Inhibition of Gluconeogenesis in Isolated Rat Liver Cells by Methylenecyclopropylpyruvate (Ketohypoglycin)

By Eccleston A. KEAN* and Christopher I. POGSON
Biological Laboratory, University of Kent, Canterbury, Kent CT2 7NJ, U.K.

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1. In isolated rat liver cells, hypoglycin is a less effective inhibitor of gluconeogenesis than its transamination product, methylenecyclopropylpyruvate (ketohypoglycin). 2. Methylenecyclopropylpyruvate at 0.3 mM inhibits gluconeogenesis from all substrates tested, except fructose. 3. Methylenecyclopropylpyruvate does not affect $^{14}\text{CO}_2$ release from $[1-^{14}\text{C}]$ palmitate, but, in the absence of lactate, inhibits ketogenesis and causes a decrease in the $[\beta\text{-hydroxybutyrate}]/[\text{acetoacetate}]$ ratio. These effects are masked when lactate (10 mM) is present. 4. In the presence of lactate and palmitate, 0.3 mM-methylenecyclopropylpyruvate produces a fall in total acid-soluble CoA and a relative increase in short-chain acyl-CoA at the expense of CoA and acetyl-CoA without changing the ATP, ADP and aspartate contents or the $[\text{lactate}]/[\text{pyruvate}]$ ratio. 5. Many of the effects of methylenecyclopropylpyruvate observed are consistent with inhibition of butyryl-CoA dehydrogenase and of specific CoA-dependent enzymes involved in gluconeogenesis.

Hypoglycin (L-2-amino-3-methylenecyclopropylpropionic acid) is the primary toxic component of the unripe arillus of the ackee *Blighia sapida*, the agent responsible for Jamaican vomiting disease. This disease is characterized by hypoglycaemia, increased glycogenolysis, isovaleric acidemia and increased hepatic fat accumulation (Bressler *et al.*, 1969; Sherratt & Osmundsen, 1976; Tanaka *et al.*, 1976). In rats *in vivo*, hypoglycin is transaminated to yield methylenecyclopropylpyruvate (ketohypoglycin). This reaction is probably catalysed by the branched-chain amino acid transaminase (EC 2.6.1.42) whose activity is highest in muscle, and rather low in liver (Felig, 1975; Kean, 1976a; Crabb & Harris, 1978). Methylenecyclopropylpyruvate is subsequently converted to methylenecyclopropylacetyl-CoA, which in turn may be hydrolysed to methylenecyclopropylacetate or conjugated with glycine to yield methylenecyclopropylacetylglycine (Kean & Rainford, 1973a; Sherratt & Osmundsen, 1976; Tanaka, 1976).

The mechanism by which hypoglycin ingestion produces hypoglycaemia remains unsettled. One hypothesis is that fatty acid oxidation is blocked by methylenecyclopropylacetyl-CoA, which inhibits short-chain acyl-CoA dehydrogenase (EC 1.3.99.2; Osmundsen & Sherratt, 1975; Kean, 1976c; Osmundsen *et al.*, 1978). Glucose utilization continues, but, since gluconeogenesis is impaired by hypoglycin, reserves of this fuel are progressively depleted and hypoglycaemia ensues. Inhibition of gluconeogenesis

is thus assigned a key role in the hypoglycaemic effect. Speculation has been directed to possible adverse effects of accumulated butyryl-CoA and decreased acetyl-CoA concentrations on the activity of pyruvate carboxylase (EC 6.4.1.1; Sherratt & Osmundsen 1976); it has been shown that both butyryl-CoA and isovaleryl-CoA may indeed compete with acetyl-CoA for the activation of pig liver pyruvate carboxylase (Billington *et al.*, 1978). An alternative hypothesis (Bressler *et al.*, 1969; Corredor, 1976), which has been strongly criticized (Sherratt & Osmundsen, 1976), is that the effects are more directly attributable to sequestration of CoA and carnitine by hypoglycin derivatives, the rates of several metabolic processes being consequently slowed.

Patrick (1962) and Billington (1976) have shown that hypoglycin is only effective in inhibiting gluconeogenesis in liver and kidney at concentrations considerably above those minimally effective *in vivo*. Kean & Rainford (1973b), however, found that methylenecyclopropylpyruvate was much more effective as an inhibitor in rat kidney slices.

