Metabolic Responses to Cardiac Hypoxia

Increased Production of Succinate by Rabbit Papillary Muscles

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SUMMARY In oxygen-deprived heart muscle tissue, alanine levels increase, whereas levels of glutamate and aspartate decline, and it is therefore postulated that free tissue amino acids participate in the metabolic response to cardiac hypoxia. Succinate is a hypothetical end product of anaerobic metabolism of glutamate and aspartate. To test this hypothesis in vitro isolated right ventricular papillary muscles from rabbits were individually incubated under oxygenated and hypoxic conditions. Lack of oxygen significantly augmented succinate, lactate, and alanine production, while levels of glutamate fell. Increased succinate production also was seen when various metabolic precursors were present in the oxygenated incubation medium. In hypoxic muscles, succinate production could be enhanced further when these precursors were present. The aminotransferase inhibitor, aminooxyacetate, reduced succinate production by hypoxic papillary muscles. This finding demonstrated a close relationship between transamination of amino acids and succinate production. In addition, it is suggested that anaerobic metabolism of the amino acids glutamate and aspartate, anaerobic glycolysis, and alanine production are quantitatively related. Moreover, the two reactions responsible for succinate production during hypoxia, 2-oxoglutarate dehydrogenase and fumarate reductase, are in oxidation-reduction balance and lead to substrate level phosphorylation in the citric acid cycle. Anaerobic mitochondrial metabolism, resulting in increased synthesis of succinate, must be considered when one estimates the energy production by oxygen-deprived heart muscle.

THE oxygen-deprived mammalian heart muscle increases glucose consumption and produces lactate in an attempt to increase anaerobic production of energy.[1] Free amino acids participate in the metabolic response to cardiac hypoxia in man,[2] although quantitatively to a lesser extent than carbohydrates. The amino acid alanine is, like lactate, produced by hypoxic myocardium in vitro while at the same time tissue levels of glutamate and aspartate decline.[3-5] Increased alanine production is thought to be the result of increased substrate availability of pyruvate and transamination of glutamate. A hypothetical end product of anaerobic metabolism of glutamate and aspartate in skeletal muscle is succinate.[6] Synthesis of succinate would (1) restore the oxidation-reduction balance of the glycolytic pathway perturbed by the synthesis of alanine instead of lactate and (2) be coupled to two energy-yielding reactions catalyzed by 2-oxoglutarate dehydrogenase and fumarate reductase, respectively.[7]

It was therefore of interest to know whether succinate is, like lactate and alanine, an end product of anaerobic myocardial metabolism, and to identify some of the factors regulating succinate synthesis by heart muscle. In addition, this study examined the role of glutamate and aspartate in myocardial succinate production during severe hypoxia.

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Methods

Papillary Muscle Preparation

The rabbit papillary muscle preparation described by Lesch et al.[8] was recently adapted for studies of intermediary metabolism.[5] In the present investigation rabbits (weight 2.2–2.5 kg) were stunned, the hearts were excised through a left thoracotomy and, within 10 seconds, right ventricular papillary muscles weighing 1.5–4 mg were dissected free. Only hearts containing three or four muscles of approximately equal size were used. One muscle was immediately frozen and homogenized in ice cold perchloric acid, a second muscle was incubated under control, and the third muscle under experimental conditions. For incubation, muscles were placed into small Pyrex test tubes containing 1 ml of Krebs-Henseleit bicarbonate saline and 5 mM glucose without amino acids. Incubations were carried out at 37°C in a Dubnoff metabolic shaker. For experimental incubations, oxygen concentration and composition of the media were altered according to the individual protocol. Metabolites were added to the Krebs-Henseleit bicarbonate saline as their Na^+ salts. The Na^+ concentration in

the medium was compensated and held constant at 142 mEq/liter.

Prior to transfer into the incubation vessel, media were warmed and equilibrated with the appropriate gas mixture. The incubation vessels were continuously aerated with a stream of humified gas directed over the meniscus of the medium. In each experiment, 12 individually paired muscles were incubated simultaneously. Most experiments were carried out over a 60-minute time period.

