De Novo Alanine Synthesis in Isolated Oxygen-deprived Rabbit Myocardium*

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De novo alanine synthesis was studied in oxygenated and hypoxic isolated rabbit right ventricular papillary muscle. Release of alanine into the incubation medium was linear for 60 min, while tissue levels remained constant following an early initial rise. Severe hypoxia (0 to 5% O₂) significantly enhanced net alanine production. Alanine release was accompanied by a fall in tissue glutamic acid levels. Proteinolysis could not account for enhanced alanine production during anoxia, as defined by estimation of the nonmetabolized amino acid tyrosine. Addition of pyruvate stimulated alanine production by oxygenated muscles. This effect was further augmented when muscles were exposed to nitrogen. Addition of aspartic acid (2 mm) to the incubation medium significantly augmented glutamate release into the medium but had no effect on alanine production. Addition of glutamic acid (0.5 mm) or leucine (0.5 mm) to the medium did not influence net production of alanine in oxygenated or anoxic papillary muscles. Inhibitors of the glycolytic pathway (2-deoxyglucose, iodoacetate, NaF) caused a decline in alanine production by approximately 50%. Alanine production was not affected by variation in media pH. De novo alanine synthesis was significantly reduced when the enzyme alanine aminotransferase was inhibited by L-cycloserine, while the inhibitor aminoxyacetic acid totally abolished alanine production by hypoxic muscles.

These studies suggest that increased alanine production in isolated right ventricular papillary muscles reflects de novo synthesis, is closely linked to intracellular pyruvate concentration, and is quantitatively related to myocardial oxygen deprivation.

De novo synthesis of alanine has been reported in a variety of skeletal muscle preparations (1-7). Interest in this phenomenon was initially prompted by the observation that large quantities of alanine appeared in venous blood draining the forearm musculature of fasting human subjects (1). This amount of alanine could not be accounted for by degradation of skeletal muscle protein, and subsequent studies have suggested that pyruvate is transaminated to form alanine. Newly synthesized alanine is then released into venous blood to be taken up by the liver where the amino group serves as a nitrogen donor for urea synthesis and the carbon skeleton is converted to glucose (8).

A second line of evidence suggesting yet another metabolic function for alanine is derived from studies of amino acid metabolism in oxygen-deprived tissues. Alanine has been identified as an end product of anaerobic metabolism in the blood of diving vertebrates (9), in ischemic rat liver (10), and in autolyzing rabbit heart (11). Similarly, enhanced myocardial alanine release and glutamate uptake were documented in patients with chronic ischemic heart disease (12). The latter observations gave rise to the hypothesis that alanine production and glutamate utilization in cardiac muscle are linked and in part regulated by factors related to the adequacy of myocardial oxygenation. The present study was designed to explore mechanisms of alanine synthesis and to quantitate the relationship between oxygen deprivation and myocardial alanine and glutamate acid metabolism.

MATERIALS AND METHODS

L-Alanine dehydrogenase (EC 1.4.1.1.), L-glutamate dehydrogenase (EC 1.4.1.3.), and beef heart L-lactate dehydrogenase (EC 1.1.1.27), as a crystalline suspension in 3.2 M ammonium sulfate, were obtained from Boehringer Mannheim Corp., Indianapolis, Ind. β-Nicotinamide adenine dinucleotide (NAD⁺), 2-iodoacetate, NaF, and aminoxyacetate were purchased from Sigma Chemical Co., St. Louis, Mo. Puromycin was obtained from Nutritional Biochemicals, Cleveland, Ohio. [U-14C]Tyrosine was obtained from New England Nuclear Corp., Boston, Mass. L-Cycloserine was a gift from Dr. W. E. Scott, Hoffmann-La Roche Inc., Nutley, N.J.

New Zealand White rabbits weighing from 2.0 to 2.5 kg were kept and fed as previously described (13). In selected experiments, food was withheld for 24 to 72 h before sacrifice. Rabbits were killed by a sharp blow to the head, the heart was excised, and right ventricular papillary muscles weighing 2 to 4 mg were dissected free. Only hearts containing three suitable papillary muscles were utilized so that a zero time control, an incubated control muscle, and an incubated experimental muscle could be obtained from a single animal to lessen differences due to inter-animal variation. Muscles were placed in small Pyrex test tubes containing 1 ml of Krebs-Ringer bicarbonate buffer without amino acids. Glucose concentration, pH, and per cent oxygen in the aerating gas was adjusted according to individual protocols. Incubations were carried out in a Dubnoff metabolic shaker at 37°. Control conditions were: pH 7.4, 90% oxygen, 5% CO₂, and 5 mm glucose. Media were equilibrated with the appropriate gas mixture and the incubation tubes were continuously stirred.

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gassed during the incubation as previously described (14). At the end of the incubation period, 0.8 ml of medium was sampled and added to 0.16 ml of cold 30% perchloric acid to destroy trace contaminations of the incubation period, 0.8 ml of medium was sampled and added to 0.16 ml of cold 30% perchloric acid to destroy trace contaminations of the incubation period. Media I represents accumulation of metabolites between 0 and 30 min and Media II represents accumulation of metabolites between 31 and 90 min of incubation. Oxygen denotes 95% O\textsubscript{2} and nitrogen denotes 95% N\textsubscript{2} in the gas phase (balance CO\textsubscript{2}).

