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THE EFFECTS OF VALPROATE ON INTERMEDIARY METABOLISM IN ISOLATED RAT HEPATOCYTES AND INTACT RATS

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Abstract—Valproate is a valuable anticonvulsant which is associated with hepatotoxicity in some patients. In concentrations in the range found in man during valproate therapy (0.1–1.0 mM), it inhibited pyruvate and palmitate oxidation, urea synthesis and gluconeogenesis by 30–50% in isolated rat hepatocytes. Valproate (100 mg/kg body weight) is also hypoglycaemic and hypoketonaemic in fasted rats. All these inhibitions can be explained in terms of the accumulation of valproyl-CoA and its further metabolites in the matrix of hepatic mitochondria. Although these inhibitions are only partial, and normally well tolerated, they could significantly impair liver function when there is an additional insult, such as may occur with multiple drug therapy or if there is already an inborn error of metabolism. Such an association with inborn errors may explain the higher incidence of valproate-associated toxicity in children. It may be of more value to measure blood urea and ammonia concentrations routinely shortly after starting valproate therapy than to do conventional liver function tests.

Valproate [dipropylacetate, 2(*n*-propyl)-pentanoate], administered as either sodium valproate or free valproic acid, is a widely used anticonvulsant, and clinical studies have shown that it is effective in both childhood [1] and adult [2] epilepsy. Valproate was thought to be relatively free from serious adverse effects, but over the past few years liver dysfunction, including fatal hepatic failure, has occurred in patients whose medication included valproate [3]. The incidence of fatal liver damage is exceedingly low [4] but when it does occur death is due to fulminant hepatic failure or a Reye-like syndrome. Hyperammonaemia without concurrent liver disease has also been reported [5]. The patients who seem most at risk are children with severe epilepsy and mental retardation, some of whom may also have an inborn error of metabolism. These patients are often taking other anticonvulsants in addition to valproate [3].

The mechanism of valproate-associated hepatic failure is unknown. Several authors [5–7] noted that valproate is a simple branched-chain fatty acid and they drew a superficial analogy with the effects of the toxic hypoglycaemic compounds hypoglycin (methylenecyclopropylalanine), the active principle of the Jamaican ackee fruit, and of pent-4-enoate. Metabolites of these toxins inhibit β-oxidation, leading to impairment of gluconeogenesis, hypoglycaemia and often death (see [8] and [9] for reviews). It was suggested that valproate, or its metabolites, may cause a similar disturbance of intermediary metabolism. We therefore investigated the effects of valproate, in concentrations similar to those found in man during valproate therapy, on some parameters of intermediary metabolism in isolated rat hepatocytes. The metabolic response of rats to a single dose of valproate was also studied to see if the changes observed in isolated hepatocytes are reflected in the intact rat.

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MATERIALS AND METHODS

Materials. Enzymes and other biochemicals were obtained from Boehringer Corp. (London); radiolabelled compounds were purchased from Amersham International Plc (Amersham, U.K.) and sodium valproate was a kind gift from Labaz, U.K. Valproyl-CoA was synthesised by the mixed anhydride method [10] and characterised by HPLC [11]; its concentration was determined by measuring the CoASH released by alkaline hydrolysis [12].

Preparation and incubation of rat hepatocytes. Hepatocytes were prepared from the livers of fed male Wistar rats (250 g) [13] and only preparations containing more than 90% of viable cells, as assessed by trypan blue exclusion, were used. Not more than a further 5–10% of these cells died during any incubation. Cells were routinely incubated in vials sealed with a rubber cap, containing, where appropriate, a small centre well [14], in a shaking water bath (100 strokes/min) at 37°, with an atmosphere of 95% O_2/5% CO_2. A bicarbonate-buffered medium [15] containing 2% defatted bovine serum albumin, 0.1 mM L-carnitine and other additions as indicated below (final volume 1.0 ml) was used throughout. Valproate at concentrations of 0.1, 0.5, 1.0 or 10 mM was added as indicated in the figures.