In the present paper we report that methylenecyclopropylpyruvate is a potent inhibitor of gluconeogenesis and ketogenesis in isolated rat hepatocytes, and discuss the possible mechanisms involved.

Materials and Methods

Animals

All rats (males, 180–220 g) were of the Sprague-Dawley CSE/ASH strain (Charles River U.K. Ltd.,

* Present address: Department of Biochemistry, University of the West Indies, Mona, Kingston, Jamaica.

Manston, Kent, U.K.) and were inbred in this laboratory. They were starved for 48 h before use.

Chemicals and enzymes

Enzymes and biochemicals were from Boehringer Corp. (London), Lewes, Sussex, U.K., with the following exceptions. Glucose oxidase (type II) and sodium palmitate were from Sigma (London) Chemical Co., Poole, Dorset, U.K., and peroxidase (RZ 0.6) was from International Enzymes Ltd., Windsor, Berks., U.K. 2-Oxoglutarate dehydrogenase (EC 1.2.4.2) and active dephosphorylated pig heart pyruvate dehydrogenase (EC 1.2.4.1) were gifts from Dr. R. M. Denton, University of Bristol; L-carnitine was a gift from Dr. G. D. Baird, National Institute for Research into Animal Diseases, Compton, Berks., U.K. Phosphoenolpyruvate was synthesized as described by Clark & Kirby (1966).

[1-¹⁴C]Palmitate was obtained from The Radiochemical Centre, Amersham, Bucks., U.K., and Norit GSX from Norit Clydesdale Co., Glasgow, Scotland, U.K. Bovine serum albumin (fraction V; Miles Laboratories, Slough, Bucks., U.K.) was freed of fatty acids and other ligands by the method of Chen (1967).

Hypoglycin, prepared from its γ -glutamyl peptide isolated from ackee seeds (Fowden, 1976), was converted to sodium methylenecyclopropylpyruvate as previously described (Kean, 1976c). From oxo acid determination (Coleman & Armstrong, 1971), the sample was assessed as 94% pure; the free acid had an equivalent weight of 141, in agreement with theory.

All other chemicals, of the purest grade available, were obtained from standard suppliers.

Incubations

Routinely, isolated rat liver cells (Elliott *et al.*, 1976) were incubated (5–6 mg dry wt. per vial), with shaking (100 cycles/min), at 37°C in stoppered siliconized glass scintillation vials containing a final volume of 2 ml of buffer (Krebs & Henseleit, 1932), supplemented with 2% (w/v) fat-free bovine serum albumin and gassed with O₂/CO₂ (19:1). Substrates were added by injection after preincubation for 40 min. Hypoglycin and methylenecyclopropylpyruvate were added 30 min after the substrate. Rates of gluconeogenesis were calculated from the amount of glucose synthesized between 30 and 90 min after addition of all substrates, except fructose, where the period was 30–60 min. Reactions were stopped by addition of 0.2 ml of 2M-HClO₄.

In experiments with [1-¹⁴C]palmitate, the substrate was bound to albumin (Garland & Randle, 1964b) and was present throughout the incubation period. Incubations (2 ml) were in 10 ml conical flasks

(Kontes Glass Co., obtained through Uniscience Ltd., Cambridge, U.K.) with stoppers and suspended plastic centre wells containing pleated filter paper. Reactions were stopped with 0.2 ml of 2M-HClO₄, and 0.25 ml of 2-phenethylamine/methanol (1:1, v/v) was injected into each centre well. After a further 60 min shaking at room temperature, wells were removed and added directly to vials containing 5 ml of scintillation fluid [0.6% (w/v) 5-(biphenyl-4-yl)-2-(4-t-butylphenyl)-1-oxa-3,4-diazole, 8% (w/v) naphthalene, 40% (w/v) 2-methoxyethanol and 60% (w/v) toluene] for counting of radioactivity.

Glucose was measured in the acid extracts (Krebs *et al.*, 1963). Ketone bodies were determined enzymically (Mellanby & Williamson, 1974; Williamson & Mellanby, 1974) after neutralization with 0.5M-triethanolamine hydrochloride, pH 7.4, containing 2M-KOH.