Table I

| Experimental conditions | Lactate | Alanine | Glutamic acid |
|-------------------------|---------|---------|---------------|
|                         | Tissue  | Media I | Media II      |
| Zero time control       | 67.6 ± 12.3 | 18.4 ± 3.4 | 44.0 ± 4.6 |
| 5 mm glucose/oxygen     | 76.8 ± 27.6 | 15.4 ± 5.8 | 20.6 ± 9.6 |
| 5 mm glucose/nitrogen   | 147.1 ± 63.0 | 25.3 ± 5.8 | 12.3 ± 2.6 |

Values are the mean ± S. D. of six individual experiments. Incubations were carried out at 37° in Krebs-Ringer bicarbonate buffer for a total of 90 min. Media changes were after 30 min. Media I represents accumulation of metabolites between 0 and 30 min and Media II accumulation of metabolites between 31 and 90 min of incubation. Oxygen denotes 95% O\textsubscript{2} and nitrogen denotes 95% N\textsubscript{2} in the gas phase (balance CO\textsubscript{2}).

### Results

#### Validation of Muscle Preparation and Analytical Techniques

The isolated right ventricular papillary muscle has been used as an in vitro model for studies of myocardial amino acid transport (13), protein, lactate, and nucleotide metabolism and mechanical function (14, 16, 22) in protocols extending up to 6 h. Other laboratories have used the same preparation to study the relationship of high energy phosphate metabolism to contractile performance (23). Since the possibility remains that the subsequent results are influenced by leakage of amino acids from the small cut surface area created by the isolation of the muscles, we compared release of alanine, glutamic acid, and lactic acid from oxygenated and hypoxic papillary muscles (16). The method was straightforward and reproducible results from 1 to 15 nmol of glutamic acid by modifying the method of Bernt and Bergmeyer (18) as follows: (a) extinctions were measured fluorometrically rather than spectrophotometrically; (b) a Tris buffer system was substituted for glycine; (c) \( \beta \)-mercaptoethanol was used in place of adenosine diphosphate as an enzyme activator/stabilizer; and (d) hydrazine present from the alanine assay served as the trap for oxoglutarate. Lactic acid was determined fluorometrically on duplicate 100-\( \mu l \) aliquots of neutralized tissue extract by the method of Passonneau (19) using a protein powder buffer system.

In selected experiments, tyrosine was assayed by the method of Waalkes and Udenfried (20) after removing 300-\( \mu l \) aliquots of glutamic acid from either tissue or medium and diluting it to 1 ml with distilled water. This method was sensitive for 0.2 to 4 nmol of tyrosine.

In all assays, standard curves were constructed covering the concentration ranges described above and each sample determination was done in duplicate.

In experiments utilizing L-cycloserine, experimental animals were injected intraperitoneally with the drug following solubilization in 0.1 n NaOH and dilution in 0.9% NaCl. The dose was 25 mg/kg body weight and injection was done 1 h before killing. Control animals were injected with an equal volume of solvent. In experiments utilizing puromycin, the compound was dissolved in incubation buffer to a final concentration of 10\( \textsuperscript{7} \) M. This concentration inhibits protein synthesis in isolated papillary muscle (16). In experiments utilizing 2-deoxyglucose, the inhibitor concentration in the medium was 15 mm. Other inhibitors were added to the medium in concentrations listed with the individual experiments.

#### Data Analysis

All values are expressed as a function of tissue protein content (nanomoles/mg protein) whether derived from medium or tissue sample. Values are means ± S. D. Net production was calculated as:

\[
\text{Tissue content + media content} - \text{zero time control tissue content}
\]

and is based on 60 min incubation unless indicated otherwise.

#### Statistical Analysis

Statistical analysis of paired samples was performed with Student's \( t \) test (21).

### Discussion

#### Dietary Regulation

The dietary state of the experimental animal is recognized as an important factor in studies of amino acid metabolism (24, 25). Odessy et al. (4) incubated diaphragms from fasted rats and found enhanced breakdown of...
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FIG. 1. Tissue levels, release into media, and net production of alanine (A) and glutamic acid (B) in oxygenated and anoxic papillary muscles as a function of the duration of incubation. Right ventricular papillary muscles from fed animals were incubated in Krebs-Ringer bicarbonate buffer containing 5 mM glucose for 0, 15, 30, and 60 min. The gas phase was either 95% O₂ (○) or 95% N₂ (●).

TABLE II

Effect of starvation on tissue alanine and glutamic acid levels in papillary muscles

Animals were fasted for the period indicated and received water ad libitum. All values are mean ± S. D. of number of individual muscles obtained at zero time control immediately after sacrifice.

| Period of starvation | Number | Alanine nmol/mg protein | Glutamic acid nmol/mg protein |
|----------------------|--------|-------------------------|-----------------------------|
| Fed control          | 32     | 18.8 ± 4.2              | 48.9 ± 17.1                 |
| 24 h                 | 10     | 12.9 ± 1.0              | 42.8 ± 6.1                  |
| 72 h                 | 8      | 15.4 ± 3.0              | 18.1 ± 4.2                  |

* Denotes p < 0.01.

