Hypoketonaemic Effect of L-Alanine

SPECIFIC DECREASE IN BLOOD CONCENTRATIONS OF 3-HYDROXYBUTYRATE IN THE RAT

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1. The injection of L-alanine (50–100 mg/kg) into 35-day-old rats that had been starved for 48 h increased blood L-alanine concentration to values observed in fed animals and lowered the blood concentration of 3-hydroxybutyrate within 2 min. 2. This hypoketonaemic action of L-alanine was specific for 3-hydroxybutyrate, since the acetoacetate concentrations did not change significantly. 3. The decrease in 3-hydroxybutyrate elicited by L-alanine was not related to changes in the blood concentrations of insulin, glucagon, growth hormone, glucose, unesterified fatty acids, lactate or pyruvate. 4. The injection of L-alanine resulted in a decrease in total ketones that was apparently unrelated to their increased peripheral utilization. These results are interpreted as an anti-ketogenic action of L-alanine. 5. The data suggest that L-alanine lowers ketone-body formation in starved rats, possibly via an alteration in hepatic redox equilibrium.

Ketone metabolism assumes significance during starvation, neonatal life and in clinical conditions associated with ketoacidosis (Williamson & Hems, 1970; Krebs, 1972; McGarry & Foster, 1972). The regulation of this metabolism is not completely understood. Several factors, such as the regulation *in vivo* of adipose-tissue lipolysis by hormones (Vaughan, 1961; Foster, 1967; Williamson, 1967; Miller & Allen, 1973; Yeh & Zee, 1976) or by ketone bodies (Madison *et al.*, 1964; Senior & Loridan, 1968; Hellman *et al.*, 1969; Balasse & Neef, 1975), the generation of reducing equivalents in liver and their transport from mitochondria (Williamson, 1967; J. R. Williamson *et al.*, 1969; Ontko, 1972), as well as altered utilization of ketone bodies by peripheral tissues (Beatty *et al.*, 1959; Winder *et al.*, 1975), have all been shown to participate in this process.

Genuth (1973) and Genuth & Castro (1974) reported a decrease in the concentration of blood 3-hydroxybutyrate after the administration of L-alanine to starved, obese and diabetic humans. An age-dependent effect of alanine on blood concentrations of 3-hydroxybutyrate was observed in 10–21-day-old ketotic rats, but not in 2-day-old rat pups (Ozand *et al.*, 1967b). The hypoketonaemic effect of alanine appears to be physiologically relevant, since this amino acid plays a major role in gluconeogenesis (Felig *et al.*, 1969, 1970) and may link these two processes. Alanine is one of several amino acids that are specifically released in substantial amounts from muscle (Ruderman & Lund, 1972; Ozand *et al.*, 1973; Garber *et al.*, 1976). In addition, alanine promotes the release of hormones (Muller *et al.*, 1971; Wise *et al.*, 1972, 1973a,b).

The results of the present study suggest that during starvation, the blood concentrations of 3-hydroxybutyrate and the ratio of [3-hydroxybutyrate]/[acetoacetate] in the rat can be altered by changes (200–400 μ M) in alanine concentrations. This effect does not appear to be related to changes in blood glucose, lactate, pyruvate, unesterified fatty acids, insulin, glucagon, or growth hormone, or to the peripheral utilization of 3-hydroxybutyrate. The results further suggest that, during starvation, blood concentrations of alanine may influence the hepatic redox state.

Experimental

Materials

Trasylol [aprotinin; 10^5 kallikrein inactivator units/ml (Webster & Prado, 1970)] and all other reagents for biochemical analyses were obtained from Sigma Chemical Co., St. Louis, MO, U.S.A., except for alanine dehydrogenase (EC 1.4.1.1; from *Bacillus subtilis*), which was obtained from Boehringer Mannheim Corp., New York, NY, U.S.A. Anti-insulin serum was purchased from Miles-Yeda Ltd., Rehovot, Israel [code no. 65-101; guinea-pig anti-(bovine insulin) serum, binding 23 ng of insulin/ μ l]. Bovine insulin was obtained from E. R. Squibb and Sons, Princeton, NJ, U.S.A.

Analyses

Glucose, lactate, pyruvate, alanine, acetoacetate and 3-hydroxybutyrate were analysed by a micro-autoanalytic procedure previously described (Ozand

et al., 1976a). L-Alanine was assayed by using alanine dehydrogenase in experiments involving dichloroacetate (Yoshida & Freese, 1970; Ozand *et al.*, 1976a). Unesterified fatty acids were measured colorimetrically (Lauwreys, 1969). Rat insulin (Albano *et al.*, 1972), glucagon (Heding, 1971; Girard *et al.*, 1972, 1973) and growth hormone (Rieutort, 1970) were measured by published procedures. Results were expressed as the average of changes from 0 min at indicated time intervals (Blackshear *et al.*, 1974) $\pm$ S.E.M. Student's *t* test was used to calculate statistical significance (White, 1951).