At the end of the experiment, 0.8 ml of medium was sampled and added to 0.16 ml of ice cold perchloric acid to destroy trace contaminations of enzyme activity. Muscles were rinsed with cold Krebs-Henseleit bicarbonate buffer, blotted, and immediately homogenized in 1.0 ml of ice cold 0.6 N perchloric acid. All homogenates were kept in the cold for 30 minutes before centrifugation at 4°C and 2000 *g*. The original supernatant fraction was removed and neutralized with an appropriate volume of cold 2 M $KHCO_3$. In most instances, neutralized tissue extracts and media samples were stored overnight at −20°C prior to enzymatic analysis. The drained precipitate from the tissue homogenate was resuspended in 0.9 ml 1% NaCl and 0.1 ml 1.25 M $NaOH/NaHCO_3$ and the protein content was determined by the method of Lowry et al.[9]

Analytical Assays

Succinate was determined enzymatically on duplicate samples by the method of Williamson and Corkey.[10] This method used the succinate thiokinase reaction, coupled to the pyruvate kinase and lactate dehydrogenase reactions, for the specific fluorometric estimation of succinate by decrease in fluorescence of NADH.

The original method was slightly modified in that the pH of the 0.1 M triethanolamine buffer used to dissolve NADH was adjusted to 8.2 with KOH and that the monosodium salt of phosphoenol pyruvate was used. The reaction was carried out at room temperature and completed at 70 minutes.

ATPase activity, thought to be present in a commercial succinate thiokinase preparation, and contamination with minute amounts of pyruvate interfered strongly with the succinate assay. After these possible sources of contamination were excluded, the assay was specific for succinate. The amount of succinate found in fresh papillary muscles from the rabbit was about half of the concentration of succinate in rat heart reported by Rodgers,[11] who used a different system for spectrophotometric estimations of this compound.

Neutralized extracts were also used for the enzymatic fluorometric determination of lactate, alanine, and glutamate by methods described previously.[5] For the purpose of this study some previously published experiments[5] were reanalyzed. In all assays, standard curves were constructed for each run covering a concentration range from 1 to 20 nmol. Each sample determination was done in duplicate (succinate, alanine, and glutamate) or in triplicate (lactate).

Data Analysis and Statistical Analysis

Concentrations of metabolites in tissue and medium were expressed as a function of total protein content of the tissue (nmol/mg protein). Values were the mean ± SD. Net production of a metabolite was calculated as (tissue content + medium content) − (zero time control tissue content) and was derived from 60-minute incubations. Supernumerary muscles were added to the control group, and therefore the mean values of differences between individual muscle groups were not always exactly the mean (tissue content + medium content) − (zero time control tissue content). Statistical analysis of paired samples was carried out with Student's *t*-test.

Enzymes and Chemicals

Succinyl CoA synthetase (EC 6.2.1.5.) was prepared from *Escherichia coli* in the laboratory of Dr. William Bridger, University of Alberta, Edmonton, Canada.[12] Two different batches of enzyme were used. The enzyme was at least 95% pure with a specific activity of 44.3 U/mg. Pyruvate kinase from rabbit muscle (EC 2.7.1.40), as crystalline suspension in 3.2 M ammonium sulphate solution, beef heart L-lactate dehydrogenase (EC 1.1.1.27.), L-alanine dehydrogenase (EC 1.4.1.1.) and L-glutamate dehydrogenase (EC 1.4.1.3.), the crystallized disodium salt of adenosine triphosphate (ATP), the trilithinum salt of acetyl CoA, and the crystallized monosodium salt of phosphoenol pyruvate were purchased from Boehringer Mannheim Corp. All other chemicals, including β-nicotinamide adenine dinucleotide in oxidized and reduced form (NAD^+ and NADH), triethanolamine hydrochloride and aminooxyacetate, were of the highest purity available and were obtained from Sigma Chemical Co.

