The mechanism of ammonia production and the effect of mechanical work load on proteolysis and amino acid catabolism in isolated perfused rat heart

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The effect of mechanical work load on net proteolysis, amino acid catabolism and ammonia production was studied in isolated perfused beating or K⁺-arrested hearts. Net proteolysis was about 16 $\mu\text{mol/g}$ dry wt. during 1 h and was not affected by the mechanical work. The combined catabolic rate of the major amino acids was 7.1 $\mu\text{mol/g}$ dry wt. in the beating heart and 2.1 $\mu\text{mol/g}$ dry wt. in the arrested heart during the 1 h experimental period. The main differences lay in the deamination of aspartate plus glutamate, which was inhibited by 60% during low energy consumption, and in net alanine synthesis, which was increased by 94%. The ammonia production plus its conversion into amide nitrogen was 9.2 and 3.4 $\mu\text{mol/g}$ dry wt. in the beating and arrested heart respectively during 1 h. The decrease in the total adenine nucleotide pool during the 1 h perfusion was very low, 1.0 and 0.5 $\mu\text{mol/g}$ dry wt. in the beating and arrested hearts respectively, and did not contribute significantly to ammonia production. Thus ammonia production is dependent on the cellular energy state, whereas net proteolysis is not. The maximal capacities of the purine nucleotide cycle and the glutamate dehydrogenase reaction towards deamination were much higher than the observed ammonia-production rates. The anaplerotic role of amino acid catabolism in the myocardium is discussed.

It has been shown previously that the myocardium produces ammonia, and that this ammonia production depends on oxygen consumption or mechanical work load (Feinberg & Alma, 1961). The sources and mechanism of the production of ammonia have nevertheless not been positively identified in the myocardium, although it has been suggested that the adenylates could be one source (Parnas, 1928; Embden & Schmidt, 1930). Previous evidence suggests that the purine nucleotide cycle acts as a source of ammonia in skeletal muscle (Goodman & Lowenstein, 1977), and that glutamate dehydrogenase activity is low (Williamson *et al.*, 1967), but the role of the purine nucleotide cycle or glutamate dehydrogenase as ammonia producers in the myocardium has not been evaluated.

Irrespective of whether the purine nucleotide cycle is functioning or not, the ultimate source of ammonia is the amino acid pool, in view of the relative constancy of the adenine nucleotide pool in the perfused heart. Another important aspect of the amino acid pool is its potential function as a source of tricarboxylic acid-cycle intermediates, the concentrations of which vary under different metabolic conditions.

In the present case, amino acids were evaluated as

precursors of ammonia and tricarboxylic acid-cycle intermediates by measuring their degradation, which, combined with measurements of the tricarboxylic acid-cycle pool, allows an estimate to be made of the anaplerotic contribution of amino acids. A balance sheet of the degradation of certain amino acids can be constructed by determining their proteolytic production and changes in the concentrations of free amino acids in the system. The former was accomplished by two different methods, which showed that the rate of proteolysis is not affected by cellular energy consumption. The rate of amino acid deamination increased on an increase in energy consumption. The influx of carbon skeletons to tricarboxylic acid-cycle intermediates was also calculated.

Determination of the maximal capacities of the purine nucleotide cycle and glutamate dehydrogenase towards deamination showed that both are higher than the observed rate of ammonia production.

Experimental

Experimental animals

Female Sprague–Dawley rats weighing 250–300 g

from the Department's own stock were used. They were fed *ad libitum* on a commercial laboratory diet (pelleted standard rodent chow; Hankkija Ltd., Helsinki, Finland).

Reagents

The enzymes were purchased from Sigma Chemical Co., St. Louis, MO, U.S.A., and Boehringer G.m.b.H., Mannheim, Germany. Phosphoenolpyruvate and nucleotides were obtained from Boehringer, except IMP, which was from Sigma. 2-Oxo[1-¹⁴C]glutarate was obtained from The Radiochemical Centre, Amersham, Bucks., U.K.; L-aspartic acid and L-leucyl-L-alanine were from Fluka A.G., Buchs, Switzerland; and standard reagents were purchased from E. Merck A.G., Darmstadt, Germany.

Heart perfusion

The rats were anaesthetized with intraperitoneal Nembutal (Abbott S.A., Ottignies, Belgium) and injected with 500 i.u. of heparin intravenously 30 s before excision of the heart. The Langendorff (1895) procedure was used. During the first 30 min the hearts were perfused without recirculation at a hydrostatic pressure of 7.85 kPa (80 cm of water), and the perfusion was then continued with recirculation for 60 min at the same hydrostatic pressure. The volume of recirculating perfusate was 60 ml. The perfusion medium was Krebs-Ringer bicarbonate buffer (Krebs & Henseleit, 1932) containing 2.5 mM-Ca²⁺ and 10 mM-glucose in equilibrium with O₂/CO₂ (19:1). K⁺-induced cardiac arrest was achieved by increasing the perfusate KCl concentration to 16 mM, the concentration of NaCl being lowered accordingly to maintain constant osmolality. In order to minimize ammonia contamination from the air, only fresh stock solutions were used and the heart chamber was closed during perfusion. No measurable evaporation of ammonia from the perfusion system was detected in control experiments in which NH₄Cl was added to the perfusate and the gas outflow from the oxygenator passed through 1 M-HCl.

