benfluorex-treated rats provided that it was expressed relative to the weight of the fat pads. However, this change was not seen when lipoprotein lipase activities were calculated per pair of epididymal fat pads. This was because the weight of the pads was lower in the benfluorex treated animals.

Table 2. Effect of benfluorex treatment on the activities of lipoprotein lipase in muscle and adipose tissue

	nmol of fatty acid released · min^{-1} · g of $tissue^{-1}$			
	Beef tallow diet		Sucrose diet	
Tissue	Controls	Benfluorex treated	Controls	Benfluorex treated
Heart	5568 ± 408 (13)	5539 ± 544 (15)	939 ± 81 (8)	911 ± 174 (7)
Diaphragm	416 ± 53 (6)	450 ± 83 (10)	311 ± 40 (8)	314 ± 33 (7)
Adipose	1766 ± 189 (12)	2369 ± 134 (14)	771 ± 97 (8)	1085 ± 115 (7)
	$P < 0.02$		$P < 0.05$	
	nmol of fatty acid released · min^{-1} · pair of epididymal fat $pads^{-1}$			
Adipose	5556 ± 605 (12)	6700 ± 409 (14)	1721 ± 299 (8)	1684 ± 200 (7)

The rats on the beef tallow diet were treated with 17 consecutive daily doses of benfluorex or suspending medium for the measurement of heart and adipose tissue activities and with 12 doses for the activity in diaphragm. All of the rats fed on the sucrose diet had 12 consecutive doses (Materials and Methods section). Rats were killed 3 hr after the last dose. The activities are given as mean ± SEM for the number of rats shown in parentheses. The significance of the differences between the results for the adipose tissue activities of treated and untreated rats were calculated by using an unpaired *t* test.

Effect of benfluorex on the clearance of [^{3}H]triacylglycerol from chylomicrons in the circulation of rats

These experiments were performed to see whether the hypotriglyceridaemic action of benfluorex could be attributed to an enhanced clearance of triacylglycerol from the circulation. The clearance of chylomicrons in rats fed beef tallow was chosen as an experimental model. This should also give an estimate for the functional lipoprotein lipase activity in the rats.

Perfusion with ^{3}H-labelled chylomicrons for 20 min produced a steady state concentration of [^{3}H]triacylglycerol in the serum [40]. The decay curve that was obtained after the infusion stopped at 20 min was biphasic in all animals tested. This curve was analysed in two ways. The first analysis used only those points between 20 and 28 min and it assumed there to be no separate terminal phase of uptake. The mean $t_{1/2}$ calculated for the 6 control and 6 benfluorex-treated rats were 3.44 ± 1.29 min and 2.48 ± 0.48 min (means ± SD). These values agree well with the $t_{1/2}$ calculated for chylomicron triacylglycerol in conscious rats that were fed fatty diets [34].

However, if a two compartment model is used starting at 20–24 min, and 24–28 min for the two biexponentials then two $t_{1/2}$ values can be obtained [40]. These were 0.61 ± 0.23 min and 6.87 ± 1.16 min for the control rats (means ± SD for 6 rats). The equivalent values for the benfluorex treated rats were 0.85 ± 0.28 min and 6.51 ± 2.11 min. The meaning of the two $t_{1/2}$ values is not entirely established from this work, but they may reflect the $t_{1/2}$ for chylomicrons, and for the remnant which would both have been detected in our experiments. This may indicate that the uptake of chylomicrons could be even quicker than that reported by Harris and Felts [34, 35]. The results, however, do not provide evidence that benfluorex treatment had increased the rate of removal of chylomicrons from the circulation.

Effect of benfluorex on the secretion of triacylglycerol

These experiments were performed to see whether benfluorex lowered the concentration of circulating triacylglycerol (Table 1) by decreasing the secretion of VLDL from the liver. Rats fed on the sucrose diet were chosen for this study since this dietary modification stimulates VLDL production. The measurement of VLDL secretion relies on the use of Triton WR-1339 which blocks the removal of VLDL from the circulation [41, 42].