Results

Effect of Hypoxia on Succinate Production by Papillary Muscles

When oxygen was replaced by nitrogen in the gas phase, there was a significant increase in succinate production by incubated papillary muscles (Fig. 1). In normal tissue, concentrations of succinate declined slightly, while there was minimal release of the compound into the incubation medium. Net production was 9.6 ± 4.1 nmol/mg protein per hour. This value was somewhat lower than those obtained in subsequent experiments (see Tables 1–3). The largest rise in succinate production occurred during the first 30 minutes of hypoxic incubation, and most of the succinate synthesized by oxygen-deprived muscles appeared in the media. This observation

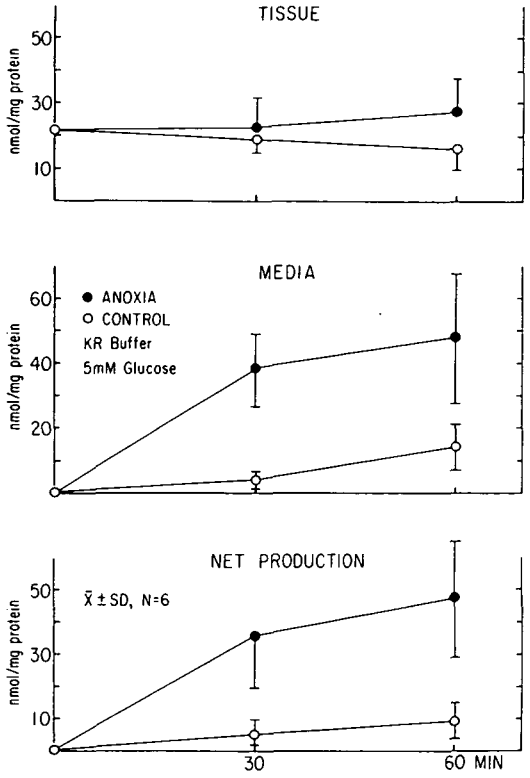

FIGURE 1 *Tissue levels, release into media, and net production of succinate by oxygenated (open circles) and severely hypoxic papillary muscles (filled circles) as a function of incubation time. Muscles from the same rabbit were paired and individually incubated in 1 ml of Krebs-Henseleit bicarbonate saline containing 5 mM glucose at 37°C for the time period indicated on the abscissa. Gas phase was either 95% O_2, 5% CO_2 (control), or 95% N_2, 5% CO_2 (anoxia). Each point represents the mean ± SD of six experiments.*

can be attributed to an effect of hypoxia on transport of succinate across cellular membrane systems.

Comparative Effect of Severe Hypoxia on Synthesis and Release of Succinate, Lactate, Glutamate, and Alanine

The effect of severe hypoxia (95% N_2, 5% CO_2, P_{O_2} in media <19 mm Hg) on tissue levels of succinate and release of this compound into the medium between 0 and 30 and 31 and 90 minutes were compared to synthesis and release of lactate, glutamate, and alanine (Fig. 2). There was continued release of succinate, lactate, and alanine from the same preparation under hypoxic conditions. Tissue levels of glutamate declined sharply with hypoxia, and glutamate release into media essentially ceased after 30 minutes, which indicated depletion and decreased availability of this compound for release into the extracellular space. The opposite effect was seen with succinate. Although this dicarboxy-acid is normally confined to the intracellular space, specifically to mitochondria, a significant release of succinate from hypoxic tissue was evident. The release of succinate was similar to the release of lactate and alanine by hypoxic muscles. The latter compounds readily pass through myocardial cell membranes.

Effect of Added Aspartate on Synthesis and Release of Succinate

In a variety of living organisms, including mammalian species, anaerobic metabolism of glutamate and aspartate provides substrate for synthesis of succinate. When glutamate was added to the medium of oxygenated papillary muscles, this compound was insufficiently transported into the cell.[5] Although transport of aspartate across myocardial cell membranes also appears to be limited,[13] high levels of aspartate in the medium (2 mM) augmented succinate production by oxygenated papillary muscles. When aspartate in the same concentration was supplied to hypoxic muscles, however, no further augmentation in succinate production was noted (Table 1).