Intracellular metabolites and medium lactate and pyruvate concentrations were determined in incubations of 4 ml of cells plus medium (15–20 mg dry wt. of cells) in 25 ml conical flasks. At 90 min after addition of substrate, cells were separated from medium in small Hems' tubes (Hems *et al.*, 1975). Samples of the supernatants were deproteinized and then neutralized as described above. Lactate (Gutmann & Wahlefeld, 1974) and pyruvate (Czok & Lamprecht, 1974) were assayed in these fractions. The cell pellets were resuspended and sonicated. After centrifugation, the supernatants were neutralized as above and 2-mercaptoethanol was added to a final concentration of 1 mM.

CoA was assayed fluorimetrically, in a final volume of 0.4 ml, with 2-oxoglutarate dehydrogenase (Garland, 1974). The sum of acetyl-CoA and CoA was obtained from assays containing, in addition, 0.25 mM-oxaloacetate and 5 μ g (0.55 unit) of citrate synthase. The sum of all short-chain acyl-CoA esters plus CoA was obtained when 0.125 mM-L-carnitine and 5 μ g (0.4 unit) of carnitine acetyltransferase were present. Results with this assay were similar to those obtained by measurement of CoA after alkaline hydrolysis. ATP (Jaworek *et al.*, 1974a), ADP (Jaworek *et al.*, 1974b) and aspartate (Lowry & Passonneau, 1972) were measured spectrophotometrically in neutralized extracts.

Results

In preliminary experiments with lactate as substrate, hypoglycin only produced appreciable inhibition of gluconeogenesis in the test system when present at concentrations above 1 mM (70% at 5 mM), and non-linear rates of glucose production were observed (Fig. 1). In contrast, methylenecyclopropylpyruvate, the product of transamination of hypoglycin, was much more effective at lower concentrations (90% inhibition at 1 mM) and was associated with linear

rates of inhibited glucose production. The relative ineffectiveness of hypoglycin in this system probably results from the low transaminase activity in liver (Kean, 1976a). We have presumed that the inhibition of gluconeogenesis *in vivo* by hypoglycin is attributable to slow formation of methylenecyclopropylpyruvate and the subsequent accumulation of toxic products, and have therefore used methylenecyclo-

propylpyruvate in short-term incubations in all succeeding experiments.

Gluconeogenesis from all substrates tested, except fructose, was inhibited by 0.3 mM-methylenecyclopropylpyruvate (Table 1). However, the degree of inhibition varied from one substrate to another. In particular, lactate and alanine were clearly classifiable together as the most sensitive. Pyruvate fell into an intermediate group, together with propionate, serine and proline, for which inhibition, expressed on a percentage basis, was about one-half that of the most sensitive group. This difference between sensitivity of pyruvate on the one hand, and lactate and alanine on the other, is unusual; the explanation may involve the detailed mechanism by which each of these substrates generates a four-carbon intermediate of gluconeogenesis.

Glycerol and glutamine ranked at the lower end of the scale, where inhibition of gluconeogenesis was relatively slight. Nevertheless, the sensitivity of glucose formation from glycerol may signify a mode of action for this inhibitor different from that for other agents whose target is phosphoenolpyruvate carboxykinase (EC 4.1.1.32; Blackshear *et al.*, 1975; Smith *et al.*, 1978).

Carnitine has been reported both to decrease hypoglycin-induced hypoglycaemia (Entman & Bresler, 1967) and to be without effect (Marley & Sherratt, 1973). Addition of 5 mM-L-carnitine to incubations neither increased the rate of gluconeogenesis nor affected the degree of inhibition by methylenecyclopropylpyruvate. Palmitate (1 mM) stimulated glucose production from lactate; the extent of inhibition by methylenecyclopropylpyruvate was, however, similar to that in the absence of fatty acid (Table 1). Similarly, acetate (10 mM) did not alter the effectiveness of methylenecyclopropylpyruvate (results not shown).

In the presence of 1 mM-palmitate, methylenecyclopropylpyruvate produced a 30% decrease in total

Fig. 1. Time course of inhibition of gluconeogenesis by hypoglycin A

Cells were preincubated for 40 min with or without inhibitors. Lactate (final 10 mM) was then added to all except control incubations. Each point is the mean of triplicate determinations. Other details were as described in the Materials and Methods section. ▲, Control; ●, lactate alone; ■, plus 5 mM-hypoglycin A; ○, plus 1 mM-methylenecyclopropylpyruvate.

Table 1. Effect of methylenecyclopropylpyruvate (0.3 mM) on gluconeogenesis from various precursors in isolated hepatocytes. Cells from 48 h-starved rats were incubated as described in the Materials and Methods section. Rates are uncorrected for control values and are means $\pm$ S.E.M. for the numbers of cell preparations given in parentheses. Inhibition is significant ($P < 0.005$; paired *t* test; Steel & Torrie, 1960) in all cases except that of fructose (not significant).