Animals

Wistar rats (5–6 weeks old) of both sexes were used. The rate of disappearance of injected alanine at this age was much faster than at other ages. Hypoketonæmia after L-alanine injection was observed in older, starved rats; however, in these animals, the elevated blood concentrations of alanine persisted for a longer period (Ozand *et al.*, 1976b).

Perfusion procedure

The animals were fed on regular laboratory chow *ad lib.* (Ralston Purina Co., St. Louis, MO, U.S.A.), and then were placed in metabolic cages and starved for 48 h, with free access to water. Sodium pentobarbital (30 mg/kg body wt. dissolved in 0.9% NaCl; Sigma Chemical Co.) was injected intraperitoneally and produced anaesthesia for 60–90 min. Surgery was performed immediately after the anaesthesia had taken effect (approx. 15 min). The arteria and vena femoralis were exposed contralaterally with the aid of a stereoscopic microscope. Bleeding was minimal (≤ 0.5 ml). The vessels were cannulated with 6 cm pieces of polyethylene catheters filled with 0.9% NaCl [Intramedic 7400; internal diam. 0.28 mm (0.011 in) outer diam. 0.61 mm (0.024 in); Clay Adams Co., Persippany, NJ, U.S.A.]. The lengths of the catheter inserted into the artery and vein were 1.5 and 3 cm respectively. The incisions were then covered with gauze and kept moist with 0.9% NaCl. Heparin (0.77 mg in 0.1 ml of 0.9% NaCl/100 g body wt.) was

injected into all the animals initially. Test solutions (pH 7.4) were administered intravenously in 0.20 ml of 0.9% NaCl/100 g body wt., followed by a volume of 0.9% NaCl equal to that displaced by the inserted catheter. The total duration of the injections was 1 min. At the indicated intervals, 50 μ l of arterial blood was quickly withdrawn and mixed with 600 μ l of 1 M-HClO₄ at 0°C. The supernatant fluids were removed and kept at -95°C until analysed (Ozand *et al.*, 1976a). Since the volume in the arterial catheter was approx. 20 μ l, this volume of blood was discarded before obtaining each experimental sample.

Results

Effects of starvation

The body weight of rats before starvation was 111.9 ± 1.9 g (mean $\pm$ S.E.M.), and 48 h starvation decreased the weight to 88.6 ± 1.1 g ($n = 137$). The concentrations of blood ketones increased, whereas those of glucose, lactate, pyruvate and alanine decreased significantly (Table 1).

Effects of alanine injection

The injection of 50–200 mg of L-alanine/kg body wt. resulted in a proportional increase in the concentration of blood L-alanine (Fig. 1). No significant change in concentration of L-alanine was observed after the injection of D-alanine. The injection of 50–200 mg of L-alanine/kg, but not of D-alanine, resulted in a proportional decrease in the concentration of blood 3-hydroxybutyrate (Fig. 2). The maximum decrease (55–65% of the initial value) in 3-hydroxybutyrate occurred 3–8 min later than the peak increment in L-alanine (Fig. 1). The concentration of 3-hydroxybutyrate returned to initial values within 20 min after the injection of 50–100 mg of L-alanine/kg, but remained significantly lower 20 min after injection of the 200 mg/kg dose (Fig. 2).

The concentrations of acetoacetate did not change after L-alanine injection (Table 2). Therefore the ratio of [3-hydroxybutyrate]/[acetoacetate] decreased. This ratio was lowest between 5 and 10 min, when the values were 52–62% of the initial ratio (Fig.

Table 1. *Changes in the blood concentrations of ketones, glucose and gluconeogenic intermediates during starvation*
Results are means $\pm$ S.E.M. for the numbers of animals indicated in parentheses. Fed values were obtained in animals 3 h after a liquid diet had been given at 09:00 h (Foster, 1967); all starvation values were significantly different ($P < 0.001$) from fed values.

	Glucose (mm)	3-Hydroxy- butyrate (μ M)	Acetoacetate (μ M)	Lactate (μ M)	Pyruvate (μ M)	Alanine (μ M)
Fed (19)	8.04 ± 0.80	103 ± 9	76 ± 5	2160 ± 35	202 ± 35	753 ± 21
Starvation, 48 h (158)	3.93 ± 0.07	1090 ± 55	241 ± 11	1080 ± 54	48 ± 3	183 ± 9

Fig. 1. Changes in L-alanine concentration in blood after alanine injection

Either L- or D-alanine was injected at zero time; the ensuing changes in the concentration of L-alanine are shown on a logarithmic scale on the ordinate. The range of L-alanine concentration for the fed state (----) represents mean $\pm$ 2 S.D. ($n=19$). Δ , D-Alanine, 100 mg/kg ($n=28$); $\blacktriangle$, L-alanine, 50 mg/kg ($n=8$); $\square$, L-alanine, 75 mg/kg ($n=9$); $\circ$, L-alanine, 100 mg/kg ($n=39$); $\bullet$, L-alanine, 200 mg/kg ($n=15$). Bar indicates S.D.