Metabolites

Samples were obtained from the heart by using aluminium clamps cooled with liquid N₂ (Wollenberger *et al.*, 1960). HClO₄ extractions were performed as described previously (Williamson & Corkey, 1969; Hiltunen, 1978). The concentrations of metabolites were assayed by enzymic methods. The formation or disappearance of NAD(P)H was calculated from $A_{340} - A_{385}$ measured in an Aminco DW-2 dual-wavelength spectrophotometer, by using $\epsilon_{340-385} = 5.33 \times 10^3 \text{ litre} \cdot \text{mol}^{-1} \cdot \text{cm}^{-1}$. ADP was determined as described by Bernt *et al.* (1970), and AMP was measured in the same cuvette by the

addition of adenylate kinase (EC 2.7.4.3). ATP was measured as described by Lamprecht & Traut-schold (1970). Citrate was measured with citrate lyase (EC 4.1.3.6) (Gruber & Moellering, 1966), 2-oxoglutarate by the method of Narins & Passonneau (1970), and malate by that of Williamson & Corkey (1969). Fumarate was measured in the same cuvette as malate after the addition of fumarate hydratase (EC 4.2.1.2). Succinate was measured as described by Williamson & Corkey (1969), with succinate thiokinase (EC 6.2.1.4), pyruvate kinase (EC 2.7.1.40) and lactate dehydrogenase (EC 1.1.1.27).

Ammonia

A sensitive method for the determination of small amounts of ammonia was obtained by measuring the ammonia enzymically with reference to glutamate synthesis from 2-oxo[1-¹⁴C]glutarate and ammonia in the glutamate dehydrogenase (EC 1.4.1.2) reaction and measuring the radioactivity of the glutamate after ion-exchange chromatography. In order to decrease the reagent-blank value, the reaction mixture, containing 2 ml of 0.4 M-Tris/HCl buffer, pH 8.0, 1 mM non-labelled 2-oxoglutarate, 2.2 mM-NADH and dialysed glutamate dehydrogenase (0.5 mg/ml), was incubated for 30 min at 37°C. This preincubation was essential to ensure reproducible results. A portion (0.6 ml) of sample or standard solution containing 0.20 nmol of ammonia was added to tubes containing 1.5 nmol of freeze-dried 2-oxo[1-¹⁴C]glutarate (specific radioactivity 600 000–800 000 d.p.m./ μmol). Then 250 μl of the preincubated reaction mixture per tube was added and the tubes were closed, stirred and incubated for 1½ h at 37°C. The reaction was stopped by adding 0.2 ml of 0.2 M-HCl. Then 25 μmol of carrier 2-oxoglutarate and 25 μmol of glutamate were added after a further 10 min and ion-exchange chromatography was carried out by using a JLC-5AH automatic amino acid analyser (Japan Electron Optics Laboratory Co., Tokyo, Japan). The radioactivities of the glutamate and 2-oxoglutarate peaks were determined in Instagel (Packard) by using a Wallac liquid-scintillation counter. The total radioactivity of the glutamate peak plus the 2-oxoglutarate peak was used as an internal standard.

In some experiments the method of Kun & Kearney (1970) was used for the determination of ammonia (see below).

Free amino acids

Freeze-dried samples of the neutralized HClO₄ extract or perfusion medium were dissolved in 0.2 M-citric acid, and the pH was adjusted to 2.2 by the addition of 1 M-NaOH. The amino acids were measured by ion-exchange chromatography by using an automatic amino acid analyser. The

phenylalanine concentration in the perfusate was measured by the fluorimetric method of McCaman & Robins (1962).

Amino acid composition of myocardial protein

The HClO_4 -insoluble precipitate was hydrolysed in 6M-HCl at 120°C for 16h and the protein hydrolysate analysed in an amino acid analyser. Amide nitrogen was determined by measuring the liberation of ammonia, aspartate and glutamate during acid hydrolysis in 2M-HCl at 100°C for 1–3h enzymically by the methods of Kun & Kearney (1970), Bergmeyer *et al.* (1970) and Bernt & Bergmeyer (1970).

Proteolysis during perfusion

(1) The release of amino acid phenylalanine not metabolizable in the myocardium (Udenfriend & Cooper, 1952) was used to estimate the rate of net proteolysis, and the frequency of phenylalanine and other amino acids in total myocardial protein was used to calculate the net amounts of the individual amino acids released during proteolysis. A potential source of error in this is the differential degradation of specific myocardial proteins, but the evidence presented below indicates that this error is small.

(2) Another method for estimating net proteolysis (x) can be devised on the basis of the measurement of the amounts (in μmol) of free individual amino acids (aa), ammonia and total adenine nucleotides (AXP), as follows:

$$x = \sum \Delta \text{Aaa} + \Delta \text{NH}_3 + \Delta \text{AXP} - \text{degradation of (Gln + Asn + His + Lys)} \quad (1)$$

where Δ is the increment in the amount of the compound in question. The four amino acids named give rise to two nitrogen equivalents, and this extra production must be subtracted in order to obtain the proteolysis rate. Degradation of (Gln + Asn + His + Lys) can be measured by first calculating their production by proteolysis (y):

$$y = \frac{\text{sum of frequencies of Gln + Asn + His + Lys}}{\text{sum of frequencies of amino acids measured}} \cdot x \quad (2)$$

where x is net proteolysis. From the numerical values from Table 5, eqn. (2) is transformed to $y = 0.224x$. Subtracting the increment of the sum of these four amino acids, $\Delta[\text{Gln} + \text{Asn} + \text{His} + \text{Lys}]$, we then obtain a transformation of equation (1) of the form:

$$x = \sum \Delta \text{Aaa} + \Delta \text{NH}_3 + \Delta \text{AXP} - 0.224x + \Delta[\text{Gln} + \text{Asn} + \text{His} + \text{Lys}] \quad (3)$$

or

$$x = \frac{\sum \Delta \text{Aaa} + \Delta \text{NH}_3 + \Delta \text{AXP} + \Delta[\text{Gln} + \text{Asn} + \text{His} + \text{Lys}]}{1.224} \quad (4)$$

As shown below, the two methods for estimating protein degradation gave essentially the same results.

