The Effects of Inhibition of Gluconeogenesis on Ketogenesis in Starved and Diabetic Rats

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1. Experiments were performed in which the effects of inhibiting gluconeogenesis on ketone-body formation were examined *in vivo* in starved and severely streptozotocin-diabetic rats. 2. The infusion of 3-mercaptopycolinate, an inhibitor of gluconeogenesis (DiTullio *et al.*, 1974), caused decreases in blood [glucose] and increases in blood [lactate] and [pyruvate] in both normal and ketoacidotic rats. 3. Patterns of liver gluconeogenic intermediates after 3-mercaptopycolinate infusion suggested inhibition at the level of phosphoenolpyruvate carboxykinase. 4. This was confirmed by measurement of hepatic oxaloacetate concentrations which were increased 5-fold after 3-mercaptopycolinate administration. 5. The infusion of 3-mercaptopycolinate caused a decrease in total ketone-body concentrations of 30% in the starved rats and 73% in the diabetic animals. 6. Blood glycerol and hepatic triglyceride concentrations remained unchanged. 7. The decreases in ketone-body concentrations were associated with increases in the calculated hepatic cytosolic and mitochondrial [NADH]/[NAD⁺] ratios. 8. The decrease in ketogenesis seen after inhibition of gluconeogenesis may have resulted from an inhibition of hepatic fatty acid oxidation by the more reduced mitochondrial redox state. 9. It was concluded that gluconeogenesis may stimulate ketogenesis by as much as 30% in severe diabetic ketoacidosis.

It has been proposed that the primary determinant of hepatic ketone-body formation is the rate of influx of free fatty acids to the liver (Fritz, 1961; Ontko & Zilversmit, 1966; Start & Newsholme, 1968) and that the exaggerated ketogenesis seen in diabetic ketoacidosis can be attributed to the increase in adipose-tissue lipolysis occurring during insulin deprivation (Ontko & Zilversmit, 1966). This has been questioned by Bieberdorf *et al.* (1970), who showed that the infusion of chylomicrons and heparin into starved rats increased the concentrations of plasma free fatty acids but not those of ketone bodies. In addition, the elevated ketogenesis seen in the perfused livers of diabetic rats is not further increased by raising the concentrations of free fatty acids in the perfusing medium (Krebs *et al.*, 1969; Van Harken *et al.*, 1969).

A variation of this hypothesis maintains that only the free fatty acids that escape re-esterification in the liver can be utilized in β -oxidation and thus lead to ketone-body formation (Fritz, 1961; Mayes & Felts, 1967). The relative amount of glycerol phos-

phate present would thus regulate the ultimate fate of the incoming free fatty acids.

Krebs (1966) has postulated that the greatly increased rate of gluconeogenesis seen in diabetes directs the available oxaloacetate to glucose and decreases its condensation with acetyl-CoA to form citrate. In this way, acetyl-CoA would accumulate at a greater rate and allow for increased ketone-body formation. Wieland *et al.* (1964), on the other hand, have also maintained that the enhanced ketogenesis is due to oxaloacetate deficiency, but from a different cause: its reduction to malate. Wieland (1965) has also reported increased concentrations of acyl-CoA thioesters and acetyl-CoA in livers from alloxan-diabetic rats, and further shown *in vitro* a competitive inhibition of citrate synthase by acyl-CoA thioesters. Thus citrate synthesis would be decreased both by lower oxaloacetate availability and by inhibition of citrate synthase, leading to a build-up of acetyl-CoA and enhanced ketogenesis.

To test these hypotheses, the effect of inhibition of gluconeogenesis on ketone-body formation has been examined. Gluconeogenesis was inhibited at the phosphoenolpyruvate carboxykinase level with quinolinic acid (Veneziale *et al.*, 1967) and 3-

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mercaptopycolinic acid (DiTullio *et al.*, 1974) in two situations associated with increased gluconeogenesis, namely starvation and severe diabetic ketoacidosis.

Experimental

Animals

Male Ash/Wistar rats weighing 185–215 g were used, except in one experiment involving rats weighing 285–300 g. They were allowed free access to water and a standard laboratory rat diet (Diet 41B; Oxoid Ltd., London SE1 9HF, U.K.) at all times except where stated in the text.

Experimental design

Rats were made diabetic by the intravenous injection of 150 mg of streptozotocin/kg and maintained on a long-acting insulin preparation (protamine-zinc insulin) for 7 days. Insulin was then withdrawn and the animals were allowed to develop ketoacidosis. The characteristics of this type of ketoacidosis have been described in detail previously (Blackshear & Alberti, 1974, 1975).

Fed normal rats or diabetic rats approx. 72 h after the last insulin injection were anaesthetized with sodium pentobarbitone (60 mg/kg body wt. intraperitoneally), and polythene cannulae (no. 1619R, Bardic I-Catheter, C.R. Bard International Ltd., Clacton-on-Sea, Essex, U.K.; no. 2FG Intravenous Cannula, Portex Ltd., Hythe, Kent, U.K.) were inserted into the left femoral artery and vein. The rats were then placed in restraining cages and allowed free access to water but not food for the next 24 h. All animals were completely conscious during the infusions.

All infusions were performed at the rate of 1.2 ml/h, and control animals received 0.9% NaCl. Sodium quinolinate (pH 7.4) was infused into both diabetic and normal animals at a concentration of 4.2 g/100 ml (250 mg/h per kg); previous studies had shown that twice this concentration proved lethal to ketoacidotic rats. Sodium 3-mercaptopycolinate (pH 7.4) was infused into diabetic animals at a concentration of 1.67 g/100 ml (100 mg/h per kg). Starved normal animals were infused with a concentration of 0.33 g/100 ml (20 mg/h per kg), higher concentrations being lethal in normal rats, owing to hypoglycaemia.

