Anaplerotic processes in human skeletal muscle during brief dynamic exercise

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1. This study examined changes in tricarboxylic acid cycle intermediates (TCAIs) in human skeletal muscle during 5 min of dynamic knee extensor exercise (~80% of maximum workload) and following 2 min of recovery.

2. The sum of the seven measured TCAIs (ΣTCAIs) increased from 1·10 ± 0·08 mmol (kg dry weight)$^{-1}$ at rest to 3·12 ± 0·24, 3·86 ± 0·35 and 4·33 ± 0·30 mmol (kg dry weight)$^{-1}$ after 1, 3 and 5 min of exercise, respectively ($P \leq 0·05$). The ΣTCAIs after 2 min of recovery (3·74 ± 0·43 mmol (kg dry weight)$^{-1}$) was not different compared with 5 min of exercise.

3. The rapid increase in ΣTCAIs during exercise was primarily mediated by large changes in succinate, malate and fumarate. These three intermediates accounted for > 90% of the net increase in ΣTCAIs during the first minute of contraction.

4. Intramuscular alanine increased after 1 min of exercise by an amount similar to the increase in the ΣTCAIs (2·33 mmol (kg dry weight)$^{-1}$) ($P \leq 0·05$). Intramuscular pyruvate was also higher ($P \leq 0·05$) during exercise, while intramuscular glutamate decreased by ~50% within 1 min and remained low despite an uptake from the circulation ($P \leq 0·05$).

5. The calculated net release plus estimated muscle accumulation of ammonia after 1 min of exercise (~60 μmol (kg wet weight)$^{-1}$) indicated that only a minor portion of the increase in ΣTCAIs could have been mediated through the purine nucleotide cycle and/or glutamate dehydrogenase reaction.

6. It is concluded that the close temporal relationship between the increase in ΣTCAIs and changes in glutamate, alanine and pyruvate metabolism suggests that the alanine aminotransferase reaction is the most important anaplerotic process during the initial minutes of contraction in human skeletal muscle.

Ambient levels of tricarboxylic acid (TCA) cycle intermediates (TCAIs) are controlled by various carboxylation and decarboxylation reactions which regulate carbon flux into and out of the cycle (Lee & Davis, 1979). Under normal conditions the cataplerotic removal of cycle intermediates is counteracted by anaplerotic processes so that the absolute concentration of TCAIs remains constant (Lehninger, Nelson & Cox, 1993). However, in mammalian skeletal muscle during contraction the concentrations of specific TCAIs have been shown to increase (Essén & Kaisjer, 1978; Aragón & Lowenstein, 1980; Sahlin, Katz & Broberg, 1990), and while no study has measured the entire pool of TCAIs, changes in seven of the eight TCAIs have been quantified in rat hindlimb during electrical stimulation (Aragón & Lowenstein, 1980) and recently in humans during prolonged, moderate cycling exercise (Gibala, Tarnopolsky & Graham, 1997). Succinyl CoA was not determined in those studies due to its extremely low concentration; however, it is likely that the remaining seven TCAIs account for > 99% of TCAIs and thus provide a quantitative index of total pool size. Those investigations clearly demonstrated that the total concentration of TCAIs in skeletal muscle can increase severalfold during the initial minutes of contraction.

Although the precise functional significance remains obscure (Williamson & Cooper, 1980; Peuhkurinen, 1984), the fact that there is a net accumulation of TCAIs indicates that flux through anaplerotic reactions greatly exceeds cataplerotic processes during the initial minutes of contraction. The increase in TCAIs could be mediated through a number of mechanisms, including the purine nucleotide cycle (PNC) as well as reactions catalysed by glutamate dehydrogenase, alanine aminotransferase, pyruvate carboxylase, phosphoenolpyruvate carboxykinase and malic enzyme (Lee & Davis, 1979; Aragón & Lowenstein, 1980). Aragón & Lowenstein (1980) proposed that the PNC is responsible for the net increase in TCAIs observed during stimulation in rodents. However, indirect evidence from human studies

suggests that the alanine aminotransferase reaction may be the major process which contributes to the increase in TCAIs at the onset of exercise (Felig & Wahren, 1971; Sahlin *et al.* 1990; van Hall, Saltin & Wagenmakers, 1995). The relative importance of the various anaplerotic processes in skeletal muscle therefore remains to be determined.

The purpose of the present investigation was to elucidate which mechanism(s) is (are) most important for the increase in TCAIs at the onset of exercise in human skeletal muscle. We utilized the knee extensor exercise model in order to quantify changes in the intramuscular concentrations and/or net uptake/release of numerous metabolites which participate in the potentially anaplerotic reactions outlined above (e.g. TCAIs, glutamate, alanine, aspartate, pyruvate and ammonia). A secondary purpose was to determine the time course for changes in individual TCAIs during the initial period of contraction. Our previous study indicated that peak expansion of the TCAI pool occurs within 5 min of moderate exercise (Gibala *et al.* 1997), and therefore biopsy samples in the present investigation were obtained at rest and after 1, 3 and 5 min of exercise. Finally, we included measurements during recovery in order to determine the extent of cataplerosis following exercise.

METHODS

Subjects

Eight healthy males, with a mean age, height and weight of 22·1 ± 1·1 years, 182·1 ± 0·8 cm and 74·8 ± 3·4 kg, respectively, volunteered for the investigation. Two subjects regularly participated in club sports (football and running), while the other six were not engaged in any form of regular physical activity. The subjects were fully informed of the purposes and associated risks of the study, and gave their written, informed consent. The experimental protocol was approved by the Ethical Committee for Copenhagen and Frederiksberg communities.

Pre-experimental procedures

The subjects were familiarized with the Krogh ergometer modified for one-legged knee extensor exercise as described previously (Anderson & Saltin, 1985). With this exercise model, electromyographic activity is absent in the hamstrings and glutei muscles and the majority of work done for knee extension is performed by the quadriceps femoris muscle. At least 3 days prior to the experiment subjects performed an incremental exercise test with their dominant leg (kicking frequency, 60 min^{-1}) in order to determine the maximal power output of the knee extensors. Subjects were instructed to consume their habitual diet and refrain from exercise or strenuous physical activity for 48 h prior to the experiment.

