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Differential inhibition of long-chain acyl-CoA hydrolases by hypolipidemic drugs *in vitro*

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Abstract—The effect of *in vitro* addition of three hypolipidemic drugs (clofibric acid, bezafibrate and gemfibrozil) on rat palmitoyl-CoA hydrolases has been studied, by using a spectrophotometric method (Berge RK, *Biochim Biophys Acta* **574**: 321–333, 1979) optimized for valoration of crude enzyme preparations. Mitochondrial and microsomal hepatic palmitoyl-CoA hydrolase activities were inhibited by the three drugs in a concentration-dependent fashion. The order of inhibitory potency was gemfibrozil > bezafibrate > clofibric acid, irrespective of the enzyme activity tested. Cytosolic rat brain palmitoyl-CoA hydrolase activity was not affected. Kinetic studies with gemfibrozil on the solubilized microsomal palmitoyl-CoA hydrolase activity point to a mixed non-competitive type of inhibition.

Long-chain acyl-CoA hydrolases are enzymes that are widely distributed in mammalian tissues [1]. They catalyse the hydrolysis of fatty acyl-CoA thioesters [2] and play an important role in controlling the chain length of synthesized fatty acids and modifying the product specificity of fatty acid synthetase [3]. Several researchers have shown the existence of at least three different long-chain fatty acyl-CoA hydrolases, two located in membranous subcellular

organelles, namely microsomal and mitochondrial long-chain fatty acyl-CoA hydrolases, and a soluble cytosolic form [4]. While the membrane enzymes are the predominant forms in some organs, such as rat liver [2, 4, 5], the cytosolic hydrolase is the main form in rat brain [6].

Clofibric acid derivatives are used clinically in the treatment of hyperlipoproteinemias [7]. Their chronic administration to rodents causes dramatic changes in the liver: hepatomegaly, proliferation of smooth endoplasmic reticulum, and peroxisomes, and the induction of several enzymes involved in lipid metabolism [8]. The mechanism of action of these compounds seems to involve both increased clearance of very low density lipoproteins and decreased production by the liver [9], although the exact biochemical mechanisms are not well understood. The quality of fatty acids (chain length and number of unsaturations), either in the free form or incorporated into glycerolipids, plays an important role controlling lipoprotein metabolism [10]. We have thus been interested in testing the effect of clofibric acid derivatives on enzymes related to the synthesis of fatty acids, either *in vitro* or *in vivo*. As a part of this project, we have studied the *in vitro* effect of clofibric acid (CFB*), bezafibrate (BFB) and gemfibrozil (GFB) on microsomal palmitoyl-CoA hydrolase (PCH) activity. In addition, comparative studies with liver mitochondrial PCH and brain cytosolic PCH have been performed.

Materials and Methods

Chemicals. Palmitoyl-CoA, fatty acid-free BSA, CFB and Hepes were obtained from the Sigma Chemical Co. (St Louis, MO, U.S.A.). BFB and GFB were a generous gift from Lab. Andreu (Barcelona, Spain) and Lab. Parke-Davis (Barcelona, Spain). DTNB was from Fluka (Buchs, Switzerland), EDTA from Merck (Darmstadt, Germany). Other general chemicals were obtained from commercial sources and were of analytical grade.

Isolation of subcellular fractions. Male Sprague–Dawley rats (200–220 g) aged 7–8 weeks were used. Following 18 hr of starvation, animals were killed by decapitation between 8 and 9 a.m. Rat livers were perfused with ice-cold NaCl (0.9%)and homogenized in ice-cold 0.25 M sucrose, 50 mM Tris–HCl buffer, pH 7.4. Subcellular fractions were obtained as described previously [11]. Microsomes were resuspended in 0.1 M phosphate buffer, pH 7.4, and mitochondria in 1 mM EDTA, 0.1 M phosphate buffer, pH 7.4. The cytosolic fraction was obtained from rat brains homogenized in a 0.35 M sucrose, 50 mM Tris–HCl buffer, pH 7.4 [12]. Protein concentration was determined by the method of Bradford [13], using BSA as standard.

