Effects of Ketone Bodies on Amino Acid Metabolism in Isolated Rat Diaphragm

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(Received 15 October 1975)

1. Diaphragms from 48h-starved rats were incubated in Krebs–Ringer bicarbonate medium at 37°C for 30min and then transferred into new medium and incubated for 1, 2 and 3h. 2. The amount of free amino acids found at the end of each time of incubation was larger than the amount at the beginning of incubation, indicating that in this system proteolysis is prevailing. 3. The diaphragms were releasing mainly alanine and glutamine into the incubation medium. 4. Within the periods of incubation the release and metabolism of free amino acids was proceeding at a constant rate. 5. Addition of sodium DL-3-hydroxybutyrate decreased the tissue content of several amino acids, among which were tyrosine and phenylalanine, suggesting that proteolysis was decreased by ketone bodies. 6. In the presence of glucose (10mM) and branched-chain amino acids (0.5mM), sodium DL-3-hydroxybutyrate at concentrations of 4 or 6mM resulted in a 30% decrease in tissue alanine content and a 20% decline in alanine release. Release of taurine and glutamine was decreased by 19 and 16% respectively with 6mM-sodium DL-3-hydroxybutyrate. Addition of sodium acetoacetate (1–3mM) also resulted in a 20–35% decrease in tissue content of alanine, glutamine and taurine and in a 15–24% decrease of alanine and glutamine release. Smaller decreases (<15%) in the release of glycine, threonine, proline, serine and aspartate were also observed in the presence of sodium DL-3-hydroxybutyrate or sodium acetoacetate. 7. Substitution of pyruvate (1.0mM) for glucose in the presence of acetoacetate restored alanine and glutamine production to control values. In the presence of acetoacetate, pyruvate also increased the tissue content of aspartate by 77% and decreased the tissue content of glutamate by 30%. 8. It is suggested that in diaphragms from starved rats, ketone bodies (a) in the absence of other substrates inhibit protein catabolism and (b) in the presence of glucose and branched-chain amino acids decrease alanine and glutamine production, by inhibiting glycolysis.

Alanine and glutamine are the predominant amino acids released by human skeletal muscle (Pozefsky *et al.*, 1969; Felig *et al.*, 1970, 1973), by isolated perfused rat hindquarter (Ruderman & Berger, 1974) and by the incubated rat diaphragm (Odessey *et al.*, 1974). Odessey *et al.* (1974) showed that the addition of branched-chain amino acids at physiological concentrations increased the production of alanine from rat diaphragm and they proposed a supplement to the 'glucose–alanine cycle' (Mallette *et al.*, 1969; Felig *et al.*, 1970; Felig, 1973), namely that the synthesis *de novo* of alanine in skeletal muscle is brought about by the coupling between catabolism of the branched-chain amino acids and alanine synthesis. In prolonged starvation a decrease in alanine output from muscle is the factor responsible for a progressive diminution in gluconeogenesis (Felig *et al.*, 1969; Palaiologos *et al.*, 1969; Felig *et al.*, 1970). Sherwin *et al.* (1975) demonstrated

that infusion of sodium DL-3-hydroxybutyrate resulted in a specific decline in plasma alanine comparable with that observed in starvation. In addition, the ketone infusion caused a 30% decline in urinary nitrogen excretion. These authors suggested that hyperketonaemia may be responsible for diminished muscle alanine output in starvation. The present study was consequently undertaken to investigate the effect of DL-3-hydroxybutyrate or acetoacetate at physiological concentrations on the production of amino acids by the diaphragm of the starved rat *in vitro*.

Materials and Methods

Animals and reagents

Male Wistar rats were from Microbiologica Associates (Bethesda, MD, U.S.A.) and starved for 48h. Water was given *ad libitum*. At the time of

death the rats weighed 70–110g. Sodium acetoacetate was prepared by hydrolysis of freshly distilled ethyl acetoacetate (Davies, 1943). The presence of ethanol in the acetoacetate solution thus produced was excluded by g.l.c. Sodium DL-3-hydroxybutyrate was from Nutritional Biochemical Corp. (Cleveland, OH, U.S.A.). Sodium pyruvate (type II) was from Sigma Chemical Co. (St. Louis, MO, U.S.A.). L-Leucine, L-isoleucine and L-valine were from Schwartz/Mann (Orangeburg, NY, U.S.A.). 3-Hydroxybutyrate dehydrogenase and NADH (disodium salt; grade II) were from Boehringer (Mannheim) Corp. (New York, NY, U.S.A.). All other chemicals were from Mallinckrodt (St. Louis, MO, U.S.A.) and were of the highest purity available.