The initial concentration of triacylglycerol (Fig. 2A) in the serum of the benfluorex-treated rats was about half of that in the control ($P < 0.02$) as expected from Table 2. The injection of Triton WR-1339 blocked the uptake of triacylglycerol from the circulation and this produced a relatively constant increase in this concentration. The respective rates of increase that were estimated from Fig. 2A for the control and benfluorex treated rats were 0.134 ± 0.033 and 0.140 ± 0.03 mM per min (means ± SD). The slopes of the lines and hence the apparent rates of VLDL secretion from the liver were not significantly different.

The glycerol concentrations in the serum (Fig. 2B) were higher than those reported in Table 2 which indicates that the anaesthetic probably stimulated lipolysis in adipose tissue. It is known that general anaesthetics including urethane produces a stress response and that this is accompanied by increases in the concentrations of circulating catecholamines [43]. There were no significant differences between the glycerol concentrations in the control and benfluorex-treated rats (Fig. 2B). However, the progressive rise in the concentration of serum glucose was significantly higher in the controls when compared with the benfluorex-treated rats at 50 min ($P < 0.05$), 150 min ($P < 0.05$), and 200 min ($P < 0.01$) after the injection of the Triton WR-1339 (Fig. 2C). This antihyperglycaemic action could indicate that benfluorex is capable of decreasing some of the metabolic consequences of stress; in this case caused by the injection of urethane and Triton WR-1339. Further experiments were therefore designed to investigate the action of benfluorex on stress responses.

Effect of benfluorex treatment on the serum concentrations of corticosterone, glycerol, triacylglycerol and glucose in response to fructose feeding

High fat diets can increase the stress responses of rats to cold, nembutal narcosis and to fructose feeding. This increased susceptibility to stress and the higher concentrations of corticosterone could contribute to the insulin resistance, hyperglycaemia and decreased energy expenditure that is observed in rats fed on high fat diets (for details see Refs 29, 30 and 44). A further indication of insulin resistance in the high fat fed model is seen in the control of lipolysis where more insulin is required to inhibit the effects of adrenalin in stimulating this process. This is demonstrated especially when rats are subjected to electrical stress [45, 46]. The effect of glucose in releasing insulin from the pancreas is also decreased after high fat feeding [46, 47]. For the present experiments with benfluorex the model of feeding rats on a corn oil diet was chosen. When these rats were fed acutely with a test meal of fructose there was a particularly pronounced and prolonged release of corticosterone compared with rats fed on the starch or sucrose diets [31]. This response for the rats fed corn oil was also more prolonged than for rats fed on the beef tallow diet [31].

This sustained release of corticosterone for rats fed corn oil was confirmed in the present work (Fig. 3A). The control rats showed about a 20-fold increase in circulating corticosterone at 50 min after feeding fructose and this was maintained for a further 100 min. The corticosterone response in the benfluorex-treated rats was decreased both in magnitude and duration. The basal concentrations of corticosterone before feeding fructose in the benfluorex treated rats were not significantly different from those in the control rats (128 ± 55 nM versus 137 ± 44 nM; means ± SEM for 4 rats per group). Feeding fructose does not alter the circulating concentrations of insulin during this period of investigation [31].