† Denotes p < 0.001.

branched chain amino acids associated with alanine formation. In contrast, starvation did not produce significant changes in alanine release but a decline of glutamate production in the rat epitrochlearis muscle preparation of Garber et al. (6). It is, however, postulated that starvation leads to enhanced protein breakdown and utilization of other fuel sources than carbohydrates. We estimated tissue alanine and glutamic acid levels in fresh papillary muscles obtained from animals deprived of food for 24 to 72 h before any intervention was carried out (Table II). Tissue alanine levels were slightly reduced after 24 and 72 h of starvation. More significant, however, was the decline of tissue glutamate levels after 72 h of fasting, indicating further metabolism of glutamate under these conditions. Because of the observed variability in metabolite levels, subsequent experiments were performed using papillary muscles from fed animals.

Effects of Total Anoxia — The term "total anoxia" refers to replacement of oxygen by nitrogen in the gas phase. Oxygen tension in the medium is less than 20 mm Hg (22). Fig. 1, A

and R depicts changes in the concentration of alanine and glutamic acid measured in tissue (upper panel), medium (middle panel), and as calculated net production (lower panel) in anoxic and fully oxygenated papillary muscles. An initial rise in tissue alanine was followed by a plateau. At all time points, mean tissue alanine levels were increased in the anoxic muscles. The concentration of alanine in the medium increased progressively over 60 min in both groups and an increased concentration of alanine in the media of anoxic muscles was noted after 60 min of incubation. Mean net ala-
nine production was greater in the anoxic muscles at all time points studied and reached statistical significance at 60 min of incubation.

Tissue glutamic acid concentration decreased over the entire time period in both muscle groups. A slight increase in the media content was noted at 15 min but no further change was seen at longer incubation periods. Calculation of net production revealed negative glutamate production at 30 min in both groups. Identical results were obtained in a control series of experiments in which puromycin was added to the medium (data not presented). No appreciable difference between groups was seen if glucose concentration in the media was raised to 15 mM and compared to 5 mM glucose.

Effects of Graded Oxygen Deprivation—Effects of graded oxygen deprivation on net production of alanine and glutamic acid are depicted in Fig. 2. Alanine production was not significantly enhanced until oxygen concentration was reduced to 5%. In contrast, glutamic acid consumption was not significantly altered until the oxygen concentration was 9%. Thus, there was a lack of 1:1 stoichiometry between the appearance of alanine and the disappearance of glutamic acid.

Contribution of Proteolysis to Alanine Release—An important consideration in this study is the contribution of free amino acids released due to proteolysis. Since tyrosine is thought not to be metabolized in skeletal muscle, release of tyrosine from isolated muscle preparations has been used to measure intracellular protein degradation (26). We incubated papillary muscles from well fed animals in the presence of [U-14C] tyrosine for 60 min and subsequently fractionated samples of tissue extracts and media by thin layer chromatography (chloroform:methanol:17% ammonium hydroxide; 40:40:20). Over 91% of the radioactivity was recovered from a narrow band characterized by the same R_P value as L-tyrosine, while approximately 5% of the radioactivity was retained in the origin. This observation was interpreted as evidence against major conversion of tyrosine in isolated papillary muscles. In an attempt to quantitate proteolysis, we measured tyrosine production from muscles under various conditions either in the presence or in the absence of an inhibitor of protein synthesis (puromycin, 10^{-3} M). The accumulation of tyrosine suggests continued degradation in oxygenated control muscles (Table III), since values for total tyrosine production were markedly increased over control tissue values. However, it is equally apparent that when the inhibitor of protein synthesis was not present, total levels of tyrosine approximated those found in control tissue which indicates that degradation and synthesis are in approximate balance. More important, there was no difference in tyrosine production in oxygenated and anoxic muscles when medium glucose concentration was 5 mM. Furthermore, alanine production remained unaltered when paired muscles were incubated in the presence and absence of puromycin (data not presented).

Effects of Pyruvate—Fig. 3 shows the effect of different medium concentrations of pyruvate on alanine and glutamic acid production in oxygenated and anoxic muscles. In both groups, increased pyruvate concentrations in the media resulted in enhanced alanine production by the muscle. Alanine production in anoxic muscles was the same as in oxygenated muscles incubated with 5 mM pyruvate (Fig. 4), but anoxia when superimposed on pyruvate incubation enhanced alanine production even further (Figs. 3 and 4). Increased alanine production was accompanied by depletion of glutamic acid. Glutamate depletion was also found when oxygenated pyruvate was added to muscle incubation media containing 5 mM glucose.