Assay of PCH activity. PCH activity was assayed spectrophotometrically as described previously [3]. The incubation medium contained, in final concentrations: 30 mM Hepes buffer, pH 7.5, 1 mM EDTA, BSA (see Results and Discussion), 100 μg of protein, and, when necessary, the drug to be tested at the desired concentration. After 5 min preincubation at 35°, 0.3 mM DTNB was added and the reaction was started by adding palmitoyl-CoA (for concentrations, see Results and Discussion and figures below). Final assay volume was 1 mL. The absorbance increase at 412 nm was recorded in a Perkin–Elmer 550S UV-Vis spectrophotometer with a kinetic recorder. Enzyme activity was obtained using an extinction coefficient of 1.36 $\times 10^4$ M^{-1} cm^{-1}. For each assay, corrections were made to subtract the unspecific hydrolysis of palmitoyl-CoA. The results were expressed as nmol palmitoyl-CoA hydrolysed/min/mg protein. The drugs to be tested were added from stock solutions adjusted to pH 8–8.5 with 0.1 N NaOH; the pH of the assay medium was not modified by this addition.

Solubilization of microsomal PCH. Solubilized microsomal PCH was obtained essentially as described by Berge [3]. Microsomes resuspended in 1.5 mM $MgCl_2$, 0.1 mM EDTA, 15 mM Hepes buffer, pH 7.4, were mixed with an equal amount of cold, water-saturated *n*-butanol under constant stirring for 2 min at 4°. The water phase and the interphase were then centrifugated at 100,000 *g* for 30 min and then dialysed at 4° for 24 hr against the same buffer used for the resuspension. A Spectrapor No. 3 membrane tubing (Spectrum Medical Inds., Los Angeles, CA, U.S.A.), 10,000 cut off, was used for the dialysis assays.

Data analysis. IC_{50} values and their 95% confidence limits were calculated by means of a "Graded Dose–Response" computer program designed following Tallarida's instructions [14]. Apparent kinetic constants were calculated by means of a non-linear regression program (ENZFITTER).

Results and Discussion

Optimal conditions of PCH assay. The kinetics of these enzymes are strongly influenced by two factors: (1) They use long-chain fatty acyl-CoAs as substrates, known to behave as natural detergents, forming micelles [15], and (2) the lipophilic membranous environment surrounding the mitochondrial and microsomal long-chain fatty acyl-CoA hydrolases is capable of incorporating part of the substrate present in the medium [16]. Berge and co-workers [3, 17] have shown that free fatty acyl-CoAs are the true substrate for the enzyme, while micellar forms act by inhibiting the hydrolase activity. The presence of BSA in the reaction medium prevents micelle formation by binding substrate, thus increasing the CMC for fatty acyl-CoAs, which counteracts the substrate inhibition.

The spectrophotometric method described by Berge [3] to determine microsomal PCH activity was initially designed to assay the purified enzyme. As we were interested in testing the effect of CFB derivatives on the PCH activity of the crude microsomal, mitochondrial and cytosolic fractions, we redefined the optimal substrate concentration and [palmitoyl-CoA]/[BSA] ratio in our assay conditions.