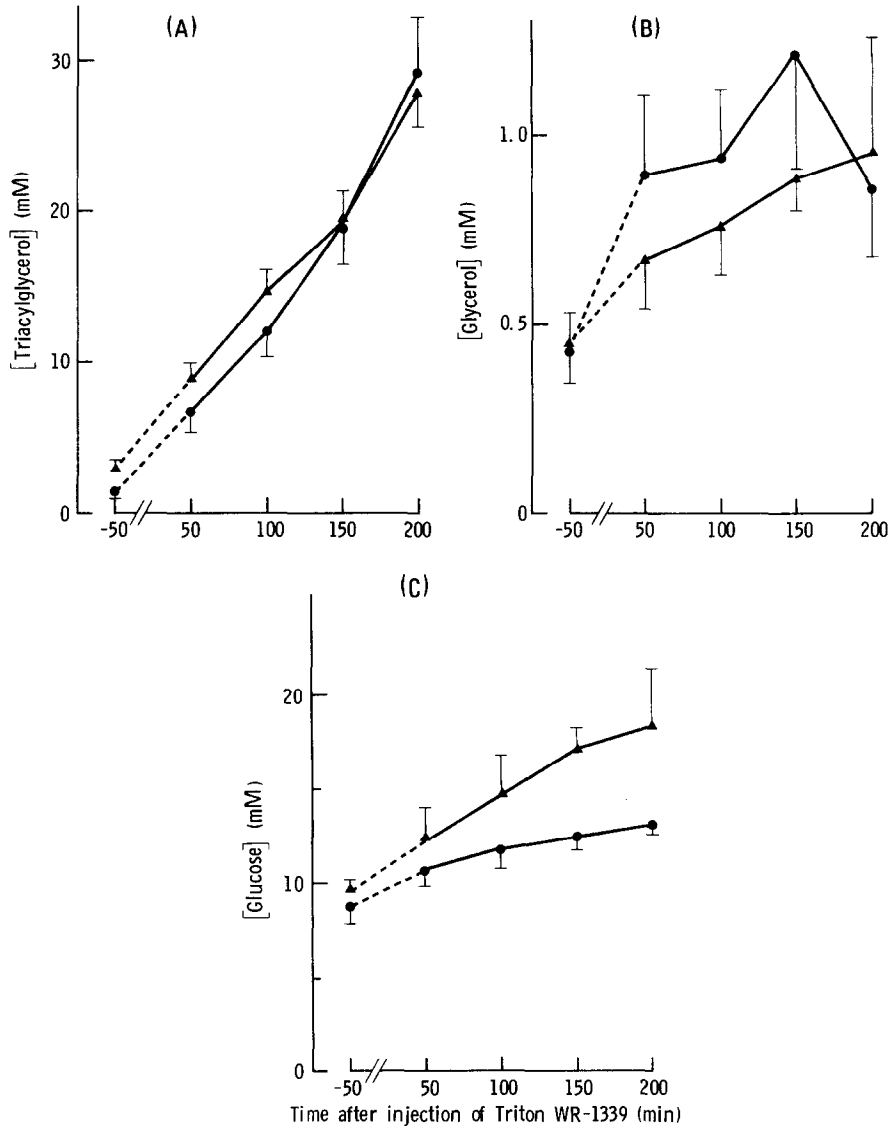

Fig. 2. Effects of benfluorex treatment upon the secretion of triacylglycerol into the circulation of rats, and upon the serum concentrations of glycerol and glucose. Rats were fed on the sucrose diet and they were given 12 consecutive daily doses of benfluorex (●) or suspending medium (▲). Two hours after the last dose the rats were injected intraperitoneally with a 50% (w/v) solution of urethane (1.18 g·kg of body wt^{-1}). The anaesthetized rats were placed on a warm operating table to maintain body temperature. The first blood sample (about 0.6 ml) was collected by cutting the end of the tail 50 min later. After a further 50 min Triton WR-1339 (200 g/l in 0.9% NaCl; 400 mg·kg of body wt^{-1}) was injected into the femoral vein. The second blood sample was collected 50 min later and three further blood samples were collected at 50 min intervals. The results are shown as means ± SEM using 6 control and 7 benfluorex-treated rats.

The concentration of glycerol tended to be lower in the benfluorex-treated group, but this was only statistically significant at 150 and 200 min after fructose feeding (Fig. 3B). The concentration of triacylglycerol was initially similar in the control rats and in those treated 2 hr previously with the last dose of benfluorex. Feeding fructose initially decreased the concentration of triacylglycerol as was observed before [28, 31]. Thereafter, the concentration in the control rats returned to the initial levels. This effect was not observed until later in the benfluorex-treated rats, and the triacylglycerol concentrations remained low at 100 and 150 min after fructose feeding (Fig. 3C). The basal concentrations of glucose before fructose feeding were lower in the benfluorex treated group (Fig. 3D) as was the rise after feeding fructose (Fig. 3D).