Experimental protocol

Subjects arrived at the laboratory after an overnight fast. Teflon catheters were inserted into the femoral artery and vein of one leg, ~2 cm proximal and distal to the inguinal ligament, respectively. A thermistor for measurement of venous blood temperature was inserted through the venous catheter and the tip advanced ~8 cm proximal to the tip of the catheter. Subjects were moved to the exercise apparatus where they rested supine for ~30 min prior to the initiation of the exercise test. During this time the area over the lateral aspect of each thigh was anaesthetized (2% lidocaine/lignocaine) and prepared for the extraction of needle biopsy samples from the vastus lateralis muscle (Bergström, 1975).

The exercise protocol consisted of kicking at ~80% of the one-legged maximal knee extension capacity (68 ± 5 W) for 5 min. In order that all blood samples, blood flow measurements and muscle biopsy samples could be obtained at the proper time points, it was necessary for both legs to perform the exercise protocol separated by a rest period of ~30 min. Leg order was randomized and counterbalanced between subjects for dominance. All blood samples and blood flow measurements were obtained during the first exercise bout. Arterial and venous blood samples were drawn simultaneously at rest, after 1, 3 and 5 min of exercise and after 2 min of recovery. Measurements of leg blood flow using the thermodilution technique (Anderson & Saltin, 1985) were made 4–6 times at rest, and immediately prior to and following each blood sample during exercise and recovery. During blood sampling and blood flow measurements, an occlusion cuff positioned just below the knee was inflated to ⩾ 220 mmHg. Needle biopsy samples during the first exercise bout were obtained at rest, after 5 min of exercise and after 2 min of recovery. Following the first exercise bout subjects remained on the exercise apparatus and rested in the supine position before beginning the second exercise bout. Muscle biopsy samples during the second bout were taken at rest, and after 1, 3 and 5 min of exercise. The mean time interruption to allow for the biopsy procedure at the 1 and 3 min sampling points was ~20 s (exercise stoppage to resumption of kicking). Heart rate and expired air measurements were made at rest and continually during each exercise bout.

Blood analyses

Blood samples were drawn with heparinized syringes. O_2 saturation, O_2 content and haemoglobin were measured using an OSM-2 Hemoximeter (Radiometer, Copenhagen, Denmark). Whole blood glucose and lactate concentrations were immediately determined using a Yellow Springs glucose–lactate analyser (Yellow Springs, OH, USA). The remainder of the arterial and venous blood samples were centrifuged and the supernatant collected and stored at −80 °C. Plasma samples were subsequently analysed for ammonia (Bergmeyer, 1974) using a fluorometer (Perkin-Elmer LS-50) and free amino acids using HPLC (Heinrickson & Meredith, 1984).

Muscle analyses

Biopsy samples were immediately frozen by plunging the needle into liquid nitrogen. The samples were removed from the needle while still frozen and subsequently freeze dried, powdered to dissect out non-muscle elements and stored at −80 °C. A 1·5–2·0 mg portion of freeze-dried muscle was homogenized for 1 min in 100 μl deionized water (Milli Q, Millipore Corp., Bedford, MA, USA) and then centrifuged for 3 min. The supernatant was used for determination of free amino acids using HPLC according to the method of Heinrickson & Meredith (1984). Aliquots of the remaining freeze-dried muscle were extracted with 0·5 M $HClO_4$ and neutralized with 2·2 M $KHCO_3$. Extracts were assayed enzymatically for citrate + oxaloacetate, malate + fumarate, isocitrate, 2-oxoglutarate and succinate (Lowry & Passoneau, 1972; Bergmeyer, 1974; Sahlin *et al.* 1990; Passoneau & Lowry, 1993) using a fluorometer as described previously (Gibala *et al.* 1997). A portion of the extract was also analysed for pyruvate (Harris, Hultman & Nordesjo, 1974) using a fluorometer.

Calculations

Thigh volume was calculated by using the thigh length, three circumferences and three skinfold measurements (Jones & Pearson,

Table 1. Cardiorespiratory, blood flow and muscle oxygen uptake data during exercise and recovery

	Heart rate (beats min^{-1})	Pulmonary $\dot{V}_{O_2}$ (l min^{-1})	Expired ventilation (l min^{-1})	Muscle blood flow (l min^{-1} kg^{-1})	Muscle $\dot{V}_{O_2}$ (ml O_2 min^{-1} kg^{-1})
Rest	70 ± 2	0·40 ± 0·06	15·6 ± 2·4	0·09 ± 0·01	5 ± 1
1 min	105 ± 3*	1·04 ± 0·09*	34·2 ± 4·4*	1·61 ± 0·13*	295 ± 30*
3 min	117 ± 2*†	1·23 ± 0·09*†	44·3 ± 4·2*†	1·86 ± 0·17*†	356 ± 36*†
5 min	129 ± 3*†	1·40 ± 0·10*†	52·9 ± 6·8*†	1·96 ± 0·17*†	382 ± 32*†
Recovery	90 ± 4	0·52 ± 0·06	20·0 ± 3·1	0·31 ± 0·05	9 ± 1

Values are means ± S.E.M. ($n = 8$). Cardiorespiratory data are from bout 1. $\dot{V}_{O_2}$, oxygen uptake.
* $P \leq 0·05$ *vs.* rest. † $P \leq 0·05$ *vs.* 1 min.

1969), and muscle mass was estimated from a regression equation (Anderson & Saltin, 1985). The active muscle mass of the leg used in bout 1, during which all blood samples and blood flow values were obtained, was 2·80 ± 0·19 kg. The active muscle mass of the leg used during bout 2 was 2·78 ± 0·18 kg, which was not different from the leg used during bout 1. The uptake and/or release of O_2, glucose, lactate, ammonia and amino acids was calculated by multiplying the blood or plasma flow by the arteriovenous difference in concentration and were expressed per kilogram of wet weight of active muscle. The exchange of lactate, ammonia and amino acids was estimated for each subject by averaging the flux between consecutive sampling points and then multiplying this value by the duration between the two points (e.g. average the flux at 1 and 3 min and then multiply by 2). The total exchange during exercise was calculated by summing the estimated exchange values between rest and 1 min, 1 and 3 min, and 3 and 5 min. In some instances the intramuscular concentrations of metabolites were converted to wet weights using the mean wet : dry ratio from biopsy samples at the corresponding time points. The branched-chain amino acids (BCAAs) were obtained by summing leucine, isoleucine and valine; the essential amino acids by summing the BCAA, threonine, methionine, phenylalanine, tryptophan and lysine; and the total amino acids by summing all amino acids except for hydroxyproline and 3-methylhistidine.

