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Role of pyruvate carboxylation in the energy-linked regulation of pool sizes of tricarboxylic acid-cycle intermediates in the myocardium

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The increase in the metabolite pool size of the tricarboxylic acid cycle in the isolated perfused rat heart after a decrease in the ATP consumption by KCl-induced arrest was used to study the anaplerotic mechanisms. During net anaplerosis the label incorporation into the tricarboxylic acid-cycle intermediates from [1-^{14}C]pyruvate increased and occurred mainly by pathways not involving prior release of the label to CO_2. A method for determination of the specific radioactivity of mitochondrial pyruvate was devised, and the results corroborated the notion that tissue alanine can be used as an indicator of the specific radioactivity of intracellular pyruvate [Peuhkurinen & Hassinen (1982) *Biochem. J.* **202**, 67–76].

The size of the tricarboxylic acid-cycle metabolite pool varies according to the availability of oxidizable substrates (Garland & Randle, 1964; Randle *et al.*, 1970; Davis *et al.*, 1972; Safer & Williamson, 1973; Davis & Bremer, 1973) and the energy expenditure of the cells (Hassinen & Hiltunen, 1975; Hiltunen & Hassinen, 1977; Spydevold *et al.*, 1977). It has been demonstrated that CO_2 is incorporated into tricarboxylic acid-cycle intermediates in isolated perfused rat skeletal muscle and heart and in mitochondria from skeletal and heart muscle (Lee & Davis, 1980; Davis *et al.*, 1980; Nuutinen *et al.*, 1981*b*), and that a small but measurable activity of pyruvate carboxylase can be detected in rat heart mitochondria (Davis *et al.*, 1980).

It has been shown more directly that during the acetate-induced net anaplerosis in the isolated perfused rat heart C-1 of pyruvate is incorporated into tricarboxylic acid-cycle intermediates (Peuhkurinen & Hassinen, 1982). Among these metabolites, citrate occupies a special position as a putative feedback regulator of glycolysis (Garland & Randle, 1964). Thus the reactions of anaplerosis and metabolite disposal may have a role not only in the regulation of glycolysis by alternative substrates, but also in the regulation of glucose oxidation to meet the cellular energy demand.

In the present paper we expand the notion of regulation of the tricarboxylic acid-cycle pool size to a situation where inhibition of glycolysis occurs owing to a decrease in energy consumption (Hiltunen & Hassinen, 1976), not to changes in the substrate supply. Here, an accumulation of the cycle intermediates occurred which was appropriate for testing the energy-linked regulation of anaplerosis.

Materials and methods

Reagents

The enzymes, coenzymes and nucleotides were from Boehringer, Mannheim, Germany, and Sigma Chemical Co., St. Louis, MO, U.S.A. Ordinary chemicals were obtained from E. Merck A.G., Darmstadt, Germany. Sodium [1-^{14}C]pyruvate was purchased from The Radiochemical Centre, Amersham, Bucks., U.K.

Animals and perfusion methods

Female Sprague–Dawley rats (from the Department's own stocks) fed *ad lib* were anaesthetized and treated as described previously (Peuhkurinen & Hassinen, 1982). The isolated hearts were perfused with Krebs–Ringer bicarbonate solution, pH 7.4 (Krebs & Henseleit, 1932), gassed with O_2/CO_2 (19:1). A modified Langendorff procedure without recirculation was used, and the aortic pressure was 7.84 kPa (80 cm H_2O). After an initial perfusion with the above medium supplemented with 10 mM-glucose and 0.2 mM-pyruvate for 15 min, perfusion was continued for a further 10 min with 0.2 mM-[1-^{14}C]-

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pyruvate (sp. radioactivity 1.8×10^5–5.2×10^5 d.p.m./μmol). Simultaneously, cardiac arrest was induced by increasing the perfusate KCl concentration to 16mM. The NaCl concentration was lowered accordingly to maintain an unchanged osmolarity.