PCH activity was measured at different palmitoyl-CoA concentrations in order to obtain the optimal concentration for the assay. The optimal palmitoyl-CoA concentration was 40 μM for the three enzymes, which is very close to the value reported by Berge and co-workers [2, 3] for the purified microsomal and mitochondrial enzymes. Further increase in substrate concentration caused inhibition of PCH activity (Fig. 1A) (except for the cytosolic enzyme), probably due to the appearance of micelles in the medium. As the influence of BSA on PCH activity is dependent on the [palmitoyl-CoA]/[protein] ratio in the assay medium, PCH was assayed at a fixed palmitoyl-CoA concentration (40 μM) and varying BSA concentration. An optimal ratio of 120 nmol palmitoyl-CoA/mg BSA was found (data not shown), practically identical to the ratios reported for the purified enzymes [2, 3]. Thereafter, PCH activity was again assayed at increasing palmitoyl-CoA concentrations but a fixed ratio of 120 nmol palmitoyl-CoA/mg protein in the assay medium was maintained. For the three enzymes, the maximum activity was achieved at 40 μM palmitoyl-CoA (Fig. 1B). In addition, BSA prevented the substrate inhibition of microsomal and mitochondrial activities at higher palmitoyl-CoA concentrations.

Liver PCH activity was mainly located in membranous particles, being maximal in microsomes (about 50% of the total hepatic activity). Initially, we also studied hepatic cytosolic hydrolase activity but it was very low, thus we followed our study with the enzyme from brain which showed the highest specific activity (230–290 nmol/min/mg).

* Abbreviations: CFB, clofibric acid; BFB, bezafibrate; GFB, gemfibrozil; PCH, palmitoyl-CoA hydrolase; BSA, bovine serum albumin; Hepes, 4-(2-hydroxyethyl)-1-piperazineethane sulphonic acid; DTNB, 5,5′-dithiobis(2-nitrobenzoic acid); CMC, critical micellar concentration.

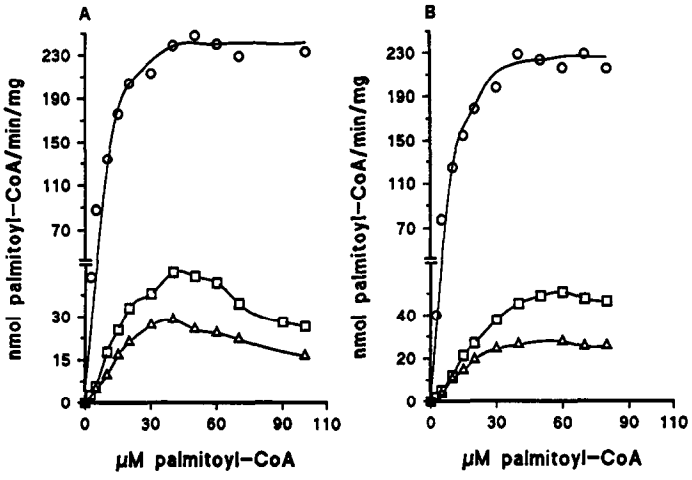

Fig. 1. Activity versus [palmitoyl-CoA] plots for brain cytosolic (○), and hepatic microsomal (□) and mitochondrial (△) palmitoyl-CoA hydrolases in the absence (A) or in the presence (B) of a fixed 120 nmol palmitoyl-CoA/mg BSA ratio. Points are means of 2–3 different experiments performed in duplicate. Each time, the samples were obtained from two pooled rat organs. Only the cytosolic enzyme values fitted to a rectangular hyperbola, giving apparent kinetic constants of K_m = 11.3 μM palmitoyl-CoA, V_{max} = 280 nmol palmitoyl-CoA/min/mg protein (A); and K_m = 12.5 μM palmitoyl-CoA, V_{max} = 270 nmol palmitoyl-CoA/min/mg protein (B).