Statistics

Blood, plasma and muscle metabolites were analysed using a one factor (1 × 5, time) repeated-measures analysis of variance. Statistical significance was accepted as $P \leq 0·05$, and significant main effects were further analysed using Tukey's honestly significant difference *post hoc* test. Data for all tables and figures are expressed as means ± S.E.M. unless otherwise noted.

Table 2. Arterial concentrations and net uptake/release of metabolites and some plasma amino acids during exercise and recovery

	Rest	1 min	3 min	5 min	Recovery
			Arterial concentration		
Glucose	4·62 ± 0·09	4·96 ± 0·17*	4·96 ± 0·15*	4·89 ± 0·15*	4·78 ± 0·14
Lactate	0·41 ± 0·04	1·30 ± 0·10*	2·25 ± 0·18*	2·77 ± 0·27*	2·93 ± 0·27*
Ammonia	21 ± 7	24 ± 6	40 ± 9*	44 ± 8*	43 ± 8*
Alanine	252 ± 23	247 ± 9	264 ± 11	303 ± 10	338 ± 14
Glutamine	564 ± 17	576 ± 19	555 ± 13	575 ± 14	600 ± 20
Glutamate	50 ± 3	44 ± 1	43 ± 2*	42 ± 3*	49 ± 4
Aspartate	2 ± 0	2 ± 0	2 ± 0	2 ± 0	2 ± 0
			Net uptake/release		
Glucose	0 ± 0	−0·1 ± 0·0	0·1 ± 0·1	0·2 ± 0·0	0·1 ± 0·0
Lactate	0 ± 0	−3·0 ± 0·5*	−3·8 ± 0·7*	−3·4 ± 0·7*	−0·4 ± 0·1
Ammonia	0 ± 0	−25 ± 8	−69 ± 13*	−94 ± 17*†	−13 ± 2
Alanine	−5 ± 2	−74 ± 13*	−90 ± 15*	−61 ± 13*	−6 ± 5
Glutamine	−6 ± 2	−91 ± 34*	−106 ± 24*	−66 ± 32*	−7 ± 8
Glutamate	2 ± 1	12 ± 2*	10 ± 3*	17 ± 2*†	4 ± 1

Values are means ± S.E.M. ($n = 8$). Arterial concentrations are in mmol l^{-1} for glucose and lactate and μmol l^{-1} for ammonia and the amino acids. Net uptake/release data are in mmol min^{-1} (kg wet weight)$^{-1}$ for glucose and lactate and μmol min^{-1} (kg wet weight)$^{-1}$ for ammonia and the amino acids. A negative value indicates a release. * $P \leq 0·05$ *vs.* rest. † $P \leq 0·05$ *vs.* 1 min.

Table 3. Summary of total uptake/release estimates for ammonia and some amino acids during exercise and recovery

	0–1 min	1–3 min	3–5 min	0–5 min	Recovery
Ammonia	−12 ± 4	−95 ± 20	−163 ± 30	−270 ± 51	−106 ± 19
Alanine	−43 ± 7	−171 ± 22	−151 ± 24	−366 ± 47	−67 ± 12
Glutamine	−43 ± 7	−214 ± 40	−172 ± 44	−444 ± 75	−73 ± 30
Glutamate	7 ± 1	21 ± 3	28 ± 5	56 ± 7	22 ± 2

Values are means ± S.E.M. with units of μmol (kg wet weight)$^{-1}$ ($n = 8$). Total uptake/release was calculated for each time period as described in the Methods. A negative value indicates a release. Recovery refers to the 2 min period immediately following exercise.

RESULTS

Cardiorespiratory, blood flow and muscle oxygen uptake

There were no significant differences between the first and second exercise bouts in the response of any cardiorespiratory variable. Changes in heart rate, pulmonary oxygen uptake and expired ventilation for the first bout, during which all blood samples and blood flow measurements were obtained, are summarized in Table 1. Muscle blood flow and muscle oxygen uptake were higher ($P \leqslant 0{\cdot}05$) after 1, 3 and 5 min of exercise compared with rest (Table 1). Muscle blood flow and muscle oxygen uptake after 2 min of recovery were lower ($P \leqslant 0{\cdot}05$) compared with 5 min of exercise and not different from rest.

Blood and plasma metabolites

The arterial glucose concentration showed a small but significant increase during exercise, and was higher at 1, 3 and 5 min compared with rest ($P \leqslant 0{\cdot}05$; Table 2). There were no significant changes in glucose uptake/release during exercise or recovery (Table 2). The arterial lactate concentration was higher ($P \leqslant 0{\cdot}05$) at 1, 3 and 5 min of exercise and after 2 min of recovery, compared with rest (Table 2). Lactate release was higher ($P \leqslant 0{\cdot}05$) at 1, 3 and 5 min of exercise compared with rest (Table 2). Arterial plasma ammonia was higher ($P \leqslant 0{\cdot}05$) at 3 and 5 min of exercise and after 2 min of recovery, compared with rest (Table 2). The release of ammonia was higher ($P \leqslant 0{\cdot}05$) at 3 and 5 min of exercise compared with rest (Table 2), and showed a progressive increase during exercise such that the value at 5 min was higher ($P \leqslant 0{\cdot}05$) compared with 1 min of exercise. A summary of total ammonia release during exercise and recovery is presented in Table 3.

Plasma amino acids and flux data

The arterial concentrations of glutamate, alanine, glutamine and aspartate are summarized in Table 2. Glutamate uptake increased during exercise and was higher ($P \leqslant 0{\cdot}05$) at 1, 3 and 5 min of exercise compared with rest (Table 2). The uptake of glutamate also showed a progressive increase during exercise, such that the value after 5 min was higher ($P \leqslant 0{\cdot}05$) compared with 1 min of exercise. Alanine and glutamine release increased during exercise and were higher ($P \leqslant 0{\cdot}05$) at 1, 3 and 5 min of exercise compared with rest (Table 2). There was no significant change in the uptake/release of aspartate during exercise or recovery, and at all times this value ranged between 0·1 and −0·1 μmol min^{-1} (kg wet weight)$^{-1}$. A summary of the total uptake/release calculations for glutamate, alanine and glutamine during exercise and recovery is presented in Table 3.