A possible reason for not observing a decrease in the rate of triacylglycerol secretion in Fig. 2A could have been that this rate was not stimulated by an increased supply of substrates. The measurements were therefore repeated by using the corn oil fed rats and then anaesthetizing them with urethane at 70 min after the last treatment with benfluorex. The

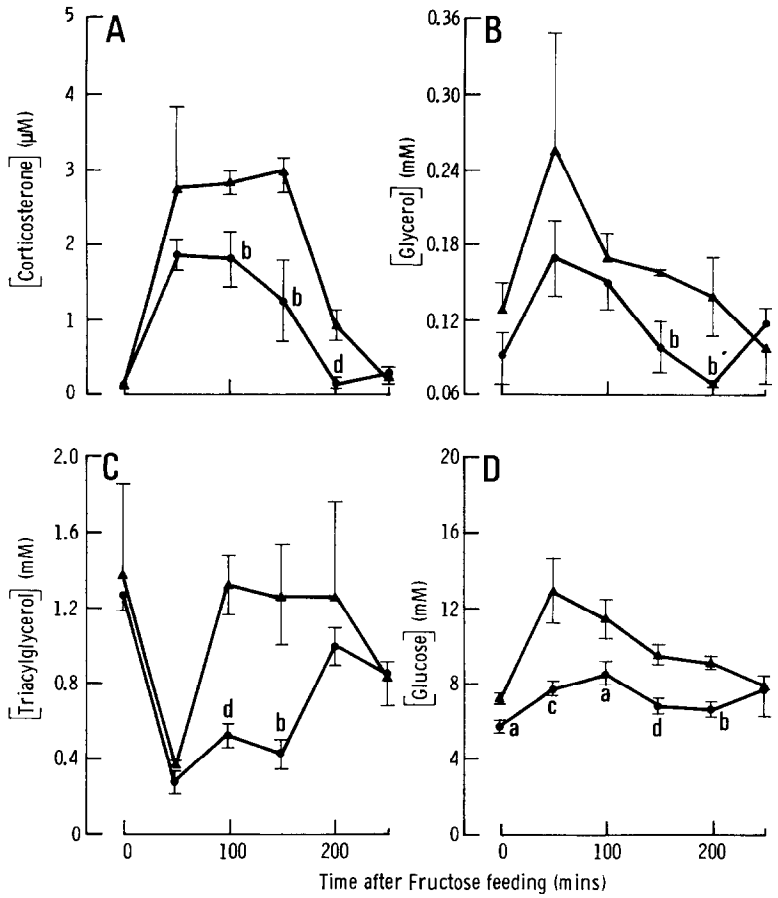

Fig. 3. Effect of benfluorex treatment on the serum concentrations of corticosterone, glycerol, triacylglycerol and glucose in response to fructose feeding. Rats were maintained on the corn oil diet and they were treated with 12 consecutive daily doses of benfluorex (●), or suspending medium (▲) as described in the Materials and Methods section. Two hours after the final dose of drug or suspending medium, they were fed by stomach tube with a fructose solution at a dose of 9.5 g per kg of body wt [31]. The rats were then killed [31] at the times indicated. Results are shown as means ± SEM for 4 rats per group, except for the control rats at 50 min and 200 min where there were 3 rats per group. The significance of the difference between the equivalent groups of control and benfluorex-treated rats as calculated by an unpaired *t* test is indicated on the figure by: *a*, $P < 0.05$; *b*, $P < 0.025$; *c*, $P < 0.01$ and *d*, $P < 0.005$.

rats were then fed by stomach tube 50 min later with 9.5 g of fructose/kg of body weight and immediately injected through the femoral vein with Triton WR-1339 (Fig. 2). Benfluorex treatment again failed to significantly decrease the rate of triacylglycerol secretion in this experimental model. However, the concentrations of glucose in the blood at 50, 100 and 150 min after the injection of Triton WR-1339 were about 20 mM for the treated rats. By contrast, the control rats had significantly higher ($P < 0.001$) mean glucose concentrations in the range 30–35 mM. If the rats were simply anaesthetized with urethane and not treated with fructose or Triton WR-1339, then the glucose concentrations were not significantly decreased in the blood at 150 min after the anaesthetic. The glucose concentrations were 6.95 ± 1.2 mM and 5.45 ± 1.1 mM for control and benfluorex treated rats (mean ± SD for 4 rats in each group).

Effect of benfluorex treatment on the metabolic responses to the injection of 2-deoxy-D-glucose

The effects of benfluorex treatment on glucose and stress metabolism were further investigated after injecting rats with 2-deoxyglucose. This compound inhibits normal glucose metabolism to provide a state of cellular glucose deprivation. Animals attempt to respond to this condition by liberating glucocorticoids and the acute acting stress hormones and by increasing the concentration of circulating glucose [48–51].