Measurement of pyruvate oxidation. This was measured by the production of $^{14}CO_2$ from 10 mM

[1-^{14}C]pyruvate (specific activity 0.05 μCi/mmole). The reaction was started by the addition of 5×10^5 cells/ml and stopped at appropriate times by the addition of 1.0 ml of 0.5 M $HClO_4$ through the rubber caps. Hyamine hydroxide [0.5 ml; 10% (w/v) in methanol] was injected into the centre wells and the vials were shaken at 0° for a further 1 hr to ensure quantitative transfer of $^{14}CO_2$ to the hyamine. This radioactivity was counted after addition to 10 ml of Unisolve scintillation fluid. It was checked that all rates of $^{14}CO_2$ production were linear with time.

Measurement of palmitate oxidation. This was measured using 1 mM [1-^{14}C]palmitate (specific activity 1 μCi/mmole) and was started by the addition of 5×10^5 cells/ml, and stopped at appropriate times by the addition of 1.0 ml of 0.5 M $HClO_4$, which precipitated unchanged palmitate and palmitate incorporated into complex lipids, together with denatured protein. The acid-soluble radioactivity remaining in the supernatant after centrifugation for 3000 g_{av} min was determined [9]. This represented the sum of [1-^{14}C]acetyl units generated by β-oxidation measured as acetoacetate, 3-hydroxybutyrate, citrate, etc. and was multiplied by 8 to give the total flux through β-oxidation. Production of $^{14}CO_2$ was consistently less than 5% of that found in the acid-soluble fraction, and was disregarded.

Measurement of urea synthesis. This was measured using 4 mM L-orthinine and 10 mM L-glutamine as substrates in the presence of 5 mM glucose [16]. The reaction was started by the addition of 1×10^6 cells/ml and stopped by rapid cooling and centrifugation at 3000 g_{av} min. The concentration of urea in the supernatants was determined [17].

Measurement of gluconeogenesis. Hepatocytes (2×10^6 cells/ml) were pre-incubated for 30 min in the presence of 5 mM glucose, 2 mM L-alanine, 0.1% bacitracin and 500 i.u. aprotinin, with or without 1 nM glucagon. A tracer amount of [U-^{14}C]alanine was then added (0.05 μCi/mmole final specific radioactivity in the incubation medium) and the cells were then incubated for a further 20 min. A sample of the cell suspension (0.5 ml) was added to 2.0 ml of 140 mM NaCl at 0° and immediately centrifuged at 3000 g_{av} min. The supernatant (1.0 ml) was deproteinised with 0.5 ml of 150 mM $ZnSO_4$ and 0.5 ml of 150 mM $Ba(OH)_2$. After centrifugation the precipitate was discarded and all ionised compounds were removed from the supernatant by passing it through a small column (1 ml bed volume) of mixed Dowex-X8 and Amberlite IR-45 resins (H^+-forms; ratio 1:4 by volume) which was then washed with 2.0 ml of water. The radioactivity not retained by the column was determined in the combined eluants (1.5 ml sample) after addition to 10 ml of Unisolve scintillation fluid. It was assumed that [^{14}C]glucose was the only molecular species present derived from [U-^{14}C]-L-alanine and that its formation was a measure of gluconeogenesis. This is essentially the same procedure as used by Claus *et al.* [18], who demonstrated linearity of [^{14}C]glucose production with time, both for the basal and glucagon-stimulated rates. However, our values are quoted as the total amount of [^{14}C]glucose formed during 20 min since linearity of glucose production with time in the presence of valproate was not checked.

Assay of valproate concentrations in blood. Four rats were given sodium valproate (100 mg/kg body weight) intraperitoneally, and blood was obtained at appropriate times by cardiac puncture under light anaesthesia for measurement of valproate concentrations by gas–liquid chromatography [19].

Effects of valproate on the concentrations of blood metabolites in rats. Male Wistar rats (200 g) fed, or deprived of food for 18 hr, were given sodium valproate intraperitoneally (100 mg/kg body weight as a 0.14 M solution), or the same volume of 0.14 M NaCl. Blood was taken from the tail vein for the determination of glucose, 3-hydroxybutyrate, pyruvate, lactate, alanine and glycerol by automated enzymic analysis [20] and acetoacetate by manual enzymic assay [21].