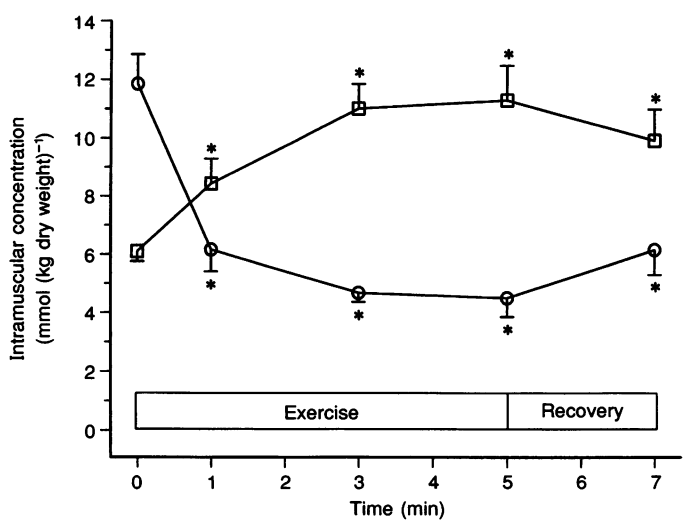

Figure 1. Intramuscular glutamate and alanine
Values for glutamate (○) and alanine (□) represent means ± S.E.M. * $P \leqslant 0{\cdot}05$ *vs.* rest. Exercise in this and all subsequent figures was performed at 80% of maximum knee extension capacity.

Table 4. Some intramuscular amino acids during exercise and recovery

	Rest	1 min	3 min	5 min	Recovery
Aspartate	1·48 ± 0·25	2·21 ± 0·53	2·58 ± 0·67	1·60 ± 0·32	1·36 ± 0·24
Hydroxyproline	0·21 ± 0·02	0·19 ± 0·02	0·20 ± 0·02	0·21 ± 0·03	0·21 ± 0·03
Serine	1·62 ± 0·05	1·47 ± 0·09	1·63 ± 0·06	1·59 ± 0·15	1·79 ± 0·10
Asparagine	1·53 ± 0·18	1·23 ± 0·14	1·37 ± 0·09	1·43 ± 0·19	1·42 ± 0·17
Glycine	3·56 ± 0·20	3·09 ± 0·20	3·60 ± 0·25	3·39 ± 0·22	3·40 ± 0·03
Glutamine	51·93 ± 2·44	46·25 ± 2·42	51·72 ± 2·20	48·00 ± 3·92	48·66 ± 2·84
Taurine	44·12 ± 2·74	39·10 ± 2·11	41·43 ± 3·36	39·22 ± 4·39	41·96 ± 5·19
Histidine	1·32 ± 0·06	1·41 ± 0·12	1·47 ± 0·07	1·56 ± 0·17	1·41 ± 0·12
Threonine	1·02 ± 0·08	0·94 ± 0·11	1·03 ± 0·14	0·98 ± 0·10	1·07 ± 0·08
Arginine	1·21 ± 0·10	1·10 ± 0·10	1·21 ± 0·08	1·25 ± 0·12	1·21 ± 0·09
Proline	4·10 ± 0·95	4·46 ± 1·15	4·38 ± 1·08	4·54 ± 1·58	3·78 ± 0·77
Tyrosine	0·27 ± 0·03	0·25 ± 0·03	0·28 ± 0·03	0·30 ± 0·03	0·29 ± 0·03
Valine	1·09 ± 0·10	1·12 ± 0·11	1·16 ± 0·10	1·22 ± 0·16	1·12 ± 0·12
Methionine	0·18 ± 0·02	0·19 ± 0·02	0·22 ± 0·02	0·25 ± 0·02	0·23 ± 0·02
Isoleucine	0·31 ± 0·03	0·31 ± 0·03	0·34 ± 0·03	0·35 ± 0·04	0·32 ± 0·03
Leucine	0·74 ± 0·07	0·79 ± 0·09	0·82 ± 0·09	0·87 ± 0·12	0·80 ± 0·08
Phenylalanine	0·30 ± 0·03	0·28 ± 0·03	0·31 ± 0·03	0·32 ± 0·04	0·31 ± 0·03
Tryptophan	0·08 ± 0·01	0·10 ± 0·02	0·10 ± 0·02	0·12 ± 0·02	0·11 ± 0·02
Ornithine	0·38 ± 0·08	0·33 ± 0·05	0·37 ± 0·06	0·37 ± 0·05	0·41 ± 0·04
Lysine	1·76 ± 0·18	1·61 ± 0·13	1·75 ± 0·13	1·79 ± 0·23	1·75 ± 0·12
BCAA	2·15 ± 0·19	2·22 ± 0·22	2·32 ± 0·21	2·44 ± 0·31	2·23 ± 0·22
EAA	5·49 ± 0·29	5·32 ± 0·39	5·73 ± 0·32	5·91 ± 0·56	5·69 ± 0·34
TAA	135·0 ± 5·1	120·8 ± 5·9	131·4 ± 6·2	124·9 ± 11·8	127·5 ± 9·4

Values are means ± S.E.M. with units of mmol (kg dry weight)$^{-1}$ ($n = 6$). There were no significant changes in these amino acids during exercise or recovery. EAA, essential amino acids; TAA, total amino acids.

Intramuscular amino acids and pyruvate

Intramuscular glutamate decreased markedly from the start of exercise and was lower ($P \leqslant 0{\cdot}05$) at 1, 3 and 5 min of exercise and 2 min of recovery, compared with rest (Fig. 1). Intramuscular alanine was higher ($P \leqslant 0{\cdot}05$) after 1, 3 and 5 min of exercise and after 2 min of recovery, compared with rest (Fig. 2). There were no significant changes in the intramuscular concentrations of the other amino acids (Table 4). Intramuscular pyruvate was higher ($P \leqslant 0{\cdot}05$) after 1, 3 and 5 min of exercise compared with rest, and increased further after exercise, such that the value after 2 min of recovery was higher ($P \leqslant 0{\cdot}05$) compared with 5 min of exercise (Fig. 2).