Treatment of the rats with benfluorex markedly decreased the rise in blood glucose at 50 and 80 min after the injection of 2-deoxyglucose. This difference was not significant at 120 min when the glucose concentration in the control rats had begun to subside (Table 3). The concentrations of triacylglycerol in the serum were lower in the benfluorex treated group at all three time points that were measured. Benfluo-

Table 3. Effect of benfluorex treatment on the metabolic responses of rats following the injection of 2-deoxy-D-glucose

	Time after injection of 2-deoxyglucose					
	50 min		80 min		120 min	
Concentration in blood serum:	Control (7)	Treated (9)	Control (8)	Treated (10)	Control (6)	Treated (10)
Glucose (mM)	27.2 ± 5.6	19.9 ± 4.7	27.0 ± 5.3	17.8 ± 9.1	19.8 ± 5.2	16.7 ± 2.2
	$P < 0.025$		$P < 0.025$			
Triacylglycerol (mM)	1.14 ± 0.59	0.60 ± 0.33	1.44 ± 0.42	0.82 ± 0.20	1.10 ± 0.13	0.71 ± 0.16
	$P < 0.05$		$P < 0.002$		$P < 0.01$	
Glycerol (μM)	155 ± 25	150 ± 40	200 ± 39	165 ± 23	219 ± 29	155 ± 19
			$P < 0.05$		$P < 0.0005$	
Fatty acid (μM)	580 ± 80	440 ± 90	690 ± 140	490 ± 90	800 ± 90	610 ± 10
	$P < 0.01$		$P < 0.005$		$P < 0.005$	
Corticosterone (nM)	928 ± 87	841 ± 323	1090 ± 110	698 ± 446	805 ± 348	902 ± 177
			$P < 0.05$			

Rats were fed on the corn-oil diet in normal cages and they were treated with 11 consecutive doses of 50 mg of benfluorex/kg or with suspending medium. At 2 hr after the final dose with drug or suspending medium they were injected intraperitoneally with 300 mg of 2-deoxyglucose/kg and they were killed at the times indicated. Results are means ± SD with the numbers of rats in parentheses and the significance of the difference was calculated by using an unpaired t test.

rex treatment also appeared to decrease the stress response after the injection of 2-deoxyglucose as indicated by the lower concentrations of circulating glycerol and fatty acids. However, corticosterone concentrations were only decreased significantly at the 80 min time point.

DISCUSSION

Treatment of rats with benfluorex produced a marked fall in body weight during the first three days but thereafter the weight gain paralleled that of the control group when they were fed with the beef tallow diet (Fig. 1), or the sucrose and corn oil diets (results not shown). Similar effects have been obtained with fenfluramine [28, 29, 52, 53]. If the rats had become tolerant to benfluorex after four days then they should have regained their lost weight, and the growth curve should have converged with that of the control rats [52, 53]. These combined observations therefore indicate that benfluorex is still exerting an antihyperphagic effect on the rats even though the food intake is similar to that of the controls after about 5 days (Fig. 1).

The rats treated with benfluorex were also leaner than the controls as indicated by the decrease in epididymal fat pad weight. For example, the weight of these pads expressed as g/100 g of body weight for a group of 21 control rats fed on the corn oil diet was 1.13 ± 0.14 (SD). This was significantly higher ($P < 0.001$) than the equivalent value of 0.84 ± 0.14 for 29 benfluorex-treated rats (see also Table 2). This loss of body fat might be partly related to the initial decrease in food intake (Fig. 1b).

The long-term effects of benfluorex treatment were measured during the period of restored food intake and weight gain to dissociate these adaptations from the effects of food deprivation. Prolonged treatment with benfluorex produced a marked decrease in the basal concentration of circulating triacylglycerol in the rats fed on the beef tallow and sucrose diets (Table 1; Fig. 3). A decreased concentration of triacylglycerol was not observed in the rats fed the corn oil diet (Fig. 3), but benfluorex did show a subsequent hypotriglyceridaemic effect when these rats were fed with fructose (Fig. 3) or injected with 2-deoxyglucose (Table 3). Benfluorex also has a hypotriglyceridaemic effect in various conditions in the rat [54, 55] and in human beings [56] including obesity and diabetes. However, benfluorex had no significant effect on the total activity of lipoprotein lipase in diaphragm, heart and adipose tissue (Table 2) and on the rate of disappearance of [^{3}H]triacylglycerol from chylomicrons. Therefore there is no evidence to suggest that an increased clearance of triacylglycerol from the circulation is causing the hypotriglyceridaemic effect.