Fig. 2. Effect of alanine injection on the concentration of 3-hydroxybutyrate and the ratio [3-hydroxybutyrate]/[acetoacetate] in blood

The experimental conditions were as in Fig. 1. All results represent the mean of changes from zero-time values $\pm$ S.E.M. (indicated by bar) for the numbers of animals in parentheses: Δ , D-alanine, 100 mg/kg (28); $\blacktriangle$, L-alanine, 50 mg/kg (8); $\square$, L-alanine, 75 mg/kg (9); $\circ$, L-alanine, 100 mg/kg (39); $\bullet$, L-alanine, 200 mg/kg (15); $*P<0.001$ when compared with D-alanine injection. The mean ratio $\pm$ S.E.M. of [3-hydroxybutyrate]/[acetoacetate] at zero time in 158 animals was 4.5 ± 0.2 .

2). The decrease in this ratio was more prolonged after injection of 200 mg of L-alanine/kg.

The changes in blood [3-hydroxybutyrate] and the ratio of [3-hydroxybutyrate]/[acetoacetate] were inversely related to the changes in the concentration of blood alanine (Fig. 2). The second injection of 100 mg of L-alanine/kg at 15 min led to a second decrease in blood 3-hydroxybutyrate and in the ratio of [3-hydroxybutyrate]/[acetoacetate]. These changes were more prolonged after the second injection of L-alanine (Fig. 3).

The concentrations of blood glucose, unesterified fatty acids and lactate did not change significantly after injection of 100 mg of L-alanine/kg, but a small significant increase was observed in blood pyruvate (Table 2). However, after the injection of 200 mg of L-alanine/kg there were significant increments in blood lactate, pyruvate and glucose (Table 2).

Effects of glucose, lactate and pyruvate administration

The injection of 100 mg of L-lactate/kg resulted in a significant increase in blood lactate and pyruvate, and the peak values of both were equivalent to the concentrations for these intermediates in fed animals (Table 3). However, no alterations in the concentration or ratio of ketones were observed after lactate injection (Table 3). In contrast, the injection of 100 mg of pyruvate/kg resulted in significant increases in blood pyruvate, lactate and alanine, and this was accompanied by a significant decrease in

blood 3-hydroxybutyrate as well as in the ratio of [3-hydroxybutyrate]/[acetoacetate] (Table 3). The increments in blood L-alanine after pyruvate injection were similar to those observed when 50 mg of L-alanine/kg was injected (Table 3 and Fig. 1). The resulting changes in blood ketones and their ratios were approximately the same as those observed after injection of 50 mg of L-alanine/kg (Table 3 and Fig. 2). The concentration of blood pyruvate increased significantly after the injection of 100 mg of lactate/kg. This had no effect on the hyperketonaemia. The injection of 100 mg of glucose/kg increased the concentration of blood glucose immediately, and caused a small increase in the blood concentrations of lactate and L-alanine after 10–15 min. No corresponding change in the concentration of ketones was observed (Table 3).

Table 2. *Changes in the concentrations of blood glucose, lactate, pyruvate, acetoacetate and unesterified fatty acids after injection of D- or L-alanine*

The number of animals is indicated in parentheses; results are the mean $\pm$ S.E.M. of change from zero-time values. * $P < 0.001$; † $0.02 > P > 0.01$; ‡ $0.05 > P > 0.02$, as compared with D-alanine injection. In the experiments where unesterified fatty acids were determined, only two 0.8 ml blood samples were obtained, the first at zero time and the second one at 2, 5, 10, 15 or 20 min. The number of rats in each set of unesterified fatty acid determinations was eight. The mean initial value $\pm$ S.E.M. for unesterified fatty acids in 80 animals at zero time was 682 ± 25 μ equiv./l.