TCA cycle intermediates

The total concentration of the of seven measured TCAIs was higher ($P \leqslant 0{\cdot}05$) after 1, 3 and 5 min of exercise compared with rest (Fig. 3). Total pool size after 2 min of recovery was

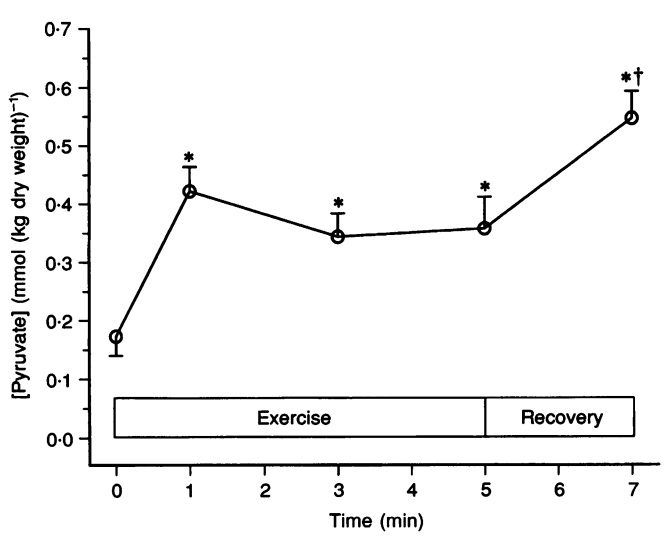

Figure 2. Intramuscular pyruvate
Values are means ± S.E.M. * $P \leqslant 0{\cdot}05$ *vs.* rest. † $P \leqslant 0{\cdot}05$ *vs.* 5 min of exercise.

higher ($P \leq 0{\cdot}05$) than the value at rest but not different compared with 5 min of exercise.

With respect to individual TCAIs, citrate did not increase above rest until 5 min of exercise, while isocitrate was higher ($P \leq 0{\cdot}05$) after 3 and 5 min compared with rest (Fig. 4*A*). Citrate was the only TCAI which showed an increase following exercise such that the value after 2 min of recovery was higher ($P \leq 0{\cdot}05$) compared with 5 min of exercise. 2-Oxoglutarate rose slightly after 1 min and then gradually declined; however, the changes in this intermediate were not statistically significant (Fig. 4*B*). Succinate, fumarate and malate showed the earliest and largest changes during exercise and were all higher ($P \leq 0{\cdot}05$) after 1, 3 and 5 min of exercise compared with their respective rest levels (Fig. 4*C–E*). The magnitude of change in these three TCAIs is further illustrated by the fact that they accounted for ~60% of the TCAI pool size at rest, but more than 80% of total pool size during exercise. The concentration of oxaloacetate was higher ($P \leq 0{\cdot}05$) after 5 min of exercise compared with rest (Fig. 4*F*).

DISCUSSION

The focus of the present investigation was the time course and processes which contribute to the net increase in TCAIs at the onset of contraction in human skeletal muscle. Our data clearly indicate that whatever mechanism(s) functions to expand the pool of TCAIs, it occurs very quickly since more than 60% of the net increase in TCAI pool size occurred within the first minute of exercise. The rapid increase in TCAIs was primarily mediated by large changes in succinate, malate and fumarate, all of which increased by two- to threefold above their respective rest values after 1 min of exercise. Changes in the other four measured TCAIs were slower and smaller in magnitude. These results are in agreement with data from the only previous study of TCAI pool size in human skeletal muscle (Gibala *et al.* 1997), but demonstrate that the expansion of the TCAI pool occurs very early during exercise and the actual rate of expansion is approximately twofold higher than previously suggested (Gibala *et al.* 1997).

A number of processes could contribute to the increase in TCAI pool size during contraction, but their physiological significance has been the subject of considerable debate (Aragón & Lowenstein, 1980; Davis, Spydevold & Bremer, 1980; Sahlin *et al.* 1990). The proposed mechanisms include the PNC, which is a series of three reactions catalysed by AMP deaminase (eqn (1)), adenylosuccinate synthase (eqn (2)) and adenylosuccinate lyase (eqn (3)), respectively:

$$\text{AMP} \rightarrow \text{IMP} + \text{NH}_3, \quad (1)$$

$$\text{IMP} + \text{aspartate} + \text{GTP} \rightarrow \text{adenylosuccinate} + \text{GTP} + \text{P}_i, \quad (2)$$

$$\text{adenylosuccinate} \rightarrow \text{AMP} + \text{fumarate}, \quad (3)$$

such that one complete 'turn' of the cycle results in the deamination of aspartate, the consumption of GTP and the production of NH_3 and fumarate. (IMP in eqns (1) and (2) is inosine monophosphate.) Other purported anaplerotic reactions include those catalysed by glutamate dehydrogenase (GDH; eqn (4)), alanine aminotransferase (AAT; eqn (5)), pyruvate carboxylase (PC; eqn (6)), phosphoenolpyruvate carboxykinase (PEPCK; eqn (7)) and malic enzyme (ME; eqn (8)):

$$\text{glutamate} + \text{NAD}^+ \rightleftharpoons \text{2-oxoglutarate} + \text{NH}_3 + \text{NADH}, \quad (4)$$

$$\text{glutamate} + \text{pyruvate} \rightleftharpoons \text{2-oxoglutarate} + \text{alanine}, \quad (5)$$

$$\text{pyruvate} + \text{CO}_2 + \text{ATP} \rightarrow \text{oxaloacetate} + \text{ADP} + \text{P}_i, \quad (6)$$

$$\text{phosphoenolpyruvate} + \text{CO}_2 + \text{IDP} \rightarrow \text{oxaloacetate} + \text{ITP}, \quad (7)$$

$$\text{pyruvate} + \text{CO}_2 + \text{NAD(P)H} + \text{H}^+ \rightleftharpoons \text{malate} + \text{NAD(P)}^+, \quad (8)$$

Aragón & Lowenstein (1980) proposed that the PNC is the major process responsible for the net increase in TCAI pool size during contraction. These authors used hadacidin, a competitive inhibitor with respect to aspartate in the adenylosuccinate synthase reaction (eqn (2)), to block PNC