RESULTS

Effects of valproate on the metabolism of isolated hepatocytes

The oxidation of [1-^{14}C]pyruvate and of [1-^{14}C]palmitate, and urea synthesis were inhibited by concentrations of valproate in the therapeutic range (Figs. 1–3). Glucagon increased the incorporation of [1-^{14}C]alanine into glucose (Fig. 4). Both basal and glucagon-stimulated gluconeogenesis were inhibited by valproate in a concentration-dependent manner (Fig. 4).

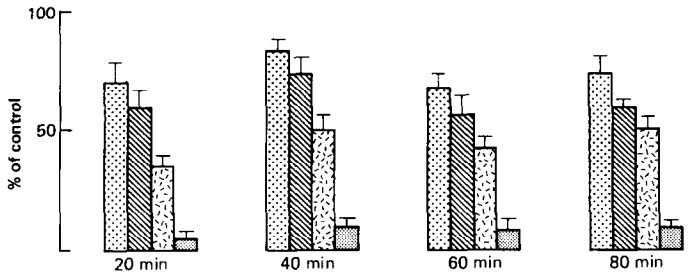

Fig. 1. Inhibition of [1-^{14}C]pyruvate oxidation in isolated hepatocytes by valproate. The results are expressed as a percentage of control values for each time point and are means ± S.E.M. for four experiments. Control mean values for 20, 40, 60 and 80 min were 73, 103, 161 and 188 nmole of pyruvate oxidised/10^6 cells, respectively. Valproate concentrations were: ⊡, 0.1 mM; ▨, 0.5 mM; ⊡, 1.0 mM; and ▩, 10 mM.

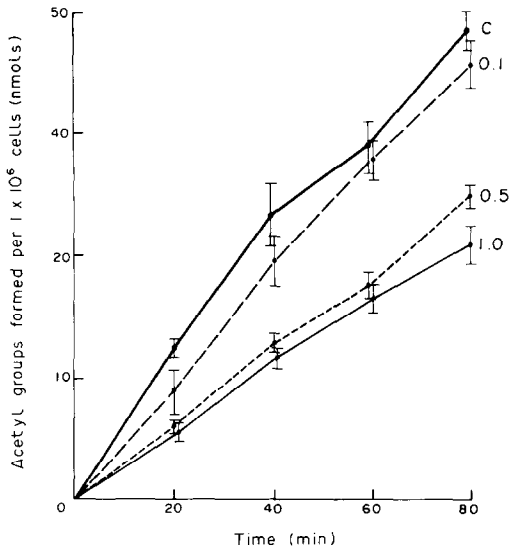

Fig. 2. Inhibition of [1-^{14}C]palmitate oxidation in isolated hepatocytes by valproate. Results are means ± S.E.M. for four experiments. Control, c; concentrations of added valproate (mM) are shown on the figure.

Valproate concentrations in blood

Intraperitoneal administration of valproate (100 mg/kg body weight) to rats gave peak plasma concentrations (Fig. 5) slightly greater than those found in humans during valproate therapy [22].

Effects of valproate on the concentrations of blood metabolites

Administration of valproate (100 mg/kg body weight) caused decreases in total ketone-body concentrations, both in fed and starved rats, with decreases in the 3-hydroxybutyrate/acetoacetate ratios. Valproate also caused decreases in blood glucose concentrations in starved rats (Fig. 6). There were no significant changes in the concentrations of pyruvate, lactate, glycerol and alanine. The maximum decreases in blood glucose and ketone-body concentrations coincided with, or occurred slightly after, the maximum blood concentration of valproate (Figs. 5 and 6).