In a final experiment, rats weighing 285–300 g were starved for 24 h, anaesthetized with sodium pentobarbitone (60 mg/kg intraperitoneally), and 0.5 ml of 0.9% NaCl or 30 mg of 3-mercaptopycolinate in 0.5 ml of 0.9% NaCl, pH 7.4, was injected into a tail vein within 2 min. Livers were removed 30 min later and freeze-clamped, as described below.

Blood and liver sampling

All animals were infused with test solutions for 240 min. During each infusion, arterial blood samples (0.25 ml) were drawn into heparinized syringes at time 0 (in duplicate), 15, 30, 60, 120 and 240 min. A portion of this blood (0.2 ml) was immediately deproteinized in 2.0 ml of ice-cold 3% (v/v) HClO_4 ; these samples were prepared for enzymic analyses as described previously (Schein *et al.*, 1971). After each infusion, the animals were killed by cervical dislocation and the livers were removed within 10 s and freeze-clamped (Wollenberger *et al.*, 1960). The frozen tissue was treated as described previously (Blackshear & Alberti, 1974), by the procedure of Williamson *et al.* (1967b). A portion of the frozen pulverized tissue was shaken with 16 vol. of chloroform-methanol (2:1, v/v) for triglyceride determination.

Assays

Enzymic assays were performed for: glucose (Slein, 1963), acetoacetate and D-3-hydroxybutyrate (Williamson *et al.*, 1962), glycerol (Eggstein & Kreutz, 1966), L-lactate (Hohorst *et al.*, 1959), pyruvate (Bücher *et al.*, 1963), glucose 6-phosphate and ATP (Lamprecht & Trautschold, 1963), ADP and AMP (Adam, 1963), glycerol phosphate (Hohorst, 1963a), phosphoenolpyruvate, D-2-phosphoglycerate and D-3-phosphoglycerate (Czok & Eckert, 1963), citrate (Gruber & Moellering, 1966), 2-oxoglutarate (Bergmeyer & Bernt, 1963), L-malate (Hohorst, 1963b), oxaloacetate (Hohorst & Reim, 1963), L-alanine (Williamson *et al.*, 1967a), L-glutamine (Lund, 1970), L-glutamate (Bernt & Bergmeyer, 1963) and L-aspartate (Pfleiderer, 1963). Pyruvate, 2-oxoglutarate, acetoacetate and oxaloacetate were determined immediately after neutralization of the acid extracts; all other hepatic metabolites were determined within 48 h. Florisil-treated tissue extracts were used for the assay of the 2-oxo acids and glucose (Williamson *et al.*, 1967b). Liver triglyceride was measured by the method of Eggstein & Kreutz (1966).

Results are expressed as means $\pm$ s.e.m.; significant differences were determined by using Student's *t* test for paired or non-paired populations.

Special chemicals

The streptozotocin was a gift from Dr. W. E. Dulin, Upjohn Co., Kalamazoo, Mich., U.S.A. Protamine-zinc insulin (40 i.u./ml) was obtained from Burroughs Wellcome and Co., London HP4 2DY, U.K. 3-Mercaptopycolinic acid was a gift from Dr. Harry L. Saunders, Smith, Kline and French Laboratories, Philadelphia, Pa. 19101, U.S.A.; quinolic acid was from Koch-Light Laboratories,

Colnbrook, Bucks., U.K. Enzymes and coenzymes were supplied by Boehringer Corp. (London) Ltd., London W5 2TZ, U.K., except for glutaminase, which was supplied by Sigma (London) Chemical Co., Kingston-upon-Thames, Surrey, U.K. Florisil was obtained from Koch-Light. Pentobarbitone sodium (Nembutal) was from Abbott Laboratories, Queenborough, Kent, U.K.

Results

Blood metabolites

(a) *Quinolate infusion.* When compared with 3-mercaptopycolinate, quinolate proved relatively ineffective in decreasing blood [glucose] in either starved or ketoacidotic rats (Fig. 1). It also failed to

Fig. 1. Blood [glucose] changes during the infusion of NaCl, quinolate or mercaptopycolinate

Both starved normal (a) and severely ketotic (b) rats were infused with NaCl (●), quinolate (▲) or 3-mercaptopycolinate (■) for 4 h as described in the text. Each point represents the mean $\pm$ S.E.M. Original blood glucose concentrations (mm) were: (a) normal rats: NaCl, 3.99 ± 0.19 ($n = 8$); quinolate, 3.93 ± 0.25 ($n = 7$); 3-mercaptopycolinate, 3.97 ± 0.21 ($n = 6$); (b) ketotic rats: NaCl, 19.6 ± 3.0 ($n = 6$); quinolate, 13.7 ± 2.4 ($n = 5$); 3-mercaptopycolinate, 16.4 ± 1.6 ($n = 8$). * $P < 0.05$, ** $P < 0.01$, when comparing mean values from animals treated with quinolate or 3-mercaptopycolinate with those from NaCl-treated rats, by using Student's t test. For other details, see the text.

cause an increase in blood [lactate] or [pyruvate] in the diabetic animals, supporting the suggestion of Alvares & Ray (1974) that quinolate does not inhibit gluconeogenesis in diabetic rats. For these reasons, the results obtained with quinolate infusions will not be discussed further.

(b) *Mercaptopycolinate infusion.* (1) 24 h-starved normal animals. The infusion of 3-mercaptopycolinate (20 mg/h per kg) resulted in significant decreases in blood [glucose] after 60, 120 and 240 min of infusion: the total decrease was 3 mm at 240 min (Fig. 1a). It also resulted in considerable increases in both arterial blood [lactate] and [pyruvate] of 4 and 0.18 mm respectively at 240 min (Figs. 2a and 2b). The blood [lactate]/[pyruvate] ratio increased almost 3-fold during the course of the 3-mercaptopycolinate infusion (Fig. 2c). These data suggest that 3-mercaptopycolinate inhibited gluconeogenesis in the starved animals.