### Table III

**Tyrosine production in papillary muscles incubated with or without puromycin (10^{-3} M) under aerobic and anaerobic conditions**

| Experimental conditions | Puromycin | Incubation time | Tyrosine production (nmol/mg protein) |
|------------------------|-----------|----------------|---------------------------------------|
| Zero time control      | –         |                | 0.8 ± 0.2                             |
| 5 mM glucose/oxygen    | –         | 60 min         | 0.5 ± 0.2                             |
|                        | +         | 30 min         | 5.2 ± 0.6                             |
|                        |           | 60 min         | 6.5 ± 0.4                             |
| 5 mM glucose/nitrogen  | –         | 60 min         | 1.7 ± 0.1                             |
|                        | +         | 30 min         | 4.9 ± 0.8                             |
|                        |           | 60 min         | 6.7 ± 0.9                             |

Fig. 3. Effect of increasing concentrations of pyruvate in the media on alanine (upper panel) and glutamic acid (lower panel) production in oxygenated (O) and anoxic (●) papillary muscles. Muscles were incubated in Krebs-Ringer bicarbonate buffer containing 5 mM glucose and 2.5, 5, or 10 mM pyruvate. Gas phase was either 95% O_2 or 95% N_2 plus 5% CO_2. Fresh nonincubated muscles served as zero time control (0) and net production was calculated as outlined under "Materials and Methods." Each value represents the mean ± S. D. of six individual experiments.

Fig. 4. A comparison of the effect of 5 mM pyruvate and anoxia on net production of alanine (upper panels) and glutamic acid (lower panels). Paired muscles were incubated in Krebs-Ringer bicarbonate buffer containing 5 mM glucose alone (open bars) or 5 mM glucose plus 5 mM pyruvate (shaded bars). O_2 refers to 95% O_2 plus 5% CO_2 and N_2 refers to 95% N_2 plus 5% CO_2 in the gas phase. Net production is calculated as outlined under "Materials and Methods." Each value represents the mean ± S. D. of six individual experiments.
vated muscles were compared to anoxic muscles (Fig. 4).

Effects of Inhibitors of Glycolytic Pathway—In well oxygenated hearts, fatty acids are the preferred substrate for the production of high energy compounds, while oxygen deprivation increases flux through the glycolytic pathway (27). We tested the hypothesis that alanine production is related to glycolytic activity and added various inhibitors to the incubation media. Fig. 5 shows the effect of glucose-free media, 2-deoxyglucose (15 mM), iodoacetate (0.5 mM), and NaF (10 mM) on lactic acid, alanine, and glutamic acid production in paired papillary muscles incubated for 60 min. Although glucose was absent in the medium, lactate and alanine production increased with anoxia, probably as a result of breakdown of endogenous glycogen stores. Inhibitors of the glycolytic pathway caused a significant suppression of lactate and alanine production. At the same time, glutamate consumption was unchanged. There were quantitative differences between the effect of 2-deoxyglucose and the effect of iodoacetate as well as NaF, and alanine production was suppressed to a lesser extent than lactate production. Table IV shows that inhibitors of glycolysis resulted in decreased tissue alanine levels, while alanine release into the medium remained unchanged. This, although related to glycolytic activity, alanine accumulation can only be partially accounted for by enhanced glucose breakdown during oxygen deprivation.

Effect of pH—When end products of anaerobic cardiac metabolism were measured, it was concluded that acidosis inhibits glycolysis (28), while alkalosis has a stimulating effect (29). Extracellular acidosis did not alter alanine production in papillary muscles under anoxic or aerobic conditions, and there was no effect of extracellular alkalosis (Table V).

Effect of Amino Acids—Addition of glutamic acid stimulated alanine production in isolated rat hemidiaphragms (5). Accordingly, glutamic acid (0.5 mM) was added to the incubation media in oxygenated and anoxic papillary muscles incubated with 15 mM glucose for 60 min. High levels of glucose were used to assure an adequate supply of carbohydrate in order to test the sensitivity of the glutamic acid-pyruvic acid transamination reaction to glutamate under conditions of pyruvate excess. No changes in alanine were detected although modest increases in tissue glutamic acid levels were found (Table VI). Since tissue levels of glutamic acid are known to be increased in skeletal muscle when aspartic acid is added to the incubation media (30), muscles were incubated with 2 mM aspartic acid and 5 mM glucose. Aspartate augmented glutamate release from papillary muscles (Table VI). This observation allowed us to test indirectly the sensitivity of the glutamic acid-pyruvic acid transaminase to increased tissue levels of glutamate. In contrast to data reported in skeletal muscle (30), no augmentation of alanine production was noted, probably reflecting a relative insensitivity of the transaminase to both exogenously added or to endogenously produced glutamate.

In experiments using isolated skeletal muscle (4, 30), perfused rat hindquarters (3), or perfused rat hearts (31), branched chain amino acids were identified as sources of alanine amino nitrogen and/or pyruvate. Therefore, the effect of leucine was studied. Leucine is known to enter intracellular pools when perfusion levels are increased in an oxygenated as well as anoxic isolated heart preparation (32). This is in contrast to most other branched chain amino acids which are less well transported into intracellular cardiac pools. Addition of leucine (0.5 mM) to the incubation media did not alter alanine or glutamic acid production (Table VII). Furthermore, net production of alanine in anoxic and oxygenated muscles was not appreciably altered from that found in experiments without leucine (compare to Fig. 1A).