The differences between cytosolic and membrane (i.e. mitochondrial and microsomal) PCHs should be pointed out. While specific activity for membranous hydrolases was increased in the presence of BSA throughout the range of palmitoyl-CoA concentration, the cytosolic enzyme was rather irresponsive to BSA, and even showed a slight decrease in specific activity in the presence of BSA, in good agreement with previous reports [6]. Furthermore, cytosolic PCH activity values fitted Michaelis–Menten kinetics (apparent V_{max} and K_m are given in Fig. 1), whereas membranous hydrolases did not, even in the presence of an optimal [palmitoyl-CoA]/[protein] ratio. Only when we used partially purified microsomal enzyme, following Berge's procedure [3], were we able to fit velocity versus [substrate] data to a rectangular hyperbola. Given that BSA was not able to restore Michaelis–Menten kinetics, we assumed the kinetic abnormality was due not only to the micellar inhibition, abolished by the presence of optimal BSA concentrations, but also to other factors such as the presence of a considerable amount of palmitoyl-CoA incorporated to the microsomal membrane [16]. As Berge's butanolic extraction virtually eliminates the membranous fraction, we can assume that the observed restoration of Michaelis–Menten kinetics may be attributed to the elimination of the membranous environment acting mainly by absorbing substrate from the medium.

From all these results, optimal assay conditions were fixed. A concentration of 40 μM palmitoyl-CoA and sufficient BSA to give a ratio of 120 nmol palmitoyl-CoA/mg BSA were used for all the assays. BSA was omitted when the activity of the cytosolic enzyme was determined. In these standard assay conditions, the enzymatic activity was proportional to the amount of sample protein added up to 200 μg and the reaction was linear for at least 6 min.

Inhibitory effect of clofibric acid derivatives on microsomal PCH: comparative studies with other cellular PCHs. Liver microsomal and mitochondrial PCH activities were inhibited markedly by the addition of CFB, BFB and GFB to the assay medium, while no inhibition was found with the brain cytosolic PCH activity (Table 1). In all cases, the inhibitory effect is concentration-related, the three drugs having IC_{50} values (Table 2) well within reported plasma concentrations (0.1–0.8 mM) after their administration either to laboratory animals or humans [18]. Moreover, given that GFB has a tendency to accumulate in liver tissue [19], marked effects may reasonably be expected after *in vivo* administration.

Table 1. *In vitro* effect of CFB, BFB and GFB on rat liver microsomal and mitochondrial palmitoyl-CoA hydrolase (PCH) and rat brain cytosolic PCH

	CFB (5 mM)	BFB (5 mM)	GFB (5 mM)
Microsomal	35.7	15.9	0.7
Mitochondrial	23.0	8.7	0.9
Cytosolic	95.7	104.2	108.4

Results are expressed as per cent activities with respect to controls (100% = 56.3, 32.2 and 293.6 nmol palmitoyl-CoA/min/mg protein for microsomal, mitochondrial and cytosolic PCH, respectively) with no drug addition.

Values are the mean of two experiments performed in duplicate, each time with subcellular fractions obtained from two rat pooled organs. Enzymatic activities were assayed as described in Materials and Methods.

Table 2. IC_{50} values for CFB derivative inhibition of rat hepatic microsomal and mitochondrial PCH

	CFB	BFB	GFB
Microsomal	2.85 (2.27–3.59)	0.97 (0.80–1.19)	0.15 (0.09–0.25)
Mitochondrial	1.49 (1.48–1.50)	0.58 (0.40–0.85)	0.13 (0.11–0.15)

IC_{50} values were calculated as described in Materials and Methods. Concentrations tested were from 0.05 to 5 mM for the three drugs.

Each curve was obtained with seven concentration points. Each point is the mean of three experiments performed in duplicate, each time with subcellular fractions from two pooled rat organs. Values in parentheses are their 95% confidence limits.