Preparation and incubation of rat diaphragms

Beginning at 9:00 a.m. each of five rats was killed by a sharp blow on the neck. The diaphragm was removed, placed in a Petri dish containing 5ml of freshly prepared Krebs–Ringer bicarbonate solution saturated with O₂+CO₂ (95:5), pH 7.4 (DeLuca & Cohen, 1964). The diaphragm was rapidly cut into quarters; each was immersed in fresh Krebs–Ringer bicarbonate solution, blotted on filter paper, weighed and placed in a 25ml Erlenmeyer flask containing 3ml of Krebs–Ringer bicarbonate. All solutions were kept at ice-bath temperature. This procedure took less than 5min for each rat from the time of death; 16 Erlenmeyer flasks were used (for each experiment involving five rats), each of which contained 30–50mg of tissue. The flasks were gassed for 3min with O₂+CO₂ (95:5), stoppered tightly and incubated in a Dubnoff metabolic shaking incubator (Precision Scientific Co., Chicago, IL, U.S.A.) for 30min at 37°C and 120rev./min. After this initial incubation (which was taken as zero time), the diaphragms were placed in 3ml of fresh Krebs–Ringer bicarbonate buffer for 0, 1, 2 or 3h, after which the tissues were homogenized and deproteinized for amino acid determination. In some of the experiments various substrates were added at zero time, as indicated in the Tables, to the following concentrations: glucose (10mM); L-valine (0.5mM); L-leucine (0.5mM) and L-isoleucine (0.5mM); sodium DL-3-hydroxybutyrate (2, 4 or 6mM); sodium acetoacetate (1 or 3mM); sodium pyruvate (1mM). After incubation with these substrates for 2h the tissues were prepared for amino acid analysis.

Treatment of tissue and media for amino acid analysis

For the determination of the free amino acid content in the tissues and in the incubation medium the tissues and media were treated by the method of Fulks *et al.* (1975). Quarter diaphragms from four flasks, consisting of approx. 160mg of tissue, were

pooled and immersed quickly in 5ml of Krebs–Ringer bicarbonate, blotted lightly and placed in a Dual glass homogenizer (size 22; Kontes Glass Co., Vineland, NJ, U.S.A.) containing 4ml of 0.01M-potassium phosphate buffer, pH 7.4, at ice-bath temperature and homogenized. The homogenate was decanted into a 12ml centrifuge tube and the homogenizer was washed twice with 2ml of potassium phosphate buffer. The combined 8ml (homogenate and washings) was deproteinized with 2ml of 50% (w/v) trichloroacetic acid. For the determination of the free amino acid concentration in the incubation medium, media from four flasks were combined. An 8ml sample was deproteinized with 2ml of 50% (w/v) trichloroacetic acid. After centrifugation (10000g-min), the supernatants of both the tissues and media were extracted twice with 12ml of water-saturated ether and freeze-dried. The residue was dissolved in 0.5ml of 67mM-sodium citrate buffer, pH 2.2, and analysed for acidic and neutral amino acids on a Beckman model 119 amino acid analyser by the method of Spackman *et al.* (1958).

Calculation of the proportion of each amino acid undergoing catabolism

From the amino acid composition of diaphragm protein, as reported by Odessey *et al.* (1974), and on the assumption that tyrosine is not metabolized in this tissue (Li *et al.*, 1973), the total amount of each free amino acid expected in the system (tissue plus medium) after incubation was calculated on the basis of the increase in free tyrosine. By comparing this predicted amount with the actual amount observed after each incubation time, the amount of each free amino acid that underwent net catabolism was estimated. Specifically, if the tissue content of a given free amino acid before incubation was A nmol/mg wet wt. of tissue and the amount of free amino acid found in the tissue plus medium at the end of incubation was B nmol/mg wet wt. of tissue then the actual amount of free amino acid added to the system would be B–A = C nmol/mg wet wt. of tissue. Since diaphragm protein contains 2.7mol per cent of tyrosine and, e.g. 9.5mol per cent of alanine, then the amount of alanine predicted to have been added to the system from proteolysis (D) may be calculated as:

$$D = \frac{9.5}{2.7} \times \text{amount of tyrosine added}$$

The percentage of alanine undergoing net catabolism (E) may be calculated as:

$$E = \frac{D - C}{D} \times 100$$

Table 1. Free amino acid production and release by the isolated rat diaphragm

Quarter diaphragms (30–50mg) were preincubated for 30min in 3ml of Krebs–Ringer bicarbonate solution, pH7.4, at 37°C and gassed with O₂+CO₂ (95:5). Incubation lasted for 0, 1, 2 and 3h in media that were identical with the preincubation media. The results (nmol/mg wet wt. of tissue) are given as the mean of 11 observations±S.E.M. N.D., concentration was not determined, because values were below the sensitivity of the assay.

Time of incubation (h)	Amino acid (nmol/mg wet wt.)						
	Tissue plus media				Media		
	... 0	1	2	3	1	2	3
Asp	0.28±0.02	0.56±0.04	0.76±0.05	0.85±0.06	0.33±0.05	0.56±0.04	0.70±0.05
Thr	0.12±0.01	0.46±0.03	0.74±0.05	0.91±0.10	0.39±0.03	0.69±0.06	0.88±0.08
Ser	0.19±0.02	0.77±0.05	1.05±0.05	1.28±0.08	0.56±0.04	0.94±0.07	1.14±0.09
Gln	0.50±0.10	0.90±0.17	1.29±0.23	1.72±0.26	0.83±0.17	1.11±0.23	1.52±0.22
Pro	N.D.	N.D.	N.D.	N.D.	0.34±0.03	0.59±0.07	0.63±0.06
Glu	0.49±0.06	0.90±0.08	1.04±0.06	1.04±0.11	0.52±0.06	0.80±0.07	0.92±0.08
Gly	0.30±0.04	0.64±0.06	0.88±0.09	1.14±0.11	0.42±0.05	0.77±0.09	1.02±0.11
Ala	0.14±0.02	0.74±0.05	1.22±0.10	1.57±0.11	0.63±0.05	1.14±0.12	1.53±0.11
Val	0.054±0.005	0.48±0.04	0.77±0.07	1.06±0.10	0.46±0.04	0.74±0.07	1.02±0.09
Met	0.048±0.009	0.19±0.01	0.40±0.03	0.46±0.05	0.16±0.02	0.37±0.03	0.42±0.05
Ile	0.040±0.005	0.34±0.03	0.61±0.05	0.76±0.03	0.28±0.03	0.56±0.05	0.73±0.04
Leu	0.059±0.007	0.61±0.06	1.12±0.08	1.45±0.08	1.52±0.05	1.05±0.08	1.38±0.08
Tyr	0.058±0.008	0.41±0.02	0.62±0.03	0.76±0.03	0.34±0.02	0.59±0.04	0.73±0.03
Phe	0.051±0.005	0.42±0.02	0.64±0.04	0.82±0.04	0.38±0.03	0.62±0.05	0.80±0.05