Determination of glutamate dehydrogenase and the activities of the enzymes of the purine nucleotide cycle

For the determination of glutamate dehydrogenase activity, rat hearts were homogenized in 6 vol. of 10mM-potassium phosphate, pH 7.4, containing 0.5mM-ADP and 10 μM -EDTA with an Ultra-Turrax homogenizer. The homogenate was sonicated three times for 1 min and centrifuged at 100000g for 15 min. The pellet was re-homogenized in 3 vol. of the homogenization buffer and the extraction procedure repeated twice. Endogenous metabolites were removed from the combined supernatants by gel filtration on a Sephadex G-15 column pre-equilibrated with the homogenization buffer. The enzyme activity was determined in the direction of oxidative deamination in a reaction mixture containing 50mM-Tris acetate, 0.1mM-EDTA, 33mM-sodium glutamate, 0.5mM-ADP and 0.6mM- NAD^+ , and linear initial rates were recorded.

For the determination of the enzyme activities of the purine nucleotide cycle, the tissue was homogenized in 2 vol of 20mM-Tris/citrate buffer, pH 7.3, containing 15mM-KCl and 1mM-dithiothreitol, by using a Ultra-Turrax homogenizer and centrifuged

at 100000g for 20 min. The pellet was re-homogenized and re-centrifuged three times. The supernatants were combined and freed of endogenous metabolites on a Sephadex G-15 column pre-equilibrated with the homogenization buffer. Glutamate dehydrogenase activity was determined as described by Schultz & Lowenstein (1976). The activities of the purine nucleotide cycle enzymes were determined as described by Schultz & Lowenstein (1976) for the brain enzymes, except for the following modifications. The protein concentration in the AMP deaminase (EC 3.5.4.6) assay was 0.1–0.5mg/ml. The adenylosuccinate synthetase (EC 6.3.4.4) reaction was monitored by measuring the IMP concentration in the HClO_4 extracts of

samples taken at 5 min intervals from the reaction mixture IMP was determined by using snake-venom 5'-nucleotidase (EC 3.1.3.5) (Schultz & Lowenstein, 1976; Rubio *et al.*, 1973), xanthine oxidase (EC 1.2.3.2) and purine nucleoside phosphorylase (EC 2.4.2.1) (Olsson, 1970). The reaction mixture for the assay of adenylosuccinate synthetase contained 1.0–2.0 mg of myocardial protein/ml. Phosphoenolpyruvate (1.1 mM) was present in the assay medium for adenylosuccinate lyase (EC 4.3.2.2).

The enzymes were assayed at 30°C, and the activities were presented on a tissue-dry-weight basis by using a wet-wt./dry-wt. ratio of 7.97.

Demonstration of the complete purine nucleotide cycle

The reaction mixture contained 0.24 mM-GDP, 0.65 mM-AMP and 0.20 mM-ATP, 4 mM-aspartate, 27 mM-imidazole/HCl buffer, pH 7.0, 8.3 mM-MgCl₂, and 100 000 g supernatant of myocardial homogenate corresponding to 121 mg fresh wt. of tissue/ml of reaction mixture. The heart extract accounted for 45% of the total volume. When about two-thirds of the adenine nucleotides had been deaminated, the amination phase was initiated by the addition of 7.9 mM-phosphoenolpyruvate. The reactions were carried out at 37°C and 0.7 ml samples were removed at 10 min intervals and mixed immediately with an equal volume of 12% (v/v) HClO₄. The concentrations of xanthine plus hypoxanthine, inosine and adenosine in the HClO₄ extracts were determined by using xanthine oxidase, purine nucleoside phosphorylase and adenosine deaminase (Olsson, 1970). Adenine nucleotides,

IMP, malate and fumarate were determined as described above. Changes in the concentration of adenylosuccinate were measured by recording changes in the u.v.-absorbance spectrum as described by Tornheim & Lowenstein (1972).

Protein

Protein was measured by the biuret method of Szarkowska & Klingenberg (1963).

Statistical analysis of data

The statistical significances of the results were calculated by using the Student's *t* test.

Results

Free amino acid content of tissue and perfusate and changes in the combined amino acid pools

The free amino acid content of the tissue is depicted in Table 1. The changes recorded were small, except for aspartate, which decreased by 6–7 µmol/g dry wt. in both the beating and K⁺-arrested hearts during 1 h. The tissue glutamate content increased in the arrested heart. The total free amino acid content of the myocardium decreased in the beating hearts, but remained unchanged in the arrested hearts.

The output of free amino acids into the perfusion medium (Table 2) was significantly higher in the beating hearts than in the arrested hearts. Of the amino acid efflux in the beating heart, 37% could be attributed to glutamine and 15% to alanine.

The initial myocardial taurine concentration was 161.6 µmol/g dry wt., and no changes were detected

Table 1. *Concentrations of free amino acids in the tissue of isolated perfused rat hearts*

Hearts from rats fed *ad libitum* were perfused by the Langendorff procedure with Krebs–Ringer bicarbonate medium containing 10 mM-glucose. The hearts were sampled after a 30-min perfusion without recirculation (initial) or perfused with recirculation for a further 60 min, and arrested when indicated by a K⁺ excess. Analytical procedures are as described in the Experimental section. The values are means ± S.E.M. for six independent biological observations.