Effect of Aminooxyacetate on Succinate, Lactate, Alanine, and Glutamate Production by Papillary Muscles

According to a metabolic scheme presented by Hochachka,[7] inhibition of alanine amino-transferase and aspartate amino-transferase should diminish substrate availability for anaerobic succinate production. Therefore, the transaminase inhibitor, aminooxyacetate (2 mM), was added to the incuba-

TABLE 1 *Synthesis and Release of Succinate by Isolated Right Ventricular Papillary Muscles (Effect of Added Aspartate and Anoxia)*

Experimental conditions	Tissue (nmol/mg protein)	Medium (nmol/mg protein)	Net production (nmol/mg protein per hr)
Control, unincubated	19.8 ± 7.9		
Oxygen	12.1 ± 5.1	27.7 ± 6.9	19.6 ± 8.4
Oxygen + aspartate (2 mM)	19.6 ± 5.7	46.9 ± 8.3	48.2 ± 7.3*
Control, unincubated	14.3 ± 1.9		
Nitrogen	18.2 ± 6.0	35.9 ± 0.8	39.8 ± 6.9
Nitrogen + aspartate (2 mM)	19.4 ± 4.3	32.8 ± 5.6	39.6 ± 6.5

Results are expressed as mean ± SD; $n = 6$.
Muscles were incubated for 60 minutes at 37°C in Krebs-Henseleit bicarbonate buffer containing 5 mM glucose. Gas phase was 95% O_2 (oxygen) or N_2 (nitrogen), balance CO_2.
* $P < 0.001$.

TABLE 2 *Synthesis and Release of Succinate by Isolated Right Ventricular Papillary Muscles (Effect of Added Malate and Anoxia)*

Experimental conditions	Tissue (nmol/mg protein)	Medium (nmol/mg protein)	Net production (nmol/mg protein per hr)
Control, unincubated	22.7 ± 4.4		
Oxygen	14.5 ± 4.5	27.6 ± 4.2	19.7 ± 1.6
Oxygen + malate (2 mM)	41.0 ± 6.1*	52.3 ± 6.9*	70.4 ± 7.2†
Control, unincubated	20.3 ± 3.6		
Nitrogen	25.8 ± 8.1	48.9 ± 8.0	54.4 ± 7.4
Nitrogen + malate (2 mM)	35.9 ± 3.9	58.4 ± 14.8	74.3 ± 14.5

Results are expressed as mean ± SD; $n = 6$.
Muscles were incubated for 60 minutes at 37°C in Krebs-Henseleit bicarbonate buffer containing 5 mM glucose. Gas phase was 95% O_2 (oxygen) or N_2 (nitrogen), balance CO_2.
* $P < 0.01$; † $P < 0.001$.

tion medium of hypoxic papillary muscles (Fig. 3). Succinate production was significantly depressed in the presence of the inhibitor, while at the same time glutamate consumption reverted to net release and alanine production completely ceased. These findings indicated that transamination of amino acids seemed to regulate anaerobic succinate production. Total rates of glycolysis were not affected by the transaminase inhibitor, as indicated by lactate production.

Effect of Citric Acid Cycle Intermediates on Succinate Production

In the course of this study, I became aware of the observations by Penney and Cascarano, who examined the effect of various metabolites on selected physiological criteria and levels of high energy phosphate compounds in anaerobic rat heart.[14] The authors postulated that succinate synthesis was linked to preservation of high energy phosphate stores in anoxic heart muscle. Our study made use of a different heart muscle preparation and employed a sensitive and specific assay for succinate to investigate this possibility. A comparison was made between oxygenated and hypoxic heart muscle. The findings were in partial agreement with those of Penney and Cascarano on three counts:

1. Addition of malate (2 mM) to the incubation medium of quiescent papillary muscles augmented succinate production in an oxygen-rich atmosphere, indicating enhanced Krebs cycle activity. When malate was supplied to hypoxic muscles, the augmentation of succinate production was of smaller magnitude (Table 2).

2. Succinate production could be augmented in both oxygenated and hypoxic muscles, when 2-oxoglutarate was present in 10 mM concentration (Table 3).

3. Finally, when malate, fumarate, and glutamate were supplied simultaneously, these compounds resulted in a significant increase in tissue succinate levels in oxygen-deprived papillary muscles. However, the increase in total succinate production under these experimental conditions was not statistically significant (Table 4), and was of smaller magnitude than the increase in succinate production by hypoxic rat heart supplied with the same substrates.[14] This observation could not be attributed to lack of mechanical activity of the papillary muscle, since hypoxia quickly leads to cessation of cardiac function in perfused rat heart.