Results

Production, release and catabolism of free amino acids by rat diaphragm

Table 1 shows the content of free amino acids in diaphragms at zero time (after the 30min preincubation), the content in diaphragms plus medium and the release of amino acids into the incubation medium at the end of 1, 2 and 3h of incubation. In Table 1 it is shown that the content of each free amino acid is increasing progressively with time of incubation and it is always larger than the initial content. The data, particularly those concerning the essential amino acids, show that under the conditions of the experiment proteolysis is prevailing. The release of free amino acids into the incubation medium increases for the first 2h and at 3h tends to stabilize. Glutamine and alanine are the amino acids released in greatest amounts.

In Table 2 the calculated (see the Materials and Methods section) proportion of each of the free amino acids undergoing catabolism is shown. For each amino acid the estimated proportion undergoing net catabolism, which varied from 11 to 73%, was fairly constant over the 3h period of observation.

Effect of ketone bodies on the tissue content and release into the medium of free amino acids

Table 3 shows the effect of DL-3-hydroxybutyrate on the tissue content and release of free amino acids. Table 3 shows that DL-3-hydroxybutyrate, in the absence of other substrates, decreases the tissue

Table 2. Calculated percentage of catabolism of each free amino acid after incubation of rat diaphragm for 1, 2 and 3h

Conditions are described in Table 1. The procedure for computing the percentage of catabolism of each free amino acid is shown in the Materials and Methods section.

Amino acid	Percentage of catabolism		
	1h	2h	3h
Ala	40	42	41
Glu+Gln	40	47	47
Leu	40	38	39
Ile	45	46	46
Val	40	42	38
Asp	73	72	72
Thr	36	37	38
Ser	11	14	16
Gly	68	71	68
Phe	14	17	18

content and release of several amino acids. Noteworthy is the 45% decrease in the tissue content of tyrosine and phenylalanine in the presence of 4 and 6mM-DL-3-hydroxybutyrate. These data suggest that DL-3-hydroxybutyrate decreases proteolysis under these conditions. The amino acid showing the largest decrease in tissue content was alanine, which fell by 56% in the presence of 6mM-DL-3-hydroxybutyrate. The release of free amino acids into the incubation medium was either not affected or minimally altered by the addition of ketones.

Table 3. *Effect of sodium DL-3-hydroxybutyrate on the content and release of free amino acids of the isolated rat diaphragm*

Quarter diaphragms were preincubated for 30min and incubated for 2h as described in Table 1 except that during the incubation the media contained 0, 2, 4 or 6mM-sodium DL-3-hydroxybutyrate. The results (nmol/mg wet wt.) are given as means of four observations $\pm$ s.e.m. *P* values refer to significance of differences from control observations as calculated by paired Student's *t* test. N.D., concentration was not determined, because the values were either below the sensitivity of the assay or the peaks were poorly resolved.