Changes from zero time	Time (min)	D-Alanine (100mg/kg) (28)	L-Alanine (100mg/kg) (39)	L-Alanine (200mg/kg) (15)
Glucose (mm)	2	-0.16 ± 0.16	0.00 ± 0.17	0.00 ± 0.28
	5	$+0.38 \pm 0.21$	$+0.44 \pm 0.08$	$+0.99 \pm 0.23$
	10	$+0.06 \pm 0.33$	$+0.44 \pm 0.28$	$+0.94 \pm 0.34$
	15	$+0.06 \pm 0.33$	$+0.16 \pm 0.16$	$+1.56 \pm 0.66^\dagger$
	20	$+0.28 \pm 0.16$	$+0.38 \pm 0.21$	$+0.28 \pm 0.16$
Pyruvate (μ M)	2	$+14.0 \pm 7.1$	$+5.0 \pm 2.1$	$+23.0 \pm 4.0$
	5	-7.0 ± 7.0	$+23.0 \pm 8.7$	$+30.0 \pm 3.0^*$
	10	-12.0 ± 6.0	$+17.0 \pm 6.0^*$	$+33.1 \pm 4.0^*$
	15	-9.0 ± 7.0	$+9.0 \pm 6.2$	$+10.0 \pm 4.0$
	20	-9.0 ± 7.0	$+8.0 \pm 6.2$	$+12.0 \pm 5.0$
Lactate (μ M)	2	$+49 \pm 65$	$+72 \pm 67$	$+124 \pm 56$
	5	-24 ± 75	-129 ± 58	$+506 \pm 150^*$
	10	-48 ± 65	$+120 \pm 75$	$+797 \pm 177^*$
	15	-65 ± 45	$+98 \pm 83$	$+491 \pm 175^*$
	20	$+60 \pm 65$	$+149 \pm 83$	$+306 \pm 170$
Acetoacetate (μ M)	2	$+3 \pm 13$	-18 ± 22	$+33 \pm 11$
	5	$+11 \pm 14$	-20 ± 23	$+1 \pm 8$
	10	-13 ± 12	-19 ± 12	-31 ± 11
	15	$+15 \pm 21$	-20 ± 22	-37 ± 28
	20	$+13 \pm 23$	-20 ± 22	-33 ± 28
Unesterified fatty acids (μ equiv./l)	2	-10 ± 25	$+25 \pm 20$	$+35 \pm 23$
	5	-30 ± 25	-29 ± 31	$+11 \pm 23$
	10	-60 ± 22	-50 ± 15	-85 ± 25
	15	Not determined	Not determined	-85 ± 30
	20	-83 ± 23	-113 ± 35	$-156 \pm 24^\ddagger$

Effect of alanine on hormones

The injection of 100 mg of D- or L-alanine/kg did not change the concentration of circulating insulin or glucagon, but 200 mg of L-alanine/kg caused a significant increase in blood insulin at 5 min, and a significant decrease in growth hormone at 20 min (Table 4).

The administration of L-alanine has been shown to increase the secretion of glucagon and insulin (Muller *et al.*, 1971; Wise *et al.*, 1972, 1973a,b; Genuth & Castro, 1974), and a hypoketonaemic effect of insulin has been documented (Foster, 1967). To demonstrate that the hypoketonaemic action of L-alanine was not due to increased blood insulin, anti-insulin serum was given to the experimental animals before L-alanine. The dose was approx. 300-fold in excess of the endogenous insulin concentration (Fig. 4), and gave rise to increasing ketonaemia. Since subsequent administration of 4 μ g of insulin/kg, which causes hyperketonaemia and hypoglycaemia in control rats had no such effect, it was clear that adequate anti-insulin serum was present to bind the

available insulin (Fig. 4). Nevertheless the subsequent injection of 100 mg of L-alanine/kg caused a significant decrease in blood 3-hydroxybutyrate (500 μ M at 5 min; Fig. 2) and the ratio of ketones (Fig. 4); both changes were similar to those seen in the absence of anti-insulin serum. In the presence of anti-insulin serum, a significant increase in blood acetoacetate and glucose occurred after L-alanine administration (Fig. 4).

Combined effects of alanine and dichloroacetate

The injection of dichloroacetate to rats decreases the utilization of ketones and increases the utilization of glucose in peripheral tissues (McAllister *et al.*, 1973; Blackshear *et al.*, 1974; Whitehouse *et al.*, 1974). In the present study injection of dichloroacetate (300 mg/kg) caused an increase in blood 3-hydroxybutyrate and acetoacetate (Fig. 5). A significant decrease in blood lactate, pyruvate and glucose also occurred (results not shown). The subsequent injection of L-alanine (100 mg/kg) elicited

Fig. 3. Effect of repeated injection of L-alanine. Rats were injected (at arrows 1 and 2) with 100 mg of L-alanine/kg (injection 1, ●), and 15 min later with either 100 mg of D-alanine (injection 2, Δ) or 100 mg of L-alanine/kg (second injection, ○). All values are the mean of changes from zero-time values $\pm$ S.E.M. (indicated by bar) for eight animals; * $P < 0.001$ for second injection of L-alanine compared with D-alanine.

significant decreases in blood 3-hydroxybutyrate and in the ratio of ketones (Fig. 5). The magnitude of these changes was similar to those observed in the absence of dichloroacetate (Fig. 2).