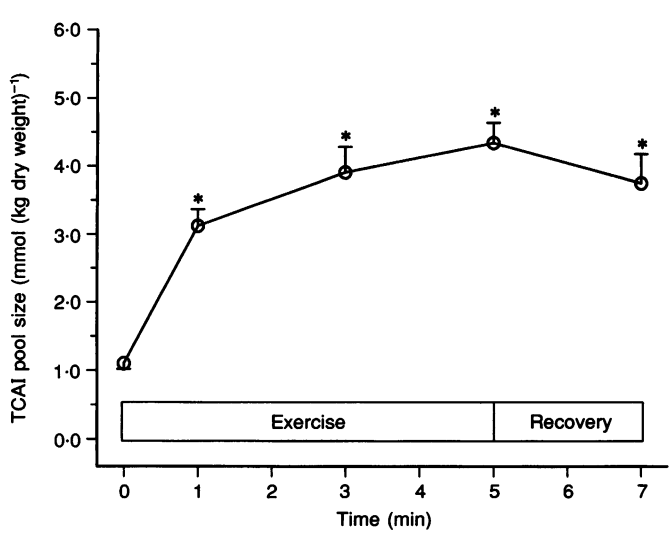

Figure 3. Total TCA cycle pool size
Total intramuscular concentration of the seven measured TCAIs at rest and during exercise and recovery. Values are means ± S.E.M. * $P \leq 0{\cdot}05$ *vs.* rest.

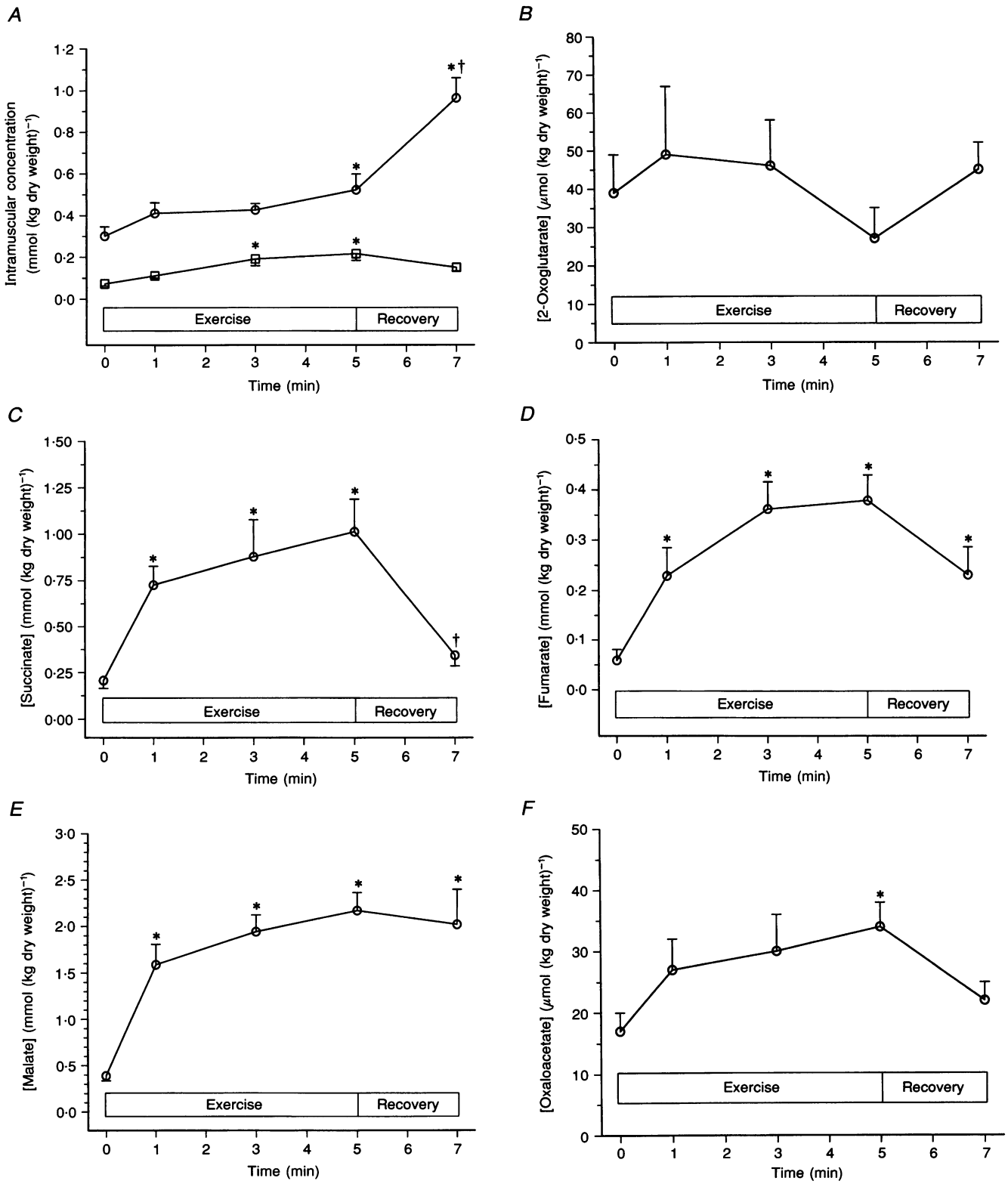

Figure 4. Intramuscular TCA cycle intermediates

A, citrate (○) and isocitrate (□). *B*, 2-oxoglutarate. *C*, succinate. *D*, fumarate. *E*, malate. *F*, oxaloacetate. All values are means ± S.E.M. * $P \leqslant 0{\cdot}05$ *vs.* rest. † $P \leqslant 0{\cdot}05$ *vs.* 5 min of exercise.

cycling in rat hindlimb muscle during electrical stimulation. They observed a 53% reduction in the accumulation of malate + fumarate compared with untreated control animals, and based on the difference in IMP accumulation between groups, calculated that the operation of the PNC accounted for at least 70% of the expansion of the TCAI pool during the first 10 min of contraction. *In vitro* studies have supported the potential role of the PNC as an anaplerotic process in skeletal muscle (Scislowski, Aleksandrowicz & Swierczynski, 1982). However, there is considerable controversy regarding the extent to which the PNC actually cycles in *active* muscle fibres (for review see Tullson & Terjung, 1991).