Sodium DL-3-hydroxybutyrate (mM) ...	Amino acid (nmol/mg wet wt.)							
	Tissue				Media			
	0 (Control)	2	4	6	0 (Control)	2	4	6
Taurine	0.47 $\pm$ 0.06	0.32 $\pm$ 0.05 <i>P</i> < 0.05	0.32 $\pm$ 0.05 <i>P</i> < 0.02	0.28 $\pm$ 0.06	0.69 $\pm$ 0.12	0.40 $\pm$ 0.07 <i>P</i> < 0.02	0.36 $\pm$ 0.11 <i>P</i> < 0.05	0.33 $\pm$ 0.08 <i>P</i> < 0.05
Asp	0.24 $\pm$ 0.03	0.18 $\pm$ 0.03	0.17 $\pm$ 0.04	0.16 $\pm$ 0.04 <i>P</i> < 0.05	0.53 $\pm$ 0.03	0.52 $\pm$ 0.01	0.47 $\pm$ 0.03	0.48 $\pm$ 0.02 <i>P</i> < 0.05
Thr	0.07 $\pm$ 0.005	0.06 $\pm$ 0.01	0.06 $\pm$ 0.01	0.05 $\pm$ 0.01	0.71 $\pm$ 0.02	0.65 $\pm$ 0.04	0.62 $\pm$ 0.04	0.61 $\pm$ 0.04 <i>P</i> < 0.05
Ser	0.12 $\pm$ 0.008	0.10 $\pm$ 0.002	0.09 $\pm$ 0.01	0.09 $\pm$ 0.02	1.04 $\pm$ 0.06	0.99 $\pm$ 0.05	0.91 $\pm$ 0.03	0.97 $\pm$ 0.06 <i>P</i> < 0.01
Gln	0.09 $\pm$ 0.01	N.D.	0.05 $\pm$ 0.01	N.D.	1.32 $\pm$ 0.05	N.D.	N.D.	0.78 $\pm$ 0.1 <i>P</i> < 0.02
Pro	N.D.	N.D.	N.D.	N.D.	0.51 $\pm$ 0.005	0.48 $\pm$ 0.04	0.47 $\pm$ 0.04	0.48 $\pm$ 0.03
Glu	0.22 $\pm$ 0.006	0.17 $\pm$ 0.02	0.17 $\pm$ 0.03	0.13 $\pm$ 0.06 <i>P</i> < 0.05	0.84 $\pm$ 0.05	0.81 $\pm$ 0.03	0.83 $\pm$ 0.08	0.85 $\pm$ 0.12
Gly	0.13 $\pm$ 0.007	0.10 $\pm$ 0.013 <i>P</i> < 0.05	0.10 $\pm$ 0.013 <i>P</i> < 0.05	0.09 $\pm$ 0.025	0.76 $\pm$ 0.04	0.66 $\pm$ 0.04	0.66 $\pm$ 0.07	0.64 $\pm$ 0.07
Ala	0.063 $\pm$ 0.006	0.047 $\pm$ 0.003	0.045 $\pm$ 0.005	0.027 $\pm$ 0.007 <i>P</i> < 0.05	1.24 $\pm$ 0.10	1.20 $\pm$ 0.07	1.15 $\pm$ 0.07	1.14 $\pm$ 0.11
Val	N.D.	N.D.	N.D.	N.D.	0.77 $\pm$ 0.03	0.76 $\pm$ 0.03	0.79 $\pm$ 0.03	0.73 $\pm$ 0.05
Met	N.D.	N.D.	N.D.	N.D.	0.34 $\pm$ 0.03	0.32 $\pm$ 0.03	0.32 $\pm$ 0.03	0.31 $\pm$ 0.02
Ile	N.D.	N.D.	N.D.	N.D.	0.57 $\pm$ 0.04	0.58 $\pm$ 0.04	0.57 $\pm$ 0.04	0.56 $\pm$ 0.03
Leu	0.042 $\pm$ 0.005	0.027 $\pm$ 0.005	0.024 $\pm$ 0.002 <i>P</i> < 0.02	0.024 $\pm$ 0.003 <i>P</i> < 0.01	1.09 $\pm$ 0.08	1.11 $\pm$ 0.07	1.11 $\pm$ 0.07	1.11 $\pm$ 0.05
Tyr	0.041 $\pm$ 0.005	0.032 $\pm$ 0.003	0.023 $\pm$ 0.001 <i>P</i> < 0.05	0.023 $\pm$ 0.001 <i>P</i> < 0.05	0.63 $\pm$ 0.03	0.62 $\pm$ 0.03	0.60 $\pm$ 0.02	0.59 $\pm$ 0.01
Phe	0.045 $\pm$ 0.005	0.031 $\pm$ 0.005	0.026 $\pm$ 0.0005 <i>P</i> < 0.05	0.021 $\pm$ 0.001 <i>P</i> < 0.05	0.66 $\pm$ 0.03	0.66 $\pm$ 0.03	0.64 $\pm$ 0.02	0.63 $\pm$ 0.01

Effect of ketone bodies on the release of alanine stimulated by glucose and branched-chain amino acids

Odessey *et al.* (1974) showed that the addition of glucose (10mM) and branched-chain amino acids (0.5mM) to the incubation medium of diaphragms from starved rats increased the production of alanine, glutamate and glutamine. This effect was observed also in the present experiments. In the presence of glucose and branched-chain amino acids, the release of alanine, glutamate and glutamine increased by 125, 50 and 54% respectively (Table 4), compared with diaphragms incubated without these substrates (Table 3). The effects of sodium DL-3-hydroxybutyrate or sodium acetoacetate on free amino acid content and release into the medium in this system are shown in Tables 4 and 5. In the presence of DL-3-hydroxybutyrate or acetoacetate the release into the media and the tissue content of alanine fell by 20–35%. The tissue content of glutamine was also decreased

by 20–30% in the presence of acetoacetate (Table 5). Smaller decreases (13–19%) were noted in the release of glutamine and taurine. The release and tissue content of the remaining amino acids was either unaffected or minimally altered (<10%).

