A possible mechanism for the anti-ketogenic action of alanine in the rat

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1. The anti-ketogenic effect of alanine has been studied in normal starved and diabetic rats by infusing L-alanine for 90 min in the presence of somatostatin ($10 \mu g/kg$ body wt. per h) to suppress endogenous insulin and glucagon secretion. 2. Infusion of alanine at 3 mmol/kg body wt. per h caused a $70 \pm 11\%$ decrease in [3-hydroxybutyrate] and a $58 \pm 9\%$ decrease in [acetoacetate] in 48 h-starved rats. [Glucose] and [lactate] increased, but [non-esterified fatty acid], [glycerol] and [3-hydroxybutyrate]/[acetoacetate] were unchanged. 3. Infusion of alanine at 1 mmol/kg body wt. per h caused similar decreases in [ketone body] (3-hydroxybutyrate plus acetoacetate) in 24 h-starved normal and diabetic rats, but no change in other blood metabolites. 4. Alanine [3 mmol/kg body wt. per h] caused a $72 \pm 9\%$ decrease in the rate of production of ketone bodies and a $57 \pm 8\%$ decrease in disappearance rate as assessed by [3-^{14}C]acetoacetate infusion. Metabolic clearance was unchanged, indicating that the primary effect of alanine was inhibition of hepatic ketogenesis. 5. Aspartate infusion at 6 mmol/kg body wt. per h had similar effects on blood ketone-body concentrations in 48 h-starved rats. 6. Alanine (3 mmol/kg body wt. per h) caused marked increases in hepatic glutamate, aspartate, malate, lactate and citrate, phosphoenolpyruvate, 2-phosphoglycerate and glucose concentrations and highly significant decreases in [3-hydroxybutyrate] and [acetoacetate]. Calculated [oxaloacetate] was increased 75%. 7. Similar changes in hepatic [malate], [aspartate] and [ketone bodies] were found after infusion of 6 mmol of aspartate/kg body wt. per h. 8. It is suggested that the anti-ketogenic effect of alanine is secondary to an increase in hepatic oxaloacetate and hence citrate formation with decreased availability of acetyl-CoA for ketogenesis. The reciprocal negative-feedback cycle of alanine and ketone bodies forms an important non-hormonal regulatory system.

An inverse correlation between blood total ketone body and alanine concentrations has been reported in diabetes both in the rat (Blackshear & Alberti, 1974) and in man (Felig *et al.*, 1970), as well as in ketotic hypoglycaemia of childhood (Pagliara *et al.*, 1972), starvation hypoglycaemia (Felig *et al.*, 1969) and pregnancy (Felig *et al.*, 1972). Recently a close relationship between alanine production rate and blood ketone-body concentrations has been reported in diabetic patients (Hall *et al.*, 1979). Although such a relationship itself does not necessarily imply causality, Sherwin *et al.* (1975) suggested that ketonaemia is the cause rather than the result of the changes in alanine concentrations. They showed that infusion of 3-hydroxybutyrate in normal and diabetic man resulted in decreased plasma alanine

concentrations. It was suggested that this was independent of hormonal effects as there was no detectable change in peripheral venous insulin concentrations (Sherwin *et al.*, 1976). This work has been criticized on the grounds that sodium hydroxybutyrate infusion causes alkalinization and the effects could be mimicked by $NaHCO_3$, the free keto acids having no effect (Fery & Balasse, 1979). The inverse hypothesis, that variations in alanine cause the variations in ketone-body concentration, therefore requires close examination.

Ozand *et al.* (1977, 1978) have reported that hepatic non-esterified fatty acid oxidation was virtually abolished in the rat by increasing blood alanine concentrations. However, in their study only the circulating concentrations of 3-hydroxybutyrate

were decreased, whereas the concentrations of acetoacetate remained unchanged, suggesting an effect of alanine on the hepatic redox state.

The results of alanine infusions *in vivo* can be difficult to interpret because of the known effects of the amino acids on both insulin and glucagon secretion (Muller *et al.*, 1971). This effect can be eliminated by simultaneous infusion of somatostatin (Alberti *et al.*, 1973). By using such a combined approach, we have recently observed that alanine infusion in normal subjects decreased both 3-hydroxybutyrate and acetoacetate concentrations without influencing non-esterified fatty acid concentrations (R. Nosadini, G. Noy, K. G. M. M. Alberti, H. Datta, & A. Hodson, unpublished work).

The present study, in the normal and diabetic rats, was designed to examine further the potential anti-ketogenic effect of alanine. The ketone-body-turnover technique has been used to assess whether the effect is primarily on ketogenesis or on ketone-body utilization.

In addition the effect of two other amino acids, aspartate and asparagine, has been examined, to help clarify the mechanisms involved.

Materials and methods

Animals

Male Ash/Wistar rats weighing 285–300 g were used. They were allowed free access to water and a standard laboratory rat diet (Labfood Number 1, B.P. Nutrition, Stepfield, Witham, Essex, U.K.) at all times except where stated in the text.

Experimental procedure

One group of rats was used after starvation for 48 h and a second group after starvation for 24 h. A third group was made diabetic by intravenous injection of streptozotocin in 0.01 M-citrate buffer, pH 4.5 (100 mg/kg body wt.). Animals were given glucose (5 g/100 ml) to drink for 24 h then maintained on protamine zinc insulin ($ZnCl_2$/protamine sulphate-modified insulin) for 4 days as described previously. Insulin was then withdrawn and the animals were used 62 h later, at which time moderate hyperketonaemia had developed (Blackshear & Alberti, 1974).

Starved normal rats or diabetic rats 48 h after the last insulin injection were anaesthetized lightly with diethyl ether and catheters (20.3 cm, lot no. 18C9E949; C. R. Bard International, Sunderland, U.K.) were inserted into the left femoral artery and vein and into the right femoral vein. The animals were placed in restraining cages and allowed to recover with free access to water but not to food for the next 12–14 h. By this time normal animals had been starved for 24 or 48 h as appropriate, whereas diabetic rats had been starved for 24 h.

