Potentialiation by ammonia of the metabolic effects of pent-4-enoate in isolated rat hepatocytes

François X. COUDÉ,* Gisèle GRIMBER, Philippe PARVY, Daniel RABIER† and
Jacqueline BARDET

Laboratoire de Biochimie Génétique, Hôpital Necker, 149 rue de Sèvres, 75743 Paris Cedex 15, France

(Received 16 July 1984/Accepted 7 August 1984)

The metabolic effects of pent-4-enoate were studied in isolated rat hepatocytes; 1 mM-pent-4-enoate did not significantly inhibit gluconeogenesis from lactate, alanine and glycerol, but significantly decreased glucose synthesis from pyruvate. The addition of 1 mM-NH₄Cl led to a drastic inhibition of glucose synthesis from all these substrates. In hepatocytes incubated with 10 mM-alanine and 1 mM-oleate, pent-4-enoate at 0.05–1 mM slightly inhibited glucose synthesis and ketogenesis. The addition of ammonia resulted in a dramatic potentiation of the metabolic effects of pent-4-enoate. Half-maximum effect of ammonia was observed at 0.2 mM concentration. Concomitant cellular concentrations of ATP and acetyl-CoA were also decreased by the addition of ammonia, as were lactate/pyruvate ratio and β -hydroxybutyrate/acetoacetate ratio. These data suggest that ammonia seriously interferes with the cellular metabolism of pent-4-enoate and leads to a dramatic potentiation of its effects.

Pent-4-enoic acid is a chemical analogue of hypoglycin, the compound which causes Jamaican vomiting sickness (Sherratt, 1969; Bressler *et al.*, 1969). The metabolism and mechanism of action of pent-4-enoate have been extensively investigated in isolated rat liver mitochondria and in perfused rat liver. Metabolism of pent-4-enoate in the liver is associated with inhibition of fatty acid oxidation (Corredor *et al.*, 1967; Holland & Sherratt, 1973), pyruvate oxidation (Senior & Sherratt, 1968), gluconeogenesis (Toews *et al.*, 1970; Williamson *et al.*, 1970) and ureogenesis (Aoyagi *et al.*, 1979). In an attempt to define the interaction between fatty acid oxidation and gluconeogenesis in rat liver, we have tested the effects of pent-4-enoate in isolated rat hepatocytes. Such experiments disclosed a striking potentiation by ammonia of the metabolic disturbances induced by pent-4-enoate. The present work was carried out to study the effects of ammonia on the metabolic effects of pent-4-enoate, in order to define more precisely the mechanism of this potentiation induced by ammonia.

Experimental

Albino Wistar male rats (150–200 g) starved for 18 h were used. After anaesthesia of the animal with Nembutal (7 mg/100 g body wt.), 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. of cells per liver. Cell viability was routinely tested by Trypan Blue (0.4%, w/v) exclusion and phase-contrast microscopy. Preparations with more than 10% damaged cells were discarded. The cells were suspended in 2 ml of Krebs bicarbonate medium (Krebs & Henseleit, 1932) gassed with O₂/CO₂ (19:1), containing 2.5 mM-CaCl₂. 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 30 min incubation. For assay of total contents of cell metabolites, 1 ml samples were added to HClO₄ (final concn. 4.5%, w/v), followed by centrifugation at 10000g for 3 min in an Eppendorf centrifuge and neutralization to pH 6 with 3 M-KOH.

* Present address: Sanofi Recherche, 195 route d'Espagne, F-31035 Toulouse, France.

† To whom reprint requests should be addressed.

The dry weight of the cells was obtained by protein precipitation and washing with trichloroacetic acid (Berry & Kun, 1972). Glucose was determined enzymically (Bergmeyer & Bernt, 1965). ATP was measured fluorimetrically by the enzymic method described by Williamson & Corkey (1969). Acetyl-CoA was measured with citrate synthase as described by Pande & Caramancion (1981). Glutamate and aspartate were determined with a Technicon TSM amino acid autoanalyser. Lactate, pyruvate, acetoacetate and β -hydroxybutyrate were determined by the spectrophotometric method of Williamson & Corkey (1969). All experiments were repeated three to six times. Results are expressed as means $\pm$ S.E.M. Statistical significance was calculated by Student's paired *t* test.