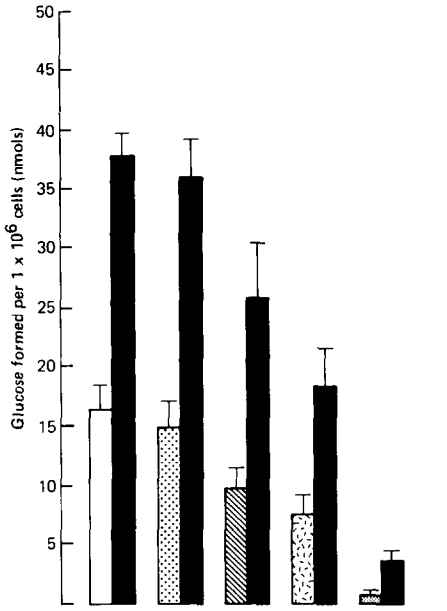

Fig. 4. Inhibition of basal and glucagon-stimulated gluconeogenesis from [U-^{14}C]-L-alanine in isolated hepatocytes by valproate. Results are means ± S.E.M. for four experiments. The black histobars represent glucagon stimulation at the appropriate valproate concentration. The valproate concentrations were: ⊠, 0.1 mM; ▨, 0.5 mM; ⊡, 1.0 mM; and ▩, 10 mM; □, control.

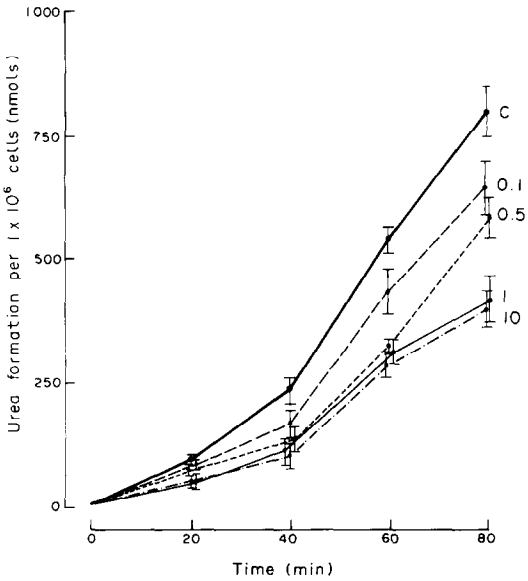

Fig. 3. Inhibition of urea synthesis from ornithine and glutamine in isolated hepatocytes by valproate. Results are means ± S.E.M. for four experiments. Control, c; concentration of added valproate (mM) are shown on the figure.

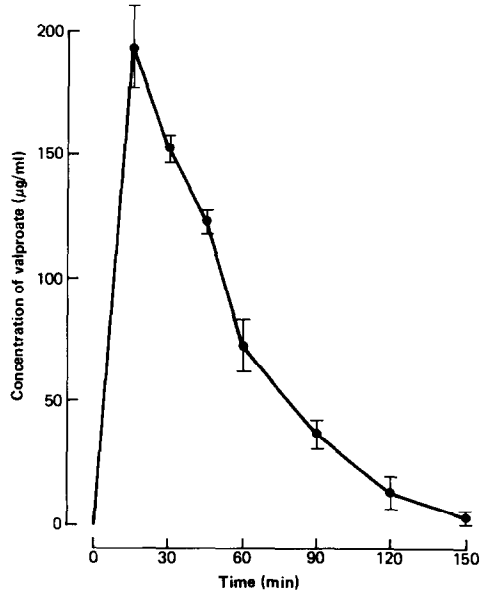

Fig. 5. Plasma valproate concentrations following intraperitoneal administration of sodium valproate (100 mg/kg body weight) in the fasted rat. Results are means ± S.E.M. for four animals.