All the infusions were given at a rate of 1.2 ml/h. Somatostatin diluted in 0.154 M-saline (0.9% NaCl) (100 μg/kg body wt. per h) was infused into the left femoral vein for 120 min. Amino acid or control saline infusions were commenced 30 min after somatostatin and continued for 90 min. Alanine (diluted in water, pH 7.4) was infused into the right femoral vein of 48 h-starved rats at a rate of 3 mmol/kg body wt. per h and in 24 h-starved normal diabetic rats at a rate of 1 mmol/kg body wt. per h. L-Asparagine and sodium L-aspartate (diluted in water) (pH 7.4) were infused into 48 h-starved rats at a rate of 6 mmol/kg body wt. per h and 3 mmol/kg body wt. per h respectively. Control rats received a 0.154 M-saline infusion instead of the amino acid infusion.

During each experiment arterial blood samples (0.3–0.4 ml) were withdrawn at 0, 30, 60, 90 and 120 min after the onset of somatostatin infusion.

Blood and liver assays

Blood was immediately put into heparinized tubes (0.1–0.2 ml), centrifuged and the plasma was deep frozen for subsequent non-esterified fatty acid (Ho, 1970) and insulin (Soeldner & Slone, 1965) assays. Other portions (0.2 ml) of blood were deproteinized with ice-cold 3% (v/v) $HClO_4$ and assays for alanine, lactate, pyruvate, 3-hydroxybutyrate, acetoacetate, glucose and glycerol were performed as previously described on the acid supernatants within 24 h (Price *et al.*, 1977; Lloyd *et al.*, 1978). After each infusion animals were killed by cervical dislocation and livers were removed within 10 s and freeze-clamped (Wollenberger *et al.*, 1960). The frozen tissue was treated by the procedure of Williamson *et al.* (1967a). Enzymic assays of liver extracts were performed for glucose (Slein, 1963), acetoacetate and 3-hydroxybutyrate (Williamson *et al.*, 1962), 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 (Moellering & Gruber, 1966), L-malate (Hohorst, 1963b), L-alanine (Williamson *et al.*, 1967b), L-glutamate (Bernt & Bergmeyer, 1963) and L-aspartate (Pfleiderer, 1963).

Measurements of total ketone-body turnover

Ethyl [3-^{14}C]acetoacetate (250 μCi, sp. radioactivity 9.9 mCi/mol) was added to 0.5 mmol of carrier ethyl acetoacetate. Hydrolysis was accomplished by addition of 300 μl of 2 M-NaOH and incubation for 60 min at 40°C. After neutralization with 0.1 M-HCl, the solution was washed five times with diethyl ether and treated for 45 min with a stream of N_2, keeping the solution in crushed ice.

The radiochemical purity of the [^{14}C]aceto-acetate was determined in each experiment (Mayes & Felts, 1967) and was between 89 and 95%. The labelled acetoacetate was diluted with 0.154 M-NaCl and infused in the 48 h-starved rats in the left femoral vein at a rate of 0.2 μCi/min with a priming dose corresponding to the amount infused in 40 min.

Ketone body specific radioactivity was determined by the method of Bates et al. (1968) with samples withdrawn 40 min after the priming dose injection to allow equilibration of the isotope. The infusion was commenced 20 min before somato-statin and continued until the end of the experiment.

Recoveries of [^{14}C]acetoacetate and 3-hydroxy-[^{14}C]butyrate were determined within each set of analyses by adding portions of diluted infusates to whole blood. Efficiency of the liquid-scintillation counting procedure was determined by using [^{14}C]-toluene as an external standard. Recoveries of acetoacetate were 82–87% when corrected for radiochemical purity and 79–86% for 3-hydroxy-butyrate. Rates of appearance and disappearance of total ketone bodies were calculated by the method of Bates (1971). Ketone-body clearance was calculated as the ratio of the rate of disappearance to blood total ketone-body concentration corrected for body weight (Riggs, 1963).

In four separate experiments, the volume of distribution of ketone bodies was determined by using a bolus injection of 5 μCi of [3-^{14}C]aceto-acetate. The value determined was 31% of body weight (range 28–33%). This mean value was used to calculate turnover rate in the non-steady state.

Chemicals

Streptozotocin was kindly provided by Upjohn Co., Kalamazoo, MI, U.S.A. Cyclic somatostatin was from Wyeth Laboratories Inc., Philadelphia, PA 19102, U.S.A. (batch no. 430A203). Protamine zinc insulin (40 i.u./ml) was obtained from Burroughs Wellcome Co., Beckenham, Kent, U.K. Enzymes and coenzymes were supplied by Boehringer Corp. L-Alanine was provided as a 10% (w/v) solution in water from McCarthys, Romford, Essex, U.K. Other chemicals were of reagent grade.

Calculations

Results were expressed as means ± S.E.M. Where appropriate, comparisons have been made by using Student's t test. Hepatic [oxaloacetate] was calculated from the formula:

$$[\text{Oxaloacetate}] = \frac{[\text{pyruvate}] \cdot [\text{malate}] \cdot K_{\text{MDH}}}{[\text{lactate}] \cdot K_{\text{LDH}}}$$

where MDH is malate dehydrogenase and LDH is lactate dehydrogenase (Hawkins et al., 1973) and K represents the equilibrium constant of the appropriate enzyme. Ketone-body turnover calculations

are as described above. Total ketone bodies refers to the sum of the concentrations of 3-hydroxybutyrate and acetoacetate. Acetone was not measured.