Regardless, if we assume the PNC was cycling in the present study, our data suggest only a minor portion of the increase in TCAI pool size during the first minute of exercise could have been mediated through this process. Based on the net equation for the PNC, it is evident that in order to reaminate IMP and produce fumarate, aspartate is needed as an NH_3 donor. In the present investigation there was no measurable change in the intramuscular aspartate concentration during exercise, and the uptake of aspartate during exercise was negligible. In fact the net increase in TCAI pool size during the first minute of exercise was larger than the resting intramuscular aspartate concentration. Therefore, in order for PNC cycling to have occurred a rapid and continuous supply of aspartate would have been required from endogenous sources (i.e. intramuscular protein catabolism or transamination). This seems unlikely since there were no significant shifts in the intramuscular essential or total amino acid pools during exercise (Table 4).

Furthermore, if the PNC was cycling and contributed to the increase in TCAIs, there should have been a stoichiometric increase in NH_3 for each fumarate that was generated. Intramuscular NH_3 was not measured in the present study, but previous investigations have shown that ~25–40% of the NH_3 produced during the initial minutes of leg extensor exercise (over a wide range of intensities) is released from the muscle (Graham, Bangsbo, Gollnick, Juel & Saltin, 1990; MacLean, Graham & Saltin, 1994, 1996). In the present study the release of NH_3 during the first minute of exercise was 12 μmol (kg wet weight)$^{-1}$ (Table 3). If we conservatively assume this represents 20% of the total NH_3 produced, then the total NH_3 production after 1 min of exercise (i.e. net release plus estimated muscle accumulation) was ~60 μmol (kg wet weight)$^{-1}$. However, the increase in TCAI pool size after the first minute of exercise was 2030 μmol (kg dry weight)$^{-1}$, or ~440 μmol (kg wet weight)$^{-1}$, which indicates ~14% of the increase in TCAIs during the first minute could have been mediated through PNC cycling.

Similarly, a portion of the NH_3 formation could have been derived from the GDH reaction (eqn (4)), and this would have further reduced the contribution of the PNC. Numerous investigators have dismissed the physiological significance of GDH due to its low activity in rodent muscle (Lowenstein, 1972). However, substantially higher GDH activities have been reported for human quadriceps (Wibom & Hultman, 1990; Wibom, Hultman, Johansson, Matherie, Constantin, Teodosiu & Schantz, 1992) and thus the enzyme could potentially be involved in anaplerosis. In the present study intramuscular glutamate declined by ~50% within the first minute of exercise (Fig. 1). However, due to the relatively small increase in NH_3 production (as outlined above for the PNC), the GDH reaction could not have contributed significantly to the rapid increase in TCAI pool size after the first minute of exercise. We recognize that in order to accurately quantify changes in NH_3 metabolism in skeletal muscle it is necessary to account for increases in glutamine and alanine production, since these compounds can function as NH_3 'carriers' (Graham & MacLean, 1992). In the present study glutamine release increased slightly during the first minute of exercise (Table 3). However, when combined with the small decrease in the intramuscular glutamine concentration (Table 4) there was no net glutamine production. Regardless, even if the glutamine released during the first minute of exercise was derived from free NH_3 and is added to our estimate of total NH_3 production, the maximum combined anaplerotic contribution of GDH and the PNC over the first minute of exercise is ~23%.

In comparison, the intramuscular concentration of alanine increased markedly during exercise (Fig. 1), and we attribute this to an increased rate of flux through the AAT reaction. AAT is a near-equilibrium enzyme, located both within the mitochondrial matrix and the cytosol (Molé, Baldwin, Terjung & Holloszy, 1973) and thus is sensitive to small changes in substrate concentrations. The activity of this enzyme is quite high in rodent (Molé *et al.* 1973) and equine (Guy & Snow, 1977) skeletal muscle, and has been shown to increase with training (Molé *et al.* 1973; Guy & Snow, 1977). The stimulus for the increased rate of flux through the AAT reaction was probably the increase in intramuscular pyruvate concentration at the start of exercise (Fig. 2) when the rate of pyruvate production from glycolysis apparently exceeded its rate of oxidation in the TCA cycle. The increase in intramuscular alanine after 1 min of exercise was quantitatively similar to the increase in TCAIs, and when viewed in conjunction with the large drop in intramuscular glutamate, we feel these data provide strong evidence that the AAT reaction was the major anaplerotic process under the present exercise conditions. These data are supported by two previous studies which demonstrated that the increase in the sum of citrate, malate and fumarate was temporally related to a decrease in glutamate and increase in alanine during exercise in healthy humans (Sahlin *et al.* 1990; Sahlin, Jorfeldt, Henriksson, Lewis & Haller, 1995). In comparison, the concentrations of alanine and pyruvate in rat hindlimb muscle *in situ* do not increase during intense electrical stimulation, nor is the release of alanine augmented (Aragón & Lowenstein, 1980). This indicates that methodological and/or species differences between the present investigation and the study by Aragón & Lowenstein (1980) could in part explain the discrepancy in the proposed mechanism of anaplerosis.

In principle, the increased rate of flux through AAT should increase the total concentration of TCAIs by providing carbon from glutamate, which would enter the cycle at the level of 2-oxoglutarate. However, despite the large increase in intramuscular alanine after the first minute of exercise we did not observe an increase in the concentration of 2-oxoglutarate (Fig. 4*B*), which suggests this intermediate is rapidly converted to other TCAIs. It has previously been demonstrated that the concentration of 2-oxoglutarate does not increase during moderate dynamic exercise in humans (Graham & Saltin, 1989; Gibala *et al.* 1997), although the explanation for the unique response of this intermediate is not clear. It is plausible that increases in the other TCAIs may occur without an increase in 2-oxoglutarate, since 2-oxoglutarate dehydrogenase is believed to catalyse a non-equilibrium, flux-generating reaction (Newsholme & Leech, 1983) and thus by definition is not regulated strictly by changes in substrate concentration. The factors which influence the concentration of 2-oxoglutarate in skeletal muscle, and the regulation of flux through 2-oxoglutarate dehydrogenase, warrant further investigation.