3-Mercaptopycolinate infusion also resulted in an increase in blood [3-hydroxybutyrate] when compared

Fig. 2. Blood [lactate] (a) and [pyruvate] (b) changes and the [lactate]/[pyruvate] ratio (c) during the infusions of NaCl or 3-mercaptopycolinate in starved normal rats

Symbols and other details are as described in Fig. 1. Original blood values were (a) [lactate] (mm): NaCl, 0.81 ± 0.06 ($n = 8$); 3-mercaptopycolinate, 0.68 ± 0.02 ($n = 6$); (b) [pyruvate] (mm): NaCl, 0.09 ± 0.01 ; 3-mercaptopycolinate, 0.10 ± 0.01 .

Fig. 3. Blood [3-hydroxybutyrate] (a) and [acetoacetate] (b) changes and the ketone-body ratio (c) during the infusion of NaCl or 3-mercaptopicolinate in starved normal rats

Symbols and other details are as described in Fig. 1. Original blood values were: (a) [3-hydroxybutyrate] (mm): NaCl, 1.65 ± 0.23 ($n=8$); 3-mercaptopicolinate, 0.99 ± 0.17 ($n=6$); (b) [acetoacetate] (mm): NaCl, 1.29 ± 0.12 ; 3-mercaptopicolinate, 0.99 ± 0.13 .

with values from the NaCl-infused control animals; this was largely due to a decrease in [3-hydroxybutyrate] of 0.5mm in the control animals by 240min (Fig. 3a). Blood [acetoacetate], however, decreased significantly by 0.5mm after only 15min of infusion (Fig. 3b). By 240min, total blood ketone-body concentrations had decreased by 30%, but this was significantly less than in control animals only for the first 60min. The infusion of 3-mercaptopicolinate resulted in a 3-fold increase in the blood [3-hydroxybutyrate]/[acetoacetate] ratio by 240min (Fig. 3c).

The infusion of 20mg/h of 3-mercaptopicolinate per kg resulted in a slight decrease in blood [glycerol] from 0.28 ± 0.01 mm at 0min to 0.21 ± 0.02 mm at 240min; these values were not significantly different from those obtained in animals infused with NaCl at either 0min (0.29 ± 0.03 mm) or 240min (0.27 ± 0.02 mm).

(2) Severely diabetic animals. The infusion of 3-mercaptopicolinate (100mg/h per kg) caused a

decrease in blood [glucose] of almost 12mm at 240min in the diabetic animals (Fig. 1b). It also caused significant increases in blood [lactate] and [pyruvate] of 6.5 and 0.25mm respectively at 240min (Figs. 4a and 4b). These changes were accompanied by a slight increase in blood [lactate]/[pyruvate] ratio.

The infusion of 3-mercaptopicolinate initially caused a 0.5mm increase in blood [3-hydroxybutyrate] at 15 and 30min; the concentration of this metabolite then decreased rapidly to 4mm less than control values at 240min (Fig. 5a). It also caused a rapid decrease in blood [acetoacetate] of 1mm at 15min, and a decrease of 3mm by 240min (Fig. 5b). Total blood ketone-body concentrations thus decreased by 73% by 240min. There was also a 5–7-fold increase in the blood [3-hydroxybutyrate]/[acetoacetate] ratio. Thus mercaptopicolinate caused a much greater absolute and percentage decrease in total blood ketone-body concentrations in the diabetic rats than in the starved animals.

Fig. 4. Blood [lactate] (a) and [pyruvate] (b) changes and the [lactate]/[pyruvate] ratio (c) during the infusion of NaCl or 3-mercaptopicolinate in severely ketotic rats

Symbols and other details are as described in Fig. 1. Original blood values were: (a) [lactate] (mm): NaCl, 0.71 ± 0.11 ($n=6$); 3-mercaptopicolinate, 0.90 ± 0.15 ($n=8$); (b) [pyruvate] (mm): NaCl, 0.07 ± 0.02 ; 3-mercaptopicolinate, 0.05 ± 0.01 .

Fig. 5. Blood [3-hydroxybutyrate] (a) and [acetoacetate] (b) changes and the ketone-body ratio (c) during the infusion of NaCl or 3-mercaptopicolinate in severely ketotic rats

Symbols and other details are as described in Fig. 1. Original blood values were: (a) [3-hydroxybutyrate] (mM): NaCl, 7.10±0.56 (n=6); 3-mercaptopicolinate, 6.24±0.88 (n=8); (b) [acetoacetate] (mM): NaCl, 3.42±0.44; 3-mercaptopicolinate, 3.40±0.44.

Blood [glycerol] increased from 0.25±0.02 mM at 0 min to 0.44±0.09 mM at 240 min after 3-mercaptopicolinate infusion; however, these values were not significantly different from those obtained in NaCl-infused animals at either 0 min (0.23±0.04 mM) or 240 min (0.23±0.05 mM).