Effect of Aminotransferase Inhibitors—Since our studies did not identify the source and metabolic fate of glutamate supplying the alanine amino nitrogen, a lack of 1:1 stoichiometry between alanine production and glutamate consumption is, therefore, not in conflict with the hypothesis that alanine is synthesized de novo via glutamic acid-pyruvic acid transamination. This hypothesis was tested using two different inhibitors of alanine aminotransferase. L-Cycloserine, a nonspecific as well as a competitive inhibitor of alanine aminotransferase (33), was injected into animals because of its poor solubility in the buffer medium. L-Cycloserine reduced endogenous alanine.
Each value represents the mean ± S. D. of six individual experiments. Muscles in each group are paired from the same animal, and net production of metabolites is calculated as described under "Materials and Methods." Muscles were incubated in Krebs-Ringer bicarbonate buffer. The gas phase was 95% N₂ and 5% CO₂. Compounds inhibiting glycolytic flux were added to the media in the concentrations indicated.

| Inhibitor added | Experimental conditions | Metabolite | Tissue | Medium | Net production |
|-----------------|------------------------|------------|--------|--------|----------------|
| None            | Nitrogen, 15 mM glucose, 60 min | Lactate | 96.5 ± 30.7 | 592.9 ± 125.5 | 619.6 ± 122.5 |
|                 |                        | Alanine   | 30.5 ± 9.5  | 35.1 ± 17.8  | 48.2 ± 8.8  |
|                 |                        | Glutamate | 15.4 ± 5.7  | 27.7 ± 12.7  | 11.3 ± 11.1 |
| 2-Deoxyglucose (15 mM) | Nitrogen, 60 min | Lactate | 43.1 ± 21.0 | 302.2 ± 102.3 | 210.4 ± 76.2* |
|                 |                        | Alanine   | 18.8 ± 5.7  | 41.1 ± 12.7  | 42.4 ± 11.1 |
|                 |                        | Glutamate | 21.7 ± 5.4  | 25.2 ± 10.5  | 11.7 ± 8.4  |
| None            | Nitrogen, 15 mM glucose, 60 min | Lactate | 29.8 ± 6.4  | 32.8 ± 4.4  | 41.5 ± 5.0  |
|                 |                        | Alanine   | 15.7 ± 5.3  | 10.4 ± 4.1  | -3.9 ± 3.9  |
| Iodoacetate (0.5 mM) | Nitrogen, 15 mM glucose, 60 min | Lactate | 20.9 ± 6.4  | 113.4 ± 20.4 | 82.2 ± 48.7* |
|                 |                        | Alanine   | 17.8 ± 2.0  | 29.1 ± 8.2  | 24.1 ± 7.4* |
|                 |                        | Glutamate | 19.5 ± 9.6  | 20.5 ± 12.5  | 7.6 ± 11.0  |
| None            | Nitrogen, 15 mM glucose, 60 min | Lactate | 81.8 ± 14.7 | 571.2 ± 50.9 | 586.6 ± 50.3 |
|                 |                        | Alanine   | 32.1 ± 11.8 | 22.1 ± 4.3  | 36.7 ± 11.4 |
|                 |                        | Glutamate | 19.8 ± 6.3  | 26.4 ± 6.9  | -12.4 ± 6.0 |
| NaF (10 mM)    | Nitrogen, 15 mM glucose, 60 min | Lactate | 18.2 ± 6.7  | 102.5 ± 31.6 | 113.3 ± 44.8* |
|                 |                        | Alanine   | 19.5 ± 4.1  | 19.8 ± 9.3  | 11.3 ± 8.9* |
|                 |                        | Glutamate | 35.5 ± 11.1 | 38.5 ± 5.8  | 11.7 ± 9.1  |

* Denotes p < 0.001.
+ Denotes p < 0.01.

**Table V**

Effect of pH on tissue levels and release of alanine and glutamic acid in oxygenated and anoxic papillary muscles

Each value is the mean ± S. D. of six experiments. Paired muscles were incubated for 60 min in Krebs-Ringer bicarbonate buffer. Media were gassed and equilibrated with 95% O₂ or 95% N₂ plus 5% CO₂ (pH 7.4), 85% O₂ or 85% N₂ plus 15% CO₂ (pH 6.8), and 100% O₂ (pH 8.9).