Discussion

Mammalian heart muscle adapts poorly to oxygen deprivation since substrate level phosphorylation from anaerobic glycolysis is inadequate to maintain normal levels of high energy phosphate compounds. However, a second mechanism for anaerobic myocardial energy production has been postulated. This mechanism involves substrate level phosphorylation in the citric acid cycle via the 2-oxoglutarate dehydrogenase and fumarate reductase reactions. A quantitative estimation of this process is fraught with difficulties, since consider-

TABLE 3 *Synthesis and Release of Succinate by Isolated Right Ventricular Papillary Muscles (Effect of Added 2-Oxoglutarate and Anoxia)*

Experimental conditions	Tissue (nmol/mg protein)	Medium (nmol/mg protein)	Net production (nmol/mg protein per hr)
Control, unincubated	19.6 ± 5.9		
Oxygen	14.3 ± 5.3	26.6 ± 6.5	19.3 ± 5.8
Oxygen + 2-oxoglutarate (10 mM)	26.9 ± 7.8*	76.3 ± 13.9†	83.5 ± 24.2*
Control, unincubated	14.0 ± 4.2		
Nitrogen	21.9 ± 5.5	33.4 ± 5.1	41.3 ± 7.8
Nitrogen + 2-oxoglutarate (10 mM)	18.5 ± 7.1	72.6 ± 6.0*	77.2 ± 10.3†

Results are expressed as mean ± SD; $n = 6$.
Muscles were incubated for 60 minutes at 37°C in Krebs-Henseleit bicarbonate buffer containing 5 mM glucose. Gas phase was 95% O_2 (oxygen) or N_2 (nitrogen), balance CO_2.
* $P < 0.01$; † $P < 0.001$.