Effect of sodium acetoacetate on free amino acid production when pyruvate is substituted for glucose

To clarify the mechanism of the inhibitory effect of ketones on alanine production, the effect of substituting pyruvate (1mM) for glucose in the incubation medium was studied. Diaphragms were incubated with 0.5mM each of L-leucine, L-isoleucine and L-valine, with the following additions: 10mM-glucose; 10mM-glucose plus 1mM-acetoacetate; 1mM-pyruvate; 1mM-pyruvate plus 1mM-acetoacetate. As shown in Table 6, in the absence of acetoacetate the production (tissue plus medium) of alanine is the same with either glucose or pyruvate. However, in contrast with its effects when glucose is present,

Table 4. *Effect of sodium DL-3-hydroxybutyrate on the content and release of free amino acids of the isolated rat diaphragm in the presence of glucose (10mM) and branched-chain amino acids (0.5mM)*

Quarter diaphragms (30–50mg) were preincubated for 30min in 3ml of Krebs–Ringer bicarbonate solution containing 10mM-glucose, pH7.4, at 37°C, and gassed with O₂+CO₂ (95:5). Incubation lasted for 2h in media that were identical with the preincubation media, with the addition of 0.5mM of each of L-leucine, L-isoleucine and L-valine and 0, 2, 4 or 6mM-sodium DL-3-hydroxybutyrate. The results (nmol/mg wet wt.) are given as means±S.E.M. of 12 observations for the control and 6mM-hydroxybutyrate experiments and of six observations for the 2 and 4mM-hydroxybutyrate experiments. *P* values refer to significance of differences from control observations as calculated by paired Student's *t* test. N.D., concentration was not determined because the values were below the sensitivity of the assay.

Sodium DL-3-hydroxybutyrate (mM) ...	Amino acid (nmol/mg wet wt.)							
	Tissue				Media			
	0 (Control)	2	4	6	0 (Control)	2	4	6
Taurine	0.97±0.06	0.86±0.10	1.03±0.10	0.97±0.10	1.05±0.08	1.08±0.10	0.94±0.12	0.85±0.06 <i>P</i> <0.05
Asp	0.32±0.02	0.34±0.05	0.34±0.05	0.28±0.01	0.40±0.01	0.37±0.03	0.38±0.01	0.36±0.01 <i>P</i> <0.01
Thr	0.054±0.004	0.062±0.005	0.053±0.008	0.061±0.006	0.85±0.02	0.84±0.04	0.80±0.03	0.84±0.04
Ser	0.12±0.01	0.09±0.01	0.08±0.01	0.11±0.01	1.16±0.06	1.10±0.08	1.00±0.07	1.05±0.05
Gln	0.19±0.01	0.18±0.03	0.15±0.02	0.16±0.02	2.03±0.15	1.80±0.26	1.50±0.19	1.70±0.18 <i>P</i> <0.02
Pro	N.D.	N.D.	N.D.	N.D.	0.65±0.02	0.59±0.02	0.57±0.05	0.61±0.03
Glu	0.68±0.03	0.74±0.09	0.68±0.11	0.71±0.08	1.24±0.06	1.24±0.08	1.09±0.07	1.15±0.06
Gly	0.22±0.01	0.20±0.02	0.23±0.02	0.21±0.01	0.98±0.04	0.93±0.03	0.93±0.08	0.88±0.03 <i>P</i> <0.02
Ala	0.27±0.01	0.20±0.02 <i>P</i> <0.02	0.20±0.02 <i>P</i> <0.02	0.18±0.02 <i>P</i> <0.005	2.82±0.10	2.51±0.21	2.15±0.11 <i>P</i> <0.005	2.23±0.11 <i>P</i> <0.001
Met	N.D.	N.D.	N.D.	N.D.	0.45±0.02	0.45±0.02	0.43±0.02	0.44±0.01
Tyr	0.030±0.006	0.029±0.002	0.025±0.005	0.026±0.002	0.57±0.01	0.55±0.03	0.55±0.02	0.55±0.02
Phe	0.028±0.002	0.028±0.002	0.024±0.001	0.028±0.002	0.62±0.02	0.58±0.04	0.59±0.02	0.59±0.02

Table 5. *Effect of sodium acetoacetate on the content and release of free amino acids of the isolated rat diaphragm in the presence of glucose (10mM) and branched-chain amino acids (0.5mM)*

Quarter diaphragms were preincubated and incubated as described in Table 4 except that the incubation medium contained 0, 1 or 3mM-sodium acetoacetate. The results are given as means of six observations±S.E.M. *P* values refer to significance of differences from control observations as calculated by paired Student's *t* test. N.D., concentrations not determined, because the values were below the sensitivity of the assay.

Sodium acetoacetate (mM) ...	Amino acid (nmol/mg wet wt.)					
	Tissue			Media		
	0 (Control)	1	3	0 (Control)	1	3
Taurine	0.92±0.07	0.68±0.15 <i>P</i> <0.05	0.86±0.06	0.89±0.05	0.66±0.01	0.81±0.05
Asp	0.30±0.03	0.27±0.04	0.26±0.02	0.42±0.01	0.38±0.01	0.39±0.02
Thr	0.060±0.006	0.058±0.007	0.057±0.006	0.96±0.06	0.84±0.05 <i>P</i> <0.005	0.85±0.03 <i>P</i> <0.05
Ser	0.12±0.01	0.10±0.01	0.11±0.01	1.25±0.09	1.12±0.07 <i>P</i> <0.01	1.14±0.05
Gln	0.20±0.01	0.14±0.02 <i>P</i> <0.05	0.16±0.02 <i>P</i> <0.05	2.25±0.14	1.87±0.26 <i>P</i> <0.05	1.95±0.16 <i>P</i> <0.05
Pro	N.D.	N.D.	N.D.	0.66±0.03	0.58±0.02 <i>P</i> <0.05	0.58±0.03
Glu	0.69±0.06	0.70±0.11	0.73±0.06	1.20±0.08	1.16±0.13	1.20±0.10
Gly	0.23±0.03	0.22±0.03	0.21±0.01	0.96±0.06	0.82±0.07 <i>P</i> <0.02	0.85±0.04
Ala	0.26±0.02	0.19±0.03 <i>P</i> <0.02	0.17±0.01 <i>P</i> <0.001	2.90±0.17	2.26±0.16 <i>P</i> <0.001	2.21±0.05 <i>P</i> <0.005
Met	N.D.	N.D.	N.D.	0.47±0.02	0.47±0.01	0.46±0.03
Tyr	0.032±0.001	0.030±0.004	0.028±0.003	0.65±0.04	0.60±0.03	0.61±0.02
Phe	0.030±0.001	0.030±0.005	0.027±0.004	0.71±0.04	0.67±0.03	0.69±0.02