Enzymes and coenzymes were purchased from Boehringer or Sigma. L-[U- 14 C]Aspartic acid (55 Ci/mol) for the measurement of acetyl-CoA was purchased from Amersham.

Results and discussion

Gluconeogenesis was determined in the presence of various substrates at 10 mM concentrations. Glucose synthesis proceeded linearly with time up to 60 min in the presence of pyruvate, alanine and glycerol, but showed a lag time with lactate, as reported elsewhere (Cornell *et al.*, 1974). Pent-4-enoate (1 mM) did not significantly affect gluconeogenesis from lactate, alanine and glycerol, but significantly decreased glucose synthesis from pyruvate (Table 1). NH_4Cl (1 mM) did not affect gluconeogenesis, but addition of this to 1 mM-pent-4-enoate led to a drastic inhibition of glucose synthesis from all these substrates. The higher sensitivity of gluconeogenesis from pyruvate to inhibition by pent-4-enoate alone should be located at the malate shuttle. When lactate or alanine is the precursor of glucose, oxaloacetate is transferred from mitochondria to cytosol by the aspartate shuttle, but, with pyruvate, oxaloacetate is transferred in the form of malate (Cornell *et al.*, 1974).

Under these conditions, the metabolism of pent-4-enoate is probably competing with malate dehydrogenase for reducing equivalents, as discussed below. This competition leads to a decreased availability of oxaloacetate for the reaction catalysed by the phosphoenolpyruvate carboxykinase.

The mechanism by which this inhibition occurs was further investigated. In hepatocytes incubated with 10 mM-alanine and 1 mM-oleate, pent-4-enoate at 0.05–1 mM slightly inhibited glucose synthesis. The addition of 1 mM- NH_4Cl resulted in a dramatic potentiation of the effect of pent-4-enoate (Fig. 1). Concomitant ketone-body production was also severely affected by the addition of NH_4Cl , as was the β -hydroxybutyrate/acetoacetate ratio (Fig. 2). The dose-dependent effect of ammonia on gluconeogenesis and ketogenesis was investigated: a half-maximum effect of ammonia was observed

Fig. 1. Effect of pent-4-enoate and NH_4Cl on gluconeogenesis in rat hepatocytes

Rat liver cells (3–5 mg dry wt./ml) isolated from 18 h-starved rats were incubated for 30 min in the presence of 10 mM-alanine and 1 mM-oleate. Glucose synthesis was calculated between 0 and 30 min without (○) and with 1 mM- NH_4Cl (●). Results are expressed as means $\pm$ S.E.M. for four experiments.

Table 1. Effect of pent-4-enoate and NH_4Cl on gluconeogenesis in rat hepatocytes

Liver cells (3–5 mg dry wt./ml) isolated from 18 h-starved rats were incubated for 30 min. Final concentration of each gluconeogenic substrate was 10 mM. Glucose synthesis was measured between 0 and 30 min. Values are means $\pm$ S.E.M. for three to five experiments: **P* < 0.01 relative to control incubated with the same substrate.

	Glucose synthesis ($\mu\text{mol/h per g dry wt.}$)			
	Controls	Pent-4-enoate (1 mM)	NH_4Cl (1 mM)	Pent-4-enoate (1 mM) + NH_4Cl (1 mM)
Lactate	208 $\pm$ 50	198 $\pm$ 39	240 $\pm$ 51	51 $\pm$ 28*
Pyruvate	325 $\pm$ 68	158 $\pm$ 33	348 $\pm$ 98	40 $\pm$ 8*
Alanine	243 $\pm$ 34	189 $\pm$ 43	245 $\pm$ 34	31 $\pm$ 6*
Glycerol	393 $\pm$ 21	445 $\pm$ 17	354 $\pm$ 48	79 $\pm$ 12*