Discussion

During starvation in 5–6-week-old rats, when an enhanced rate of ketogenesis and gluconeogenesis is present, a hypoketonaemic action of L-alanine has been observed. An increase in blood [alanine] from 200 to 400 μ M was sufficient to produce a significant decrement in blood [3-hydroxybutyrate] within 2 min (Figs. 1 and 2).

The hypoketonaemic action of L-alanine was not associated with changes in the concentrations of blood glucose, lactate or pyruvate (Tables 2 and 3). The hypoketonaemic changes after pyruvate injection were most likely related to associated changes

Table 3. Changes in the concentrations of ketone bodies, glucose and gluconeogenic intermediates after the injection of 100 mg of lactate, pyruvate or glucose/kg. The results are the mean $\pm$ S.E.M. for the numbers of experiments in parentheses; * $P < 0.001$ compared with results of injection of 100 mg of D-alanine/kg shown in Figs. 2 and 3 and Table 2.

Compound	Time (min)	[3-Hydroxybutyrate] (μ M)	[Acetoacetate] (μ M)	[3-Hydroxybutyrate]/[Acetoacetate]	[Lactate] (μ M)	[Pyruvate] (μ M)	[Alanine] (μ M)	[Glucose] (mM)
L-Lactate (100 mg/kg) (10)	2	-62 $\pm$ 45	-33 $\pm$ 21	+0.1 $\pm$ 0.5	+1210 $\pm$ 106*	+137 $\pm$ 18*	+39 $\pm$ 23	-0.38 $\pm$ 0.28
	5	-144 $\pm$ 85	-37 $\pm$ 26	-0.8 $\pm$ 0.7	+733 $\pm$ 195*	+72 $\pm$ 17*	+40 $\pm$ 20	+0.22 $\pm$ 0.28
	10	-127 $\pm$ 65	-27 $\pm$ 29	-1.0 $\pm$ 0.3	+468 $\pm$ 145*	+35 $\pm$ 7*	+42 $\pm$ 22	+0.50 $\pm$ 0.22
	15	-152 $\pm$ 65	-31 $\pm$ 35	-0.7 $\pm$ 0.5	+385 $\pm$ 96*	+32 $\pm$ 5*	+42 $\pm$ 22	+0.44 $\pm$ 0.22
	20	-83 $\pm$ 45	-29 $\pm$ 35	-0.4 $\pm$ 0.5	+381 $\pm$ 61*	+27 $\pm$ 5	+42 $\pm$ 20	+0.22 $\pm$ 0.17
Pyruvate (100 mg/kg) (12)	2	-221 $\pm$ 90*	+36 $\pm$ 36	-0.8 $\pm$ 0.3	+905 $\pm$ 132*	+204 $\pm$ 21	+235 $\pm$ 82*	-0.33 $\pm$ 0.28
	5	-277 $\pm$ 80*	-32 $\pm$ 39	-1.0 $\pm$ 0.3	+468 $\pm$ 117*	+108 $\pm$ 26*	+145 $\pm$ 6*	-0.28 $\pm$ 0.33
	10	-232 $\pm$ 63*	-49 $\pm$ 28	-1.5 $\pm$ 0.2*	+201 $\pm$ 62*	+37 $\pm$ 5*	+83 $\pm$ 5*	-0.33 $\pm$ 0.33
	15	-202 $\pm$ 102	-29 $\pm$ 27	-0.2 $\pm$ 0.2	+253 $\pm$ 76*	+23 $\pm$ 5	+61 $\pm$ 11*	-0.06 $\pm$ 0.28
	20	-83 $\pm$ 35	-5 $\pm$ 21	-0.2 $\pm$ 0.4	+239 $\pm$ 82*	+15 $\pm$ 12	+56 $\pm$ 9*	-0.00 $\pm$ 0.28
Glucose (100 mg/kg) (11)	2	-3 $\pm$ 45	-8 $\pm$ 19	-0.1 $\pm$ 0.3	+59 $\pm$ 57	-7 $\pm$ 6	+23 $\pm$ 16	+1.56 $\pm$ 0.17*
	5	-90 $\pm$ 28	+20 $\pm$ 21	-0.6 $\pm$ 0.3	+64 $\pm$ 36	+2 $\pm$ 7	+27 $\pm$ 13	+0.72 $\pm$ 0.06*
	10	-169 $\pm$ 52	-9 $\pm$ 31	-0.7 $\pm$ 0.3	+209 $\pm$ 89*	+15 $\pm$ 8	+48 $\pm$ 17	-0.06 $\pm$ 0.17
	15	-194 $\pm$ 67	-27 $\pm$ 31	-0.4 $\pm$ 0.1	+336 $\pm$ 73*	+23 $\pm$ 8	+56 $\pm$ 9*	-0.11 $\pm$ 0.17
	20	-163 $\pm$ 65	-21 $\pm$ 26	-0.4 $\pm$ 0.3	+491 $\pm$ 58*	+15 $\pm$ 9	+66 $\pm$ 17*	-0.11 $\pm$ 0.17