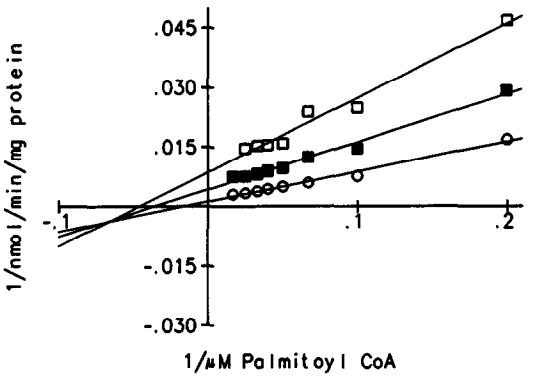

Fig. 2. Lineweaver–Burk plots of velocity versus palmitoyl-CoA concentration for the solubilized microsomal palmitoyl-CoA hydrolase in the absence (○) and in the presence of 0.25 (■) and 0.5 mM (□) of gemfibrozil (GFB). A 120 nmol palmitoyl-CoA/mg BSA ratio is used in all the assays. Values are the means of three experiments performed in duplicate, each time with microsomal enzyme obtained from two pooled rat livers. Apparent kinetic constants are: V_{max} = 480, 183 and 100 nmol palmitoyl-CoA/min/mg protein for control, 0.25 mM and 0.5 mM GFB, respectively; K_m = 26.9, 16.9 and 15.9 μM palmitoyl-CoA for control, 0.25 mM and 0.5 mM GFB, respectively.

The mitochondrial enzyme was more affected by the presence of the three drugs than the microsomal enzyme, with lower IC_{50} values for the former (Table 2). For both enzymes, the order of drug inhibitory potency was identical, being maximal with GFB, followed by BFB, and, at a great distance, by CFB. It is interesting to note that the same order of potency has been reported for the effect of these drugs *in vitro* on other enzymatic activities related to fatty acid synthesis [20]. Further, a similar relationship is found in their therapeutic use [7, 19]. As the inhibitory assays were performed in the presence of BSA, the inhibition could be attributed to an indirect effect, due to a hypothetical displacement of palmitoyl-CoA from BSA binding, thus lowering palmitoyl-CoA CMC and producing micellar inhibition (CFB derivatives have a high affinity for BSA binding [21]). Nevertheless, similar assays with the three drugs performed in the absence of BSA gave practically similiar inhibition percentages for a wide range of drug concentrations (data not shown), ruling out the possible BSA interference. Further, we also found that inhibition of microsomal PCH activity was independent of microsomal protein concentration in the assay medium (25–100 μg/mL) which also indicated that inhibition is not influenced by protein concentration. Moreover, inhibition was independent of the time of preincubation (e.g. about 64% inhibition at 0.25 mM GFB, from 0 to 15 min preincubation), and was reversible upon centrifugation of microsomes which had been pretreated with the drug at 100,000 *g* for 30 min (data not shown).

Kinetics of inhibition of microsomal PCH by GFB. As has been mentioned above, velocity versus substrate concentration data for microsomal PCH enzyme did not show Michaelis–Menten behaviour. To achieve this goal, microsomal enzyme was solubilized as described by Berge [3]. An 8-fold increase of specific activity was obtained (35.9 ± 10.4 vs 304.1 ± 16.6 nmol/min/mg protein for the microsomal and the solubilized preparation, respectively; mean recovery of total activity was 68%). Velocity versus substrate concentration data for the solubilized enzyme now fitted Michaelis–Menten kinetics.

Figure 2 shows Lineweaver–Burk plots of velocity versus palmitoyl-CoA concentration for the solubilized microsomal PCH in the absence and in the presence of 0.25 and 0.5 mM of GFB. The addition of GFB to the assay medium induced a decrease in apparent K_m and V_{max} values. Thus, we can assume that GFB behaves as a mixed non-competitive inhibitor [22]. The decrease in the apparent K_m value points to a higher enzyme affinity for the substrate in the presence of GFB, although the reaction rate is ultimately decreased (lower apparent V_{max}). The drug may interact with the enzyme at a different point from the catalytic site, producing a change in the protein, which leads to a higher affinity for the substrate but, at the same time, impairs product formation, thus decreasing the reaction rate. As the three drugs are closely related structurally, we can presuppose a similar mechanism for CFB and BFB. The differences in potency order could be attributed to differences in affinity for the enzyme. It is remarkable that, while the three drugs share the terminal isobutyrate structure, GFB has the most lipophilic side chain and CFB the least, BFB being in a half way position. Should a hydrophobic interaction be responsible for the drug affinity for the enzyme, this would explain the order of potency found for the three drugs *in vitro*. Moreover, the fact that we found practically the same inhibition percentages with the microsomal and the solubilized enzyme (data not shown) points to a direct interaction between the drug and the enzyme. The interaction is also reversible (the inhibition is eliminated simply by centrifuging the microsomes and resuspending them in a drug-free buffer), which also points to a non-covalent type interaction.