Fig. 2. Effect of pent-4-enoate and NH_4Cl on ketogenesis in rat hepatocytes

Rat liver cells (3–5 mg dry wt./ml) isolated from 18 h-starved rats were incubated for 30 min in the presence of 1 mM-oleate. Rates of formation of β -hydroxybutyrate and acetoacetate were calculated between 0 and 30 min without (○) and with 1 mM- NH_4Cl (●). Results are expressed as means $\pm$ S.E.M. for four experiments.

at 0.2 mM (Fig. 3). In view of the rather long incubation period and the cell concentration used, it is likely that the effective concentration of ammonia was much lower than the concentration added. As shown in Table 2, cellular concentrations of ATP and acetyl-CoA were also drastically modified by the addition of ammonia, as was the lactate/pyruvate ratio, which markedly decreased.

To a first approximation, flux through pyruvate carboxylase in hepatocytes incubated with alanine and oleate can be estimated from the sum of twice the glucose production and the net accumulation of glutamate and aspartate (Williamson *et al.*, 1981).

Fig. 3. Effect of ammonia on ketogenesis and glucose synthesis in rat hepatocytes incubated in the presence of 1 mM-pent-4-enoate

Rat liver cells (3 mg dry wt./ml) isolated from 18 h-starved rats were incubated for 30 min in the presence of 10 mM-alanine, 1 mM-oleate and 1 mM-pent-4-enoate. Glucose synthesis (●) and ketone-body production (□) were calculated between 0 and 30 min. 'Ketone bodies' refers to the sum of β -hydroxybutyrate and acetoacetate. Results (means $\pm$ S.E.M.) are expressed as percentages of controls incubated without ammonia.

Fig. 4 shows the relationship between the calculated flux through pyruvate carboxylase measured after 30 min incubation with different concentrations of pent-4-enoate, in the presence or absence of NH_4Cl , and acetyl-CoA concentrations. Over the range of observed tissue acetyl-CoA concentrations, there was an approximately linear relationship between flux through pyruvate carboxylase and acetyl-CoA concentrations, suggesting that the decreased gluconeogenesis was mainly due to a fall in flux through pyruvate carboxylase, which is probably brought about by the lower concentrations of acetyl-CoA. On the other hand, the rate of ketogenesis in hepatocytes mainly depends on the mitochondrial acetyl-CoA concentration (McGarry & Foster, 1980). As expected, ketone-body production and cellular acetyl-CoA were also significantly correlated (Fig. 5).

Our data fit the hypothesis that the impairment of gluconeogenesis induced by pent-4-enoate was secondary to the inhibition of fatty acid oxidation. As shown by Williamson *et al.* (1970), impaired β -oxidation would cause a decrease in mitochondrial acetyl-CoA, resulting in a block at the pyruvate carboxylase step and a decreased supply of reducing equivalents, resulting in another block in the conversion of 1,3-bisphosphoglycerate into triose phosphates. The dramatic potentiation by

Table 2. *Effect of pent-4-enoate and NH₄Cl on metabolite concentrations in isolated rat hepatocytes*

Hepatocytes (14mg dry wt./ml) isolated from 18h-starved rats were incubated for 30min in the presence of 10mM-alanine and 3mM-ornithine. Rates of formation of lactate, pyruvate, aspartate and glutamate were measured between 0 and 30min. ATP and acetyl-CoA concentrations were determined at the end of the incubation period. All metabolite measurements were performed on neutralized HClO₄ extracts as described in the Experimental section. Results are expressed as means $\pm$ S.E.M. for four experiments: * $P < 0.01$ relative to control incubated without addition.