TABLE 4. *Alterations in concentrations of hormones after alanine injection*

The rats were treated as described in the Experimental section. Approx. 0.8 ml of blood was collected at zero time; a second sample of 0.8 ml of blood was collected either 5 or 20 min after the injection of alanine. Trasylol (400 units in 40 μ l) was added to blood samples placed in chilled tubes, and the mixtures were promptly centrifuged at 1200g at 4°C for 10 min. The plasma was separated and stored frozen at -78°C until assayed. Only two blood samples were obtained from each rat; the number of rats in each set (either 0 and 5 min or 0 and 20 min) was six. The means $\pm$ S.E.M. of the initial values for hormones in 36 animals were: insulin 619 $\pm$ 98 pg/ml, glucagon 521 $\pm$ 30 pg/ml and growth hormone 46.6 $\pm$ 8.3 ng/ml. The results represent the mean change $\pm$ S.E.M. from zero time; *0.05 < P < 0.10; †0.05 > P > 0.02 when L-alanine (either 100 mg or 200 mg/kg) is compared with D-alanine.

Compound injected	Time (min)	Changes from zero time		
		Insulin (pg/ml)	Glucagon (pg/ml)	Growth hormone (ng/ml)
D-Alanine	5	-86 $\pm$ 152	+30 $\pm$ 87	-4.9 $\pm$ 9.3
(100 mg/kg)	20	+156 $\pm$ 53	+13 $\pm$ 72	+6.9 $\pm$ 11.9
L-Alanine	5	-8 $\pm$ 103	+63 $\pm$ 35	-11.3 $\pm$ 10.8
(100 mg/kg)	20	+160 $\pm$ 57	-77 $\pm$ 61	-27.3 $\pm$ 11.3*
L-Alanine	5	+718 $\pm$ 281*	+38 $\pm$ 65	-6.3 $\pm$ 6.6
(200 mg/kg)	20	+144 $\pm$ 205	-68 $\pm$ 72	-36.7 $\pm$ 11.7†

in blood alanine. No significant hyperglycaemia was observed after injection of 50 or 100 mg of L-alanine/kg, although a small increment in blood glucose occurred after the injection of 200 mg/kg (Table 2).

Although unesterified fatty acids and the rate of lipolysis in adipose tissues are clearly related to the regulation of ketogenesis, the influx of unesterified fatty acids is not solely responsible for the regulation of ketone-body synthesis (J. R. Williamson *et al.*, 1969; D. H. Williamson *et al.*, 1969; Meier *et al.*, 1972; Yeh & Zee, 1976). In the present report, the injection of 100 mg of L-alanine/kg did not alter the concentration of unesterified fatty acids, although 200 mg/kg caused a small decrement after 20 min (Table 2). This suggests that changes in concentration of unesterified fatty acids occur subsequent to the changes in 3-hydroxybutyrate.

An inverse relationship between the concentration of blood ketones and insulin has been demonstrated (Foster, 1967). Insulin inhibits lipolysis, decreases fatty acid oxidation, gluconeogenesis and ketone formation (Vaughan, 1961; Foster, 1967; Miller & Allen, 1973). The concentration of insulin required to decrease plasma ketones is lower than that required for increased glucose uptake and inhibition of lipolysis (Yeh & Zee, 1976). The prior injection of anti-insulin serum completely inhibited the hypoketonaemic action of exogenous insulin, but did not prevent the hypoketonaemic effect of L-alanine injection (Fig. 4). Therefore it is concluded that the hypoketonaemic action of injected L-alanine *in vivo* was not related to associated changes in the concentrations of blood insulin or glucagon. Genuth & Castro (1974) also suggested that alanine-induced hypoketonaemia was not associated with an increase in insulin release.

Ketone-body utilization by extrahepatic tissues might have been influenced by the administration of alanine. In the present study the prior injection of dichloroacetate did not prevent the hypoketonaemic action of administered L-alanine (Fig. 5). This result indicated that the hypoketonaemic action of alanine was probably not related to increased utilization of 3-hydroxybutyrate by peripheral tissues.

Our results establish a specific relationship between physiological concentrations of blood alanine and 3-hydroxybutyrate during starvation (Figs. 1 and 2), and suggest that the duration and degree of ketonaemia is inversely related to the concentration of circulating alanine in these starved rats (Figs. 1-3). The physiological importance of this finding is apparent, since the hypoketonaemic effect of alanine is observed for 3-hydroxybutyrate, the major ketone body present, during a physiological state when the alanine concentration is low and increased alanine release from muscle occurs (Karl *et al.*, 1976).

