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Inhibition of ureagenesis by valproate in rat hepatocytes

Role of *N*-acetylglutamate and acetyl-CoA

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Valproate (0.5–5 mM) strongly inhibited urea synthesis in isolated rat hepatocytes incubated with 10 mM-alanine and 3 mM-ornithine. Valproate at the same concentrations markedly decreased concentrations of *N*-acetylglutamate, an essential activator of carbamoyl-phosphate synthetase I (EC 6.3.4.16), in parallel with the inhibition of urea synthesis by valproate. This compound also lowered the cellular concentration of acetyl-CoA, a substrate of *N*-acetylglutamate synthase (EC 2.3.1.1); glutamate, aspartate and citrulline were similarly decreased. Valproate in a dose up to 2 mM did not significantly affect the cellular concentration of ATP and had no direct effect on *N*-acetylglutamate synthesis, carbamoyl-phosphate synthetase I and ornithine transcarbamoylase (EC 2.1.3.3) activities.

The sodium salt of valproic acid (2-propylpentanoic acid) is an anti-epileptic drug which has been successfully used in the treatment of several types of epilepsy, particularly in childhood (Pinder *et al.*, 1977). It has subsequently been shown that valproate induced hyperammonaemia in patients (Coulter & Allen, 1980; Sius *et al.*, 1980). Moreover, an encephalopathic disorder resembling Reye's syndrome has been attributed to valproate (Gerber *et al.*, 1979; Young *et al.*, 1980). Patients with organic acidaemias also have episodes of hyperammonaemia with disordered hepatic function and encephalopathy (Coude *et al.*, 1982). Because the structure and reaction products of valproate are similar to short-chain fatty acids, which accumulate in organic acidaemias (Kuhara & Matsumoto, 1974; Matsumoto *et al.*, 1976), the mechanism of hyperammonaemia in valproate therapy may be similar to the cause of hyperammonaemia in propionic acidaemia. In propionic acidaemia it has been proposed that propionyl-CoA either directly inhibits carbamoyl-phosphate synthetase I (EC 6.3.4.16) (Gruskay & Rosenberg, 1979) or indirectly influences it by interfering with the synthesis of its activator, *N*-acetylglutamate (Stewart & Walser, 1980; Coude *et al.*, 1979). Moreover, Martin-Requero *et al.* (1983) have simulated organic acidaemia in hepatocytes and found inhibition of both pyruvate carboxylase and carbamoyl-phosphate synthetase by methyl-malonyl-CoA, propionyl-CoA and isobutyryl-CoA. In a previous study we observed that citrullinogenesis and *N*-acetylglutamate synthesis measured in intact mitochondria from livers of valproate treated rats were significantly decreased (Coude *et al.*, 1981). The purpose of the present work was to study the effect of valproate on ureagenesis in isolated rat hepatocytes in order to define more precisely the mechanism of its action.

Experimental

Albino Wistar male rats (150–200 g), starved for 18 h, were used. The animal was anaesthetized with Nembutal (7 mg/100 g body wt.), and the liver was cannulated, isolated and mounted in a recirculation perfusion system as described by Williamson *et al.* (1969). The procedure used for the cell isolation was similar to that of Berry & Friend (1969) with some minor modifications (Meijer *et al.*, 1975). The yield was usually 500 mg dry wt. per liver. Cell viability was routinely tested by Trypan Blue (0.4%, w/v) exclusion and phase-contrast microscopy. The cells were suspended in 2 ml of Krebs bicarbonate medium (Krebs & Henseleit, 1932) gassed with O_2/CO_2 (19:1), containing 1.3 mM-Ca^{2+}. Incubations were performed in 25 ml Erlenmeyer flasks, which were shaken at 80 oscillations/min in a water bath at 37°C. The final concentration of cells was 3–5 mg dry wt./ml of incubation medium. Experiments were usually terminated after incubation for 30 min. For assay of total contents of cell metabolites., 1 ml samples were added to $HClO_4$ (final concn.