Addition	Rate of formation ($\mu\text{mol/h per g dry wt.}$)		Lactate/pyruvate ratio	Content ($\mu\text{mol/g dry wt.}$)		Rate of formation ($\mu\text{mol/h per g dry wt.}$)	
	Lactate	Pyruvate		ATP	Acetyl-CoA	Aspartate	Glutamate
None	882 $\pm$ 116	172 $\pm$ 20	5.1 $\pm$ 0.8	11.8 $\pm$ 1.7	0.59 $\pm$ 0.06	75 $\pm$ 16	265 $\pm$ 82
Pent-4-enoate (1 mM)	1082 $\pm$ 96	386 $\pm$ 64*	2.7 $\pm$ 0.5*	9.2 $\pm$ 2.9	0.43 $\pm$ 0.04	42 $\pm$ 10	197 $\pm$ 30
NH ₄ Cl (1 mM)	1106 $\pm$ 75	153 $\pm$ 22	7.5 $\pm$ 1.3	9.0 $\pm$ 2.7	0.50 $\pm$ 0.02	91 $\pm$ 21	295 $\pm$ 93
Pent-4-enoate (1 mM) + NH ₄ Cl (1 mM)	850 $\pm$ 12	737 $\pm$ 93*	1.2 $\pm$ 0.3*	4.0 $\pm$ 1.7*	0.13 $\pm$ 0.03*	11 $\pm$ 2*	117 $\pm$ 24*

Fig. 4. *Relationship between flux through pyruvate carboxylase and cellular acetyl-CoA content*

Liver cells (14mg dry wt./ml) isolated from 18h-starved rats were incubated for 30min with 10mM-alanine and 1mM-oleate in the presence of different concentrations of pent-4-enoate without (○) and with 1mM-NH₄Cl (●) ($r = 0.9069$, $y = 1.66x + 244.77$; $P < 0.01$).

Fig. 5. *Relationship between ketone-body production and cellular acetyl-CoA content*

Liver cells (14mg dry wt./ml) isolated from 18h-starved rats were incubated for 30min with 10mM-alanine and 1mM-oleate in the presence of different concentrations of pent-4-enoate without (○) and with 1mM-NH₄Cl (●) ($r = 0.7686$, $y = 1.13x + 496.89$; $P < 0.01$).

ammonia of the toxicity of pent-4-enoate clearly appeared as an accentuation of the effects of pent-4-enoate alone on mitochondrial acetyl-CoA content, oxidoreduction state and ATP production. Although the reason for this effect of ammonia is not obvious, it must be stressed that pent-4-enoate can be metabolized intramitochondrially by two pathways. Common to both pathways are the activation of pent-4-enoate to pent-4-enoyl-CoA, presumably catalysed by medium-chain acyl-CoA synthetase, and the dehydrogenation of pent-4-enoyl-CoA to penta-2,4-dienoyl-CoA, catalysed by butyryl-CoA dehydrogenase. The first pathway involves the β -oxidation of penta-2,4-dienoyl-CoA, which produces 3-oxopent-4-enoyl-CoA, the metabolite responsible for the inhibition of fatty acid oxidation (Schulz, 1983). The second pathway involves the crucial NADPH-dependent reduction of penta-2,4-dienoyl-CoA (Borrebaek *et al.*, 1980; Van Hoof *et al.*, 1979), producing pent-2-enoyl-CoA, which can be degraded via β -oxidation to propionyl-CoA and acetyl-CoA. It appears that the first pathway is quantitatively minor, as supported by the observation that the inhibition of fatty acid oxidation by pent-4-enoate in rat hepatocytes is relieved by feeding of clofibrate, which causes a several-fold increase in the activity of the NADPH-dependent 2,4-dienoyl-CoA reductase (Borrebaek *et al.*, 1980). Since 2,4-dienoyl-CoA reductase requires NADPH as a cofactor (Kunau & Dommes, 1978; Cuebas & Schulz, 1982) and since, apart from a stimulation of carbamoyl phosphate formation, the primary effect of ammonia addition to hepatocytes is a stimulation of reductive amination of α -oxoglutarate via glutamate dehydrogenase, causing a large oxidation of the mitochondrial NADP system (Tischler *et al.*, 1977; Sies *et al.*, 1977), addition of ammonia would therefore impair the activity of 2,4-dienoyl-CoA reductase, with the resulting accumulation of 3-oxopent-4-enoyl-CoA, the metabolite presumed responsible for the inhibition of fatty acid oxidation by pent-4-enoate.