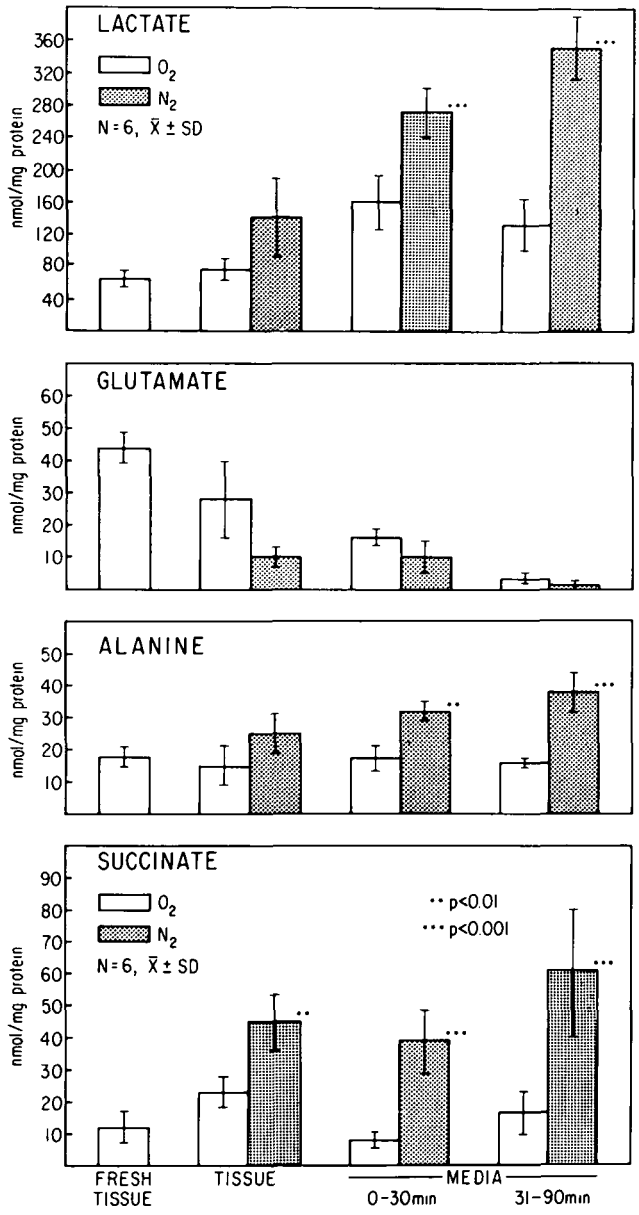

FIGURE 2 *Tissue levels and release into media of lactate, glutamate, alanine, and succinate by oxygenated (unshaded bars) and severely hypoxic papillary muscles (shaded bars). Paired muscles were individually incubated in 1 ml of Krebs-Henseleit bicarbonate saline containing 5 mM glucose. Media were changed once after 30 minutes. Total incubation time was 90 minutes. Gas phase was either 95% O_2 and 5% CO_2 (designated O_2) or 95% N_2 and 5% CO_2 (designated N_2). Each bar represents the mean ± SD of six experiments.*

able changes in levels of citric acid cycle intermediates occur within the first minutes of anaerobic mitochondrial metabolism.[15]

The present study evaluated the significance of substrate level phosphorylation in the citric acid cycle at later time points under quasi steady state conditions. It was based on a previous report that hypoxia stimulated synthesis and release of alanine by heart muscle.[5] Augmented rates of anaerobic glycolysis could result in increased substrate availability for both the lactate dehydrogenase and the alanine amino transferase reactions. High levels of pyruvate increased alanine release by the perfused guinea pig heart.[16] Whereas hypoxia stimulated alanine production in heart muscle, levels of glutamate and aspartate declined.[3-5] The observed synthesis of alanine through transamination would result in an imbalance of the oxidation-reduction state of the glycolytic pathway.

The synthesis of alanine by oxygen-deprived heart muscle requires therefore not only the presence of pyruvate and glutamate as substrates but also a system to reoxidize NADH. Principally, two cytosolic reactions are capable of restoring the perturbed oxidation-reduction balance: the glycerolphosphate dehydrogenase reaction leading to synthesis of α-glycerolphosphate in the cytosol, and the malate dehydrogenase reaction leading to synthesis of malate as precursor for mitochondrial succinate synthesis. In the latter scheme, aspartic acid serves a dual function as donor of amino nitrogen for glutamate and as donor of the carbon skeleton for oxaloacetate and malate. Further breakdown of aspartate (and, to a lesser extent, probably also of glutamate) would result in accumulation of succinate, as illustrated in Figure 4.

The following three observations reported in the literature would support the view that succinate production by hypoxic papillary muscles is the result of anaerobic metabolic activity in mitochondria:

1. Oxidation of fumarate, derived from aspartate, was coupled to the synthesis of ATP and succinate in cyanide-treated mitochondrial particles from rat heart.[17] Although this finding has not been confirmed, the present study suggests that it could represent a mechanism for anaerobic myocardial energy production.

2. A second energy-yielding reaction was found to accompany oxidation of 2-oxoglutarate by anaerobically incubated kidney and liver mitochondria,[18] and resulted in succinate accumulation.

3. Finally, evidence has been presented that anoxic perfusion of rat hearts with citric acid cycle intermediates and glucose could stimulate mitochondrial synthesis of ATP and enhance anaerobic cardiac function by increasing the energy expenditure per mole of glucose utilized or lactate produced.[14] This stimulation of mitochondrial ATP synthesis was, however, inadequate to sustain normal physiological performance of the heart.

In the latter study, anaerobic synthesis of ATP by mitochondria was related to increased synthesis of succinate which appeared in tissue and perfusate of anoxic rat hearts perfused with succinate precursors. The present study provides further direct evidence for accumulation of succinate in oxygen-de-