Benfluorex might produce its hypolipidaemic action by altering the secretion of triacylglycerol into the circulation. This could partly result from a decrease in fat absorption from the intestine for the beef tallow diet (for details, see Ref. 26). However, this could not explain the hypolipidaemic action in the rats on the sucrose diet where most of the triacylglycerol would have been present in VLDL. However, benfluorex did not significantly alter the apparent rate of appearance of triacylglycerol in the circulation when its removal was blocked with Triton WR-1339 (Fig. 2). This result was unexpected since fenfluramine and benfluorex inhibit hepatic triacylglycerol synthesis and secretion when added directly to incubations [39, 57–59]. Kaye *et al.* [60] also demonstrated a decreased secretion of VLDL after treatment of rabbits with fenfluramine, norfenfluramine or benfluorex when protamine was used to suppress the uptake of triacylglycerol. They did not take steps to stimulate triacylglycerol synthesis, and their animals were only treated for three days with the drugs so that food intake might have been depressed.

In other experiments the rate of triacylglycerol synthesis in the liver of rats treated with benfluorex

for five days was not significantly altered [61] unless the rats were fed with glucose, or especially ethanol, in order to stimulate triacylglycerol synthesis [62, 63]. The effects of benfluorex on phosphatidate phosphohydrolase activity and triacylglycerol synthesis were indirect and mediated by partially preventing an ethanol-induced release of corticosterone or other stress hormones [9–12, 62, 63]. However, rats fed with fructose to stimulate triacylglycerol synthesis instead of ethanol did not show a significant increase in VLDL secretion up to 150 min after the injection of Triton WR-1339. Although benfluorex treatment should again have decreased the release of corticosterone (Fig. 3) the effects of this hormone on the phosphohydrolase would not have been expressed for 5–6 hr. However, the decrease in the stress response as expressed through a lower concentration of glycerol (and presumably fatty acid) in the blood (Fig. 3) should have prevented the acute activation of phosphatidate phosphohydrolase by its fatty acid-induced translocation to the endoplasmic reticulum [12, 59]. This could explain why benfluorex prevented the rise in the concentration of circulating triacylglycerols between 100 and 200 min after feeding fructose (Fig. 3).

A major problem of the experiments that were performed to study the secretion and clearance of triacylglycerol is that the effects of the experimental procedure may mask the pharmacological action of benfluorex. It is difficult to provide a more stable experimental model, and at the same time to ensure maintenance of normal food intake, physiological function and endocrine balance. The extent to which an increased hepatic secretion of VLDL is responsible for a hypertriglyceridaemia depends on the extent to which fatty acids liberated by adipose tissue are oxidized in muscle and liver and this determines whether they accumulate in the liver to stimulate triacylglycerol synthesis. Their subsequent secretion also depends upon the ability to co-ordinate the synthesis of phospholipids and apolipoproteins needed to produce the VLDL. The stimulation of this process by glucocorticoids is also a long-term event [13].

Figures 2 and 3 do, however, demonstrate that benfluorex has a marked ability to prevent an increase in blood glucose concentrations. In Fig. 3 an acute fructose load was used to promote a "dietary stress" in rats that had been fed a high fat diet in order to exaggerate this response [31]. Benfluorex diminished the effect of fructose in increasing the concentration of circulating corticosterone, and it also lowered the concentrations of glycerol, triacylglycerol and glucose in the blood. This experimental model may be particularly useful in investigating dietary interactions. High fat diets promote the actions of fructose and ethanol in producing a hypertriglyceridaemia and a fatty liver, and the release of glucocorticoids may be partly responsible for these effects [31]. In human beings cortisol concentrations increase after meals [20–22]. These responses are likely to be exaggerated by eating nutrients such as fructose, sorbitol, glycerol, ethanol and fat which at the same time have little effect on the concentrations of circulating insulin [21, 31].