Although injected L-alanine lowered blood [3-hydroxybutyrate], it did not affect [acetoacetate]. This observation raises several possibilities. Since alanine has no inhibitory action on 3-hydroxybutyrate dehydrogenase (EC 1.1.1.30) (results not shown), the mechanism for the observed decrease in concentration of ketone bodies and the ratio of [3-hydroxybutyrate]/[acetoacetate] may be related to the availability of unesterified fatty acids for ketone-body formation, the availability of NADH or the alteration of the mitochondrial redox state. Under the present experimental conditions, the administration of L-alanine may have caused a redistribution of reducing equivalents, shifting the reducing power to the cytosol, and thereby restricting the mitochondria from reducing acetoacetate to 3-hydroxybutyrate.

Fig. 4. Combined effects of anti-insulin serum and alanine

Rats were injected (arrows 1 and 2) at zero time with sufficient anti-insulin serum to bind $57 \mu\text{g}$ of insulin/kg body wt. Then 10 min later either 100 mg of L-alanine/kg ($\circ$), or $4 \mu\text{g}$ of insulin/kg ($\blacktriangle$) was injected; control rats were injected with 100 mg of D-alanine/kg ($\triangle$). The results represent the average of changes from zero-time values $\pm$ S.E.M. (indicated by bar), in eight animals; * $P < 0.001$ when L-alanine is compared with D-alanine injection.

Fig. 5. Combined effects of dichloroacetate and alanine

The rats were injected (arrows 1 and 2) with 300 mg of sodium dichloroacetate, pH 7.4/kg at zero time. Either 100 mg of L-alanine/kg ($\circ$) or 100 mg of D-alanine/kg ($\triangle$) was injected at 20 min. The results represent the average of changes from zero-time values $\pm$ S.E.M. (indicated by bar), in eight animals; * $P < 0.001$ when L-alanine is compared with D-alanine injection.

Although no direct evidence has been provided in the present studies, ample evidence exists in the literature to support this working hypothesis. Alanine serves both as a gluconeogenic precursor (Aikawa *et al.*, 1972; Snell & Walker, 1973) and stimulates gluconeogenesis in the rat (Friedrichs & Schoner, 1974). The rate of gluconeogenesis is considered to be regulated in part by the availability of reducing equivalents in the cytosol (J. R. Williamson *et al.*, 1969; D. H. Williamson *et al.*, 1969). When alanine is the gluconeogenic substrate, these reducing equivalents are generated in the mitochondria and transported to the cytoplasm indirectly by the malate-aspartate shuttle (Williamson, 1967; J. R. Williamson *et al.*, 1969; D. H. Williamson *et al.*, 1969). From these studies it

seems likely that during starvation (i.e. increased gluconeogenesis and ketogenesis) the transient shift of the metabolic equilibrium by experimental elevation of blood, [alanine] results in transient redox changes in the liver (Figs. 2 and 3).

The acute decrease in ketone-body synthesis caused by injection of L-alanine (Figs. 2 and 3) and the subsequent decrease in unesterified fatty acids in blood suggests that alanine has multiple regulatory actions on ketogenesis. The participation of endocrine mechanisms in this process must be included, since L-alanine injection caused an increase in blood concentrations of acetoacetate, if circulatory insulin was depleted by the prior injection of anti-insulin serum (Fig. 4).

These observations require further investigations with respect to each of these possibilities.