Substrate (10mM)	Rate of gluconeogenesis (nmol/h per mg dry wt.)		Percentage inhibition
	Substrate alone	+ Methylenecyclopropylpyruvate	
None (5)	14 $\pm$ 2	9 $\pm$ 2	41 $\pm$ 4
Lactate (13)	211 $\pm$ 13	87 $\pm$ 7	59 $\pm$ 3
Pyruvate (4)	183 $\pm$ 11	131 $\pm$ 12	29 $\pm$ 2
Alanine (4)	72 $\pm$ 6	26 $\pm$ 6	64 $\pm$ 8
Propionate (5)	44 $\pm$ 6	32 $\pm$ 6	29 $\pm$ 5
Glutamine (3)	110 $\pm$ 4	90 $\pm$ 2	18 $\pm$ 1
Serine (4)	58 $\pm$ 15	41 $\pm$ 11	31 $\pm$ 1
Proline (4)	46 $\pm$ 11	32 $\pm$ 7	30 $\pm$ 5
Glycerol (7)	218 $\pm$ 33	166 $\pm$ 22	22 $\pm$ 4
Fructose (3)	552 $\pm$ 36	482 $\pm$ 63	14 $\pm$ 6
Lactate + 1 mM-palmitate (5)	335 $\pm$ 39	177 $\pm$ 37	48 $\pm$ 7

Table 2. *Effect of methylenecyclopropylpyruvate on the disposition of [1-¹⁴C]palmitate by isolated rat liver cells*
Cells from 48h-starved rats were incubated with 1 mM-[1-¹⁴C]palmitate as described in the Materials and Methods section. All values represent the amounts formed between 70 and 130 min of incubation. Where relevant, lactate (10 mM) and methylenecyclopropylpyruvate (0.3 mM) were added at 40 and 70 min respectively. Results are means $\pm$ s.e.m. for three independent experiments. Significance between means was tested with the paired *t* test (Steel & Torrie, 1960); NS, not significant.

Additions	Production (nmol/mg dry wt.)				Ratio [hydroxybutyrate] [acetoacetate]
	¹⁴ CO ₂	Acetoacetate	β -Hydroxybutyrate	Total ketone bodies	
I None	6.7 $\pm$ 0.1	126 $\pm$ 16	167 $\pm$ 37	298 $\pm$ 37	2.06 $\pm$ 0.28
II Lactate	20.6 $\pm$ 1.0	123 $\pm$ 30	-33 $\pm$ 51	90 $\pm$ 63	1.30 $\pm$ 0.27
<i>P</i> (versus I)	<0.005	NS	<0.005	<0.01	<0.01
III Methylenecyclopropylpyruvate	8.7 $\pm$ 0.7	144 $\pm$ 13	56 $\pm$ 53	203 $\pm$ 54	1.47 $\pm$ 0.21
<i>P</i> (versus I)	NS	NS	<0.05	<0.05	<0.05
IV Lactate+methylenecyclopropylpyruvate	18.3 $\pm$ 1.2	113 $\pm$ 17	-21 $\pm$ 42	92 $\pm$ 53	1.39 $\pm$ 0.30
<i>P</i> (versus II)	NS	NS	NS	NS	NS
<i>P</i> (versus III)	<0.05	NS	NS	<0.05	NS

ketone-body output. This decrease was accompanied by a decrease in the [β -hydroxybutyrate]/[acetoacetate] ratio, as a result of a marked fall in, or even a reversal of, β -hydroxybutyrate output (Table 2). These results are consistent with the lowered [β -hydroxybutyrate]/[acetoacetate] ratio seen *in vivo* in starved rats treated with the related compound pent-4-enoate (Senior & Sherratt, 1968), and support the suggestion by Williamson & Wilson (1965) that, in such circumstances, ketosis results from decreased peripheral oxidation rather than increased hepatic production of ketone bodies.

Ketone-body output from palmitate was significantly decreased, as was the ratio of [β -hydroxybutyrate] to [acetoacetate], in the presence of 10 mM-lactate [the well-known anti-ketogenic effect of lactate, confirmed by Exton *et al.* (1972)]. Methylenecyclopropylpyruvate caused no further changes in these parameters (Table 2).