Tissue sampling and determination of metabolite concentration and specific radioactivities

The hearts were quick-frozen and extracted with $HClO_4$, and the extracts neutralized, as described previously (Peuhkurinen & Hassinen, 1982). Metabolite concentrations were determined by conventional enzymic methods, as listed by Nuutinen *et al.* (1981*b*) and Peuhkurinen & Hassinen (1982).

The metabolites were isolated from the $HClO_4$ extracts by ion-exchange chromatography on a Dowex-1 (formate form) column (LaNoue *et al.*, 1970) and by an automatic amino acid analyser, and their radioactivities were determined as described previously (Peuhkurinen & Hassinen, 1982).

The results of the label-incorporation experiments were normalized to a perfusate [1-^{14}C]pyruvate specific radioactivity of 10^5d.p.m./μmol. The purity of the [1-^{14}C]pyruvate substrate was tested on a Dowex-1 (formate form) column, where 89–92% of the radioactivity was eluted in a single peak. A minor peak was eluted just before malate, but this did not interfere with determination of the radioactivity of the metabolites. The recovery was tested throughout the analytical procedure in respect of the organic acids under investigation, and appropriate corrections were applied.

Results and discussion

In this special case, where the label is introduced in the form of [1-^{14}C]pyruvate, the complicated fates of the individual carbon atoms of the tricarboxylic acid-cycle metabolites can be followed with sufficient accuracy in a relatively simple way, since label input to the cycle in the form of acetyl-CoA is avoided. The principal modes of behaviour of the label have been summarized previously (Peuhkurinen & Hassinen, 1982). The sampling schedule was selected in order to record an estimate of the initial rate of label influx into the metabolites studied and the steady-state value of the specific radioactivity of the metabolites.

Malate, citrate and 2-oxoglutarate

The specific radioactivity of malate in the KCl-arrested heart reached a value equivalent to 5% of the specific radioactivity of the pyruvate in the perfusion medium (Table 1), i.e. 4 times that in beating control hearts (Peuhkurinen & Hassinen, 1982). The malate concentrations did not change significantly (Table 1).

The myocardial citrate concentration tripled within 10min after arrest of the heart, and its specific radioactivity reached a value 70% higher than in the beating heart (Table 1). The 2-oxoglutarate concentration also increased in the arrested heart in the presence of pyruvate (Table 1), but the small total amount of this metabolite in the heart did not allow accurate determination of specific radioactivity.

Bicarbonate

The output of label in bicarbonate in the KCl-arrested heart diminished rapidly after 1min, and its specific radioactivity was thereafter less than 0.1% of the pyruvate specific radioactivity. In view of this, labelling of the tricarboxylic acid-cycle metabolites could not have occurred through the mediation of bicarbonate prelabelled by the decarboxylation of [1-^{14}C]pyruvate. The outflow of ^{14}C label as HCO_3^- is shown in Table 2.

Glutamate and aspartate

Because of the large sizes of the glutamate and aspartate pools in the myocardium, labelling of these must be taken into consideration when assessing the rate of label influx into tricarboxylic acid-cycle metabolites. Label can be incorporated into glutamate and aspartate by the aminotransferase reactions and into glutamate also by glutamate dehydrogenase, which has an appreciable activity in rat heart mitochondria (Nuutinen *et al.*, 1981*a*). The specific radioactivity of aspartate reached about 50% of that of malate in the KCl-arrested hearts (Table 1). There was a transient increase in the glutamate concentration after arrest of the heart, but no significant decrease in the aspartate pool size was observed.

Alanine

It has previously been shown that when [1-^{14}C]-lactate is used as the label source, myocardial alanine reaches 70% of the specific radioactivity of tissue pyruvate (Peuhkurinen & Hassinen, 1982). Since it has been similarly demonstrated with [1-^{14}C]pyruvate that myocardial lactate reaches a much lower specific radioactively than alanine, it has been suggested that alanine would be a more appropriate indicator of intracellular pyruvate specific radioactivity (Peuhkurinen & Hassinen, 1982) than the lactate used previously (Scholz *et al.*, 1978).