Liver metabolites

(a) 24 h starved normal rats. The concentrations of liver metabolites from normal starved rats infused with NaCl or 3-mercaptopicolinate are shown in Table 1. 3-Mercaptopicolinate caused significant elevations in liver [malate], [2-oxoglutarate], [citrate], [lactate] and [pyruvate]; the [lactate]/[pyruvate] ratio was also increased. Hepatic [phosphoenolpyruvate], [2-phosphoglycerate], [3-phosphoglycerate], [glucose 6-phosphate] and [glucose] were all significantly decreased in the normal animals. The effect of 3-mercaptopicolinate on the pattern of gluconeogenic intermediates is shown graphically in

Fig. 6(a), and indicates a point of inhibition between malate and phosphoenolpyruvate, i.e. at the level of phosphoenolpyruvate carboxykinase. The infusion of 3-mercaptopicolinate caused a decrease in hepatic [ADP], and the mass-action ratio of the adenylate kinase system, [ATP][AMP]/[ADP]², remained relatively unchanged after the infusion, being 0.20 and 0.36 in the animals infused with NaCl or 3-mercaptopicolinate respectively. Of the amino acids measured, 3-mercaptopicolinate caused an increase in hepatic [glutamine], a 4-fold increase in [aspartate], and a 10-fold increase in liver

Table 1. Hepatic metabolite concentrations after the infusion of NaCl or 3-mercaptopicolinate in normal starved rats

Normal 24h-starved rats were infused with NaCl or 3-mercaptopicolinate for 240 min. After each infusion, the animals were killed by cervical dislocation and the livers removed within 10 s and freeze-clamped. Significant differences were determined by using Student's *t* test. **P*<0.05, ***P*<0.01, when comparing results from animals infused with 3-mercaptopicolinate with those from animals receiving NaCl. The number of animals in each group is shown in parentheses. For other details see the text. Values are means±S.E.M.

Type of infusion	Metabolite (μmol/g)	
	0.9% NaCl (9)	3-Mercaptopicolinate (7)
Glucose	5.83 ± 0.42	1.09 ± 0.17**
Glucose 6-phosphate	0.08 ± 0.02	0.02 ± 0.00**
3-Phosphoglycerate	0.23 ± 0.04	0.10 ± 0.01*
2-Phosphoglycerate	0.10 ± 0.01	0.04 ± 0.00**
Phosphoenolpyruvate	0.09 ± 0.02	0.04 ± 0.01*
Pyruvate	0.04 ± 0.00	0.19 ± 0.02**
Lactate	0.76 ± 0.12	4.95 ± 0.30**
Citrate	0.30 ± 0.02	1.27 ± 0.08**
2-Oxoglutarate	0.06 ± 0.01	0.08 ± 0.01*
Malate	0.36 ± 0.06	4.15 ± 0.24**
Glycerol phosphate	0.22 ± 0.01	0.26 ± 0.02
3-Hydroxybutyrate	1.74 ± 0.24	1.49 ± 0.12
Acetoacetate	0.66 ± 0.06	0.32 ± 0.02**
Triglyceride	30.4 ± 4.20	37.9 ± 4.92
ATP	2.07 ± 0.10	2.38 ± 0.19
ADP	2.61 ± 0.13	1.97 ± 0.05**
AMP	0.67 ± 0.04	0.59 ± 0.06
Total adenine nucleotides	5.20 ± 0.16	4.95 ± 0.14
Glutamate	2.15 ± 0.10	1.99 ± 0.06
Glutamine	5.24 ± 0.43	7.54 ± 1.06*
Aspartate	0.85 ± 0.08	3.43 ± 0.04**
Alanine	0.56 ± 0.06	5.94 ± 0.41**
[Lactate]	21.3 ± 2.07	28.5 ± 3.17*
[Pyruvate]		
[3-Hydroxybutyrate]	2.59 ± 0.26	4.77 ± 0.60**
[Acetoacetate]

Fig. 6. Patterns of hepatic gluconeogenic intermediates after 3-mercaptopycolinate infusion in (a) normal starved rats and (b) diabetic rats

Each point represents the percentage of the control value, taken from NaCl-infused animals. The abbreviations used were as follows: Ala, alanine; Lac, lactate; Pyr, pyruvate; Cit, citrate; Mal, malate; Asp, aspartate; Pyr-P, phosphoenolpyruvate; 2P-Gri, 2-phosphoglycerate; 3P-Gri, 3-phosphoglycerate; Glc-6-P, glucose 6-phosphate; Glc, glucose. For other details see the text.

[alanine]. The mass-action ratio of the glutamate-pyruvate transaminase system, $\frac{[\text{alanine}][2\text{-oxoglutarate}]}{[\text{pyruvate}][\text{glutamate}]}$, was increased from 0.39 in the NaCl-infused animals to 1.26 in the 3-mercaptopycolinate-infused animals, indicating a movement toward the equilibrium value *in vitro* of 1.52 (Krebs & Veech, 1970). Williamson *et al.* (1967a) have concluded that the reactants in this system are near equilibrium in rat liver; thus the markedly increased [alanine] probably derives from the increases in [pyruvate] seen after inhibition of gluconeogenesis. The glutamate-oxaloacetate transaminase system also appears to be at equilibrium in rat liver (Williamson *et al.*, 1967a; Parrilla & Goodman, 1974), and increased oxaloacetate concentrations would be expected to result in an increase in [aspartate]; this may be the cause of the striking elevation in hepatic [aspartate] seen after the infusion of 3-mercaptopycolinate.

[Glycerol phosphate] was unchanged in livers from the 3-mercaptopycolinate-infused animals, but [acetoacetate] was significantly decreased and the ketone-body ratio was correspondingly elevated. Mercaptopycolinate must therefore have caused increases in both the mitochondrial and cytosolic $[\text{NADH}]/[\text{NAD}^+]$ ratios in starved rats.

Table 2 shows the hepatic [oxaloacetate] and the components of three redox-state couples 30 min after the intravenous injection of 30 mg of 3-mercaptopycolinate/kg body wt. in anaesthetized starved rats. Differences in control values between these experiments in anaesthetized rats and those in conscious animals probably reflect the different experimental treatment of the animals as well as the effects of pentobarbitone anaesthesia (Dale, 1965). [Oxaloacetate] was elevated 5-fold by this treatment, confirming a site of inhibition of gluconeogenesis at the level of phosphoenolpyruvate carboxykinase. Increases in [lactate], [pyruvate] and [malate], but a slight decrease in total ketone-body concentration, were found. The cytosolic redox state, as reflected in the [lactate]/[pyruvate] ratio, remained constant. However, the [malate]/[oxaloacetate] ratio increased 7-fold, and the [3-hydroxybutyrate]/[acetoacetate] ratio, reflecting the mitochondrial redox state, increased 2.5-fold.