To our knowledge, CFB is the only one of the three drugs that has been tested *in vivo* for its effects on PCH [1, 4, 8, 23, 24]. After CFB administration *in vivo* there is a marked increase in hepatic PCH activity. Other inhibitors of enzymes involved in lipid metabolism, such as hydroxy methyl-glutaryl-CoA reductase inhibitors, increase the total hepatic activity of the enzyme after their administration *in vivo* [25]. Thus, it is possible that CFB may act by producing a similar situation *in vivo*.

Nevertheless, at this point, it is difficult to establish a relationship between the *in vitro* and *in vivo* effects. The modification of total hepatic palmitoyl-CoA hydrolase activity by CFB *in vivo* comprises a moderate increase in the mitochondrial fraction, no increase or even decrease in the microsomal fraction, and an astonishing increase in the cytosolic activity. Kawashima and co-workers [1, 24] attributed the induced hepatic cytosolic activity mainly to

the so-called hydrolase I, that reported to be similar to the rat brain cytosolic PCH, which is completely resistant to the *in vitro* inhibition by CFB (see Table 1). In any case, it must be mentioned that the similarity between the two enzymes is not perfect, thus rat brain cytosolic activity is not affected after CFB administration *in vivo* [5], although no data was given about CFB distribution in brain tissue.

On the other hand, Berge *et al* [4] argued that the cytosolic-induced activity is derived from membrane hydrolases (mainly from microsomes and peroxisomes), given that the enzyme is more releasable from the structurally altered membranes after the drug administration. Should this situation be found, there would not be such a large discrepancy with the *in vitro* results. Further, the activity induced *in vivo* is not due to an increase in clofibroyl-CoA hydrolase activity [23]. Given that the *in vivo* data is reduced to the less potent of the three drugs tested *in vitro*, we are now conducting *in vivo* experiments with the three clofibric acid derivatives in order to gain some insight into the complex relationship between *in vitro* inhibition and *in vivo* induction of liver PCH activity.

In conclusion, the effect of *in vitro* addition of three hypolipidemic drugs (CFB, BFB and GFB) on rat PCHs has been studied, by using a spectrophotometric method optimized for the use of crude enzyme preparations. Mitochondrial and microsomal hepatic PCH activities were inhibited by the three drugs in a concentration-dependent fashion. The order of inhibitory potency was GFB > BFB > CFB, irrespective of the enzyme activity tested. Cytosolic rat brain PCH activity was not affected. Kinetic studies with GFB on the solubilized microsomal PCH activity point to a mixed non-competitive type of inhibition.

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Cytochrome aa_3 depletion is the cause of the deficient mitochondrial respiration induced by chronic valproate administration

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Abstract—Liver mitochondria from rats fed 1% (w/w) valproic acid for 75 days displayed an approximate 30% decrease in coupled respiration rates with substrates entering the respiratory chain at complexes I and II. Uncoupling the respiration from proton-pumping, or measuring the respiration without complex IV removed this inhibition. The treatment induced a loss of activity of cytochrome oxidase consistent with a decrease in the mitochondrial content of cytochrome aa_3. The inhibition induced by long lasting administration of valproate is apparently located at the site of the proton-pumping activity of complex IV. Furthermore, the capacity of electron transport through complex IV, being far in excess of that required for normal functioning in coupled mitochondria, seems to be controlled by the coupling to proton-pumping in which cytochrome aa_3 appears to play a major role.