The specific radioactivity of alanine reaches a plateau within 5min (Peuhkurinen & Hassinen, 1982) in the beating heart, but in the arrested heart a small but statistically non-significant increase in the labelling occurred between 5 and 10min. It is shown below by appropriate treatment of the present results that a near-equilibrium in the alanine aminotransferase reaction probably prevails in the myo-

cardium, corroborating the notion that alanine can be used as an indicator of the specific radioactivity of pyruvate in the heart.

Correlation between labelling kinetics and net anaplerosis

Initial labelling rate. After the K^+-induced arrest of the heart, the combined citrate, and malate and 2-oxoglutarate pool increased by 1.9 μmol/g dry wt. from the initial value of 1.6 ± 0.1 to 3.5 ± 0.3 μmol/g dry wt. ($P < 0.001$). The initial labelling rate for the tricarboxylic acid-cycle metabolites plus aspartate and glutamate can be used as an estimate for the unidirectional carboxylation rate of pyruvate. During the first 2 min the total labelling rate of this combination of metabolites were 15000 d.p.m./min per g dry wt., equal to 0.83 ± 0.05 μmol/min per g dry wt. in terms of pyruvate incorporation when the specific radioactivity of alanine is taken to reflect that of cellular pyruvate (see below).

Table 1. *Concentrations and specific radioactivities of tricarboxylic acid-cycle intermediates and related metabolites in beating and KCl-arrested isolated perfused rat hearts*

The values are means ± S.E.M. from four to ten independent experiments and are normalized to a [1-^{14}C]pyruvate specific radioactivity of 100000 d.p.m./μmol. The hearts were perfused with 10 mM-glucose and 0.2 mM-pyruvate. [1-^{14}C]Pyruvate tracer and 16 mM-KCl (in the K^+-arrested hearts) were present in the perfusion medium from zero time onwards. The metabolite pool sizes are given as μmol/g dry wt. and the units of specific radioactivity are 10^3 d.p.m./μmol. The data of the beating hearts are taken from Peuhkurinen & Hassinen (1982).

		K^+-arrested heart						Beating heart	
Time (min) ...	0	2		5		10		10	
	Pool size	Pool size	Sp. radio-activity	Pool size	Sp. radio-activity	Pool size	Sp. radio-activity	Pool size	Sp. radio-activity
Malate	0.76 ± 0.08	0.85 ± 0.04	3.90 ± 1.68	0.78 ± 0.07	5.17 ± 1.14	0.87 ± 0.14	5.13 ± 1.03	0.62 ± 0.07	1.24 ± 0.04
Citrate	0.61 ± 0.02	1.20 ± 0.19	2.13 ± 0.41	2.05 ± 0.23	3.32 ± 0.19	2.01 ± 0.26	3.19 ± 0.22	0.77 ± 0.10	1.87 ± 0.26
2-Oxoglutarate	0.27 ± 0.02	0.40 ± 0.02		0.63 ± 0.08		0.62 ± 0.09		0.24 ± 0.02	
Aspartate	8.14 ± 0.66	7.41 ± 0.56	2.30 ± 0.26	8.13 ± 0.67	2.38 ± 0.21	8.27 ± 0.36	2.20 ± 0.51	7.51 ± 1.01	0.382 ± 0.045
Glutamate	21.5 ± 0.9	25.9 ± 1.0	0.204 ± 0.060	24.1 ± 1.9	0.580 ± 0.209	21.0 ± 1.1	0.783 ± 0.209	21.0	0.149 ± 0.11
Pyruvate	0.33 ± 0.03	0.66 ± 0.02		0.59 ± 0.08		0.51 ± 0.06		0.33 ± 0.02	
Alanine	4.34 ± 0.47	2.95 ± 0.46	18.9 ± 1.1	4.06 ± 0.49	25.4 ± 3.2	4.11 ± 0.45	32.5 ± 4.5	5.47 ± 0.68	20.7 ± 2.7

Table 2. *$^{14}CO_2$ production and calculated tricarboxylic acid-cycle flux in isolated perfused rat hearts*

The production of $^{14}CO_2$ was determined in 1-min fractions of the effluent perfusion fluid, as described in the Materials and methods section, and is given as the mean ± S.E.M. from four independent experiments. The tricarboxylic acid-cycle was calculated from the oxygen consumption and information on the contribution of glucose oxidation taken from Hiltunen & Hassinen (1976). The remaining oxygen consumption was taken to represent endogenous palmitate. In separate experiments 0.2 mM-pyruvate had no effect on the oxygen consumption.