(b) *Severely diabetic rats.* The concentrations of liver metabolites from ketoacidic rats infused

Table 2. Hepatic [oxaloacetate] and redox couples after 3-mercaptopycolinate injection

Rats starved for 24 h were anaesthetized and injected with 0.5 ml of 0.9% NaCl or 30 mg of 3-mercaptopycolinate in 0.5 ml of NaCl as described in the text. Exactly 30 min later the livers were removed and freeze-clamped; the values presented below are the means $\pm$ S.E.M. * $P < 0.05$, ** $P < 0.01$, when comparing 3-mercaptopycolinate-treated rats with those receiving NaCl, by using Student's *t* test. Three animals were used in each group. For other details, see the text.

Substance injected...	Metabolite ($\mu\text{mol/g}$)	
	NaCl	3-Mercaptopycolinate
Malate	0.12 $\pm$ 0.02	4.36 $\pm$ 0.22**
Oxaloacetate	0.012 $\pm$ 0.001	0.061 $\pm$ 0.005**
Lactate	0.32 $\pm$ 0.01	0.95 $\pm$ 0.25*
Pyruvate	0.056 $\pm$ 0.006	0.161 $\pm$ 0.021
3-Hydroxybutyrate	1.68 $\pm$ 0.40	1.14 $\pm$ 0.36
Acetoacetate	1.09 $\pm$ 0.19	0.29 $\pm$ 0.05*
Total ketone bodies	2.77 $\pm$ 0.59	1.43 $\pm$ 0.42
[Malate]		
[Oxaloacetate]	10.9 $\pm$ 1.6	72.3 $\pm$ 7.9**
[Lactate]		
[Pyruvate]	5.7 $\pm$ 0.8	5.7 $\pm$ 0.7
[3-Hydroxybutyrate]		
[Acetoacetate]	1.50 $\pm$ 0.13	3.73 $\pm$ 0.50**

Table 3. *Hepatic metabolite concentrations after the infusion of NaCl or 3-mercaptopycolinate in severely ketoacidotic rats*

Severely ketoacidotic rats were infused with NaCl or 3-mercaptopycolinate for 240 min. Other details are as in Table 2.

Type of infusion ...	Metabolite ($\mu\text{mol/g}$)	
	0.9% NaCl (8)	3-Mercaptopycolinate (6)
Glucose	17.0 ± 0.86	2.57 ± 0.39
Glucose 6-phosphate	0.04 ± 0.01	0.03 ± 0.01
3-Phosphoglycerate	0.22 ± 0.03	$0.12 \pm 0.02^*$
2-Phosphoglycerate	0.05 ± 0.01	0.04 ± 0.01
Phosphoenolpyruvate	0.10 ± 0.02	$0.03 \pm 0.01^{**}$
Pyruvate	0.09 ± 0.01	$0.20 \pm 0.02^{**}$
Lactate	1.03 ± 0.07	$9.82 \pm 1.53^{**}$
Citrate	0.31 ± 0.05	$1.37 \pm 0.50^*$
2-Oxoglutarate	0.05 ± 0.01	$0.09 \pm 0.02^*$
Malate	0.39 ± 0.07	$3.54 \pm 0.55^{**}$
Glycerol phosphate	0.52 ± 0.06	$0.20 \pm 0.04^{**}$
3-Hydroxybutyrate	8.67 ± 1.07	$1.97 \pm 0.28^{**}$
Acetoacetate	4.38 ± 0.50	$0.21 \pm 0.02^{**}$
Triglyceride	63.2 ± 10.6	60.7 ± 11.9
ATP	1.91 ± 0.22	1.88 ± 0.25
ADP	1.62 ± 0.07	1.23 ± 0.22
AMP	0.35 ± 0.04	0.38 ± 0.10
Total adenine nucleotides	3.88 ± 0.25	3.49 ± 0.30
Glutamate	1.69 ± 0.24	$2.75 \pm 0.26^{**}$
Glutamine	4.56 ± 0.45	$6.04 \pm 0.31^*$
Aspartate	0.47 ± 0.08	$3.20 \pm 0.27^{**}$
Alanine	0.34 ± 0.07	$6.87 \pm 0.90^{**}$
[Lactate]		
[Pyruvate]	13.7 ± 2.40	$61.6 \pm 18.5^{**}$
[3-Hydroxybutyrate]		
[Acetoacetate]	2.08 ± 0.23	$10.4 \pm 1.82^{**}$

with NaCl or 3-mercaptopycolinate are listed in Table 3. 3-Mercaptopycolinate infusion resulted in a 9-fold elevation in hepatic [lactate], and [pyruvate] was increased 2-fold with a more than 4-fold increase in hepatic [lactate]/[pyruvate] ratio. As in the starved animals, the hepatic [phosphoenolpyruvate], [2-phosphoglycerate] and [3-phosphoglycerate] were decreased after 3-mercaptopycolinate infusion, and liver [glucose] was decreased almost 5-fold. [Citrate], [2-oxoglutarate] and [malate] were significantly elevated in livers from the 3-mercaptopycolinate-infused animals. The pattern of gluconeogenic intermediates seen after the infusion of this compound in the severely diabetic animals is shown in Fig. 6(b).

The concentrations of all three adenine nucleotides were relatively unchanged in livers from diabetic animals infused with 3-mercaptopycolinate, as was the mass-action ratio of the adenylate kinase system, being 0.25 and 0.47 in the animals treated with NaCl

and 3-mercaptopycolinate respectively. The concentrations of all four amino acids were elevated after this treatment, with [alanine] increased more than 20-fold over control values. The mass-action ratio of the alanine aminotransferase system was 0.11 in the NaCl-infused rats and increased to 1.12 in rats receiving 3-mercaptopycolinate.