The experiments with 2-deoxyglucose were performed to provoke a stress response by stimulating the release of corticotropin and corticosterone in rats [48–50], to produce hyperglycaemia [48, 50] and possibly hypertriglyceridaemia [50]. The increases in corticotropin, corticosterone and glucose are thought to be mediated through the hypothalamus since they are decreased by lesioning with gold thioglucose [48], or by hypothalamic deafferentiation [51]. The glucocorticoid secretion was not directly responsible for the increase in circulating glucose [51], and this is also expected from the relatively long time that is normally required for glucocorticoids to produce their effects. Table 3 demonstrates that there was a pronounced hypoglycaemic effect of benfluorex that is accompanied by a decreased stress response as indicated by the lowered concentrations of circulating fatty acids and glycerol. This effect was probably mediated through the acute acting stress hormones rather than by corticosterone. This latter hormone was only decreased at the 80 min time point in the benfluorex treated rats (Table 3). In the report where a hypertriglyceridaemic effect of 2-deoxyglucose was observed this result was attributed to an action on lipoprotein lipase [50]. Since these rats had been deprived of food overnight this should have decreased this activity in adipose tissue thus limiting its ability to clear circulating triacylglycerol. However, the rats used in Table 3 were not starved overnight and therefore this mechanism might not have operated.

The effects of benfluorex and fenfluramine on energy balance [64, 65] and on the stress reaction [66, 67] are thought to be mediated through effects on the metabolism of 5-HT. When administered acutely benfluorex and fenfluramine cause a decrease in food intake, they increase the concentrations of corticosterone [27, 66, 67] and fatty acids in the circulation [25, 26]. However, in the longer-term food intake recovers (e.g. Fig. 1) and there is a decreased release of corticosterone in rats after the ingestion of ethanol [27, 63] or fructose [28, 29] (Fig. 3). Basal concentrations of corticosterone were also not significantly different from the control animals (Table 1, Fig. 3, Ref. 27). This could represent an adaptation of the animals to overcome the initial stress response by depressing their sensitivity to further stress stimuli. This effect is probably specific to some compounds that operate through the serotonergic system since similar experiments with rats treated chronically with 5 mg of D-amphetamine/kg demonstrated an exaggerated corticosterone response after fructose feeding (results not shown).

Although D-fenfluramine shows similar effects to benfluorex in decreasing the rise in corticosterone, glycerol and triacylglycerol after fructose feeding it did not prevent the increase in circulating glucose [28, 29]. There was also no suppression of the hyperglycaemia at 80 min after the injection of 300 mg of 2-deoxy-glucose/kg in rats treated chronically with 10 mg D-fenfluramine/kg (results not shown). By contrast, benfluorex seemed to be particularly effective at counteracting the hyperglycaemia caused by the injection of urethane and Triton WR-1339 (Fig. 2), 2-deoxyglucose (Table 3) and by the ingestion of fructose (Fig. 3). Benfluorex is rapidly hydrolysed to 1-(3-trifluoromethylphenyl) - 2 - [N-(2-hydroxy-

ethyl)amino]propane and benzoic acid. The former compound is an effective inhibitor of gluconeogenesis with rat hepatocytes and benzoic acid modifies metabolism by sequestering CoA and by the effects of its CoA ester or one of its metabolites [58].

The present work demonstrates that benfluorex has persistent hypoglycaemic and hypotriglyceridaemic effects that occurred when food intake and growth rates were similar to the control rats. These effects are probably a combination of the direct effect of the metabolites of benfluorex on metabolism and of indirect actions on hormonal balance. Benfluorex might partly act by decreasing exaggerated stress responses. This should improve insulin-sensitivity by decreasing the effects of the counter-regulatory hormones (Introduction) and fatty acids [67] on metabolism. Furthermore, it is postulated that this mechanism could be beneficial in alleviating some of the adverse metabolic complications of obesity, diabetes and atherosclerosis.

Acknowledgements—We thank Dr K. Palin for help in preparing chylomicrons, Drs S. Gardiner and T. Bennett for catheterizing animals for the measurement of chylomicron uptake, Dr D. B. Campbell for the interpretation of the kinetics of chylomicron uptake and Mrs J. Edson for her assistance in performing the fatty acid analysis.

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