	Content (µmol/g dry wt.)			<i>P</i> <		
	Initial	Beating heart	Arrested heart	Beating versus initial	Arrested versus initial	Beating versus arrested
Alanine	3.41 ± 0.64	2.70 ± 0.30	4.82 ± 0.64			0.01
Arginine	1.35 ± 0.15	1.35 ± 1.10	1.50 ± 0.11			
Asparagine	2.09 ± 0.27	1.99 ± 0.28	2.38 ± 0.50			
Aspartate	12.00 ± 1.55	5.39 ± 0.70	5.75 ± 0.94	0.001	0.001	
Glutamate	18.36 ± 1.35	16.35 ± 0.12	21.43 ± 0.94		0.05	0.001
Glutamine	16.39 ± 0.46	10.85 ± 0.12	15.78 ± 0.88	0.001	N.S.	0.001
Glycine	2.13 ± 0.33	1.62 ± 0.12	2.41 ± 0.52			
Lysine	2.47 ± 0.25	2.35 ± 0.40	2.14 ± 0.12			
Serine	1.18 ± 0.15	0.87 ± 0.15	1.04 ± 0.12			
Threonine	1.06 ± 0.22	0.81 ± 0.08	0.88 ± 0.31			
Total	60.55 ± 1.89	44.28 ± 1.50	58.23 ± 2.11	0.001	N.S.	0.001

Table 2. *Amino acid release into perfusion medium from isolated rat hearts*

The experimental conditions are as in Table 1. The volume of the recirculating perfusion medium was 60 ml and the amino acid increment during 60 min is shown. The values are means $\pm$ S.E.M. from six perfusions. Analytical procedures are as described in the Experimental section. *P* (versus beating heart) * <0.05 , *** <0.001 .

Amino acid	Content ($\mu\text{mol/g dry wt.}$)	
	Beating heart	Arrested heart
Alanine	3.51 ± 0.42	3.01 ± 0.47
Arginine	0.66 ± 0.10	$0.32 \pm 0.05^*$
Asparagine	1.61 ± 0.22	1.01 ± 0.17
Aspartate	0.16 ± 0.05	0.10 ± 0.02
Glutamate	0.73 ± 0.18	0.53 ± 0.07
Glutamine	8.59 ± 1.07	$3.99 \pm 0.50^{***}$
Glycine	2.05 ± 0.33	1.26 ± 0.09
Histidine	0.53 ± 0.98	$0.32 \pm 0.05^*$
Isoleucine	0.37 ± 0.12	$0.29 \pm 0.8^*$
Leucine	0.37 ± 0.22	0.52 ± 0.07
Lysine	0.97 ± 0.17	$0.41 \pm 0.08^*$
Methionine	0.18 ± 0.03	0.17 ± 0.04
Phenylalanine	0.61 ± 0.05	0.60 ± 0.06
Proline	0.69 ± 0.12	0.32 ± 0.06
Serine	0.72 ± 0.09	$0.47 \pm 0.03^*$
Threonine	0.78 ± 0.10	0.47 ± 0.03
Valine	0.55 ± 0.04	0.38 ± 0.40
Total	23.00 ± 2.30	$14.12 \pm 1.33^*$

Table 3. *Changes in the combined tissue and perfusate pools of individual amino acids during Langendorff perfusion of isolated rat hearts*

The experimental conditions are as in Table 1. The values are the means $\pm$ S.E.M. for the changes in six hearts during 60 min of perfusion. *P* (versus beating heart) * <0.05 .

Amino acid	Change ($\mu\text{mol/g dry wt.}$)	
	Beating heart	Arrested heart
Alanine	$+3.03 \pm 0.63$	$+4.42 \pm 1.30$
Arginine	$+0.68 \pm 0.30$	$+0.45 \pm 0.20$
Asparagine	$+1.51 \pm 0.62$	$+1.30 \pm 0.63$
Aspartate	-6.45 ± 1.72	-6.16 ± 1.95
Glutamate	-1.30 ± 1.19	$+3.55 \pm 1.55^*$
Glutamine	$+3.05 \pm 1.51$	$+3.37 \pm 1.87$
Glycine	$+1.54 \pm 0.49$	$+1.54 \pm 0.72$
Histidine	$+0.24 \pm 0.03$	$+0.24 \pm 0.03$
Isoleucine	$+0.37 \pm 0.05$	$+0.29 \pm 0.06$
Leucine	$+0.37 \pm 0.07$	$+0.53 \pm 0.07$
Lysine	$+0.79 \pm 0.59$	$+0.08 \pm 0.37$
Methionine	$+0.18 \pm 0.03$	$+0.17 \pm 0.04$
Phenylalanine	$+0.61 \pm 0.05$	$+0.60 \pm 0.06$
Proline	$+0.49 \pm 0.12$	$+0.32 \pm 0.06$
Serine	$+0.41 \pm 0.20$	$+0.41 \pm 0.11$
Threonine	$+0.53 \pm 0.31$	$+0.30 \pm 0.21$
Valine	$+0.61 \pm 0.04$	$+0.38 \pm 0.04$
Total	$+6.67 \pm 2.36$	$+11.78 \pm 2.11$

in the combined tissue plus perfusate pool of taurine in the beating or arrested hearts. This is in accord with the negligible capacity of the myocardium for metabolizing taurine (Peck & Awapara, 1967). Taurine was therefore not investigated further.

As shown in Table 3, the combined pool (tissue + perfusate) of most amino acids increased during the perfusion, although a noticeable decrease was observed in the pool size of aspartate. The total amino acid pool tended to increase more in the arrested hearts, but the difference compared with the beating hearts was not significant.