Hepatic [glycerol phosphate], [3-hydroxybutyrate] and [acetoacetate] were all decreased after 3-mercaptopycolinate infusion, but liver [triglyceride] remained unchanged. The [3-hydroxybutyrate]/[acetoacetate] ratio was increased more than 5-fold. As in the starved normal animals, there was an increase in the [NADH]/[NAD⁺] ratio in both the cytosol and the mitochondrion, but the magnitude of these changes was much greater in the diabetic rats.

Discussion

Although gluconeogenesis is supposedly inhibited at the phosphoenolpyruvate carboxykinase level by L-tryptophan (Foster *et al.*, 1966; Ray *et al.*, 1966) and one of its metabolites, quinolinic acid (Veneziale *et al.*, 1967), the present studies indicate that quinolinate is much less effective than 3-mercaptopycolinate in decreasing blood [glucose] both in starved and severely diabetic rats. Indeed, a report by Alvares & Ray (1974) suggests that quinolinate may not be an effective inhibitor of gluconeogenesis in the diabetic state.

In contrast, DiTullio *et al.* (1974) have shown that 3-mercaptopycolinic acid caused hypoglycaemia in both starved and alloxan-diabetic rats. In the perfused liver this compound decreased the rates of gluconeogenesis from lactate, pyruvate and alanine, but not from fructose or dihydroxyacetone; a cross-over plot further indicated inhibition of phosphoenolpyruvate carboxykinase as its site of action (DiTullio *et al.*, 1974). The present study confirms these findings (Fig. 6b) and the elevation in measured hepatic [oxaloacetate] (Table 2) further supports an inhibitory effect on this enzyme. 3-Mercaptopycolinate infusion also caused marked decreases in blood [glucose] and increases in blood [lactate] and [pyruvate] in both diabetic and normal animals (Figs. 1, 2 and 4).

The results presented in this paper indicate that inhibition of gluconeogenesis is accompanied by a decrease in total blood ketone-body concentration in the severely diabetic animals, with only minor changes in the starved animals. In agreement with these results, Bässler & Brinkroff (1971) showed that L-tryptophan caused decreased blood ketone-body concentrations in starved rats with or without the simultaneous infusion of octanoate. Williamson (1974), on the other hand, found only a small decrease in hepatic ketone-body concentrations

after quinolinate administration to starved normal rats. The present results also indicate that the magnitude of the ketone-body decrease after inhibition of gluconeogenesis was much greater in the severely diabetic animals than in the starved rats. Since 3-mercaptopycolinate has been shown to inhibit only hepatic phosphoenolpyruvate carboxykinase to date, it is assumed in this discussion that decreases in blood glucose and ketone-body concentrations *in vivo* reflect the inhibition of hepatic gluconeogenesis and ketogenesis respectively. The possibility that a decrease in ketogenesis could be secondary to a decrease in free fatty acid supply has been discounted as blood [glycerol] tended to rise rather than fall.

The increased hepatic [oxaloacetate] after inhibition of phosphoenolpyruvate carboxykinase (Table 2) suggests that the decrease in ketone-body concentrations may have been caused by increased oxaloacetate condensation with acetyl-CoA. Decreased oxaloacetate availability from enhanced gluconeogenesis might allow for the stimulation of ketogenesis because of the accumulation of acetyl-CoA (Krebs, 1966), and the reverse might occur during a build-up of oxaloacetate. However, treatment of starved rats with asparagine, which should increase the flux of carbon through oxaloacetate, did not cause a significant decrease in liver [ketone bodies] (Williamson, 1974). Similarly, asparagine infusion in severely ketotic rats did not decrease total ketone-body concentrations in either blood or liver, although increased flux through the gluconeogenic pathway was suggested by a 3 mm increase in blood [glucose] (P. J. Blackshear, P. A. H. Holloway & K. G. M. M. Alberti, unpublished work). This failure of asparagine administration to decrease ketone-body concentrations in either starved or severely diabetic rats suggests that the above theory proposed by Krebs (1966) may not be tenable.

A second possible mechanism is suggested by the accumulation of reducing equivalents in both the cytosol and the mitochondrion after 3-mercapto-

picolinate administration, as indicated by the increased hepatic [lactate]/[pyruvate] and [3-hydroxybutyrate]/[acetoacetate] ratios. Treatment of starved rats with 3-mercaptopycolinate caused increases in these ratios of 30 and 200% respectively (Table 1). However, in the diabetic animals (Table 3), the increases in the [lactate]/[pyruvate] and [3-hydroxybutyrate]/[acetoacetate] ratios were appreciably greater, concomitant with the much larger decreases in blood ketone-body concentration. These reducing equivalents are probably derived from hepatic free fatty acid oxidation (Williamson *et al.*, 1971). Ontko (1973) suggests that ethanol-induced increases in the mitochondrial [NADH]/[NAD⁺] ratio could decrease free fatty acid oxidation by inhibiting mitochondrial acyl-CoA dehydrogenase and 3-hydroxyacyl-CoA dehydrogenase. A similar mechanism could be responsible for the inhibition of ketogenesis by 3-mercaptopycolinate. Since reducing equivalents would be unnecessary for reduction of 1,3-diphosphoglycerate after the inhibition of gluconeogenesis at the level of phosphoenolpyruvate carboxykinase, their accumulation in the mitochondrion could in turn inhibit their formation via the oxidation of free fatty acids. Conversely, the increased rates of gluconeogenesis characteristic of diabetes (Exton *et al.*, 1972) could relay this demand for reducing equivalents to the mitochondrion in the form of a decreased [NADH]/[NAD⁺] ratio, thus stimulating fatty acid oxidation and ketone-body formation.