Table 6. Effect of sodium acetoacetate on the content and release of free amino acids of the isolated rat diaphragm when pyruvate is substituted for glucose

Quarter diaphragms were preincubated and incubated as described in Table 4. The incubation medium contained 0.5 mM of each of L-leucine, L-isoleucine and L-valine and: 10 mM-glucose; 10 mM-glucose plus 1 mM-sodium acetoacetate; 1 mM-sodium pyruvate; 1 mM-sodium pyruvate plus 1 mM-sodium acetoacetate. The results (nmol/mg wet wt.) are given as means of six observations $\pm$ S.E.M. *P* values refer to significance of differences as calculated by paired Student's *t* test.

	Amino acid (nmol/mg wet wt.)							
	Tissue				Media			
	Glucose (10 mM)		Pyruvate (1 mM)		Glucose (10 mM)		Pyruvate (1 mM)	
	Control	+Acetoacetate (1 mM)	Control	+Acetoacetate (1 mM)	Control	+Acetoacetate (1 mM)	Control	+Acetoacetate (1 mM)
Taurine	1.13 $\pm$ 0.07	0.91 $\pm$ 0.08 <i>P</i> ₁ * < 0.02	0.78 $\pm$ 0.05	0.99 $\pm$ 0.08 <i>P</i> ₂ * < 0.01 <i>P</i> ₃ * < 0.02	1.38 $\pm$ 0.20	0.94 $\pm$ 0.13 <i>P</i> ₁ < 0.02	0.97 $\pm$ 0.15	1.08 $\pm$ 0.13 <i>P</i> ₂ < 0.05
Asp	0.38 $\pm$ 0.02	0.31 $\pm$ 0.03	0.46 $\pm$ 0.03	0.54 $\pm$ 0.04 <i>P</i> ₄ * < 0.001	0.40 $\pm$ 0.02	0.36 $\pm$ 0.02	0.39 $\pm$ 0.01	0.40 $\pm$ 0.02
Thr	0.064 $\pm$ 0.005	0.048 $\pm$ 0.003	0.053 $\pm$ 0.003	0.060 $\pm$ 0.007	0.85 $\pm$ 0.03	0.84 $\pm$ 0.02	0.85 $\pm$ 0.02	0.85 $\pm$ 0.03
Ser	0.11 $\pm$ 0.01	0.10 $\pm$ 0.01	0.11 $\pm$ 0.01	0.10 $\pm$ 0.01	1.03 $\pm$ 0.04	1.04 $\pm$ 0.04	1.09 $\pm$ 0.04	1.06 $\pm$ 0.02
Gln	0.41 $\pm$ 0.03	0.33 $\pm$ 0.03 <i>P</i> ₁ < 0.05	0.27 $\pm$ 0.02	0.34 $\pm$ 0.03 <i>P</i> ₂ < 0.01	4.48 $\pm$ 0.20	3.89 $\pm$ 0.20 <i>P</i> ₁ < 0.01	3.70 $\pm$ 0.25	3.77 $\pm$ 0.22 <i>P</i> ₂ < 0.05
Glu	0.72 $\pm$ 0.04	0.72 $\pm$ 0.04	0.43 $\pm$ 0.03 <i>P</i> ₂ < 0.01	0.48 $\pm$ 0.03 <i>P</i> ₄ < 0.001	1.15 $\pm$ 0.05	1.09 $\pm$ 0.05	0.89 $\pm$ 0.05 <i>P</i> ₂ < 0.01	0.92 $\pm$ 0.03
Gly	0.24 $\pm$ 0.01	0.23 $\pm$ 0.01	0.24 $\pm$ 0.02	0.22 $\pm$ 0.01	1.02 $\pm$ 0.04	0.97 $\pm$ 0.03	0.96 $\pm$ 0.04	0.96 $\pm$ 0.02
Ala	0.32 $\pm$ 0.03	0.22 $\pm$ 0.02 <i>P</i> ₁ < 0.01	0.28 $\pm$ 0.03	0.27 $\pm$ 0.02 <i>P</i> ₄ < 0.02	2.78 $\pm$ 0.11	2.41 $\pm$ 0.07 <i>P</i> ₁ < 0.05	3.11 $\pm$ 0.18	2.96 $\pm$ 0.04 <i>P</i> ₂ < 0.05 <i>P</i> ₄ < 0.001
Met	0.036 $\pm$ 0.003	0.035 $\pm$ 0.003	0.038 $\pm$ 0.003	0.032 $\pm$ 0.004	0.46 $\pm$ 0.01	0.47 $\pm$ 0.02	0.51 $\pm$ 0.03	0.48 $\pm$ 0.01
Tyr	0.041 $\pm$ 0.002	0.037 $\pm$ 0.001	0.038 $\pm$ 0.002	0.034 $\pm$ 0.001	0.60 $\pm$ 0.03	0.63 $\pm$ 0.02	0.66 $\pm$ 0.02	0.63 $\pm$ 0.01
Phe	0.039 $\pm$ 0.002	0.035 $\pm$ 0.001	0.038 $\pm$ 0.002	0.031 $\pm$ 0.002	0.64 $\pm$ 0.03	0.67 $\pm$ 0.03	0.71 $\pm$ 0.02	0.67 $\pm$ 0.02