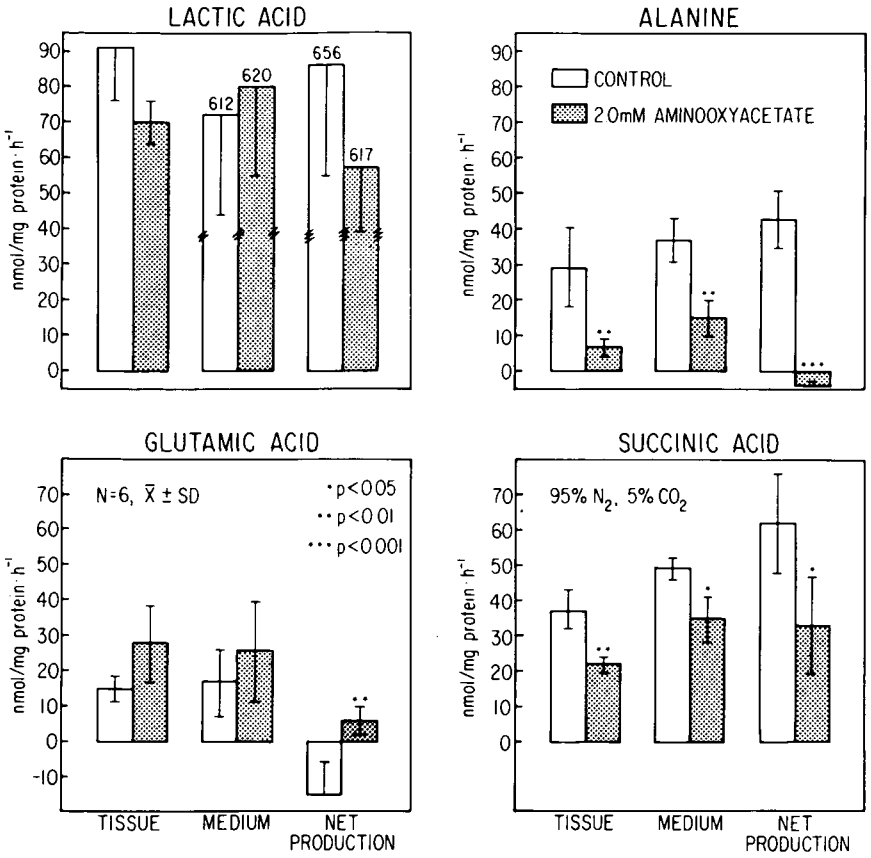

FIGURE 3 *Tissue levels, release into media, and net production of lactate, alanine, glutamate, and succinate by severely hypoxic right ventricular papillary muscles incubated in the absence (unshaded bars) or presence (shaded bars) of the aminotransferase inhibitor, aminooxyacetate (2.0 mM). Paired muscles were individually incubated for 60 minutes at 37°C in 1 ml of Krebs-Henseleit bicarbonate saline containing 5 mM glucose. Each bar represents the mean ± SD of six experiments.*

prived heart muscle and release of this compound from hypoxic tissue. From experiments with isolated mitochondria, it was suggested that accumulation of succinate in the extra-mitochondrial space occurred by anion exchange, chiefly with malate.[19] The appearance of succinate in the extracellular space (medium) may be the result of membrane damage during hypoxia.

Davis and Bremer[20] have reported that amino acid catabolism appears to regulate the level of citric acid cycle intermediates in aerobic heart muscle. Lesch and his colleagues recently showed that papillary muscles degrade glutamate under anaerobic conditions.[21] The authors suggested that succinate was an end product of anaerobic glutamate metabolism. Inhibition of succinate and alanine formation by aminooxyacetate during hypoxia provides further evidence for amino acid breakdown in the metabolic response to cardiac hypoxia. Aside from hypoxia, several other factors seem to affect synthesis of succinate by heart muscle. These factors include: (1) the presence of availability of the precursor amino acids, glutamate and aspartate and (2) transamination of these compounds via substrate specific transaminases.

Substrate level phosphorylation occurring during conversion of succinyl CoA to succinate and the reported oxidation of NADH by fumarate in heart muscle mitochondria[22] are energy-yielding mitochondrial reactions (see Fig. 4). These nonglycolytic sources of energy probably contribute to the adaptation of diving animals to hypoxia,[23] and there is some evidence that they may play a similar role in man.[24, 25]

TABLE 4 *Synthesis and Release of Succinate by Isolated Right Ventricular Papillary Muscles (Effect of Added Fumarate, Malate, and Glutamate in Presence of Severe Hypoxia)*

Experimental conditions	Tissue (nmol/mg protein)	Medium (nmol/mg protein)	Net production (nmol/mg protein per hr)
Control, unincubated	20.4 ± 5.7		
Nitrogen	22.1 ± 5.4	33.9 ± 6.4	35.6 ± 9.6
Nitrogen + FMG	39.6 ± 7.3*	37.1 ± 10.6	56.2 ± 13.9

Results are expressed as mean ± SD; $n = 6$.