An alternative hypothesis derives from the observation of Garland & Randle (1964) that increased [NADH]/[NAD⁺] ratios can inhibit the pyruvate dehydrogenase complex. In this event, the flux of pyruvate through both pyruvate carboxylase and pyruvate dehydrogenase would decrease after the inhibition of phosphoenolpyruvate carboxykinase, resulting in large increases in hepatic [lactate] and [alanine] in liver (Tables 1 and 3). If this occurred, then acetyl-CoA derived from free fatty acids would be oxidized rather than converted into ketone bodies; this may explain the decrease in blood [ketone

Table 4. Comparisons of inhibition of gluconeogenesis and ketogenesis by 3-mercaptopycolinate and 3,5-dimethylpyrazole with total inhibition caused by functional hepatectomy

Values were taken from maximum rates of decrease of both blood glucose and total ketone-body concentrations. Values from functionally hepatectomized animals were taken from Blackshear *et al.* (1974, 1975). See the text for other details.

Metabolite	Treatment	Rate of decrease (mm/h)		% of maximum rate of decrease (after functional hepatectomy)	
		Normal	Ketotic	Normal	Ketotic
Glucose	3-Mercaptopycolinate	1.2	2.7	33	52
	Functional hepatectomy	3.6	5.2		
Total ketone bodies	3-Mercaptopycolinate	1.1	3.2	15	27
	3,5-Dimethylpyrazole	5.0	6.5	69	56
	Functional hepatectomy	7.2	11.7		

bodies] observed without a massive increase in liver [triglyceride] after 3-mercaptopycolinate administration. Further studies *in vitro* are necessary to test this possible mechanism for the antiketogenic effect of 3-mercaptopycolinate.

It is useful to compare the magnitude of the antiketotic affect of 3-mercaptopycolinate with the decrease in ketogenesis caused by other compounds, e.g. antilipolytic agents. The maximum rates of decrease of the total ketone-body and glucose concentrations have therefore been calculated. In previous studies (Blackshear *et al.*, 1974, 1975), blood metabolite concentrations were measured in 24h-starved and severely diabetic rats after functional hepatectomy. Although these animals were anaesthetized, the results obtained after functional hepatectomy provide a useful estimate of the effects of total inhibition of gluconeogenesis and ketogenesis in the two situations. Table 4 compares the relative inhibitory action of 3-mercaptopycolinate on these processes with the maximal inhibition obtained by functional hepatectomy. 3-Mercaptopycolinate caused 33 and 52% of maximum inhibition of gluconeogenesis in the starved and ketotic animals respectively, but the inhibition of ketogenesis was only 15 and 27% of maximum in the two groups. This inhibition of ketogenesis was less than half that found after the administration of 3,5-dimethylpyrazole, an antilipolytic agent (Bizzi *et al.*, 1966; P. J. Blackshear, unpublished work). Thus the inhibition of ketogenesis caused by 3-mercaptopycolinate is not only half the magnitude of its inhibition of gluconeogenesis, but it also inhibits ketone-body formation much less than an antilipolytic agent. This implies that the flux of free fatty acids to the liver is the main determinant of the rate of ketogenesis in most cases. However, it also demonstrates that ketogenesis could be increased by approx. 15 and 27% in starvation and severe ketoacidosis respectively, by high rates of gluconeogenesis alone.

In summary, the data presented in this paper show that inhibition of gluconeogenesis at the level of phosphoenolpyruvate carboxykinase with 3-mercaptopycolinate resulted in decreased total blood ketone-body concentrations in both starved and severely diabetic ketoacidotic rats. The absolute and proportional decreases were greater in the severely diabetic animals, as was the degree of reduction in both cytosol and mitochondria. 3-Mercaptopycolinate did not cause a decrease in blood [glycerol], suggesting that decreased adipose-tissue lipolysis was not a cause of decreased ketone-body formation. The infusion of asparagine resulted in increases in blood [glucose] and [acetoacetate], suggesting that increased oxaloacetate formation alone is not sufficient to inhibit ketogenesis in diabetic ketoacidosis. These results suggest that inhibition of gluconeogenesis at the phosphoenolpyruvate carboxykinase level may

decrease ketogenesis through the inhibition of free fatty acid oxidation caused by the more reduced mitochondrial redox state. This in turn implies that ketone-body formation could be stimulated as much as 30% by the increased rates of gluconeogenesis seen in severe diabetic ketoacidosis.