* *P*₁, comparison between glucose and glucose plus acetoacetate; *P*₂, comparison between pyruvate and glucose in the absence of acetoacetate; *P*₃, comparison between pyruvate and pyruvate plus acetoacetate; *P*₄, comparison between pyruvate plus acetoacetate and glucose plus acetoacetate.

acetoacetate fails to cause a decrease in the tissue content and release of alanine in the presence of pyruvate. It is noteworthy that there is an increase of 77% in the tissue content of aspartate in the presence of pyruvate plus acetoacetate when compared with tissues incubated in the presence of glucose plus acetoacetate. On the other hand, when pyruvate replaces glucose in the incubation medium, in the absence of acetoacetate, the tissue content and release of glutamine and glutamate are decreased by 20–40%.

Discussion

The present findings demonstrate that in the incubated rat diaphragm proteolysis is prevailing and that net catabolism of individual amino acids is proceeding at a fairly constant rate. In confirmation of earlier studies *in vivo* in man (Felig *et al.*, 1970, 1973) and previous studies *in vitro* with incubated rat diaphragm (Odessey *et al.*, 1974), alanine and glutamine were observed to be the amino acids released in greatest amounts.

Of particular interest was the observation that the addition of ketone bodies decreases the free amino acid content of the tissue as well as the release of amino acids into the medium. Since tyrosine, which is not catabolized in muscle, was among the amino acids affected, augmented amino acid catabolism cannot be invoked as an explanation for the ketone-induced decrease in free amino acid content. The alternative explanation, a decrease in net protein catabolism, is in keeping with the demonstration that the infusion of ketone bodies results in protein conservation in fasted human subjects (Sherwin *et al.*, 1975). Hyperketonaemia has also been associated with decreased protein breakdown in traumatized man (Smith *et al.*, 1975) and in post-operative patients (Hoover *et al.*, 1975). The current findings provide no data as to whether the protein-sparing effect of ketones involves stimulation of protein synthesis, decreased proteolysis or both. It should be noted, however, that Buse & Buse (1967) failed to observe an increase in [¹⁴C]leucine incorporation into diaphragm protein in the presence of sodium DL-3-hydroxybutyrate. The current findings thus may be explained on the

basis of ketone-induced inhibition of protein catabolism.

The stimulatory effect of glucose and branched-chain amino acids on net alanine release from diaphragm is consistent with previous observations by Odessey *et al.* (1974). These findings support the conclusion that alanine formation is related to glucose utilization (Felig, 1973), as well as branched-chain amino acid oxidation (Odessey *et al.*, 1974). The formation of alanine from glucose and branched-chain amino acids can be shown by the following reactions (Odessey *et al.*, 1974):

The glycolytic enzymes of reaction (1), the major portion of the enzyme system in reaction (2a) (Taylor & Jenkins, 1966; Adibi *et al.*, 1975) and part of the enzyme system involved in reaction (2b) reside in the cytosol. 4-Methyl-2-oxopentanoate and pyruvate formed by these reactions in the cytosol must enter the mitochondria to be further oxidized. 4-Methyl-2-oxopentanoate has been shown to inhibit competitively pyruvate transport into the mitochondria (Halestrap *et al.*, 1974; Mowbray, 1974; Land & Clark, 1974). Oxidation of branched-chain amino acids may thus contribute to alanine synthesis not only by providing a source of amino groups, but by increasing the pyruvate concentration in the cytosol.

In the presence of ketone bodies, stimulation of alanine production by glucose and branched-chain amino acids was substantially decreased. This observation is consistent with the demonstration that the plasma alanine concentration is decreased in man by infusion of ketones (Sherwin *et al.*, 1975). In addition, an inverse relation between plasma alanine concentration and blood ketones has been observed in traumatized patients (Smith *et al.*, 1975). Possible mechanisms by which ketone bodies decrease alanine production include: (a) inhibition of glycolysis; (b) stimulation of pyruvate oxidation; (c) inhibition of branched-chain amino acid catabolism. The present experiments (Table 6) showed that the inhibitory effect of ketone bodies on alanine synthesis is overcome by the substitution of pyruvate (1 mM) for glucose (10 mM). Further, in the absence of ketones the stimulatory effect of pyruvate on alanine accumulation and release is comparable with that of glucose (Table 6). These findings are thus compatible with the conclusion