Results

Effect of alanine infusion on blood alanine and ketone-body concentrations in normal starved and diabetic rats (Fig. 1, Table 1)

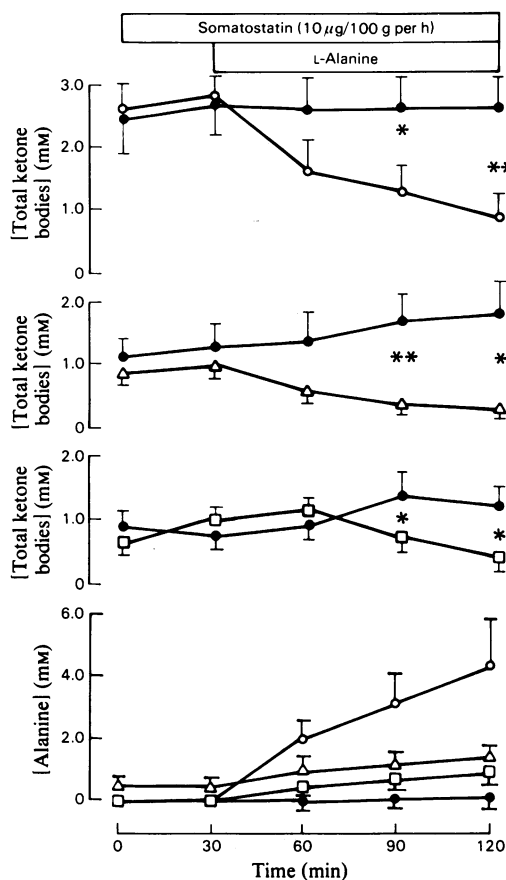Alanine infused at 3 mmol/kg body wt. per h in the presence of somatostatin in 48 h-starved rats

Fig. 1. *Effect of alanine and somatostatin infusion on blood alanine and ketone-body concentrations in normal and diabetic rats*

All rats received somatostatin as described in the text. Symbols: O, 48 h-starved rats infused with alanine (3 mmol/kg body wt. per h) ($n = 5$); △, 24 h-starved rats (1 mmol of alanine/kg body wt. per h) ($n = 5$); □, 24 h-starved diabetic rats (1 mmol of alanine/kg body wt. per h); ●, appropriate control group infused with somatostatin and saline ($n = 5$ in each case). Results are presented as means ± S.E.M. *$P < 0.05$; **$P < 0.02$; ***$P < 0.01$, as assessed by Student's t test when compared with the appropriate control value.

Table 1. *Alanine and ketone-body concentrations in normal and diabetic rats before and after 30 and 120 min of somatostatin infusion*

Alanine or saline were infused from 30 to 120 min.

	Rate of alanine infusion (mmol/kg body wt. per h)	Time (min) ...	Blood [alanine] (mM)			Blood [3-hydroxybutyrate] (mM)			Blood [acetoacetate] (mM)		
			0	30	120	0	30	120	0	30	120
24 h-starved normal rats	0		0.256 ±0.066	0.260 ±0.035	0.258 ±0.040	0.75 ±0.19	0.90 ±0.27	1.19 ±0.32	0.35 ±0.12	0.49 ±0.17	0.58 ±0.21
	1		0.238 ±0.070	0.239 ±0.022	1.89 ±0.03	0.58 ±0.11	0.59 ±0.00	0.22 ±0.04	0.28 ±0.07	0.33 ±0.04	0.09 ±0.00
48 h-starved normal rats	0		0.233 ±0.050	0.233 ±0.055	0.230 ±0.059	1.73 ±0.32	1.80 ±0.28	1.92 ±0.21	0.81 ±0.29	0.88 ±0.33	0.79 ±0.12
	3		0.230 ±0.054	0.233 ±0.055	4.51 ±1.41	1.80 ±0.29	1.97 ±0.31	0.57 ±0.15	0.85 ±0.19	0.91 ±0.24	0.31 ±0.09
24 h-starved diabetic rats	0		0.066 ±0.007	0.060 ±0.005	0.068 ±0.006	0.61 ±0.12	0.49 ±0.08	0.89 ±0.19	0.30 ±0.09	0.27 ±0.09	0.36 ±0.10
	1		0.058 ±0.008	0.055 ±0.007	1.15 ±0.32	0.44 ±0.09	0.73 ±0.11	0.29 ±0.05	0.26 ±0.08	0.38 ±0.07	0.11 ±0.07

caused a prompt rise in blood alanine concentration with values finally reaching 4.5 ± 1.4 mM. In control infusions (somatostatin plus 0.154 M-NaCl) there was no significant change. Blood ketone-body concentrations that were stable at 2.83 ± 0.42 mM in the pre-alanine phase decreased sharply and significantly $(P < 0.01)$ to 0.92 ± 0.28 mM after 90 min of alanine infusion. Both 3-hydroxybutyrate and acetoacetate decreased to a similar degree so that there was no significant change in the [3-hydroxy-butyrate]/[acetoacetate] ratio (2.3 ± 0.8 versus 2.0 ± 0.3). In control infusions ketone-body concentrations did not change.

In the 24 h-starved normal and diabetic rats, blood alanine concentrations increased to 0.6–1.5 mM with an alanine infusion rate of 1 mmol/kg body wt per h together with somatostatin. In both groups blood ketone-body concentrations decreased to less than 0.5 mM after alanine from starting values of 0.97 ± 0.15 mM in the non-diabetic and 1.07 ± 0.18 mM in the diabetic rats. Again the [3-hydroxybutyrate]/[acetoacetate] ratio was unaltered.