Production of ammonia

There were no significant differences in the tissue ammonia content between the beating and arrested hearts (Table 4). The ammonia output, however, was significantly greater from the beating heart than from the K^+ -arrested heart, and the rate of ammonia production was also greater in the former case (Table 4). The net synthesis of glutamine plus asparagine, converting free ammonia into amide nitrogen (Table 6), must be taken into account when calculating the total production of ammonia. The values obtained for ammonia production compare favourably with the calculated catabolism of amino acids.

Tissue content of adenine nucleotides

The net deamination of adenine nucleotides can be determined by measuring the net production of hypoxanthine compounds (IMP, inosine, hypoxanthine plus xanthine) or the net degradation of adenine nucleotides. In the present instance the total concentration of adenine nucleotides (ATP, ADP and AMP) was measured. This decreased slightly, but not significantly, during the perfusion (Table 4), probably indicating that the net deamination of adenine nucleotides to IMP and other hypoxanthine compounds does not play any major role in the production of the amounts of ammonia observed, and that amino acids must be the major source for this ammonia production in the rat myocardium.

Net proteolysis during perfusion

As shown in Table 3, the liberation of the non-metabolizable amino acid phenylalanine was the same ($0.6 \mu\text{mol}$ in 1 h) in both beating and arrested hearts. Table 5 shows the frequencies of individual amino acids in total myocardial proteins, and these data allow the calculation of net proteolysis, which was $16 \mu\text{mol}$ of amino acids in 1 h in both the beating and the arrested hearts.

The rate of proteolysis can also be estimated from the changes in the ammonia, amino acid and adenine nucleotide pools during the perfusion, as explained in the Experimental section. The increase in ammonia in the beating heart was $6.8 \mu\text{mol/g dry wt.}$ during

Table 4. *Contents of ammonia and adenine nucleotides in isolated perfused rat hearts*

Hearts from rats fed *ad libitum* were perfused by the Langendorff procedure with Krebs–Ringer bicarbonate medium containing 10 mM-glucose. After pre-perfusion for 30 min in a non-circulating system, the hearts were either arrested with K⁺ or allowed to beat and perfusion was continued for 60 min in a recirculating system. At the end of the perfusion period they were freeze-clamped and the ammonia content of the tissue and perfusate and tissue adenine nucleotides were measured as described in the Experimental section. The values are means $\pm$ S.E.M. from the numbers of independent biological observations in parentheses. *P* (beating versus arrested) ††† < 0.001; *P* (arrested versus initial) ** < 0.01.

	Amount (μ mol/g dry wt.)		
	Initial	Beating heart	Arrested heart
Free ammonia in tissue	1.90 (5) ± 0.12	1.72 (5) ± 0.15	1.46 (5) ± 0.25
Free ammonia released to perfusate		7.00 (5) ± 1.00	1.34 (5) ± 0.34
Total free ammonia	1.90 ± 0.12	8.78††† ± 0.82	2.80** ± 0.25
Net production of free ammonia		6.87	0.90
Conversion of ammonia into amide nitrogen		2.35	2.49
Total ammonia production		9.22	3.39
Adenine nucleotides in tissue	28.26 ± 1.54 (10)	27.28 ± 0.70 (9)	27.80 ± 0.40 (8)
Adenine nucleotide degraded		0.98	0.46

Table 5. *Frequencies of individual amino acids in total myocardial protein and amino acid liberation in net proteolysis*
It is assumed that phenylalanine is not metabolized further in the perfused heart, and the contribution of the other amino acids to net proteolysis is calculated from the amino acid composition of total protein.

Amino acid	Content (residues/100 residues)	Liberation (μ mol/g dry wt.)	
		Beating heart	Arrested heart
Alanine	9.4	1.54	1.53
Arginine	4.7	0.77	0.76
Aspartate + glutamate	9.4	1.54	1.53
Asparagine + glutamine	13.4	2.20	2.18
Glycine	10.3	1.69	1.67
Histidine	1.9	0.31	0.31
Isoleucine	8.9	1.46	1.45
Leucine	4.6	0.76	0.75
Lysine	6.3	1.03	1.02
Methionine	2.1	0.34	0.34
Phenylalanine	3.7	0.61	0.60
Proline	4.9	0.81	0.80
Serine	5.4	0.89	0.88
Threonine	5.1	0.84	0.83
Valine	6.3	1.04	1.02
Measured	96.4	15.84	15.66
Total	100	16.43	16.24

the 1 h period, and the decrease in the adenylate pool 1.0 μ mol/g (Table 4). The concomitant change in the combined pools of glutamine, asparagine, histidine and lysine was 5.6 μ mol, and the change in the total amino acid pool 6.7 μ mol, during the 1 h perfusion time (Table 3).

The net proteolysis in the beating heart is thus $(6.7 + 6.8 - 1.0 + 5.6)/1.224 \mu$ mol/g = 14.8 μ mol/g.

Similarly, a change of 11.8 μ mol/g in the total amino acid pool in the arrested heart (Table 3),

0.9 μ mol/g in ammonia, 0.5 μ mol/g in the adenine nucleotide pool and 5.0 μ mol/g in the combined pool of glutamine, asparagine, histidine and lysine give a proteolytic rate of 14.1 μ mol/g in total amino acids in 1 h. Thus the rate of proteolysis is obviously not affected by the mechanical work load of the heart.

As the net proteolysis calculated from the phenylalanine release was 16.4 μ mol/g dry wt. and the rate calculated from the ammonia production as described above was 14.8 μ mol/g dry wt. under

similar conditions, the error in the phenylalanine method introduced by the use of average amino acid frequencies in total myocardial protein is quite small.