that ketone bodies decrease alanine production in the isolated diaphragm by inhibiting glycolysis. Pertinent to the present findings are the studies of Newsholme & Randle (1964), Garland *et al.* (1964) and Randle *et al.* (1966) that ketone bodies inhibit glycolysis in the perfused rat heart. The possibility must also be considered that ketone bodies exert their effect on alanine synthesis in muscle by inhibiting branched-chain amino acid catabolism. Buse *et al.* (1972) showed that in diaphragms from fed rats 4 mM-sodium DL-3-hydroxybutyrate caused a mild but significant inhibition of $^{14}\text{CO}_2$ production from $[1\text{-}^{14}\text{C}]\text{leucine}$ and $[1\text{-}^{14}\text{C}]\text{valine}$.

The substitution of pyruvate for glucose in the incubation medium also resulted in an increase in tissue content of aspartate, which was accentuated by the presence of acetoacetate, and a decrease in tissue content and release of glutamine and glutamate. These findings may be explained on the basis of the fall in the NADH/NAD⁺ ratio of the cytosol induced by an increase in pyruvate availability. Such a change would favour the production of oxaloacetate by way of the cytosolic malate dehydrogenase reaction. Transamination of the oxaloacetate thus produced would result in an increase in cytosolic aspartate at the expense of glutamate and glutamine.

This investigation was supported by a United States Public Health Service Grant AM-13526 from the National Institutes of Health. We thank William Sweet and Ralph Jacob for their expert technical assistance.

References

- Adibi, S. A., Peterson, J. A. & Krzysik, B. A. (1975) *Am. J. Physiol.* **228**, 432–435
- Buse, M. G. & Buse, J. (1967) *Diabetes* **16**, 753–764
- Buse, M. G., Biggers, J. F., Friderici, K. H. & Buse, J. F. (1972) *J. Biol. Chem.* **247**, 8085–8096
- Davies, R. (1943) *Biochem. J.* **37**, 230–238
- DeLuca, H. F. & Cohen, P. P. (1964) in *Manometric Techniques* (Umbreit, W. W., Burris, R. H. & Stauffer, J. F., eds.), 4th edn., pp. 131–133, Burgess Publishing Company, Minneapolis
- Felig, P. (1973) *Metabolism* **22**, 179–207
- Felig, P., Owen, O. W., Wahren, J. & Cahill, G. F., Jr. (1969) *J. Clin. Invest.* **48**, 584–594
- Felig, P., Pozefsky, T., Marliss, E. & Cahill, G. F., Jr. (1970) *Science* **167**, 1003–1004
- Felig, P., Wahren, J. & Räf, L. (1973) *Proc. Natl. Acad. Sci. U.S.A.* **70**, 1775–1779
- Fulks, R. M., Li, J. B. & Goldberg, A. L. (1975) *J. Biol. Chem.* **250**, 290–298
- Garland, P. B., Newsholme, E. A. & Randle, P. J. (1964) *Biochem. J.* **93**, 665–678
- Halestrap, A. P., Brand, M. D. & Denton, R. M. (1974) *Biochim. Biophys. Acta* **367**, 102–108
- Hoover, H. C., Jr., Grant, J. P., Gorschboth, C. & Ketcham, A. S. (1975) *N. Engl. J. Med.* **293**, 172–175
- Land, J. M. & Clark, J. B. (1974) *FEBS Lett.* **44**, 348–351

- Li, J. B., Fulks, R. M. & Goldberg, A. L. (1973) *J. Biol. Chem.* **248**, 7272-7275
- Mallette, L. E., Exton, J. H. & Park, C. R. (1969) *J. Biol. Chem.* **244**, 5713-5723
- Mowbray, J. (1974) *FEBS Lett.* **44**, 344-347
- Newsholme, E. A. & Randle, P. J. (1964) *Biochem. J.* **93**, 641-651
- Odessey, R., Khairallah, E. A. & Goldberg, A. L. (1974) *J. Biol. Chem.* **249**, 7623-7629
- Palaiologos, G., Muntwyler, E. & Kesner, L. (1969) *Proc. Soc. Exp. Biol. Med.* **132**, 270-271
- Pozefsky, T., Felig, P., Tobin, J. D., Soeldner, J. S. & Cahill, G. F., Jr. (1969) *J. Clin. Invest.* **48**, 2273-2282
- Randle, P. J., Garland, P. B., Hales, C. N., Newsholme, E. A., Denton, R. M. & Pogson, C. I. (1966) *Recent Progr. Horm. Res.* **21**, 1-48
- Ruderman, N. B. & Berger, M. (1974) *J. Biol. Chem.* **249**, 5500-5506
- Sherwin, R. S., Hendler, R. G. & Felig, P. (1975) *J. Clin. Invest.* **55**, 1382-1390
- Smith, R., Fuller, D. J., Wedge, J. H., Williamson, D. H. & Alberti, K. G. G. M. (1975) *Lancet* **ii**, 1-3
- Spackman, D. H., Stein, W. H. & Moore, S. (1958) *Anal. Chem.* **30**, 1190-1206
- Taylor, R. T. & Jenkins, W. T. (1966) *J. Biol. Chem.* **241**, 4391-4395