The large variation in the values for ketone-body output between 70 and 130 min may be attributed partly to the small size of the changes occurring, but also to the non-linearity of the effects of both lactate and methylenecyclopropylpyruvate. The time course of ketone-body output is shown in an experiment (Fig. 2) where inhibition was particularly marked.

The data for release of ¹⁴CO₂ from [1-¹⁴C]palmitate (Table 2) indicate that the inhibitor had no effect on processes that generate acetyl units from the fatty acid and oxidize them in the tricarboxylic acid cycle. Measurement of ¹⁴CO₂ as an index of the overall rate of fatty acid oxidation has been criticized (Sherratt & Osmundsen, 1976) on the grounds that ¹⁴CO₂ is by no means the only labelled end product during short-term metabolism of carboxy-labelled fatty acid by liver preparations; a significant

Fig. 2. *Effect of methylenecyclopropylpyruvate on ketogenesis from 1 mM-palmitate*

Where appropriate, lactate (final concn. 10 mM) was added after 40 min, and methylenecyclopropylpyruvate (final concn. 0.3 mM) after 70 min of incubation. Each point is the mean of triplicate determinations in one experiment, which is typical of three. Other details were as described in the Materials and Methods section. (A) Total ketone bodies; (B) β -hydroxybutyrate; (C) acetoacetate. $\circ$, Palmitate alone; $\bullet$, plus lactate; Δ , plus methylenecyclopropylpyruvate.

proportion of the label appears in acid-insoluble products (triacylglycerols and other lipids), although this is appreciably lower in cells from starved animals (Christiansen *et al.*, 1976). However, the present data are at least consistent with an unaltered rate of palmitate transit through the first turn of the β -oxidation spiral, and oxidation of acetyl units in the tricarboxylic acid cycle.

Results shown in Table 3 are from measurements of intracellular metabolites in cells incubated with lactate, both with and without palmitate. There was a substantial effect of the inhibitor upon the distribution of intracellular CoA between free CoA and its various combined forms. A marked decrease in free CoA and acetyl-CoA, and an increase in short-chain acyl-CoA was evident. The increase in the latter species did not fully compensate for the fall in free CoA and acetyl-CoA concentrations, since total acid-soluble CoA decreased. The short-fall is most likely accounted for by a concurrent increase in acid-insoluble (i.e. long-chain acyl) CoA species, similar to the findings of Williamson *et al.* (1970b) with the related inhibitor pent-4-enoate. The accumulation of CoA esters represents a balance between the rates of their synthesis and deacylation within the mitochondrion. Such deacylation, leading to production of free butyrate and allowing recycling of CoA, has been seen both *in vivo* with hypoglycin A and *in vitro* with methylenecyclopropylacetate (Sherratt & Osmundsen, 1976).

The components contributing to the increase in the short-chain acyl-CoA pool have not been investigated. It can be assumed that methylenecyclopropylpyruvate was metabolized to methylenecyclopropylacetyl-CoA, which then specifically inhibited butyryl-CoA dehydrogenase. Therefore, it is likely the CoA esters of both methylenecyclopropylacetic acid and butyric acid would accumulate under these conditions.

No change was detected in aspartate concentrations (Table 3), which probably indicates that oxaloacetate concentrations were similarly unaffected. Table 3 also shows that no changes occurred in the concentrations of ATP and ADP, or of lactate and pyruvate.

Discussion

The evidence suggests the following interpretation. The generation of methylenecyclopropylacetyl-CoA from methylenecyclopropylpyruvate specifically inhibits butyryl-CoA dehydrogenase (Kean, 1976c; Billington *et al.*, 1978) in the mitochondrial compartment. This primary action triggers secondary effects. Of those observed in these experiments, one or more may be responsible for the decrease in gluconeogenesis observed with the several substrates.

Table 3. Effect of methylenecyclopropylpyruvate on metabolite concentrations in isolated rat liver cells

Conditions of incubations and assays were as described in the Materials and Methods section. All incubations contained lactate (10 mM) with additions as stated. Lactate and pyruvate were assayed in the medium, other metabolites in cells. Results are means $\pm$ s.e.m. for three independent observations; significance between means was tested with the paired *t* test (Steel & Torrie, 1960). *P* (for II versus III and IV versus III): * < 0.05; ** < 0.01; *** < 0.001; other differences are not significant.