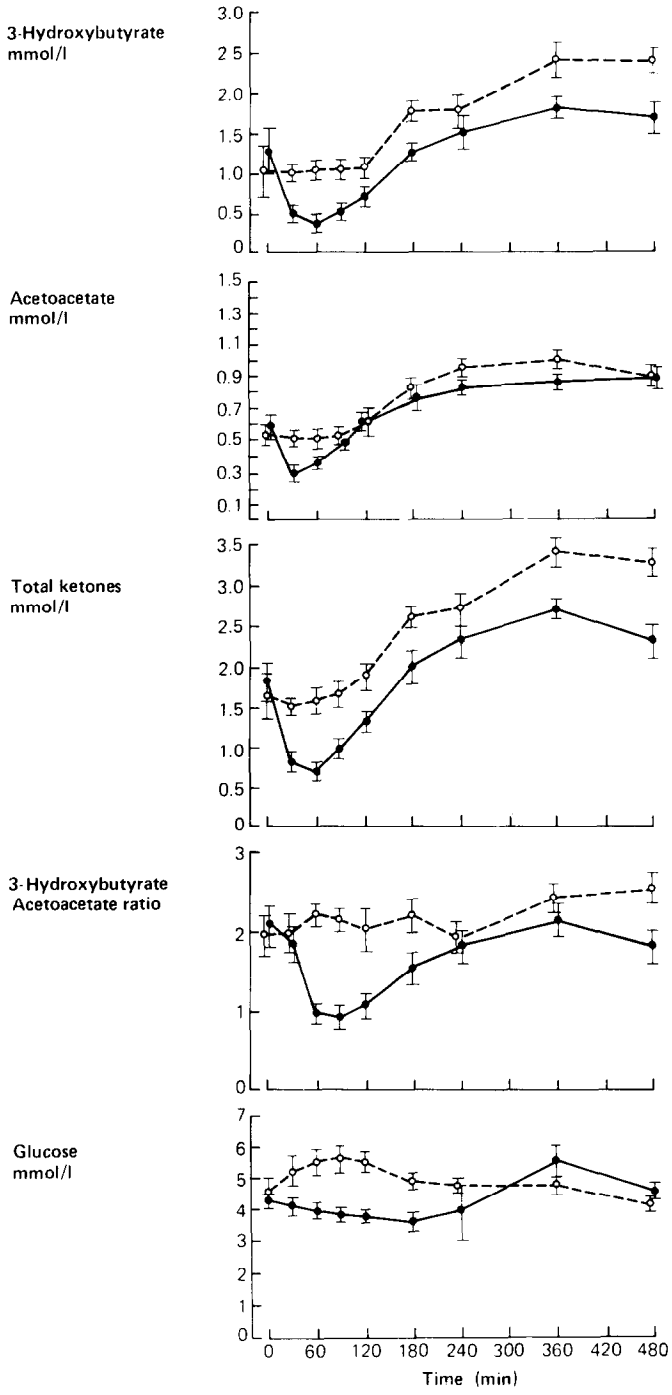

Fig. 6. Blood metabolite concentrations following intraperitoneal administration of sodium valproate (100 mg/kg body weight) in 18-hr fasted rats. Results are means ± S.E.M. for four animals. Values ± S.E.M.: controls (○), valproate-treated (●).

DISCUSSION

The data presented in this paper demonstrate that valproate has a significant effect on the metabolism of isolated rat hepatocytes. Pyruvate and palmitate oxidation, urea synthesis and gluconeogenesis were inhibited by 30–80% by valproate at concentrations between 0.1 and 10 mM. The concentrations used were 0.1 mM (16.6 μg/ml), 0.5 mM (83 μg/ml), 1.0 mM (166 μg/ml) and 10 mM (1.66 mg/ml).

The pathways of valproate metabolism include partial β-oxidation to 3-oxodipropylacetate, which requires that it is first converted to valproyl-CoA and then to further metabolites in the mitochondrial

matrix [23] so that a significant fraction of the total mitochondrial CoA is acylated. The contributions of these competing pathways differ quantitatively in man and rat, and formation of 3-oxodipropylacetate is greater in man [23]. The mitochondrial fraction from rat liver was obtained by differential centrifugation [24] and incubated with 1.0 mM valproate for 5 min. When examined by high pressure liquid chromatography [11], $HClO_4$ extracts of the mitochondria gave a peak corresponding to valproyl-CoA, and the peak corresponding to acetyl-CoA and free CoASH was greatly diminished when compared with control incubations (not shown).