Effect of alanine infusion on blood metabolite and plasma insulin concentrations in normal starved and diabetic rats

In 48-h-starved rats alanine infusion at 3 mmol/kg body wt. per h caused a significant increase in blood glucose concentrations compared with somatostatin alone (Fig. 2). Blood lactate concentrations also increased by more than 2-fold. Blood glycerol and plasma fatty acid concentrations were, however, similar in control and alanine infusions as were the
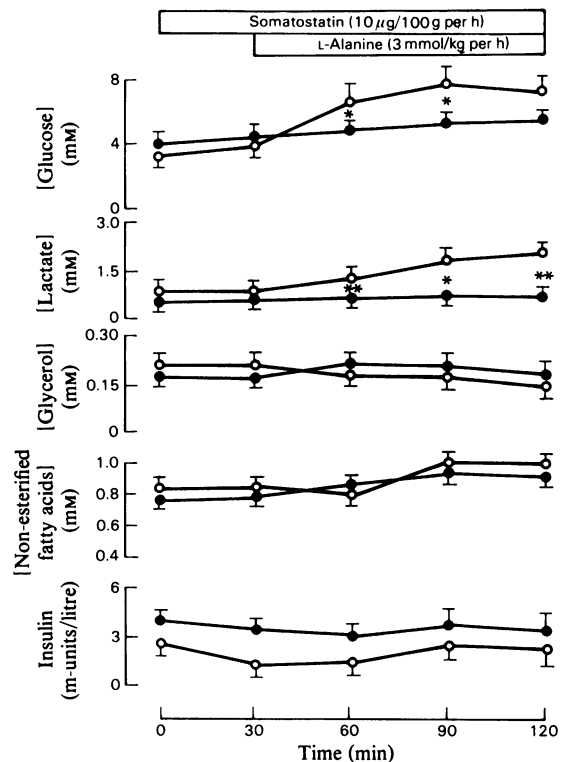

Fig. 2. *Effect of alanine (3 mmol/kg body wt. per h) and somatostatin infusion on blood glucose, lactate, glycerol, non-esterified fatty acids and serum insulin concentrations in 48 h-starved rats*

Symbols: O, somatostatin-plus-alanine infusion $(n = 5)$; ●, somatostatin-plus-saline infusion $(n = 5)$. Other symbols are as defined in the legend to Fig. 1.

[lactate]/[pyruvate] ratios. Plasma insulin concentrations were low before alanine infusion due to the effect of somatostatin and remained low throughout.

In the alanine (1 mmol/kg body wt.) infusions no significant changes occurred in [glucose], [lactate] or [pyruvate] compared with control infusions. In the 24-h-starved non-diabetic rats, glucose was 4.3 ± 1.8 mM before alanine infusion and 5.9 ± 0.9 mM at the end, compared with 3.6 ± 0.5 mM and 5.1 ± 0.7 mM in the rats infused with somatostatin alone. In the diabetic rats, blood glucose values were 15.1 ± 2.1 mM before and 16.2 ± 2.7 mM after alanine infusion plus somatostatin and 12.9 ± 3.2 mM and 11.9 ± 2.1 mM in the saline-plus-somatostatin infusions.

Effect of alanine infusion (3 mmol/kg body wt. per h) on ketone-body turnover in 48-h-starved rats

Two groups of 48-h-starved rats were infused with [3-^{14}C]acetoacetate for 130 min. Somatostatin was infused from 20 to 130 min: one group was infused with NaCl (0.154 M) from 50 to 130 min, and the other group received alanine. The specific radioactivities of blood 3-hydroxybutyrate and acetoacetate were 5.3 ± 1.4 and 15.9 ± 4.2 d.p.m./nmol at 50 min and 6.9 ± 1.8 and 20.6 ± 3.8 d.p.m./nmol at 130 min in the saline group with total specific radioactivity varying between 21.5 and 30.8 d.p.m./nmol at intermediate time points (Fig. 3). This indicated the presence of an isotopic steady state. In the alanine group specific radioactivities of 3-hydroxybutyrate and acetoacetate were 7.5 ± 1.0 and 25.9 ± 6.2 d.p.m./nmol respectively at the onset of alanine infusion (50 min), increasing to 40.0 ± 2.5 and 73.8 ± 18.9 d.p.m./nmol at the end of infusion (130 min).

During alanine infusion there was a steady increase of specific radioactivity of both ketone bodies (Fig. 3). This was reflected in a prompt and marked decrease in the rate of appearance and rate of disappearance of total ketone bodies with no significant change in metabolic clearance rate (9.6 ± 2.8 ml/min before alanine infusion and 11.3 ± 6.8 ml/min after 80 min of alanine infusion, compared with 8.5 ± 2.7 and 5.4 ± 1.3 ml/min in the somatostatin-plus-saline-infused controls).

Effect of aspartate and asparagine infusion on blood metabolite concentrations in 48 h-starved rats

To establish whether oxaloacetate might be involved in the anti-ketogenic effect of alanine, groups of 48 h-starved rats were infused with aspartate at 3 and 6 mmol/kg body wt. per h. Despite the marked increase in blood aspartate concentrations, which had already reached 2.9 ± 0.1 mM after 30 min infusion, the 3 mmol/kg body wt. per h infusion did not have any significant
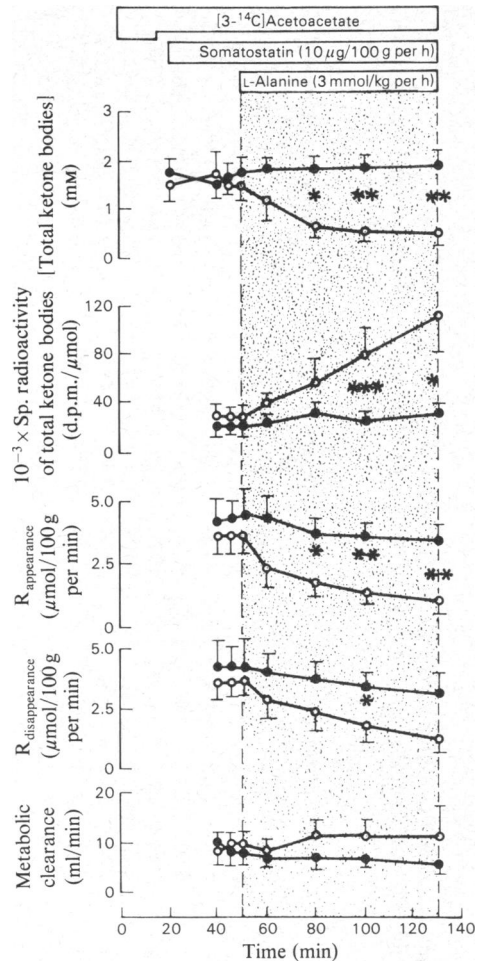