	Additions (mM)	Cell content (nmol/mg dry wt.)										Concentration (mM)		[Lactate] [pyruvate]
		CoA	Acetyl-CoA	Short-chain acyl-CoA	Acid-soluble CoA	Aspartate	ATP	ADP	[ADP] [ATP]	Lactate	Pyruvate			
I	None	0.50 $\pm$ 0.09	0.11 $\pm$ 0.01	0.02 $\pm$ 0.01	0.63 $\pm$ 0.08	1.42 $\pm$ 0.14	8.01 $\pm$ 0.46	3.46 $\pm$ 0.25	2.32 $\pm$ 0.09	4.72 $\pm$ 0.31	0.65 $\pm$ 0.04	7.3 $\pm$ 0.02		
II	Methylenecyclopropylpyruvate (0.3)	0.16 $\pm$ 0.03*	0.05 $\pm$ 0.02	0.24 $\pm$ 0.02***	0.44 $\pm$ 0.05*	1.45 $\pm$ 0.30	8.41 $\pm$ 0.58	3.86 $\pm$ 0.05	2.18 $\pm$ 0.13	5.24 $\pm$ 0.32	0.87 $\pm$ 0.03	6.0 $\pm$ 0.04		
III	Palmitate (1.0)	0.47 $\pm$ 0.07	0.17 $\pm$ 0.01**	0.03 $\pm$ 0.01	0.67 $\pm$ 0.08	1.32 $\pm$ 0.19	8.03 $\pm$ 0.72	3.49 $\pm$ 0.08	2.29 $\pm$ 0.16	4.14 $\pm$ 0.52	0.62 $\pm$ 0.07	6.7 $\pm$ 0.2		
IV	Palmitate (1.0) + methylenecyclopropylpyruvate (0.3)	0.16 $\pm$ 0.03*	0.03 $\pm$ 0.02***	0.23 $\pm$ 0.05*	0.43 $\pm$ 0.06**	0.97 $\pm$ 0.21	6.90 $\pm$ 1.18	3.49 $\pm$ 0.07	1.98 $\pm$ 0.32	4.94 $\pm$ 0.75	0.71 $\pm$ 0.06	7.2 $\pm$ 1.7		

In mitochondria, the ratio $[NADH]/[NAD^+]$ decreased, as indicated from data on the ratio of $[\beta\text{-hydroxybutyrate}]$ to $[\text{acetoacetate}]$ (Williamson *et al.*, 1967) after incubations that included 1 mm-palmitate. It is not clear whether this shift of redox state is an outcome of the powerful inhibition of β -oxidation exerted at the level of four- and six-carbon chain length (Osmundsen & Sherratt, 1975; Kean, 1976c). The lowering by methylenecyclopropylpyruvate of ketone-body formation from 1 mm-palmitate (32% in the absence of lactate; III versus I in Table 2) was not seen in the presence of lactate (IV versus II in Table 2). Nevertheless, in the latter case, gluconeogenesis was inhibited by 48% (last line, Table 1). In neither case was there a decrease in the oxidation rate of the first acetyl unit cleaved from the carboxy end of palmitate. These observations suggest that intramitochondrial NAD^+ reduction was not primarily restricted by a decreased rate of β -oxidation caused by the inhibitor, but more likely by the decreased extent of this process. (This does not imply that hypoglycin fails to inhibit β -oxidation *in vivo*, since the lactate concentration used here was considerably higher than those occurring physiologically.)

However caused, the shift of nicotinamide nucleotide to a more highly oxidized state in mitochondria implies a decrease in the concentration gradient of malate between mitochondrial matrix and cytosol; as a result, the efflux of malate to the cytosol, a mechanism important for gluconeogenesis from pyruvate and several other substrates (Lardy *et al.*, 1965; Williamson *et al.*, 1970a), would be retarded. On the other hand, as Rognstad & Katz (1970) point out, gluconeogenesis from lactate involves mitochondrial transamination of oxaloacetate to aspartate and translocation of that amino acid rather than malate to the cytosol. The same is true for gluconeogenesis from alanine. Therefore the observed shift of the $[NADH]/[NAD^+]$ ratio is most probably unrelated to the inhibition of gluconeogenesis from lactate and alanine.