4.5%, w/v), followed by centrifugation (10000*g*) and neutralization to pH 6 with 3M-KOH containing 0.5M-4-morpholinopropanesulphonic acid.

The dry weight of the cells was obtained by protein precipitation and washing with trichloroacetic acid (Berry & Kun, 1972). Urea was determined colorimetrically (Ceriotti & Spandrio, 1965) with an automatic apparatus. ATP was measured fluorimetrically by the enzymic method described by Williamson & Corkey (1969). Acetyl-CoA was measured by using citrate synthase as described by Pande & Caramancion (1981). Glutamate, aspartate and citrulline were determined with a Technicon TSM amino acid autoanalyser. The assay of *N*-acetylglutamate was based on measurement of the activation of carbamoyl-phosphate synthetase I by the neutralized cell extract. The procedure was that described by Zollner (1981). Batches of mitochondria prepared by the method of Hogeboom (1955) were disrupted by addition of 4vol. of distilled water and used for the assay of enzyme activities. Carbamoyl-phosphate synthetase I (EC 6.3.4.16) and ornithine transcarbamoylase (EC 2.1.3.3) were measured by the methods of Brown & Cohen (1959) and Snodgrass (1968) respectively. *N*-Acetylglutamate synthase (acetyl-CoA:L-glutamate *N*-acetyltransferase, EC 2.3.1.1) was determined as previously described (Coude *et al.*, 1979). Mitochondrial protein was determined by the biuret method (Layne, 1957). All experiments were repeated three to six times. Results are expressed as means ± S.E.M. Statistical significance was calculated by using Student's paired *t* test.

Enzymes and coenzymes were purchased from Boehringer or Sigma. L-[U-^{14}C]Aspartic acid (55Ci/mol) for the measurement of acetyl-CoA, $NaH^{14}CO_3$ (56Ci/mol) for the measurement of *N*-acetylglutamate and L-[1-^{14}C]glutamic acid (0.5mCi/mol) were purchased from Amersham.

Results and discussion

Ureagenesis was determined in the presence of 10mM-alanine and 3mM-ornithine. It proceeded linearly with time up to 60min at a rate of 620 ± 75μmol/h per g dry wt. Urea synthesis in isolated hepatocytes was strongly inhibited by valproate at concentrations of 0.05–5mM (Fig. 1). The mechanism by which this inhibition occurs was further investigated. The first step of urea synthesis, catalysed by carbamoyl-phosphate synthetase I, requires adequate concentrations of its allosteric activator *N*-acetylglutamate and sufficient production of ATP. Valproate at 5mM did not significantly inhibit the activities of carbamoyl-phosphate synthetase I and ornithine carbamoyltransferase (61 ± 14 and 2040 ± 725nmol/min per mg of protein respectively). Valproate produced in hepatocytes

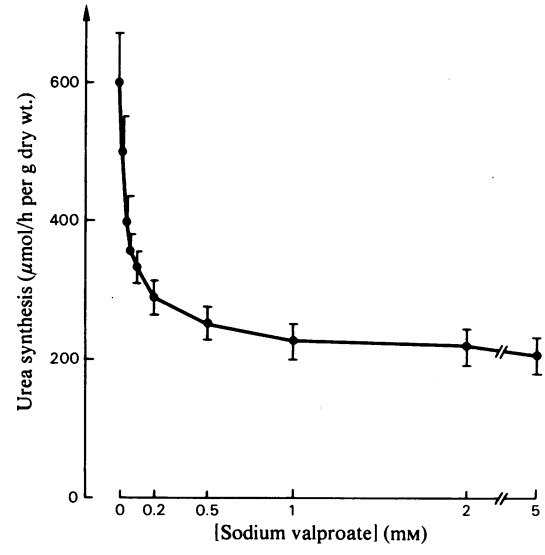

Fig. 1. *Effect of valproate on urea synthesis in isolated hepatocytes*

Rat liver cells (3mg dry wt./ml) isolated from 18h-starved rats were incubated for 30min in the presence of 10mM-alanine and 3mM-ornithine. Values shown are means ± S.E.M. for at least three experiments.