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References

- Aikawa, T., Matsukata, H., Takezawa, K. & Ishikawa, E. (1972) *Biochim. Biophys. Acta* **279**, 234–244
- Albano, J. D. M., Ekins, R. P., Maritz, G. & Turner, R. C. (1972) *Acta Endocrinol.* **70**, 487–509
- Balasse, E. O. & Neef, M. A. (1975) *Metabolism* **24**, 999–1007
- Beatty, C. H., Peterson, R. D., Bocek, R. M. & West, E. S. (1959) *J. Biol. Chem.* **234**, 11–16
- Blackshear, P. J., Holloway, P. A. H. & Alberti, K. G. M. M. (1974) *Biochem. J.* **142**, 279–286
- Felig, P., Marliss, E., Owen, O. E. & Cahill, G. F. (1969) *Adv. Enzyme Regul.* **7**, 41–46.
- Felig, P., Pozefsky, T. & Marliss, E. (1970) *Science* **167**, 1003–1004
- Foster, D. W. (1967) *J. Clin. Invest.* **46**, 1283–1296
- Friedrichs, D. & Schoner, W. (1974) *Biochim. Biophys. Acta* **343**, 341–355
- Garber, A. J., Karl, I. E. & Kipnis, D. M. (1976) *J. Biol. Chem.* **251**, 826–835
- Genuth, S. M. (1973) *Metabolism* **22**, 927–937
- Genuth, S. M. & Castro, J. (1974) *Metabolism* **23**, 375–386
- Girard, J., Bal, D., & Assan, R. (1972) *Horm. Metab. Res.* **4**, 168–170
- Girard, J. R., Cuendet, G. S., Marliss, E. B., Kervran, A., Rieutort, M. & Assan, R. (1973) *J. Clin. Invest.* **52**, 3190–3200
- Heding, L. G. (1971) *Diabetologia* **7**, 10–19
- Hellman, D. E., Senior, B. & Goodman, H. M. (1969) *Metabolism* **18**, 906–915
- Karl, I. E., Garber, A. J. & Kipnis, D. M. (1976) *J. Biol. Chem.* **251**, 844–850
- Krebs, H. A. (1972) *Adv. Enzyme Regul.* **10**, 392–420
- Lauwreys, R. R. (1969) *Anal. Biochem.* **32**, 331–333
- Madison, L. L., Mebane, D., Unger, R. H. & Lochner, A. (1964) *J. Clin. Invest.* **43**, 408–415
- McAllister, A., Allison, S. P. & Randle, P. J. (1973) *Biochem. J.* **134**, 1067–1081
- McGarry, J. D. & Foster, D. W. (1972) *Metabolism* **21**, 471–489
- Meier, J. M., McGarry, J. D., Faloona, G. R., Unger, R. H. & Foster, D. W. (1972) *J. Lipid Res.* **13**, 228–233
- Miller, E. A. & Allen, D. O. (1973) *J. Lipid Res.* **14**, 331–336
- Muller, W. A., Faloona, G. R. & Unger, R. H. (1971) *J. Clin. Invest.* **50**, 2215–2218
- Ontko, J. A. (1972) *J. Biol. Chem.* **247**, 1788–1800
- Ozand, P. T., Tildon, J. T., Wapnir, R. A. & Cornblath, M. (1973) *Biochem. Biophys. Res. Commun.* **53**, 251–257
- Ozand, P. T., Hawkins, R. L., Collins, Jr., R. M., Tildon, J. T. & Cornblath, M. (1976a) *Biochem. Med.* **14**, 170–183
- Ozand, P. T., Tildon, J. T., Stevenson, J. H., Reed, W. D. & Cornblath, M. (1976b) *Am. J. Physiol.* **230**, 1385–1388
- Rieutort, M. (1970) *C.R. Hebd. Séances Acad. Sci. Ser. D* **274**, 3589–3592
- Ruderman, N. B. & Lund, P. (1972) *Isr. J. Med. Sci.* **8**, 295–302
- Senior, B. & Loridan, L. (1968) *Nature (London)* **219**, 83–84
- Snell, K. & Walker, D. G. (1973) *Enzyme* **15**, 40–81
- Vaughan, M. (1961) *J. Biol. Chem.* **236**, 2196–2199
- Webster, M. E. & Prado, E. S. (1970) *Methods Enzymol.* **19**, 681–699
- White, C. (1951) *Med. J. Austr.* **2**, 797–804
- Whitehouse, S., Cooper, R. H. & Randle, P. J. (1974) *Biochem. J.* **141**, 761–774
- Williamson, D. H. & Hems, R. (1970) in *Essays in Cell Metabolism* (Bartley, W., Kornberg, H. L. & Quayle, J. R., eds.), pp. 257–281, Wiley-Interscience, New York
- Williamson, D. H., Veloso, D., Ellington, E. V. & Krebs, H. A. (1969) *Biochem. J.* **114**, 575–584
- Williamson, J. R. (1967) *Adv. Enzyme Regul.* **5**, 229–255
- Williamson, J. R., Browning, E. T. & Scholz, R. (1969) *J. Biol. Chem.* **244**, 4607–4616
- Winder, W. W., Baldwin, K. M. & Holloszy, J. O. (1975) *Can. J. Physiol. Pharmacol.* **53**, 86–91
- Wise, J. K., Hendler, R. & Felig, P. (1972) *Science* **178**, 513–514
- Wise, J. K., Hendler, R. & Felig, P. (1973a) *N. Engl. J. Med.* **288**, 487–490
- Wise, J. K., Lyall, S. S., Hendler, R. & Felig, P. (1973b) *J. Clin. Endocrinol. Metab.* **37**, 341–344
- Yeh, Y. Y. & Zee, P. (1976) *Pediat. Res.* **10**, 192–197
- Yoshida, A. & Freese, E. (1970) *Methods Enzymol.* **17A**, 176–181