Fig. 3. *Effect of alanine (3 mmol/kg body wt. per h) on ketone-body turnover in 48 h-starved rats*
Symbols: O, somatostatin-plus-alanine infusion ($n = 6$): ●, somatostatin-plus-saline infusion ($n = 6$). [3-^{14}C]Acetoacetate was infused from time 0, somatostatin from 20 to 130 min and alanine from 50 to 130 min. $R_{appearance}$ = rate of appearance of total ketone bodies; $R_{disappearance}$ = rate of disappearance of ketone bodies; *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$. Other details are as described in the text.

effect on blood ketone-body concentrations (Table 2). However, at 6 mmol/kg body wt. per h blood ketone-body concentrations decreased linearly from 2.01 ± 0.43 mM to 0.85 ± 0.22 mM (Table 2). At the same time minor increases in blood glucose, lactate, pyruvate and alanine concentrations were noted, compared with controls (data not shown). Asparagine at 3 mmol/kg body wt. per h caused no

Table 2. *Aspartate and ketone-body concentration in 48-h-starved normal rats infused with aspartate*

Somatostatin (100 μg/kg body wt. per h) was infused from 0 to 120 min and aspartate or saline (0.154 M) from 30 to 120 min. **, $P < 0.001$ compared with control; *, $P < 0.05$.

Rate of aspartate infusion (mmol/kg body wt. per h)	n	Blood [aspartate] (mM)			Blood [3-hydroxybutyrate] (mM)			Blood [acetoacetate] (mM)			Blood [total ketone bodies] (mM)		
Time (min)		0	30	120	0	30	120	0	30	120	0	30	120
0	6	0.016 ±0.019	—	—	1.73 ±0.32	1.80 ±0.28	1.92 ±0.21	0.81 ±0.29	0.88 ±0.33	0.79 ±0.12	2.64 ±0.55	2.70 ±0.52	2.71 ±0.47
3	5	0.094 ±0.018	0.099 ±0.027	2.44 ±0.38**	1.28 ±0.28	1.67 ±0.14	1.38 ±0.13	0.67 ±0.19	0.79 ±0.15	0.67 ±0.11	1.96 ±0.48	2.42 ±0.33	2.08 ±0.31
6	5	0.180 ±0.023	0.170 ±0.035	4.57 ±0.80**	1.44 ±0.30	1.28	0.56 ±0.17*	0.71 ±0.11	0.63 ±0.09	0.28 ±0.09**	2.15 ±0.43	2.01 ±0.43	0.85 ±0.22**

significant change in ketone-body concentrations (2.45 ± 0.39 and 2.15 ± 0.42 mM).

Liver metabolite concentrations after infusion of alanine, aspartate and asparagine with somatostatin

Liver metabolite content after infusion with somatostatin plus or minus alanine (3 mmol/kg body wt. per h) is shown in Table 3 with percentage changes shown in Fig. 4. It can be seen that hepatic [alanine] was increased 10-fold. This was associated with a 60% increase in [lactate] ($P < 0.05$) but no change in pyruvate. However, the concentrations of the triose phosphates, phosphoenolpyruvate, 2-phosphoglycerate and 3-phosphoglycerate were all markedly increased as was the tricarboxylic acid-cycle intermediate malate. The calculated concentration of oxaloacetate was increased 75%. [Glutamate] was increased more than 2-fold, citrate nearly 2-fold and [aspartate] 4-fold. [3-Hydroxybutyrate] and [acetoacetate] decreased by more than half, whereas the ratio of the two ketone bodies as well as [lactate]/[pyruvate] were not significantly altered. The adenine nucleotides were unchanged. Table 4 shows the concentrations of malate, aspartate and ketone bodies in liver from rats infused with somatostatin
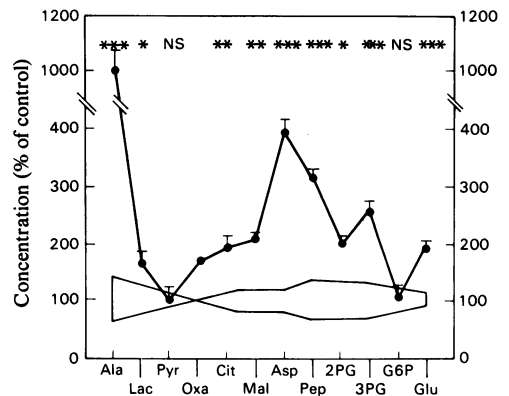

Fig. 4. *Patterns of hepatic gluconeogenic intermediates after somatostatin and alanine infusion (3 mmol/kg body wt. per h) in 48 h-starved rats (n = 6)*
Symbols: ●, mean ± S.E.M. of the percentage of the control value, taken from somatostatin-plus-saline-infused animals. Abbreviations used: Lao, lactate; Pyr, pyruvate; Oxa, oxaloacetate (calculated mean value); Cit, citrate; Mal, malate; Pep, phosphoenolpyruvate; 2PG, 2-phosphoglycerate; 3PG, 3-phosphoglycerate; G6P, glucose 6-phosphate; Glu, glucose. The enclosed area represents the value of controls ± S.E.M. *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$; NS, $P > 0.05$. Statistical analysis was not performed for the calculated oxaloacetate data.