from 18h-starved rats a concentration-dependent inhibition of the accumulation of glutamate, aspartate and citrulline, which paralleled its effect on ureagenesis (Fig. 2). *N*-Acetylglutamate concentrations, which were measured at the end of the urea-synthesis assay, markedly decreased with addition of valproate (Table 1), and the rate of urea synthesis paralleled concentrations of *N*-acetylglutamate (Fig. 3). Valproate (5mM) did not significantly affect *N*-acetylglutamate synthase activity (22 ± 4nmol/h per mg of protein). As shown in Table 1, no significant decrease in ATP was observed in the presence of valproate at concentrations up to 2mM. Since it has been demonstrated that variations in the *N*-acetylglutamate content affect the urea cycle (Shigesada & Tatibana, 1971; McGivan *et al.*, 1976; Saheki *et al.*, 1977; Martin-Requero *et al.*, 1983), these results indicate that the decrease in *N*-acetylglutamate is at least partly responsible for inhibition of urea synthesis by valproate. This was further confirmed by the dramatic fall in the concentration of citrulline, which normally accumulates in hepatocytes incubated with alanine and ornithine.

Valproate at 0.2mM and 2mM significantly decreased acetyl-CoA in hepatocytes incubated wtih 3mM-ornithine and 10mM-alanine (Table 1). Acetyl-CoA is not directly connected with the pathway of

Table 1. *Effects of valproate on metabolic rates in isolated rat liver cells*

Hepatocytes (7mg dry wt./ml) isolated from 18h-starved rats were incubated for 30min in the presence of 10mM-alanine and 3mM-ornithine. *N*-Acetylglutamate and acetyl-CoA were determined in neutralized $HClO_4$ extracts as described in the Experimental section.

Measurement Valproate (mM) ...	0	0.2	2
Urea production (μmol/h per g dry wt.)	602 ± 73	298 ± 34*	240 ± 24*
ATP (μmol/g dry wt.)	11.4 ± 0.7	9.0 ± 1.4	9.2 ± 0.9
Acetyl-CoA (nmol/g dry wt.)	542 ± 86	280 ± 49*	205 ± 26*
N-Acetylglutamate (nmol/g dry wt.)	351 ± 24	138 ± 24*	64 ± 21*

* Significantly different from controls incubated without valproate ($P < 0.01$).

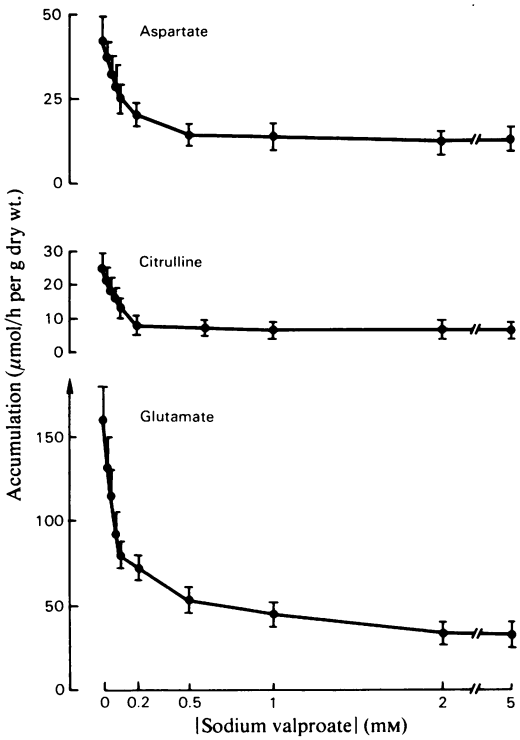

Fig. 2. *Effect of valproate on accumulation of aspartate, glutamate and citrulline in isolated hepatocytes*

Rat liver cells (3mg dry wt./ml) isolated from 18h-starved rats were incubated for 30min in the presence of 10mM-alanine and 3mM-ornithine. Rates of accumulation of aspartate, citrulline and glutamate were calculated between 0 and 30min. Values are means ± S.E.M. for at least three experiments.