The efficacy of sodium valproate in the treatment of generalized epileptic seizures is well established [1]. Its use, however, occasionally causes severe and even fatal toxicity [2, 3]. Liver complications resembling that in Reye's syndrome have been reported in a small number of patients [4]. The appearance of dicarboxylic acids in urine during valproate therapy [5] suggests interference with the function of mitochondria.

Inhibitory effects of valproate on the mitochondrial respiratory chain *in vitro* have been reported [6] but not confirmed [7]. We have shown recently that the addition of valproate to a mitochondrial fraction isolated from the liver of control rats decreases the rate of oxygen consumption not by inhibiting the respiratory chain directly, but by impairing the oxidation of substrates in the mitochondrial matrix [8].

The purpose of the present work was to study the effects of chronic *in vivo* administration of valproate on the respiratory chain of rat liver mitochondria. We report that this treatment results in decreased rates of oxidative phosphorylation in the liver and that the site of the inhibtion lies within complex IV of the respiratory chain.

Materials and Methods

Valproic acid and rotenone were from Janssen Chimica (Beerse, Belgium); glutamic and succinic acids from Merck (Darmstadt, F.R.G.); and pyruvate kinase (EC 2.7.1.40), lactate dehydrogenase (EC 1.1.1.27) and oligomycin from Boehringer (Mannheim, F.R.G.). FCCP* and TMPD were from the Sigma Chemical Co. (St Louis, MO, U.S.A.). Other chemicals were of the best grades available from local suppliers.

Adult male Wistar rats (approx. 150 g) were fed a standard laboratory rat chow (A-03, UAR, France) with or without 1% (w/w) neutralized valproic acid. After 75 days, the animals were killed by decapitation and mitochondrial fractions prepared as described by Veitch and Van Hoof [8].

Oxidative activities were measured at 30° with a Clark-type oxygen electrode (Yellow Springs Inst., OH, U.S.A.) as described by Veitch and Van Hoof [8] with either 10 mM succinate or 10 mM glutamate/1 mM malate as substrates. Coupled conditions were obtained by addition of 0.33 mM ADP and uncoupled conditions with 1 μM FCCP. Succinate oxidation rates were also measured spectrophotometrically at 30° as the antimycin-sensitive reduction of ferricyanide [9]. Cytochrome *c* oxidase (complex IV, EC 1.9.3.1) was assayed both spectrophotometrically at 25° by the oxidation of cytochrome *c* at 550 nm [10] and polarographically at 30° with 10 mM ascorbate (+250 μM TMPD) and 50 μM cytochrome *c* [11].

The content of cytochromes aa_3, *b*, *c* and c_1 was calculated using the appropriate wavelength pairs [12] from dithionite reduced–oxidized difference spectra obtained with an Aminco DW-2 spectrophotometer [13].

ATPase (EC 3.6.1.3) activity was measured at 30° in mitochondrial fractions stored previously at −80° with an ATP regenerating system in a medium containing 33 mM Tris–acetate, 83 mM sucrose, 20 mM $MgCl_2$, 1 mM EDTA, 4 mM ATP, 1.5 mM phosphoenolpyruvate, 0.17 mM NADH, 6 U pyruvate kinase, 12 U lactate dehydrogenase and 2.5 μg/mL rotenone, as described by Pullman *et al.* [14] with slight modifications. ATPase activity was calculated from the difference in the rates of NADH oxidation followed at 340 nm in the presence and in the absence of 5 μM oligomycin. Mitochondrial enzymes citrate synthase (EC 4.1.3.7) [15] and succinate dehydrogenase (EC 1.3.99.1) [16] were assayed as described.

Results and Discussion

After 75 days of valproate treatment, the animals displayed no modification in behaviour or body weight gain

* Abbreviation: FCCP, carbonyl cyanide-*p*-trifluoromethoxyphenylhydrazone; TMPD, tetramethyl-*p*-phenylenediamine.