Table 3. *Effect of alanine infusion on hepatic metabolite content in 48 h-starved rats*

Rats were infused with somatostatin (100 μg/kg body wt. per h) for 120 min. After 30 min saline (0.154 M) or alanine (3 mmol/kg body wt. per h) was infused for 90 min. Livers were then freeze-clamped and metabolite concentrations were measured as described in the text. $n = 6$ for each group. Significance of differences was assessed with Student's t test: $*P < 0.05$; $**P < 0.01$; $***P < 0.001$. [Oxaloacetate] was calculated according to the formula of Hawkins *et al.* (1973). Results are given as means ± S.E.M.

| | Metabolite content (μmol/g wet wt.) | |
Metabolite	Saline infusion	Alanine infusion
Alanine	0.48 ± 0.16	4.94 ± 1.61***
Glutamate	1.60 ± 0.28	3.72 ± 0.74*
Malate	0.38 ± 0.07	0.76 ± 0.08**
Aspartate	0.46 ± 0.06	1.78 ± 0.23***
Oxaloacetate	0.004	0.007
Citrate	0.205 ± 0.020	0.387 ± 0.057**
Pyruvate	0.109 ± 0.011	0.116 ± 0.010
Lactate	0.96 ± 0.17	1.56 ± 0.20*
Phosphoenolpyruvate	0.081 ± 0.023	0.254 ± 0.033***
2-Phosphoglycerate	0.042 ± 0.011	0.081 ± 0.011*
3-Phosphoglycerate	0.24 ± 0.05	0.61 ± 0.07***
2-Glycerophosphate	0.79 ± 0.13	0.59 ± 0.06
Glucose 6-phosphate	0.360 ± 0.052	0.380 ± 0.075
Glucose	7.2 ± 0.5	13.6 ± 0.7***
[Lactate]/[pyruvate]	10.0 ± 2.2	13.4 ± 1.5
[Malate]/[aspartate]	2.93 ± 1.42	0.47 ± 0.05
3-Hydroxybutyrate	1.89 ± 0.17	0.68 ± 0.11***
Acetoacetate	0.45 ± 0.05	0.23 ± 0.04***
Total ketone bodies	2.34 ± 0.20	0.92 ± 0.11***
[3-Hydroxybutyrate]/[acetoacetate]	4.55 ± 0.47	3.48 ± 0.42
ATP	2.01 ± 0.20	1.96 ± 0.15
ADP	1.75 ± 0.26	1.80 ± 0.14
AMP	1.72 ± 0.16	1.82 ± 0.29
Total adenine nucleotides	5.48 ± 0.60	5.27 ± 0.55
[ATP]/[ADP]	1.15 ± 0.06	1.10 ± 0.11

Table 4. *Effect of asparagine and aspartate infusion on hepatic aspartate, malate and ketone-body concentrations in 48 h-starved rats*

Rats were treated as described in the legend to Table 3, except that L-aspartate (3 mmol/kg body wt. per h or 6 mmol/kg body wt. per h) or L-asparagine (3 mmol/kg body wt. per h) were infused instead of alanine. Other symbols are defined in the legend to Table 3. Numbers of animals are shown in parentheses. Statistical comparisons were made only between amino acid infusions and saline controls. Results are given as means ± S.E.M.

| | Metabolite concn. (μmol/g wet wt.) | | | |
Metabolite	Saline (3 mmol/kg body wt. per h) (6)	Asparagine (3 mmol/kg body wt. per h) (6)	Aspartate (3 mmol/kg body wt. per h) (6)	Aspartate (6 mmol/kg body wt. per h) (6)
Malate	0.38 ± 0.07	0.24 ± 0.02	0.33 ± 0.04	0.66 ± 0.05**
Aspartate	0.46 ± 0.06	0.76 ± 0.08*	0.87 ± 0.19**	1.45 ± 0.18**
3-Hydroxybutyrate	1.89 ± 0.17	1.59 ± 0.13	1.55 ± 0.11	0.96 ± 0.18***
Acetoacetate	0.45 ± 0.05	0.95 ± 0.22	0.70 ± 0.16	0.31 ± 0.06***
Total ketone bodies	2.34 ± 0.20	2.53 ± 0.11	2.25 ± 0.25	1.25 ± 0.26***
[3-Hydroxybutyrate]/[acetoacetate]	4.55 ± 0.47	2.45 ± 0.75	2.52 ± 0.34	3.25 ± 0.59***

and aspartate or asparagine. Asparagine and aspartate (3 mmol/kg body wt. per h) caused a small increase in aspartate but no change in ketone-body concentration. Aspartate at 6 mmol/kg body wt. per

h, however, caused a more than 3-fold increase in [aspartate] to concentrations similar to those found after 3 mmol of alanine/kg body wt. per h and a similar decrease in [ketone bodies].

Discussion

In 1973 Genuth reported that oral administration of alanine to fasting obese subjects caused a decrease in blood 3-hydroxybutyrate concentration. Genuth & Castro (1974) later reproduced this observation in normal and diabetic subjects. It was deduced that this was independent of an effect on insulin. The decrease in blood ketone-body concentration caused by alanine was confirmed in the rat by Ozand et al. (1977), who showed that the effect was unchanged by the presence of anti-insulin serum, suggesting more directly that insulin was not involved in the phenomenon. A notable finding of Ozand et al. (1977, 1978) was that the anti-ketogenic effect was restricted to 3-hydroxybutyrate with blood acetoacetate concentrations remaining unchanged or even increasing.

In recent experiments in man using a continuous intravenous infusion of alanine we have also found a decrease in blood ketone-body concentrations. In these experiments, somatostatin was infused before and during the alanine infusion to inhibit insulin and glucagon secretion (Alberti et al., 1973; Assan, 1976), both of which can be increased by alanine (Muller et al., 1971). Physiological increments in blood alanine concentration were associated with sharp decreases in both blood [3-hydroxybutyrate] and [acetoacetate] without any alteration in the [3-hydroxybutyrate]/[acetoacetate] ratio (R. Nosadini, G. Noy, K. G. M. M. Alberti, H. Datta & A. Hodson, unpublished work). This has been confirmed in the present work in the rat, where hypoketonaemia was noted at two different doses of alanine in starved normal and mildly diabetic animals. In all cases somatostatin was also infused to eliminate changes in insulin or glucagon as causative factors. At the higher rate of infusion marked changes were found in blood glucose, lactate and pyruvate concentrations, but these were not found at the lower dose of alanine. Ozand et al. (1977) also found that the hypoketonaemic effect of alanine could occur in the absence of significant changes in these other metabolites.