| Experimental conditions | pH | Tissue | Media | Net production | Tissue | Media | Net production |
|------------------------|----|--------|-------|----------------|--------|-------|----------------|
| 5 mM glucose/oxygen    | 7.4| 14.8 ± 2.0 | 25.9 ± 3.1 | 21.2 ± 4.5 | 20.7 ± 6.4 | 8.9 ± 1.3 | -15.3 ± 9.6 |
|                        | 6.8| 14.4 ± 3.4 | 29.7 ± 2.1 | 24.7 ± 3.1 | 18.9 ± 8.7 | 10.1 ± 1.7 | -13.0 ± 8.2 |
| 5 mM glucose/nitrogen  | 7.4| 26.6 ± 4.8 | 32.3 ± 6.8 | 38.5 ± 3.5 | 16.3 ± 3.3 | 7.1 ± 2.4 | -20.8 ± 7.6 |
|                        | 6.8| 27.7 ± 7.6 | 28.9 ± 7.3 | 36.3 ± 6.1 | 15.2 ± 4.6 | 12.3 ± 5.8 | -16.6 ± 7.8 |
| 5 mM glucose/oxygen    | 7.4| 18.8 ± 2.7 | 19.3 ± 3.9 | 24.5 ± 4.3 | 20.0 ± 15.3 | 10.4 ± 9.5 | -27.7 ± 2.6 |
|                        | 8.9| 18.0 ± 3.7 | 18.0 ± 7.3 | 10.2 ± 10.6 | 26.6 ± 16.6 | 14.1 ± 12.1 | -17.6 ± 17.0 |

**DISCUSSION**

It has been suggested that alanine released from skeletal muscle serves as a gluconeogenic precursor and is intimately related to carbohydrate metabolism (8). More recent studies by Garber et al. (6) have questioned this postulate, since alanine synthesis in the rat epitrochlearis muscle appeared not to be related to the rates of glucose metabolism or pyruvate production. This indicates that alanine production in rat epitrochlearis muscle can be mediated by glutamate-pyruvate transamination, but the reaction is not regulated by the rate of pyruvate production from glycolysis.

In the heart, oxygen deprivation is the most potent stimulus to glycolysis (27). Alanine accumulation in autolyzing rabbit myocardium and enhanced alanine release found in patients with chronic ischemic heart disease were reported in previous studies (11, 12) and tentatively attributed to an increase in glycolytic flux.

Data from the present study seem to partially support this
**TABLE VI**

Effect of added amino acids on alanine and glutamic acid levels in tissue and medium of papillary muscles

Incubations were carried out in Krebs-Ringer bicarbonate buffer with the additions as indicated. Gas phase was either 95% oxygen or 95% nitrogen (balance CO₂). Each value is the mean ± S. D. of six individual experiments. Duration of incubation is indicated under "Experimental Conditions".

| Amino acid added | Experimental conditions | Free amino acid | Tissue | Medium | Net production |
|------------------|------------------------|----------------|--------|--------|----------------|
|                  |                        |                | nmol/mg protein |        |                |
| None             | Oxygen, 15 mM glucose, 60 min | Alanine       | 12.9 ± 6.4 | 17.5 ± 3.2 | 14.6 ± 6.9 |
| Glutamic acid, 0.5 mM | Oxygen, 15 mM glucose, 60 min | Alanine       | 11.9 ± 3.8 | 21.6 ± 2.9 | 17.7 ± 5.4 |
| None             | Nitrogen, 15 mM glucose, 60 min | Alanine       | 13.6 ± 3.1 | 15.5 ± 1.3 | 22.1 ± 8.1 |
| Glutamic acid, 0.5 mM | Nitrogen, 15 mM glucose, 60 min | Alanine       | 13.3 ± 1.9 | 14.5 ± 1.9 | 21.1 ± 8.6 |
| None             | Oxygen, 15 mM glucose, 60 min | Glutamic acid | 17.4 ± 4.5 | 22.1 ± 2.9 | -10.5 ± 2.9 |
| Glutamic acid, 0.5 mM | Oxygen, 15 mM glucose, 60 min | Glutamic acid | 20.1 ± 1.9 | NE⁺ | NE⁺ |
| None             | Nitrogen, 15 mM glucose, 60 min | Glutamic acid | 15.6 ± 1.8 | 17.6 ± 0.9 | -6.9 ± 3.3 |
| Glutamic acid, 0.5 mM | Nitrogen, 15 mM glucose, 60 min | Glutamic acid | 23.1 ± 4.5³ | NE⁺ | NE⁺ |
| None             | Oxygen, 5 mM glucose, 30 min | Alanine       | 17.0 ± 5.6 | 10.3 ± 1.4 | 13.9 ± 5.9 |
| Aspartic acid, 2 mM | Oxygen, 5 mM glucose, 30 min | Alanine       | 14.2 ± 2.3 | 14.4 ± 2.2 | 15.6 ± 4.5 |
| None             | Oxygen, 5 mM glucose, 30 min | Glutamic acid | 19.2 ± 3.2 | 10.4 ± 3.4 | -15.2 ± 6.1 |
| Aspartic acid, 2 mM | Oxygen, 5 mM glucose, 30 min | Glutamic acid | 19.8 ± 1.5 | 23.3 ± 4.9³ | -1.5 ± 0.6³ |

* Not estimated.
* p < 0.01.