	10^{-3} × $^{14}CO_2$ production (d.p.m./min per g dry wt.)			
Time (min) ...	1	2	5	10
Beating heart	160 ± 53	182 ± 45	189 ± 45	210 ± 74
KCl-arrested heart	151 ± 23	101 ± 27	37 ± 5	31 ± 3
	Tricarboxylic acid-cycle flux (μmol/min per g dry wt.)			
Beating heart	7.9	7.9	7.9	7.9
KCl-arrested heart	7.8	7.6	3.7	3.3

The initial rate of label influx after its introduction to the system in a metabolic steady state would also serve as an estimate of the reversal rate of these reactions, i.e. decarboxylation of malate or oxaloacetate, since net anaplerosis should not exist. From the values reported by Peuhkurinen & Hassinen (1982) for beating hearts in a metabolic steady state, the reversal of the anaplerotic reaction(s) would be $0.41 \pm 0.13\,\mu$mol/min per g dry wt. when calculated from the initial labelling rates as described above for the present results.

Isotopic and metabolic steady state. Since the label in C-1 and C-4 of malate is lost as CO_2 during the first turn of the cycle, the isotopic and metabolic steady state at 10min can be used to estimate the rate of pyruvate carboxylation provided that the tricarboxylic acid-cycle flux is known. The specific radioactivity of malate (Table 1) at 10min has reached a steady state of 5130 ± 1030 d.p.m./μmol, which by multiplication by the cycle flux (Table 2), $3.3\,\mu$mol/min, gives a label flux into the cycle of 16900 ± 3400 d.p.m./min. By using x (μmol/min) as the carbon influx from pyruvate to malate and that from malate to pyruvate in a metabolic steady state, the label influx to malate ($x \cdot p$) should equal the label efflux from malate $[(x+c)m]$, where p and m are the specific radioactivities of pyruvate and malate respectively, and c is the tricarboxylic acid-cycle flux. When alanine is used as an indicator of p, solving for x gives a value of $0.62 \pm 0.18\,\mu$mol/min per g dry wt. for the unidirectional carbon fluxes between pyruvate and malate. Identical treatment of the previously reported data (Peuhkurinen & Hassinen, 1982) in the isotopic steady state at 10min gives unidirectional rates of pyruvate carboxylation and malate decarboxylation in a beating heart of $0.50 \pm 0.07\,\mu$mol/min per g dry wt. This value is of the same order of magnitude as the value estimated from the 'initial' labelling data in the beating heart (see above). However, the latter value should be corrected for label lost in the tricarboxylic acid cycle and malate decarboxylation during the first 2min, because the 2-min values are not strictly initial. By approximation, the specific radioactivity of malate can be taken to increase linearly during that time, and, by using the values of Peuhkurinen & Hassinen (1982) and the cycle flux of $7.8\,\mu$mol/min per g dry wt. from Table 2, unidirectional label influx rate to malate in the metabolic steady state in the beating heart would be $0.92 \pm 0.30\,\mu$mol/min per g dry wt. When a similar correction to the 'initial' metabolite labelling in the arrested heart is made, by using the cycle flux values from Table 2 and the malate decarboxylation rate of $0.50\,\mu$mol/min per g dry wt. (although the contribution of this is negligible), the unidirectional label influx to malate during net anaplerosis becomes $1.56 \pm 0.14\,\mu$mol/min per g dry wt. One should note also that these values are minimum values, because correction is not made for the lag in labelling of intracellular pyruvate.

The reported maximum activity of pyruvate carboxylase, $1.36\,\mu$mol/min per g dry wt., calculated from the reported mitochondrial content of the enzyme (Davis *et al.*, 1980) and the content of mitochondria in rat myocardium (Kinnula & Hassinen, 1977), is close to the observed unidirectional label influx from pyruvate to malate as calculated above.