The inhibitory effect of NH_4Cl + pent-4-enoate on gluconeogenesis from glycerol could be compared with that observed with 2-tetradecylglycidic acid, another hypoglycaemic agent (Tutwiler & Delleveigne, 1979). In both cases fatty acid oxidation was inhibited and NADH generation limited. As a consequence, gluconeogenesis would be expected to be limited only insofar as triose phosphate is diverted from glucose formation to lactate and pyruvate formation.

References

- Aoyagi, K., Mori, M. & Tatibana, M. (1979) *Biochim. Biophys. Acta* **587**, 515–521
- Bergmeyer, H. U. & Bernt, E. (1965) In *Methods of Enzymatic Analysis* (Bergmeyer, H. U. ed.), pp. 123–130, Academic Press, New York
- Berry, M. N. & Friend, D. S. (1969) *J. Cell Biol.* **43**, 506–520
- Berry, M. N. & Kun, E. (1972) *Eur. J. Biochem.* **27**, 395–400
- Borrebaek, B., Osmundsen, H. & Bremer, J. (1980) *Biochem. Biophys. Res. Commun.* **93**, 1173–1180
- Bressler, R., Corredor, C. & Brendel, K. (1969) *Pharmacol. Rev.* **21**, 105–130
- Cornell, N. W., Lund, P. & Krebs, H. A. (1974) *Biochem. J.* **142**, 327–337
- Corredor, C., Brendel, K. & Bressler, R. (1967) *Proc. Natl. Acad. Sci. U.S.A.* **58**, 2299–2306
- Cuebas, D. & Schulz, M. (1982) *J. Biol. Chem.* **257**, 14140–14144
- Holland, P. C. & Sherratt, H. S. A. (1973) *Biochem. J.* **136**, 157–171
- Krebs, H. A. & Henseleit, K. (1932) *Hoppe-Seyler's Z. Physiol. Chem.* **210**, 33–66
- Kunau, W. H. & Dommes, P. (1978) *Eur. J. Biochem.* **91**, 533–544
- McGarry, J. D. & Foster, D. W. (1980) *Annu. Rev. Biochem.* **49**, 395–420
- Meijer, A. J., Gimpel, J. A., Deleeuw, G. A., Tager, J. M. & Williamson, J. R. (1975) *J. Biol. Chem.* **250**, 7728–7738
- Pande, S. V. & Caramancion, N. (1981) *Anal. Biochem.* **112**, 30–38
- Schulz, H. (1983) *Biochemistry* **22**, 1827–1832
- Senior, A. E. & Sherratt, H. S. A. (1968) *Biochem. J.* **110**, 499–509
- Sherratt, H. S. A. (1969) *Br. Med. Bull.* **25**, 250–255
- Sies, H., Akerboom, T. P. M. & Tager, J. (1977) *Eur. J. Biochem.* **72**, 301–307
- Tischler, M. E., Friedrichs, D., Coll, K. & Williamson, J. R. (1977) *Arch. Biochem. Biophys.* **184**, 222–236
- Toews, C. J., Lowy, C. & Ruderman, N. B. (1970) *J. Biol. Chem.* **245**, 818–824
- Tutwiler, G. F. & Delleveigne, P. (1979) *J. Biol. Chem.* **254**, 2935–2941
- Van Hoof, F., Hue, L. & Sherratt, H. S. A. (1979) *Biochem. Soc. Trans.* **7**, 163–165
- Williamson, J. R. & Corkey, B. E. (1969) *Methods Enzymol.* **13**, 434–513
- Williamson, J. R., Browning, E. T. & Scholz, R. (1969) *J. Biol. Chem.* **244**, 4607–4616
- Williamson, J. R., Rostand, S. G. & Peterson, M. J. (1970) *J. Biol. Chem.* **245**, 3242–3251
- Williamson, J. R., Martin-Requero, A., Corkey, B. E., Brandt, M. & Rothman, R. (1981) In *Metabolism and Clinical Implications of Branched Chain Amino and Ketoacids* (Walser, T. M. & Williamson, J. R., eds.), pp. 105–117, Elsevier/North-Holland, Amsterdam