All the metabolic disturbances caused by valproate in rat hepatocytes described in this paper can be explained by an increase in the acyl-CoA/CoASH ratio in the mitochondrial matrix, which may also be associated with a lower acetyl-CoA concentration. (i) Pyruvate oxidation is inhibited at the stage of pyruvate dehydrogenase by several short-chain and medium-chain acyl-CoA esters [25], and valproyl-CoA may inhibit similarly. We have shown that purified pig heart pyruvate dehydrogenase [26] is inhibited by 70% by 0.4 mM valproyl-CoA with 2 mM pyruvate, 0.1 mM CoASH and 2.5 mM NAD^+ in the assay system. In addition, valproate has also been shown to impair the uptake of pyruvate by rat brain mitochondria [27]. (ii) β-Oxidation of fatty acids, which involves several acyl-CoA esters as obligatory intermediates, is weakly competitively inhibited by several short-chain fatty acids which form acyl-CoA esters in the mitochondrial matrix [24] and this mechanism may be invoked to explain inhibition of palmitate oxidation. (iii) Decreased urea synthesis is probably due to inhibition of an enzyme involved in urea synthesis, the most likely site being carbamoylphosphate synthase, since the conversion of ornithine to citrulline is the only step of the urea cycle which occurs in the mitochondrial matrix. This enzyme is inhibited by high acyl-CoA/CoASH ratios and, indeed, propionate and pent-4-enoate, which generate acylated metabolites in the matrix impair urea synthesis [24, 28, 29]. (iv) Inhibition of gluconeogenesis probably occurs at the stage of pyruvate carboxylase which has an absolute requirement for acetyl-CoA as an allosteric activator. Antagonism of this activation by some other acyl-CoA esters is an important site of inhibition of gluconeogenesis and there is strong evidence that hypoglycaemia, both in some inborn errors of metabolism and that caused by some poisons, is due to this effect (see [8] and [9]). Further, inhibition of pyruvate carboxylase may also contribute to the impairment of urea synthesis indirectly by limiting the synthesis of oxaloacetate, and hence of aspartate [30]. Pyruvate carboxylase in sonicated mitochondria, assayed by the pyruvate-dependent fixation of $^{14}CO_2$ [31], is inhibited by 75% by 0.4 mM valproyl-CoA in the presence of 0.2 mM acetyl-CoA. Further, incubation of intact mitochondria with 1.0 mM valproate resulted in almost complete inhibition of pyruvate-dependent $^{14}CO_2$-fixation (not shown).

Other unusual short-chain and medium-chain fatty acids that are not readily metabolised have some non-specific effects of intermediary metabolism similar to those described here for valproate [9, 24], although these compounds are not used therapeutically. Too much weight should not be given to doubtful analogies with hypoglycin or pent-4-enoate, which are metabolised to methylenecyclopropylacetyl-CoA and 3-oxopent-4-enoyl-CoA, respectively. Methylenecyclopropylacetyl-CoA inactivates butyryl-CoA and isovaleryl-CoA dehydrogenases, and 3-oxopent-4-enoyl-CoA inactivates 3-oxoacyl-CoA thiolase, and these specific enzyme inhibitions are the primary causes of the toxic effects of these compounds (see [8] and [9]).

Intraperitoneal administration of sodium valproate to starved rats caused significant decreases in blood glucose and ketone-body concentrations with a lowering of the 3-hydroxybutyrate/acetoacetate ratios, which is often associated with inhibition of β-oxidation [32]. These metabolite concentrations in the fasted state may reflect impairment of gluconeogenesis and of β-oxidation, respectively. They are probably due to a greater inhibition of the production of these metabolites than of their utilisation, although in this study the effects of valproate on extrahepatic oxidations were not determined.

An important factor in valproate-associated toxicity could be impairment of liver metabolism similar to that found in rat hepatocytes, and there are indications that this occurs in man [5, 33]. Preliminary evidence suggesting some inhibition of hepatic fatty acid oxidation is that dicarboxylic aciduria occurs in patients on valproate therapy [33]. Further, one of us (D.M.T.) received sodium valproate (400 mg i.v.) after fasting for 18 hr and this caused a decrease of more than 50% in total ketone-body concentrations in blood after 90 min. Although metabolic inhibition caused by therapeutic concentrations of valproate are only partial and are normally well tolerated, they could nevertheless significantly impair liver function when there is an additional insult, such as may occur with multiple drug therapy or if there is already an inborn error of metabolism. Indeed, two cases of valproate-associated hepatotoxicity have been reported in patients with inborn errors of urea synthesis [34, 35]. Such putative association with inborn errors may account for the increased incidence of valproate-associated toxicity in children. If our interpretation is correct, it may be of more value to measure blood urea and ammonia concentrations routinely shortly after starting valproate therapy rather than the indiscriminate use of conventional liver function tests.

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