It is noteworthy that the results indicate that a transient increase in the unidirectional reaction carboxylating pyruvate coincided with the phase of net anaplerosis.

The steady-state decarboxylation rates of malate in beating and arrested hearts were not significantly different. If this were the case also during net anaplerosis, the net carboxylation rate of pyruvate would be between the values $1.56-0.50 = 1.06$ (see above) and $1.56-0.92 = 0.64\,\mu$mol/min per g dry wt. This value should be compared with the net increase in the total combined pool of cycle metabolites plus aspartate and glutamate, which was $2.27 \pm 0.81\,\mu$mol/min per g dry wt. ($P < 0.005$) (Table 1) during the first 2min after cardiac arrest.

Specific radioactivity of mitochondrial pyruvate. Knowledge of the pyruvate oxidation rate in the perfused heart, which is known to oxidize only external glucose (or pyruvate) under these conditions (Hiltunen & Hassinen, 1976), allows calculation of the specific radioactivity of the intramitochondrial pyruvate from the rate of $^{14}CO_2$ production (Table 2). Hence, the total $^{14}CO_2$ production rate is 31000 ± 3000 d.p.m./min, and since $^{14}CO_2$ production in the cycle is 16900 ± 3400 d.p.m./min (see above), the $^{14}CO_2$ production in the pyruvate dehydrogenase reaction must be 14100 ± 4600 d.p.m./min. A pyruvate dehydrogenase flux of $0.74\,\mu$mol/min [twice the glucose oxidation rate, $0.37\,\mu$mol/min per g dry wt., from Hiltunen & Hassinen (1976)] would then give 19100 ± 6100 d.p.m./μmol, which is lower than the specific radioactivity of alanine. In the beating heart, the values from Peuhkurinen & Hassinen (1982) and from Table 2 give a specific radioactivity of 25300 ± 9400 d.p.m./μmol for intramitochondrial pyruvate, which is quite close to the 20700 ± 2700 d.p.m./μmol for alanine obtained. These data justify the use of the specific radioactivity of alanine as an approximate indicator for that of intracellular pyruvate in the myocardium.

Another important point is revealed by the evaluation of the above data, namely that at low rates of pyruvate dehydrogenase flux, as observed here in the arrested heart and elsewhere in hearts perfused with fatty acids (Olson *et al.*, 1978), more than 50% of the $^{14}CO_2$ production from [1-^{14}C]-pyruvate occurs in the tricarboxylic acid cycle, and

the use of total $^{14}CO_2$ production as a measure of the pyruvate dehydrogenase flux, although in common practice (see, e.g., Olson *et al.*, 1978), gives values which are in error by a factor of 2 even when corrected for the cellular pyruvate specific radioactivity by some means. Therefore the specific radioactivity of malate should be known and an estimate of the tricarboxylic acid-cycle flux obtained in order to measure the pyruvate dehydrogenase flux in isotope experiments.

Anaplerosis as a metabolic regulator. The present results emphasize that appreciable changes in the key metabolites occur after metabolic changes involving only the cellular energy state without change in substrate availability. It is characteristic of the current view of the regulation of cellular respiration in intact tissues, including myocardium (Hassinen & Hiltunen, 1975; Nishiki *et al.*, 1978) that this regulation operates according to the cellular energy state, not by substrate limitation. Therefore the present approach for studying the regulation of anaplerotic mechanisms via perturbations in the cellular energy state might be more appropriate than experiments on the effects of added substrates (Peuhkurinen & Hassinen, 1982). The possibility of energy-linked regulation of the concentrations of the tricarboxylic acid-cycle intermediates affords a mechanism not only for controlling the tricarboxylic acid cycle, but also for the feed-back regulation of cytosolic processes by mitochondria, as exemplified by the inhibition of glycolysis during fatty acid oxidation (Newsholme *et al.*, 1962; Garland & Randle, 1964) and the decrease in ATP consumption (Hiltunen & Hassinen, 1976).

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