Metabolism of amino acids in the myocardium

The catabolic rates of the individual amino acids are summarized in Table 6. Glutamate and aspartate form a pool characterized by a mutual exchange of ammonia by transamination and the release of ammonia by deamination by means of glutamate dehydrogenase or the purine nucleotide cycle. The frequencies of glutamate and aspartate in protein cannot be measured by conventional amino acid determination methods, owing to the decomposition of the corresponding amino acid amides. This aspartate + glutamate pool showed the highest rate of catabolism, and the myocardium demonstrated a net synthesis of alanine and asparagine + glutamine. In Table 6 the amino acid catabolism is also presented in nitrogen equivalents to take into account the differences in the ammonia (or amino acid) yielded from mono- and di-amino acids.

The combined catabolic rate in nitrogen equivalents of the amino acids studied was 7.05 $\mu\text{mol/g}$ dry wt. in the beating heart and 2.11 $\mu\text{mol/g}$ dry wt. in the arrested heart. Comparison of the high- and low-energy-consumption states shows the main difference to lie in the deamination rate of aspartate plus glutamate, which is inhibited by 60% during low energy consumption, and in the net synthesis of

alanine, which is 94% higher during low energy consumption.

Tricarboxylic acid-cycle intermediates and amino acid metabolism

Catabolism of certain amino acids produces intermediates of the tricarboxylic acid cycle, whereas other amino acids are metabolized by the consumption of cycle intermediates.

Glutamate can be deaminated by glutamate dehydrogenase and aspartate by the purine nucleotide cycle to result in a net yield of tricarboxylic acid-cycle intermediates. Alanine synthesis by alanine aminotransferase produces cycle intermediates at the expense of glutamate. Lysine catabolism consumes two molecules of cycle intermediates per molecule of lysine and yields two molecules of glutamate, whereas leucine degradation produces one molecule of glutamate and three of acetyl-CoA at the expense of one of cycle intermediates per molecule of leucine metabolized, and methionine yields one molecule of tricarboxylic acid-cycle intermediates per molecule metabolized. The above reactions and the present data allow the construction of a metabolic reaction rate map of the connexions between amino acids and the tricarboxylic acid cycle (Fig. 1). The total net anaplerosis owing to amino acid degradation during 1 h in the beating isolated perfused rat hearts was 9.0 $\mu\text{mol/g}$

Table 6. Catabolism of amino acids in isolated perfused rat heart

The sum of the amount of amino acid liberated in net proteolysis (Table 4) and the decrease in the pool size of that amino acid (Table 3) during 60 min of perfusion was taken as representing the catabolic rate of the amino acid. The nitrogen (or amino acid) equivalencies of the amino acids are those of Fig. 1.

Amino acid	Amino acid degraded ($\mu\text{mol/g}$ dry wt.)		Nitrogen equivalency	Nitrogen equivalents produced ($\mu\text{mol/g}$ dry wt.)	
	Beating heart	Arrested heart		Beating heart	Arrested heart
Alanine	-1.49	-2.89	1	-1.49	-2.89
Arginine	0.09	0.31	—	—	—
Aspartate + glutamate	9.29	4.13	1	9.29	4.13
Asparagine + glutamine	-2.35	-2.49	2	-4.70	-4.98
Glycine	0.15	0.13	1	0.15	0.13
Histidine	0.07	0.07	2	0.14	0.14
Isoleucine	1.09	1.19	1	1.09	1.19
Leucine	0.39	0.22	1	0.39	0.22
Lysine	0.24	0.94	2	0.48	1.88
Methionine	0.16	0.17	1	0.16	0.17
Phenylalanine	0.00	0.00	—	0.00	0.00
Proline	0.32	0.48	1	0.32	0.48
Serine	0.48	0.43	1	0.48	0.47
Threonine	0.31	0.53	1	0.31	0.53
Valine	0.43	0.64	1	0.43	0.64
Total	9.18	3.86		7.05	2.11

dry wt., which is 1.5% of the tricarboxylic acid-cycle flux in acetyl units, and that in the arrested perfused hearts was $4.2\mu\text{mol/g}$, or 2% of the tricarboxylic acid-cycle flux in acetyl units. When both the tissue

Fig. 1. Replenishment and consumption of tricarboxylic acid-cycle intermediates by amino acid metabolism in beating and arrested isolated perfused rat hearts

The arrows depict the stoichiometry between amino acid catabolism and the elimination or production of tricarboxylic acid (T.C.A.)-cycle metabolites. [OX] depicts the sink of cycle metabolites used as oxidizable fuel. Metabolic fluxes (in $\mu\text{mol/g}$ dry wt. during the 60min experimental period) are shown for the beating heart and (in parentheses) for the arrested heart.

concentration and the leakage into the perfusion medium of the major intermediates of the tricarboxylic acid cycle (Table 7) are taken into account, the increase in the total pool size of the cycle intermediates emerges as $1.2\mu\text{mol/g}$ dry wt. in the beating heart and $1.8\mu\text{mol/g}$ dry wt. in the arrested heart (Table 7.).

The tissue succinate concentration was $0.38 \pm 0.08\mu\text{mol/g}$ dry wt. (S.E.M., $n = 5$) after perfusion of the beating hearts for 1 h. Leakage of succinate into the perfusion medium during the 1 h experimental period was below the detection limit ($<0.4\mu\text{mol/g}$). Since previous data (Hiltunen & Hassinen, 1977) indicate that the metabolic transitions in myocardial succinate concentration are small, this was not investigated further.