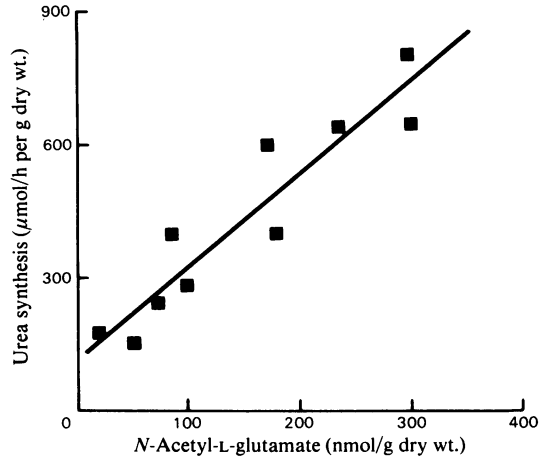

Fig. 3. *Relationship between urea synthesis and N-acetylglutamate concentrations in hepatocytes incubated with different concentrations of valproate*

Rat liver cells (7mg dry wt./ml) isolated from 18h-starved rats were incubated for 30min in the presence of 10mM-alanine and 3mM-ornithine. ($r = 0.938$; $y = 2.1x + 114.4$; $P < 0.001$.)

valproate metabolism; hence the question arises why hepatocyte concentrations of acetyl-CoA fall. Under the conditions of the present experiments, with alanine as substrate, endogenous fatty acid oxidation is largely suppressed (Meijer *et al.*, 1978) and acetyl-CoA is generated primarily by the pyruvate dehydrogenase reaction (Walajtys-Rode & Williamson, 1980). The fall in the acetyl-CoA content might therefore be the consequence of an inhibition of pyruvate dehydrogenase. Valproyl-CoA could inhibit pyruvate dehydrogenase activity, since it accumulates in hepatocytes treated with valproate (Harris & Becker, 1982) and since valproate inhibits the oxidation of [1-^{14}C]pyruvate to $^{14}CO_2$ in intact hepatocytes (Turnbull *et al.*, 1983). However, since acetyl-CoA and glutamate are substrates for *N*-acetylglutamate synthase, and since cellular *N*-acetylglutamate concentration is mainly determined by the rate of its synthesis (Shigesada & Tatibana, 1971; Stewart & Walser, 1980), it seems likely that depletion of cellular *N*-acetylglutamate by valproate is concomitantly due to decreased concentrations of acetyl-CoA and glutamate. On the other hand it is well recognized that the activity of pyruvate car-

boxylase is strongly influenced by acetyl-CoA, which acts as an allosteric activator (Utter & Scrutton, 1969). As pyruvate carboxylase is involved in the synthesis of aspartate (via transamination from oxaloacetate), and as pyruvate dehydrogenase and pyruvate carboxylase are involved in the synthesis of glutamate (via citrate synthase, isocitrate dehydrogenase and transamination from α-oxoglutarate), any lowering of the activities of pyruvate carboxylase and pyruvate dehydrogenase would seriously decrease the rates of accumulation of glutamate and aspartate in hepatocytes incubated with alanine (Meijer *et al.*, 1975).

Although numerous studies, including those *in vivo* (Stewart & Walser, 1980; Shigesada *et al.*, 1978; Kawamoto *et al.*, 1982), with isolated hepatocytes (Hensgens *et al.*, 1980) and isolated mitochondria (McGivan *et al.*, 1976; Meijer & Van Woerkom, 1978; Cheung & Raijman, 1980), have demonstrated that rates of synthesis of urea or citrulline are mainly determined by *N*-acetylglutamate concentrations, nevertheless, since stoichiometric amounts of aspartate and citrulline are needed for the operation of the urea cycle (Krebs *et al.*, 1973), and since valproate decreases aspartate and citrulline to the same extent, our data suggest that the inhibition of ureagenesis by valproate is concomitantly due to defective synthesis of carbamoyl phosphate and aspartate, which is probably brought about by lowered concentrations of *N*-acetylglutamate and acetyl-CoA.

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