Krebs-Henseleit bicarbonate buffer, 15 mM glucose, 95% N_2/5% CO_2 in the gas phase; 60-minute incubation at 37°C; FMG = fumarate (12.5 mM), malate (12.5 mM); glutamate (10 mM).

* $P < 0.005$.

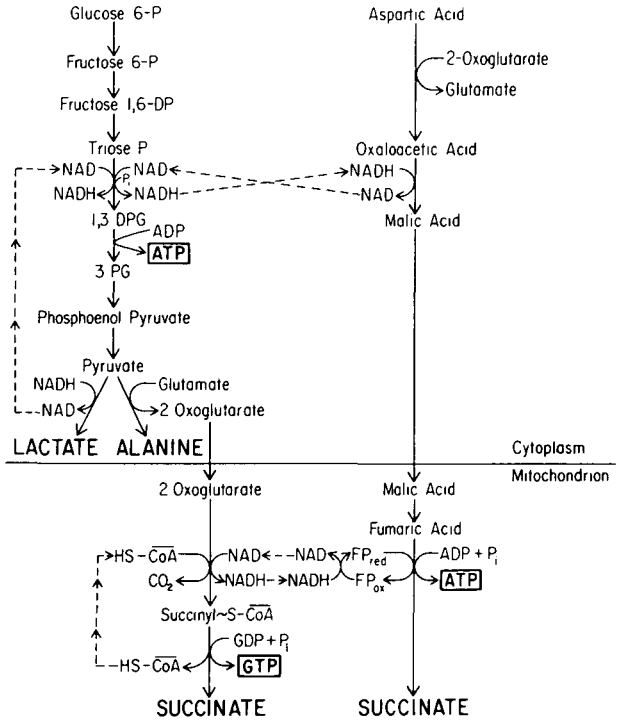

FIGURE 4 *Metabolic pathway illustrating the synthesis of succinate from aspartate and glutamate. If pyruvate is diverted to alanine, 2-oxoglutarate appears from transamination of glutamate and NADH is regenerated by the malate dehydrogenase reaction. 2-Oxoglutarate and malate pass the mitochondrial membrane and are converted to succinate through the 2-oxoglutarate dehydrogenase and fumarate reductase reactions. Production of succinate is linked to substrate level phosphorylation in the mitochondrion. For reasons of clarity, the synthetic pathway leading to formation of α-glycerolphosphate during hypoxia was omitted. Adapted from Hochachka et al.*[7]

The present study shows that provision of substrate would further enhance succinate production during anaerobiosis in heart muscle. A significant augmentation of anaerobic succinate production could be achieved with 2-oxoglutarate as substrate, and here mainly by increasing the amount of succinate released into the medium. 2-Oxoglutarate readily enters heart muscle cells,[20] whereas myocardial membrane kinetics of other compounds, such as glutamate, aspartate, fumarate, and malate, are either less favorable or not well known.

Last, the quantitative importance of amino acid breakdown and increased succinate production during cardiac hypoxia should be considered and compared to lactate production from anaerobic glycolysis. From Figure 3, net production of lactate from glucose by severely hypoxic papillary muscles (656 nmol/mg protein per hour) would yield 656 nmol ATP/mg protein per hour, while alanine production from glucose and succinate production from either aspartate or glutamate (44 and 64 nmol/mg protein per hour) would yield another 108 nmol ATP/mg protein per hour. Therefore, anaerobic alanine formation through glycolysis and succinate formation in the citric acid cycle would amount to approximately 16% of ATP production when compared to ATP derived from lactate production through anaerobic glycolysis alone. Anaerobic ATP production through substrate level phosphorylation could be further enhanced by providing appropriate substrates.

Augmented succinate production from breakdown of amino acids suggests a metabolic reaction of heart muscle in response to oxygen deprivation. Since succinate production during hypoxia also indicates anaerobic mitochondrial function, any ATP synthesized is likely to be found in the mitochondrial space. The physiological importance of nonglycolytic energy production through substrate level phosphorylation in the citric acid cycle by heart muscle remains unknown.

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