The changes in disposition of intracellular CoA presumably resulted from inhibition of butyryl-CoA dehydrogenase and were likely to be of importance in determining utilization of several substrates for gluconeogenesis. Substantial accumulation of short-chain acyl-CoA was found, despite the limitation imposed by intramitochondrial deacylase activity (Osmundsen & Sherratt, 1975; Sherratt & Osmundsen, 1976). These changes could affect both pyruvate carboxylase and pyruvate dehydrogenase. Both enzymes are sensitive to the $[\text{acetyl-CoA}]/[\text{CoA}]$ ratio (Garland & Randle, 1964a; Barritt *et al.*, 1976; Roche & Cale, 1977; Siess *et al.*, 1978), and the former at least will be further inhibited by a rise in $[\text{short-chain acyl-CoA}]/[\text{acetyl-CoA}]$ ratio (Barritt *et al.*, 1976). Gluconeogenesis from lactate, pyruvate,

alanine and serine would thus be inhibited directly at the level of pyruvate carboxylase. With 10 mm-pyruvate supplied as substrate, pyruvate dehydrogenase may be activated by a direct action of pyruvate on pyruvate dehydrogenase kinase (Kerbey *et al.*, 1976). The resultant increase in acetyl-CoA would then partially overcome the inhibition of pyruvate carboxylase, as suggested by the relevant data in Table 1.

An alternative explanation, that the inhibitor may block pyruvate uptake into mitochondria, as shown to occur with other pyruvate analogues (Land & Clark, 1974), is less likely, since methylenecyclopropylacetate, which has otherwise similar effects, does not affect pyruvate oxidation in isolated mitochondria (Billington *et al.*, 1978).

The structural similarity of methylenecyclopropylpyruvate to pyruvate suggested that pyruvate dehydrogenase might either catalyse the formation of methylenecyclopropylacetyl-CoA or be otherwise susceptible to the inhibitor. Experiments with highly purified active (dephosphorylated) pig heart pyruvate dehydrogenase revealed, however, that neither of these possibilities occurs. Furthermore, there was no evidence for competition between pyruvate and methylenecyclopropylpyruvate (C. I. Pogson, unpublished experiments). It thus remains most likely that the oxidative decarboxylation of methylenecyclopropylpyruvate involves the branched-chain oxo acid dehydrogenase (EC 1.2.4.3).

We are unable to account fully for the varied degrees of inhibition observed with the different substrates. This would require fairly precise information, not presently available, on the response of the rate-limiting step in each separate case. Furthermore, although we propose the pyruvate carboxylase step as a major locus of inhibition, other reactions could be sensitive. This is well illustrated by results with glycerol, glutamine, proline and propionate for which the gluconeogenic pathway does not involve pyruvate carboxylase. Inhibition was minimal with glutamine and to a lesser extent with glycerol. However, there is a requirement for participation of CoA in gluconeogenesis from glutamine, proline and propionate; decreased availability of the cofactor would be expected to affect flux through the pathway to glucose in a manner quantitatively different in each case, as was observed.

The inhibition of gluconeogenesis from glycerol, which has been found in previous work (Kean, 1976b), has been confirmed with rat liver cells. Earlier experiments on the effect of methylenecyclopropylpyruvate in slices of rat kidney cortex gave results consistent with an inhibition at fructose 1,6-bisphosphatase (EC 3.1.3.11; Kean & Rainford, 1973b). Further experiments are needed to confirm this possibility and to evaluate the importance of altered CoA distribution when glycerol is the pre-

cursor [glucose synthesis from glycerol requires continued mitochondrial ATP production and NADH oxidation (Siess & Wieland, 1976), and therefore the participation of CoA].

The absence of change in adenine nucleotide content and [ATP]/[ADP] ratio in cells incubated with the inhibitor argues against the hypothesis that ATP may be a limiting factor in the inhibition (von Holt *et al.*, 1966). Although changes in distribution of adenine nucleotides across the mitochondrial membrane are possible without there being overall change in tissue content, we have found, using the technique of Zuurendonk & Tager (1974), (results not shown) that this does not apply when lactate is the substrate. The constancy of the [lactate]/[pyruvate] ratios similarly does not support the suggestion (Senior *et al.*, 1976) that cytoplasmic NADH may be limiting.

We conclude that the effects of methylenecyclopropylpyruvate on gluconeogenesis are largely attributable to changes in acyl-CoA/CoA distribution with its consequent enzymic effects (Billington *et al.*, 1978), although the limitation of the concentration of CoA as a factor cannot be excluded for all substrates.

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