**TABLE VII**

Effect of leucine on alanine and glutamic acid levels in oxygenated and anoxic papillary muscles

All values are mean ± S. D. of six experiments carried out with papillary muscles from animals fasted for 24 h. Incubations were carried out for 60 min in Krebs-Ringer bicarbonate buffer, pH 7.4, 37°. Oxygen denotes 95% O₂, nitrogen denotes 95% N₂, balance CO₂ in the gas phase. 0.5 mM leucine was present in the media.

| Experimental conditions | Alanine | Glutamic acid |
|------------------------|---------|---------------|
|                        | Tissue | Medium | Net production | Tissue | Medium | Net production |
|                        | nmol/mg protein |        |        | nmol/mg protein |        |        |
| Oxygen                 | 8.9 ± 4.1 | 22.9 ± 2.4 | 18.4 ± 3.4 | 26.8 ± 6.3 | 7.9 ± 2.4 | -1.5 ± 1.0 |
| Nitrogen               | 10.7 ± 4.4⁶ | 23.6 ± 2.6 | 29.9 ± 3.3⁶ | 15.7 ± 1.2 | 10.4 ± 1.3 | 10.1 ± 1.7⁺ |

* p < 0.001.

**TABLE VIII**

Effect of L-cycloserine on tissue levels and release of alanine and glutamic acid in oxygenated and anoxic papillary muscles

Each value is the mean ± S. D. of four individual experiments. L-Cycloserine (25 mg/kg) was injected intraperitoneally 60 min before sacrifice. Muscles were incubated in Krebs-Ringer bicarbonate buffer containing 5 or 15 mM glucose. Media were gassed with 95% oxygen or 95% nitrogen (balance CO₂) and incubation was carried out over 60 min at 37°. Zero time control relates to nonincubated control muscles obtained immediately after sacrifice.

| Experimental conditions | Cycloserine | Alanine | Glutamic acid |
|------------------------|------------|---------|---------------|
|                        | Tissue | Media | Net production | Tissue | Media | Net production |
|                        | nmol/mg protein |        |        | nmol/mg protein |        |        |
| Zero time - control    | -      | 13.1 ± 0.8 | 38.3 ± 7.3 | 51.0 ± 6.2⁺ |
| +                      | 4.2 ± 0.8³ |        |        |        |        |        |
| 5 mM glucose/oxygen    | -      | 13.4 ± 3.7 | 23.5 ± 3.4 | 23.8 ± 3.5 | 21.0 ± 2.1 | 17.7 ± 6.8 | -3.3 ± 8.0 |
| +                      | 6.9 ± 1.0³ | 7.8 ± 3.1² | 9.3 ± 3.9³ | 24.3 ± 1.2 | 21.9 ± 5.8 | +1.8 ± 9.2 |
| 15 mM glucose/oxygen   | -      | 12.5 ± 3.6 | 17.2 ± 4.3 | 22.5 ± 7.0 | 23.4 ± 4.8 | 11.8 ± 2.7 | -8.1 ± 6.4 |
| +                      | 16.5 ± 1.2 | 11.7 ± 2.2² | 20.3 ± 3.3 | 16.0 ± 0.6 | 13.3 ± 4.1 | -18.2 ± 5.4 |
| 15 mM glucose/nitrogen | -      | 24.2 ± 3.6 | 19.3 ± 5.9 | 31.4 ± 3.4 | 16.6 ± 2.6 | 12.3 ± 2.1 | -13.9 ± 2.9 |
| +                      | 16.5 ± 2.0 | 8.5 ± 1.8³ | 20.3 ± 1.7³ | 23.4 ± 4.8 | 11.9 ± 2.7 | -12.2 ± 10.0 |

* p < 0.001 compared to control animals not treated with L-cycloserine.
* p < 0.01.
Alanine Synthesis in Oxygen-deprived Papillary Muscles

Effect of aminooxycetic acid on tissue levels and release of alanine, glutamic acid, and lactic acid in hypoxic papillary muscles

All values are the mean ± S. D. of six individual experiments. Incubations were carried out at 37° in Krebs-Ringer bicarbonate buffer over 60 min. Nitrogen denotes 95% N, and 5% CO2 in the gas phase. Aminooxycetate was added to the media as indicated. Net production of metabolites was calculated as indicated under "Materials and Methods" (nanomoles/mg of protein h-1).

| Experimental conditions | Ami- noxycetic acid | Alanine | Glutamic acid | Lactic acid |
|-------------------------|--------------------|---------|---------------|------------|
|                         | Tissue             | Media   | Tissue        | Media      | Tissue     | Media   | Tissue       | Media   |
| 15 mm Glucose/ nitrogen | None               | 28.7 ± 12.0 | 37.7 ± 6.1 | 43.4 ± 8.9 | 14.7 ± 2.8 | 17.4 ± 9.4 | -15.0 ± 9.1 | 91.6 ± 13.9 | 612.6 ± 74.4 | 656.3 ± 46.9 |
| 15 mm Glucose/ nitrogen | 2 mm               | 6.5 ± 2.4  | 14.6 ± 4.7  | -4.8 ± 2.2 | 27.9 ± 11.8 | 25.8 ± 14.4 | 6.3 ± 3.8  | 69.7 ± 6.4  | 620.0 ± 87.0 | 617.0 ± 49.4 |

contention. Alanine release from isolated rabbit right ventricular papillary muscles represents de novo synthesis of this amino acid by the alanine transaminase reaction with glutamic acid as amino nitrogen donor. This conclusion is based upon the following observations: (a) tyrosine release, used as a marker of proteolysis, was not increased in anoxic tissue incubated in 5 mm glucose, although total alanine production was markedly increased over control values under these conditions; (b) in oxygenated, as well as in anoxic muscles, alanine release is preceded by an initial increase in tissue alanine levels at 15 min followed by continued amino acid release without changes in the tissue alanine pool for up to 60 min; and (c) inhibition of the enzyme alanine aminotransferase with cycloserine and aminooxycetate resulted in abolition of anoxia-induced increases in alanine production. Although preferential degradation of an alanine-rich myocardial protein or further metabolism of alanine was not studied, it is suggested that net production of alanine is a good approximation of newly synthesized alanine.