The decrease in circulating ketone-body concentrations could be due either to inhibition of production or to increased utilization. It has been suggested that utilization of ketone bodies is unaltered by alanine (Ozand et al., 1978) and that formation of both 3-hydroxybutyrate and acetoacetate from [^{14}C]oleate is decreased. The latter effect was disproportionately greater than the effects on circulating ketone-body concentrations, suggesting some formation of ketone bodies from sources other than circulating fatty acids. Our own results showed clearly a decreased rate of appearance of ketone bodies with a decreased rate of disappearance, but no change in metabolic clearance rate. A

peripheral effect of alanine on ketone-body utilization is thus ruled out and the anti-ketogenic effect is confirmed. This must be a direct effect on the liver as substrate supply, i.e. plasma non-esterified fatty acids, was unchanged during experiments.

Our finding that the anti-ketogenic effect involved acetoacetate as much as 3-hydroxybutyrate in the rat as well as in man is in direct contrast with the reports of Ozand et al. (1977, 1978). The only major differences in experimental protocol were that our rats were older and, perhaps more importantly, we used a simultaneous infusion of somatostatin. The latter will have resulted in a decrease in glucagon concentration as well as in insulin, which could affect hepatic metabolism.

The failure to observe a change in ketone-body ratio is important in that Ozand et al. (1977, 1978) have based their explanation of the anti-ketogenic effect of alanine on this change. In the starved state the reducing equivalents needed for gluconeogenesis are generated in the mitochondria and transported to the cytosol by the malate–aspartate shuttle (D. H. Williamson et al., 1967a, 1969; J. R. Williamson et al., 1969). Alanine stimulates gluconeogenesis (Snell & Walker, 1973; Friedrichs & Schoner, 1974) and it was suggested that alanine causes a transient increase in the flow of reducing equivalents to the cytosol, leaving the mitochondria in a relatively oxidized state. This would explain the apparent disequilibrium between 3-hydroxybutyrate and acetoacetate, as acetoacetate formation would be favoured. It is also possible that alanine causes preferential formation of acetoacetate from C_3 units such as pyruvate (Lopes-Cardozo and Van den Bergh, 1972). These suggestions do not, however, explain satisfactorily the absolute decrement in ketone-body formation. Our own failure to demonstrate changes in either the hepatic ketone-body ratio or the [lactate]/[pyruvate] ratio indicate that redox changes must be of minor significance. Alternative explanations must therefore be sought.

The livers of alanine-treated rats showed a trend towards increased citrate concentration, a 4-fold increase in [aspartate] and a 75% increase in the calculated oxaloacetate concentration. Oxaloacetate availability may be of considerable importance in determining acetyl-CoA disposal and hence ketogenesis. Krebs (1966) suggested that in diabetes the greatly enhanced rate of gluconeogenesis directed available oxaloacetate to gluconeogenesis and hence decreased citrate formation, allowing the excess acetyl-CoA to form ketone bodies. Wieland et al. (1964) also maintained that increased ketogenesis was due to oxaloacetate deficiency. We would contend that the opposite situation obtains with alanine infusion. Oxaloacetate increases, resulting in enhanced citrate formation and decreased intramitochondrial acetyl-CoA availability for

ketone-body formation. The decrease in ketogenesis from [^{14}C]oleate observed by Ozand et al. (1978) is in keeping with this, as are the observations of Blackshear et al. (1975), although a diversion of long-chain fatty acids to esterified products cannot be totally ruled out. The latter authors showed that inhibition of gluconeogenesis caused inhibition of ketogenesis. This could have been due to oxaloacetate accumulation, although alternative explanations are also possible.

A major difficulty in proving our hypothesis is the difficulty in measuring intramitochondrial oxaloacetate. In an attempt to circumvent this difficulty both Williamson (1974) and we have infused asparagine into rats. This should theoretically increase the flux of carbon through oxaloacetate. No effect, however, was noted on hepatic ketone-body concentrations. Our own data show that despite the relatively high dose of asparagine there was a relatively small change in hepatic [aspartate]. Similar changes were found after infusing aspartate at 3 mmol/kg body wt. per h. These findings may be explained by the recent finding of Milman et al. (1979), who reported that after injection of [^{14}C]asparagine and [^{14}C]aspartate into mice, the majority of the radioactivity was recovered in pancreas, small intestine and lung, and only small amounts in liver. In contrast alanine is taken up avidly by liver. When we increased the aspartate infusion to twice that of alanine, i.e. 6 mmol/kg body wt. per h, a marked anti-ketogenic effect was again found, and hepatic aspartate concentrations were similar to those found after alanine (3 mmol/kg body wt. per h).

It is also possible that pyruvate formed from alanine in the cytosol directly increases mitochondrial oxaloacetate, whereas aspartate and asparagine influence primarily cytosolic oxaloacetate formation.

Our hypothesis leaves unexplained the finding of Ozand et al. (1977) that infusions of lactate and pyruvate, in equimolar concentrations to those they used for alanine, did not influence ketone-body concentrations. Again this could be due to active uptake by tissues other than liver. Lactate is well known to have an anti-ketogenic effect in vitro (Exton et al., 1969). The anti-ketogenic effects of fructose in vivo (Dietze et al., 1978) and in vitro (Prager & Ontko, 1976) could, in part, be explained on the same basis.