In addition to AAT, the reactions catalysed by PC, PEPCK and ME are also dependent on the availability of pyruvate; however, the relative importance of these processes in anaplerosis is equivocal (Aragón & Lowenstein, 1980; Brodal & Hjelle, 1990). Skeletal muscle contains only residual amounts of PC (Crabtree, Higgins & Newsholme, 1972; Brodal & Hjelle; 1990), and the activity of this enzyme is probably too low to be of any physiological significance. Davis *et al.* (1980) concluded that the PC reaction could account for the increase in TCAIs observed in rat muscle over the course of 10–15 min, but the activity of the enzyme is certainly too low to have played a significant role in anaplerosis in the present study. In addition, the PC reaction is energetically costly and since it is controlled by the ATP : ADP ratio (Davis *et al.* 1980), one would expect a decrease in enzyme activity during the rest to work transition in skeletal muscle. The maximal activity of PEPCK has been reported to be ~0·3–0·5 mmol (kg wet weight)$^{-1}$ min^{-1} in human and rodent quadriceps (Newsholme & Leech, 1983). However, these values are at least 100-fold lower than the maximal activity of AAT (Molé *et al.* 1973; Guy & Snow, 1977), and since both enzymes catalyse near-equilibrium reactions one would expect the latter mechanism to predominate under conditions of increased pyruvate availability. In addition, the method used to determine PEPCK activity in these earlier studies has been questioned, and more recent investigations have concluded that skeletal muscle contains only trace amounts of PEPCK (Brodal & Hjelle, 1990). Significant amounts of ME are present in skeletal muscle; however, the reductive carboxylation of pyruvate seems unlikely for enzyme kinetic reasons (Aragón & Lowenstein, 1980; Davis *et al.* 1980). NAD-specific ME converts malate to pyruvate irreversibly, and while the NADP-linked ME reaction is reversible, the conversion of pyruvate to malate is extremely slow since the enzyme possesses a very high K_m for pyruvate (Davis *et al.* 1980).

An alternative explanation for the increase in TCAIs which must be considered is an increase in pyruvate dehydrogenase (PDH) activity and lack of cataplerotic TCA cycle losses. However, we feel that such a mechanism is unlikely given that the activity of PDH is under strict metabolic control and functions to regulate the flux of pyruvate-derived acetyl CoA into the TCA cycle (Hultman, 1996). In addition, in order for PDH to directly contribute to anaplerosis, a continuous source of oxaloacetate would be required to condense with acetyl CoA and form citrate. This seems unlikely given the extremely low concentration of oxaloacetate present in mammalian skeletal muscle (Aragón & Lowenstein, 1980; Gibala *et al.* 1997). However, it is possible that PDH may indirectly contribute to anaplerosis by *limiting* the rate of entry of pyruvate into the mitochondrion. That is, the increase in intramuscular [pyruvate], which occurs when its rate of production from glycolysis exceeds its rate of entry into the TCA cycle (the latter being determined by PDH), could be important in order to drive some of the near-equilibrium anaplerotic reactions outlined above (e.g. AAT) towards the production of TCAIs.

After the first minute of exercise the total concentration of TCAIs continued to increase; however, the rate of pool expansion was markedly reduced and it is difficult to identify the predominant mechanism(s) of anaplerosis during this time. It is possible that GDH and/or the PNC may have played a relatively larger anaplerotic role compared with the first minute of exercise, since the release of NH_3 increased progressively during the bout. Alternatively, if only the deaminating portion of the PNC was functioning during exercise (Tullson & Terjung, 1991), this would have resulted in the generation of NH_3 and IMP but no concomitant increase in fumarate. Intramuscular IMP was not measured in the present investigation. However, a previous study showed an increase in this metabolite after 5 min of leg extensor exercise at a slightly lower work intensity (MacLean *et al.* 1994). It is also plausible that the AAT reaction continued to predominate after the first minute of exercise, since the decrease in intramuscular glutamate and increase in alanine during this time were larger than the increase in the pool of TCAIs.

Following exercise, most individual TCAIs tended to decline. However, citrate showed a large increase and was the only TCAI which was higher during recovery compared with the end of exercise. As a result the overall rate of cataplerosis was relatively slow and the total concentration of TCAIs after 2 min of recovery was not different compared with 5 min of exercise. Essén & Kaijser (1978) also noted an increase in muscle citrate during recovery from intense cycle exercise, and suggested this was due to inhibition of the isocitrate dehydrogenase reaction. Inhibition of isocitrate dehydrogenase would also be expected to result in an increase in isocitrate, since the aconitase reaction is purportedly near equilibrium (Newsholme & Leech, 1983) and thus the relative changes in isocitrate should reflect changes in citrate. However, we did not observe an increase

in isocitrate during recovery, which indicates that flux through aconitase *in vivo* may not simply be regulated by changes in citrate concentration. Nevertheless, a discussion of the operational control mechanisms in the cycle is beyond the scope of this paper and was not the focus of the present investigation. This topic is further complicated by the general lack of information regarding metabolite concentrations in the mitochondria of skeletal muscle. In cardiac muscle for example, cytosolic and mitochondrial fractions are known to exist for citrate, isocitrate, 2-oxoglutarate, malate and oxaloacetate, as well as the TCA cycle enzymes aconitase, isocitrate dehydrogenase and malate dehydrogenase (Randle & Tubbs, 1979).

In summary, this study demonstrated that the total concentration of TCAIs in human skeletal muscle increases approximately twofold within 1 min of moderate, dynamic exercise. It has been proposed that the PNC is the major anaplerotic process at the onset of contraction in skeletal muscle. However, these data indicate that only a minor portion of the increase in TCAI pool size could have been mediated through this process. Similarly, due to the relatively small increase in NH_3 accumulation after 1 min of exercise, the glutamate dehydrogenase reaction could not have contributed significantly to the rapid increase in TCAIs. The increase in TCAI pool size after 1 min was quantitatively similar to the increase in intramuscular alanine, and when viewed in conjunction with the increase in intramuscular pyruvate and decrease in intramuscular glutamate, these data suggest that the alanine aminotransferase reaction is the major anaplerotic process during the initial minutes of contraction in human skeletal muscle.

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Acknowledgements

The authors thank Dr Thomas Rohde, Lynda Powell, Karin Juel, Charlotta Mortensen and Hana Villmusen for excellent technical assistance. This work was supported by the Natural Sciences and Engineering Research Council of Canada (NSERC) and the Danish National Research Foundation. M. J. Gibala was the recipient of a NSERC Postgraduate Scholarship and a Gatorade Sports Science Institute Student Research Award. D. A. MacLean was supported by a postdoctoral fellowship from the Medical Research Council of Canada.

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Received 7 January 1997; accepted 25 April 1997.