Severe hypoxia and addition of pyruvate stimulated the synthesis of alanine in the isolated right ventricular papillary muscle, whereas glutamate, the branched chain amino acid leucine, and variation in pH were without effect. The effect of anoxia and/or graded hypoxia on alanine production in skeletal muscle is unknown. Since myocardial glycolysis is maximally stimulated during anoxia and severe hypoxia (28), our findings are at variance with those reported by Garber et al. (6), disputing the relationship between glycolytic activity and alanine production in skeletal muscle. These differences between cardiac and skeletal muscle may be quantitative rather than qualitative, as it is suggested by the observation that in papillary muscles, incubated at 25% O2, ATP content is reduced and lactate levels are elevated, indicating enhanced anaerobic metabolism (22). Since alanine production was not significantly augmented at 25% O2 (Fig. 2), it seems that alanine synthesis in cardiac muscle is neither directly proportional to the degree of hypoxia nor to the rate of glycolysis. Possible explanations for this observation are as follows. During moderately severe hypoxia, glycolysis is insufficiently stimulated in heart muscle to augment alanine synthesis, or the aminotransferase reaction is not exclusively regulated by the concentration of reactants and a secondary regulatory factor is activated only under conditions of severe hypoxia or total anoxia. Since the $K_m$ of alanine aminotransferase for pyruvate is reported to decrease with decreasing pH (35), moderately severe hypoxia may produce insufficient intracellular acidosis to augment the reaction. It has been suggested that alanine aminotransferase in skeletal muscle operates at near equilibrium (6) and factors perturbing the equilibrium (e.g., addition of pyruvate and glutamate) produce direct and reciprocal changes in each component of the reaction (6). Enhanced alanine synthesis by pyruvate has been described in the human forearm (36) and the isolated guinea pig heart (37).

The lack of response to elevated glutamate levels cannot be explained by inadequate transport of this amino acid into the cell alone. Studies on the kinetics of purified beef heart glutamate pyruvate transaminase showed that at concentrations 2- and 5-fold greater than their absolute $K_m$ values, both alanine and glutamic acid demonstrated substrate inhibition effects. In contrast, pyruvate at levels 100 times greater than its absolute $K_m$ value showed no inhibitory effects (38). The same studies revealed that alanine was a competitive inhibitor of the reaction with respect to glutamic acid. Since pyruvate levels are elevated under anoxic conditions in cardiac muscle tissue (29, 39-41), our results are consistent with but do not prove a direct relationship between glycolysis and alanine synthesis.

Odessey et al. (4) reported that branched chain amino acids increase alanine and glutamic acid production in isolated rat diaphragms. It was suggested that degradation of branched chain amino acids provides amino groups and stimulates de novo synthesis of alanine from exogenous glucose, providing an efficient source of ATP production. Although branched chain amino acids had no such effect in the isolated rat heart, the addition of 2-oxoacids (branched chain amino acid analogues) to the media promoted an increase of citric acid cycle intermediates and a decline in glutamic acid (31). Although leucine is transported into heart muscle cells (32), we were unable to demonstrate further enhancement of alanine production beyond that already observed during anoxia. Moreover, under anaerobic conditions, oxidation of branched chain amino acids is unlikely, and availability of glutamic acid through transamination of other amino acids is probably limited. Since free ammonia accumulates rapidly in oxygen-deprived muscle (11), a possible role of alanine could be to serve as a nontoxic carrier of ammonia from oxygen-deprived heart muscle. It is presently not known whether amino acids participate in this process in myocardial tissue.

Regardless of the mechanism regulating aminotransferase activity in cardiac muscle, the present study demonstrates enhanced activity with resultant alanine synthesis and glutamate depletion during anoxia. As such, alanine is an end product of anaerobic carbon metabolism. The concomitant generation of $\alpha$-ketoglutarate from glutamate and alanine from three carbon fragments derived from glycolysis during anoxia could theoretically diminish the deleterious effects of anoxia on cardiac muscle by one of two mechanisms. (a) $\alpha$-ketoglutarate could be transaminated with aspartate and,
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thus, enhance substrate levels for the formation of high energy phosphates in the mitochondrion, as has been proposed in diving mammals (9); and (b) the shunting of pyruvate to alanine rather than lactate might reduce lactate levels in oxygen-deprived tissue and relieve the inhibition of glycolysis that occurs when these levels become too high.

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