References

Adam, H. (1963) Methods of Enzymatic Analysis (Bergmeyer, H. U., ed.), pp. 573–577, Academic Press, New York

Alberti, K. G. M. M., Christensen, N. J., Christensen, S. F., Hansen, Aa. P., Iversen, J., Lundbaek, K., Seyer-Hansen, K. & Ørskov, H. (1973) Lancet ii, 1299–1301

Assan, R. (1976) Diabete Metab. 2, 135–146

Bates, M. W. (1971) Am. J. Physiol. 221, 984–991

Bates, M. W., Krebs, H. A. & Williamson, D. H. (1968) Biochem. J. 110, 655–661

Bernt, E. & Bergmeyer, H. U. (1963) Methods of Enzymatic Analysis (Bergmeyer, H. U., ed.), pp. 384–388, Academic Press, New York

Blackshear, P. J. & Alberti, K. G. M. M. (1974) Biochem. J. 138, 107–117

Blackshear, P. J., Holloway, P. A. H. & Alberti, K. G. M. M. (1975) Biochem. J. 148, 353–362

Bücher, T., Czok, R., Lamprecht, W. & Latzko, E. (1963) Methods of Enzymatic Analysis (Bergmeyer, H. U., ed.), pp., 253–259, Academic Press, New York

Czok, R. & Eckert, L. (1963) Methods of Enzymatic Analysis (Bergmeyer, H. U., ed.), pp. 229–233, Academic Press, New York

Dietze, G., Wicklmayr, M. & Mehnert, H. (1978) Diabetes 27, 709–714

Exton, J. H., Corbin, J. G. & Park, C. R. (1969) J. Biol. Chem. 244, 4095–4102

Felig, P., Owen, O. E. & Wahren, J. (1969) J. Clin. Invest. 48, 584–594

Felig, P., Marliss, E., Ohman, J. L. & Cahill, G. F. Jr. (1970) Diabetes 19, 727–729

Felig, P., Kim, Y. J. & Lynch, V. (1972) J. Clin. Invest. 51, 1195–1202

Fery, F. & Balasse, E. O. (1979) Excerpta Med. Found. Int. Congr. Ser. n 481, 63–64

Friedrichs, D. & Schoner, W. (1974) Biochim. Biophys. Acta 343, 341–344

Genuth, S. M. (1973) Metabolism 22, 927–934

Genuth, S. M. & Castro, J. (1974) Metabolism 23, 375–386

Hall, S. E. H., Braaten, J. T., McKendry, J. B. R., Bolton, T., Foster, D. & Berman, M. (1979) Diabetes 28, 737–745

Hawkins, R. A., Houghton, R. S. & Williamson, D. H. (1973) Biochem. J. 132, 19–25

Ho, R. H. (1970) Anal. Biochem. 36, 105–113

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. Y., ed.), pp. 328–332, Academic Press, New York

Hohorst, H. J., Kreutz, F. H. & Bücher, T. (1959) Biochem. Z. 332, 18–46

Krebs, H. A. (1966) Adv. Enzyme Regul. 4, 339–353

Lamprecht, W. & Trautschold, I. (1963) in *Methods of Enzymatic Analysis* (Bergmeyer, H. U., ed.), pp. 543–551, Academic Press, New York

Lloyd, B., Burrin, J., Smythe, P. & Alberti, K. G. M. M. (1978) *Clin. Chem.* **24**, 1724–1729

Lopes-Cardozo, M. & Van den Bergh, S. G. (1972) *Biochim. Biophys. Acta* **283**, 1–15

Mayes, P. A. & Felts, J. M. (1967) *Biochem. J.* **102**, 230–235

Milman, H. A., Cooney, D. A. & Young, D. M. (1979) *Am. J. Physiol.* **236**, E746–E753

Moellering, H. & Gruber, W. (1966) *Anal. Biochem.* **17**, 369–376

Muller, W. A., Faloona, G. R. & Unger, R. H. (1971) *J. Clin. Invest.* **50**, 2215–2218

Ozand, P. T., Reed, W. D., Girard, J., Hawkins, R. L., Collins, R. M., Tildon, J. T. & Cornblath, M. (1977) *Biochem. J.* **164**, 557–564

Ozand, P. T., Reed, W. D., Hawkins, R. L., Stevenson, J. H., Tildon, J. T. & Cornblath, M. (1978) *Biochem. J.* **170**, 583–591

Pagliara, A. S., Karl, I. E. & DeVivi, D. C. (1972) *J. Clin. Invest.* **51**, 1440–1449

Pfleiderer, G. (1963) in *Methods of Enzymatic Analysis* (Bergmeyer, H. U., ed.), pp. 381–383, Academic Press, New York

Prager, G. N. & Ontko, I. A. (1976) *Biochim. Biophys. Acta* **424**, 386–395

Price, C. P., Lloyd, B. & Alberti, K. G. M. M. (1977) *Clin. Biochem.* **23**, 1893–1897

Riggs, D. S. (1963) in *The Mathematical Approach to Physiological Problems: A Critical Primer*, pp. 196–198, The Williams and Wilkins Company, Baltimore

Sherwin, R. S., Hendler, R. G. & Felig, P. (1975) *J. Clin. Invest.* **22**, 1382–1390

Sherwin, R. S., Hendler, R. G. & Felig, P. (1976) *Diabetes* **25**, 776–784

Slein, M. W. (1963) in *Methods of Enzymatic Analysis* (Bergmeyer, H. U., ed.), pp. 117–173, Academic Press, New York

Snell, K. & Walker, D. G. (1973) *Enzyme* **15**, 40–81

Soeldner, J. S. & Slone, D. (1965) *Diabetes* **14**, 771–779

Wieland, O., Wiess, L. & Eger-Neufeldt, I. (1964) *Adv. Enzyme Regul.* **2**, 85–99

Williamson, D. H. (1974) *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-Vieira, D. & Walker, B. (1967b) *Biochem. J.* **104**, 497–502

Williamson, D. H., Veloso, D., Ellington, E. V. & Krebs, H. A. (1969) *Biochem. J.* **114**, 575–584

Williamson, J. R., Browning, E. T. & Scholz, R. (1969) *J. Biol. Chem.* **244**, 4607–4616

Wollenberger, A., Ristau, O. & Schoffa, G. (1960) *Pflueger's Arch. Gesamte Physiol. Menschen Tiere* **270**, 399–412