Mechanisms of ammonia production

(1) *The purine nucleotide cycle.* The maximal activities of the myocardial purine nucleotide-cycle enzymes are shown in Table 8. If the values are expressed at 38°C by multiplying by a factor of $1.8/10^\circ\text{C}$, maximal activities of $19.5\mu\text{mol/min per g}$ dry wt. for AMP deaminase, $1.70\mu\text{mol/min per g}$ for adenylosuccinate synthetase and $1.18\mu\text{mol/min per g}$ for adenylosuccinate lyase are obtained.

The function of the purine nucleotides in a cell-free extract of rat myocardium is presented in Fig. 2. The cycling experiment was started at the deamination phase. The disappearance of adenine nucleotides is almost equivalent to the appearance of hypoxanthine compounds (IMP, inosine, hypoxanthine plus xanthine) and ammonia in the incubation mixture. When about two-thirds of the adenine nucleotides had been deaminated, the reaction mixture was energized by adding phosphoenolpyruvate, which resulted in the conversion of

Table 7. Concentrations of tricarboxylic acid-cycle intermediates in tissue of isolated rat hearts and their accumulation in the perfusion medium

The experimental conditions are as in Table 2. The tissue samples were obtained after a once-through pre-perfusion of 30min, or else the tissue and perfusion medium concentrations were determined after a further perfusion of 60min with recirculation. The values are means $\pm$ S.E.M. from the numbers of independent biological observations in parentheses. P (versus beating heart) $* < 0.05$.

	Amount ($\mu\text{mol/g}$ dry wt.)		
	Initial	Beating	Arrested
In tissue			
Citrate	0.59 ± 0.09 (9)	0.49 ± 0.09 (8)	0.81 ± 0.09 (7)*
2-Oxoglutarate	0.25 ± 0.04 (9)	0.21 ± 0.03 (8)	0.31 ± 0.05 (7)
Malate + fumarate	0.61 ± 0.08 (9)	0.53 ± 0.07 (8)	0.70 ± 0.08 (7)
Total	1.44 ± 0.16	1.24 ± 0.17	1.82 ± 0.18 *
In perfusion medium			
Citrate		0.24 ± 0.04 (4)	0.33 ± 0.05 (5)
2-Oxoglutarate		0.68 ± 0.13 (5)	0.64 ± 0.13 (5)
Malate + fumarate		0.49 ± 0.06 (3)	0.45 ± 0.06 (3)
Total		1.41	1.42
Total accumulation		1.21	1.80

Table 8. *Activities of the purine nucleotide-cycle enzymes and glutamate dehydrogenase in rat heart*
The enzyme activities in heart muscle extracts were determined as described in the Experimental section. The values are means $\pm$ S.E.M. from three independent biological observations.

Enzyme	pH	Maximum activity (μ mol/min per g dry wt.)
AMP deaminase	7.0	12.2 $\pm$ 3.6
Adenylosuccinate synthetase	7.0	1.06 $\pm$ 0.21
Adenylosuccinate lyase	7.4	0.74 $\pm$ 0.06
Glutamate dehydrogenase	7.0	0.48 $\pm$ 0.16
	7.5	2.47 $\pm$ 0.24
	8.0	7.17 $\pm$ 2.39

Fig. 2. *One turn of the purine nucleotide cycle in rat heart*

The initial reaction mixture contained 0.24 mM-GDP, 0.65 mM-AMP and 0.20 mM-ATP, 4 mM-aspartate, 27 mM-imidazole/HCl buffer, pH 7.0, myocardial 100000g supernatant corresponding to 121 mg fresh wt. of tissue/ml final volume, and other components as indicated in the Experimental section. The reaction was run at 37°C. When about two-thirds of the adenine nucleotides had been deaminated, the amination phase was initiated by adding 7.9 mM-phosphoenolpyruvate (PEP). Samples were withdrawn for enzymic analysis at the times indicated. A typical experiment is shown. Symbols: (a) O, adenine nucleotides (ATP, ADP and AMP), ●, hypoxanthine compounds (IMP, inosine, xanthine and hypoxanthine); (b) O, fumarate and malate, ●, ammonia.

adenine nucleotides mostly into ATP, and guanine nucleotides into GTP. The concentrations of the hypoxanthine compounds were decreased in a ratio almost equimolar to the increase in adenine nucleotides and in malate plus fumarate. No measurable amounts of adenylosuccinate were detected during this phase.

The actual activities of the enzymes during the cyclic experiment were 2.3 $\pm$ 0.3, 19 $\pm$ 3 and 27 $\pm$ 5% (mean $\pm$ S.E.M., n = 3) of the maximal activity for AMP deaminase, adenylosuccinate synthetase and adenylosuccinate lyase respectively. That the activities in tissue extracts are lower than the maximum activities has been also demonstrated in cell-free extracts of rat brain by Schultz & Lowenstein (1976), but direct comparison of the actual fractional activities in cycling experiments of different design is not feasible, since the enzyme activities depend on the initial conditions set by the cycle phase at the start.

(2) *Glutamate dehydrogenase.* Although the maximum activity of glutamate dehydrogenase is

usually measured in the direction of amination, it is the maximum activity towards deamination that is more relevant in the present context. The maximal deamination rate at pH 7.5 was 2.47 μ mol/min per g dry wt. for cardiac muscle at 30°C, but when the activity is converted into that at 38°C by using the factor 1.6 (1.8/10°C), a value of 3.9 μ mol/min per g dry wt. is obtained.