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References

- Adam, H. (1963) in *Methods of Enzymatic Analysis* (Bergmeyer, H.-U., ed.), pp. 573-577
- Alvares, F. L. & Ray, P. D. (1974) *J. Biol. Chem.* **249**, 2058-2062
- Bässler, K. H. & Brinkrolf, H. (1971) *Z. Gesamte Exp. Med.* **156**, 52-60
- Bergmeyer, H.-U. & Bernt, E. (1963) in *Methods of Enzymatic Analysis* (Bergmeyer, H.-U., ed.), pp. 324-327, Academic Press, New York
- Bernt, E. & Bergmeyer, H.-U. (1963) in *Methods of Enzymatic Analysis* (Bergmeyer, H.-U., ed.), pp. 384-388, Academic Press, New York
- Bieberdorf, F. A., Chernick, S. S. & Scow, R. O. (1970) *J. Clin. Invest.* **49**, 1685-1693
- Bizzi, A., Tacconi, M. T. & Garattini, S. (1966) *Experientia* **22**, 664
- Blackshear, P. J. & Alberti, K. G. M. M. (1974) *Biochem. J.* **138**, 107-117
- Blackshear, P. J. & Alberti, K. G. M. M. (1975) *Am. J. Physiol.* **228**, in the press
- Blackshear, P. J., Holloway, P. A. H. & Alberti, K. G. M. M. (1974) *Biochem. J.* **142**, 279-286
- Blackshear, P. J., Holloway, P. A. H. & Alberti, K. G. M. M. (1975) *Biochem. J.* **146**, 447-456
- Bücher, T., Czok, R., Lamprecht, W. & Latzko, E. (1963) in *Methods of Enzymatic Analysis* (Bergmeyer, H.-U., ed.), pp. 253-259, Academic Press, New York
- Czok, R. & Eckert, L. (1963) in *Methods of Enzymatic Analysis* (Bergmeyer, H.-U., ed.), pp. 229-233, Academic Press, New York
- Dale, R. A. (1965) *J. Physiol. (London)* **181**, 701-711
- DiTullio, N. W., Berkoff, C. E., Blank, B., Kostos, V., Stack, E. J. & Saunders, H. L. (1974) *Biochem. J.* **138**, 387-394
- Eggstein, M. & Kreutz, F. H. (1966) *Klin. Wochenschr.* **44**, 262-267
- Exton, J. H., Corbin, J. G. & Harper, S. C. (1972) *J. Biol. Chem.* **247**, 4996-5003
- Foster, D. O., Ray, P. D. & Lardy, H. A. (1966) *Biochemistry* **5**, 563-569
- Fritz, I. B. (1961) *Physiol. Rev.* **41**, 52-129
- Garland, P. B. & Randle, P. J. (1964) *Biochem. J.* **91**, 6c-7c
- Gruber, W. & Moellering, H. (1966) *Biochem. Z.* **346**, 85-88

- Hohorst, H. J. (1963a) in *Methods of Enzymatic Analysis* (Bergmeyer, H.-U., ed.), pp. 215–219, Academic Press, New York
- Hohorst, H. J. (1963b) in *Methods of Enzymatic Analysis* (Bergmeyer, H.-U., ed.), pp. 328–332, Academic Press, New York
- Hohorst, H. J. & Reim, M. (1963) in *Methods of Enzymatic Analysis* (Bergmeyer, H.-U., ed.), pp. 335–339, Academic Press, New York
- Hohorst, H. J., Kreutz, F. H. & Bücher, T. (1959) *Biochem. Z.* **332**, 18–46
- Krebs, H. A. (1966) *Advan. Enzyme Regul.* **4**, 339–353
- Krebs, H. A. & Veech, R. L. (1970) in *Pyridine Nucleotide Dependent Dehydrogenases* (Sund, H., ed.), pp. 413–434, Springer-Verlag, Berlin, Heidelberg and New York
- Krebs, H. A., Wallace, P. G., Hems, R. & Freedland, R. A. (1969) *Biochem. J.* **112**, 595–600
- Lamprecht, W. & Trautschold, I. (1963) in *Methods of Enzymatic Analysis* (Bergmeyer, H.-U., ed.), pp. 543–551, Academic Press, New York
- Lund, P. (1970) in *Methoden der Enzymatischen Analyse* (Bergmeyer, H.-U., ed.), pp. 1670–1673, Verlag Chemie, Weinheim/Bergstr.
- Mayes, P. A. & Felts, J. M. (1967) *Nature (London)* **215**, 716–718
- Ontko, J. A. (1973) *J. Lipid Res.* **14**, 78–86
- Ontko, J. A. & Zilversmit, D. B. (1966) *Proc. Soc. Exp. Biol. Med.* **121**, 319–321
- Parrilla, R. & Goodman, M. N. (1974) *Biochem. J.* **138**, 341–348
- Pfleiderer, G. (1963) in *Methods of Enzymatic Analysis* (Bergmeyer, H.-U., ed.), pp. 381–383, Academic Press, New York
- Ray, P. D., Foster, D. O. & Lardy, H. A. (1966) *J. Biol. Chem.* **241**, 3904–3908
- Schein, P. S., Alberti, K. G. M. M. & Williamson, D. H. (1971) *Endocrinology* **89**, 827–834
- Slein, M. W. (1963) in *Methods of Enzymatic Analysis* (Bergmeyer, H.-U., ed.), pp. 117–123, Academic Press, New York
- Start, C. & Newsholme, E. A. (1968) *Biochem. J.* **109**, 37P–38P
- Van Harken, D. R., Dixon, C. W. & Heimberg, M. (1969) *J. Biol. Chem.* **244**, 2278–2285
- Veneziale, C. M., Walter, P., Kneer, N. & Lardy, H. A. (1967) *Biochemistry* **6**, 2129–2138
- Wieland, O. (1965) in *On the Nature and Treatment of Diabetes* (Leibel, B. S. & Wrenshall, G. A., eds.), pp. 533–544, Excerpta Medica Foundation, Amsterdam
- Wieland, O., Weissel, L. & Eger-Neufeldt, I. (1964) *Advan. Enzyme Regul.* **2**, 85–99
- Williamson, D. H. (1974) in *Diabetes* (Malaisse, W. J. & Pirart, J., eds.), pp. 271–281, Excerpta Medica Foundation, Amsterdam
- Williamson, D. H., Mellanby, J. & Krebs, H. A. (1962) *Biochem. J.* **82**, 90–96
- Williamson, D. H., Lund, P. & Krebs, H. A. (1967a) *Biochem. J.* **103**, 514–527
- Williamson, D. H., Lopes-Vierira, O. & Walker, B. (1967b) *Biochem. J.* **104**, 497–502
- Williamson, D. H., Mayor, F. & Veloso, D. (1971) in *Regulation of Gluconeogenesis* (Söling, H. D. & Willms, B., eds.), pp. 92–102, Academic Press, New York and London
- Wollenberger, A., Ristau, O. & Schoffa, G. (1960) *Pflügers Arch. Gesamte Physiol. Menschen Tiere* **270**, 399–412