Discussion

In order to investigate the effect of the energy state on the net proteolysis and the carbon and nitrogen metabolism of amino acids in the isolated rat heart, the mechanical work was varied by means of K⁺-induced cardiac arrest. K⁺ affects the contractility of the heart by shortening the action potentials and suppressing the pacemaker activity (Weidman, 1951). Reversible regulation of the automaticity of excitable myocardial tissue by an external K⁺ concentration can also be demonstrated in isolated Purkinje fibres (Vassalle, 1965). This K⁺-induced

cardiac arrest results in a decrease in glucose and oxygen consumption and an inhibition of respiration (Hassinen & Hiltunen, 1975), but the myocardium remains well oxygenated, as can be demonstrated by NADH fluorescence photography, for instance (Peuhkurinen *et al.*, 1979).

High and low cellular energy-consumption states result in changes in the oxygen consumption and tricarboxylic acid-cycle flux, and in changes in the concentrations of metabolites which also play a part in feedback regulation of the energy metabolism.

Amino acids are an important source of replenishment of the dicarboxylic acid intermediates of the tricarboxylic acid cycle (Davis & Bremer, 1973), and it may be expected that amino acid metabolism is closely connected with energy-yielding metabolism and anaplerotic mechanisms.

The present data show that the catabolism of amino acids is indeed connected with the activity of oxidative metabolism. There is nevertheless no significant correlation between the changes in the pool size of the tricarboxylic acid-cycle intermediates in myocardial cells and amino acid catabolism. The ratio of amino acid catabolism to oxygen consumption is almost constant irrespective of the energy state. Since the accumulation of tricarboxylic acid-cycle intermediates is only a fraction of the anaplerotic rate produced by the net catabolism of amino acids, there must be continuous usage of the cycle intermediates for the synthesis of other non-nitrogenous compounds. This rate of elimination from the combined pool of tricarboxylic acid-cycle intermediates must be $7.8 \mu\text{mol/g dry wt.}$ in the beating heart and $2.4 \mu\text{mol/g dry wt.}$ in the arrested heart within the 1 h experimental period.

The routes for the elimination of tricarboxylic acid-cycle intermediates to be used as oxidizable fuels in the myocardium have not been positively identified. It is known that the decarboxylating malate dehydrogenase (EC 1.1.1.40) reaction is poised towards decarboxylation, and reversal of the reaction is kinetically unfavourable (Frenkel, 1972; Lin & Davis, 1974). This reaction could represent a link between tricarboxylic acid-cycle intermediates and pyruvate. Some activity of phosphoenolpyruvate carboxykinase (EC 4.1.1.32), although very low, has also been detected in cardiac muscle (Crabtree *et al.*, 1972), so that the possibility exists that oxaloacetate could be converted into phosphoenolpyruvate. Anaplerotic sources other than amino acids also exist, e.g. there is evidence that CO_2 -fixation reactions are involved in the replenishment of tricarboxylic acid-cycle intermediates in skeletal (Spydevold *et al.*, 1976) and cardiac muscle (M. Nuutinen, K. Peuhkurinen, P. Pictitainen, K. Hiltunen & I. Hassinen, unpublished work).

The metabolism fluxes of amino acids presented in

Table 6 were calculated by using the approximation that proteolysis involves all the myocardial proteins at the same rate. The usefulness of this approximation is supported by the finding that practically the same proteolytic rates were obtained by the phenylalanine-liberation method and the method involving measurements of ammonia, amino acids and adenine nucleotides. These methods are not totally independent, as the latter method involves an addendum of 22% of the total which is dependent on the amino acid composition of the protein degraded. The contribution of this variable is therefore small.

The constancy of net proteolysis in the isolated heart perfused in the absence of added amino acids, in spite of large differences in cellular energy consumption, indicates that proteolysis does not arise from a loss of cellular integrity because of a lack of ATP. One reason for the net proteolysis is the low free amino acid concentration in the cell, owing to a leak into the perfusion medium (Morgan *et al.*, 1971). The use of physiological amino acid concentrations in the perfusion medium was not feasible, however, because of a loss of sensitivity in the measurement of the changes in amino acid concentrations. It has been shown previously that insulin can prevent net proteolysis in isolated perfused rat heart in the presence of normal plasma concentrations of amino acids (Rannels *et al.*, 1974).

The measured ammonia production (Table 4) was very close to the calculated net deamination of amino acids, indicating that amino acids are the main source of ammonia. The absence of any significant decrease in the combined pool size of adenine nucleotides (Table 4) also fits with the other data.

It has been demonstrated that the production of ammonia from AMP in the AMP deaminase reaction in skeletal muscle is accelerated by increased energy consumption (Goodman & Lowenstein, 1977). IMP accumulates during exercise, and this is followed by aspartate consumption during recovery, when IMP is converted into AMP by a pathway involving adenylosuccinate synthetase and adenylosuccinate lyase. The purine nucleotide cycle, but not the glutamate dehydrogenase reaction, has the capacity to cover the total rate of ammonia production in skeletal muscle (Goodman & Lowenstein, 1977). There are previous indications that the activity of glutamate dehydrogenase is higher in cardiac muscle than in skeletal muscle (Pette *et al.*, 1962; Williamson *et al.*, 1967). The present study demonstrates that the maximal capacities of both these ammonia-producing pathways are greater than the observed ammonia production in the myocardium. Although the maximum activity of a pathway can be used as a rough indicator of its metabolic significance, its contribution *in situ*

depends on the actual activity under physiological conditions.

The results indicate that the net proteolytic rate in an isolated perfused heart is independent of energy consumption, although the deamination of the glutamate plus aspartate pool to tricarboxylic acid-cycle intermediates and ammonia is dependent on the cellular energy state.

The present results also demonstrate that it is possible, given a simple straightforward method for measuring the catabolic fluxes of individual amino acids, to estimate the anaplerotic requirements of myocardial energy metabolism.

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