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Pyruvate carboxylation as an anaplerotic mechanism in the isolated perfused rat heart

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1. The role of pyruvate carboxylation in the net synthesis of tricarboxylic acid-cycle intermediates during acetate metabolism was studied in isolated rat hearts perfused with [1-^{14}C]pyruvate. 2. The incorporation of the ^{14}C label from [1-^{14}C]pyruvate into the tricarboxylic acid-cycle intermediates points to a carbon input from pyruvate via enzymes in addition to pyruvate dehydrogenase and citrate synthase. 3. On addition of acetate, the specific radioactivity of citrate showed an initial maximum at 2 min, with a subsequent decline in labelling. The C-6 of citrate (which is removed in the isocitrate dehydrogenase reaction) and the remainder of the molecule showed differential labelling kinetics, the specific radioactivity of C-6 declining more rapidly. Since this carbon is lost in the isocitrate dehydrogenase reaction, the results are consistent with a rapid inactivation of pyruvate dehydrogenase after the addition of acetate, which was confirmed by measuring the $^{14}CO_2$ production from [1-^{14}C]pyruvate. 4. The results can be interpreted to show that carboxylation of pyruvate to the C_4 compounds of the tricarboxylic acid cycle occurs under conditions necessitating anaplerosis in rat myocardium, although the results do not identify the enzyme involved. 5. The specific radioactivity of tissue lactate was too low to allow it to be used as an indicator of the specific radioactivity of the intracellular pyruvate pool. The specific radioactivity of alanine was three times that of lactate. When the hearts were perfused with [1-^{14}C]lactate, the specific radioactivity of alanine was 70% of that of pyruvate. The results suggest that a subcompartmentation of lactate and pyruvate occurs in the cytosol.

Intermediates of the tricarboxylic acid cycle are continuously regenerated by 'anaplerotic' mechanisms (Kornberg, 1966). Under some conditions net anaplerosis occurs, leading to an increase in the metabolite pool of the tricarboxylic acid cycle. In cardiac muscle *in vitro*, this occurs upon an increase in the supply of external substrates such as glucose (Garland & Randle, 1964; Safer & Williamson, 1973), ketone bodies (Bowman, 1966), propionate (Davis & Quastel, 1964), pentenoate (Hiltunen, 1978) and acetate (Randle *et al.*, 1970), and upon aerobic arrest of the heart (Hiltunen & Hassinen, 1977), and in the brain during ischaemia (Folbergrová *et al.*, 1974).

Potential anaplerotic pathways include coupled transaminations between aspartate and pyruvate (Davis & Bremer, 1973), the purine nucleotide cycle (Lowenstein, 1972), and oxidative deamination of glutamate or carboxylation of pyruvate (Spydevold *et al.*, 1976). The mechanism of the last reaction has not been identified in the heart, where the activity of pyruvate carboxylase (EC 6.4.1.1) is considered to be low (Scrutton & Utter, 1968). It has been suggested that reversal of the decarboxylating reaction of malate dehydrogenase (EC 1.1.1.39) may be involved, at least in skeletal muscle (Spydevold *et al.*, 1976).

In the present study metabolic perturbations induced by acetate in the pyruvate-perfused rat heart were employed to investigate the routes by which pyruvate carbon enters the tricarboxylic acid-cycle intermediates. The heart was perfused with pyruvate and the acetate-induced changes in the metabolite-labelling kinetics from [1-^{14}C]pyruvate were observed. The results can be interpreted as showing that pyruvate carboxylation is of metabolic significance in heart muscle.

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Materials and methods

Reagents

The enzymes were from Sigma Chemical Co., St. Louis, MO, U.S.A., and Boehringer G.m.b.H., Mannheim, Germany. Standard chemicals were obtained from E. Merck A.G., Darmstadt, Germany, and the nucleotides and coenzymes from Boehringer. Sodium [1-^{14}C]pyruvate was purchased from The Radiochemical Centre, Amersham, Bucks., U.K., and sodium L-[1-^{14}C]lactate from International Chemical and Nuclear Corp., Irvine, CA, U.S.A. [6-^{14}C]Citric acid was from New England Nuclear, Boston, MA, U.S.A., and its purity was tested before use by paper chromatography with butan-1-ol/formic acid/water (10:2:5, by vol.).

Animals and perfusion methods

Female Sprague–Dawley rats from the Department's own stocks were used, with no starvation period before the experiments. The rats were anaesthetized with diethyl ether and injected intravenously with 500i.u. of heparin 1min before excision of the heart. The hearts were perfused with Krebs–Ringer bicarbonate solution, pH7.4 (Krebs & Henseleit, 1932), containing 2.5mM-$CaCl_2$, 10mM-glucose and 0.2mM-pyruvate in equilibrium with O_2/CO_2 (19:1), by the Langendorff procedure without recirculation, at a hydrostatic pressure of 7.84kPa (80cm of water). The glassware of the perfusion apparatus (Takala, 1981) was equipped with water jackets and thermostatically maintained at 37°C with a recirculating water bath. Coronary flow was measured with a calibrated drop-frequency counter with an analogue output, and the oxygen concentration with a Radiometer E5046 electrode. The beating frequency remained stable at 4–5Hz during the 25-min experimental period. Oxygen consumption was calculated from the measured arterio-venous oxygen concentration difference and the measured coronary flow. In some experiments the intraventricular pressure was monitored by a Statham P231D pressure transducer through a needle penetrating the ventricular wall.

After an initial perfusion with Krebs–Ringer solution containing 10mM-glucose and 0.2mM-pyruvate for 15min, the perfusion was continued with Krebs–Ringer solution containing 10mM-glucose, 0.2mM-[1-^{14}C]pyruvate and 5mM-sodium acetate. In the control experiments no acetate was present. The specific radioactivity of [1-^{14}C]-pyruvate, determined for each experiment, was in the range 2.4×10^5–5.3×10^5d.p.m./μmol. The results of the label-incorporation experiments were normalized to a [1-^{14}C]pyruvate specific radioactivity of 100000d.p.m./μmol.

Heart extracts

At the end of the perfusions the hearts were frozen with aluminium tongs cooled with liquid nitrogen (Wollenberger *et al.*, 1960). The frozen pulverized sample was extracted with 8% (v/v) $HClO_4$ in 40% (v/v) ethanol, pre-cooled to −20°C (Williamson & Corkey, 1969). Extraction was repeated with 6% (v/v) $HClO_4$ and the filtrate neutralized to pH 6 with 3.75M-K_2CO_3 containing 0.5M-triethanolamine hydrochloride.

Metabolite and specific-radioactivity determinations

The metabolites were determined by enzymic methods, by measuring the appearance or disappearance of NADH in an Aminco DW-2 dual-wavelength spectrophotometer by using an $\varepsilon_{340}-\varepsilon_{385}$ value of 5.33×10^3 litre·mol^{-1}·cm^{-1}. Citrate was measured with citrate lyase (EC 4.1.3.6) (Gruber & Möllering, 1966), malate essentially by the method of Williamson & Corkey (1969), glutamate with glutamate dehydrogenase (EC 1.4.1.4) (Bernt & Bergmeyer, 1970), and aspartate with aspartate aminotransferase (EC 2.6.1.1) and malate dehydrogenase (EC 1.1.1.37) (Bergmeyer *et al.*, 1970). Lactate was measured enzymically as described by Hohorst (1963), pyruvate by the method of Bücher *et al.* (1963), 2-oxoglutarate as described by Narins & Passonneau (1970), and alanine as described by Grassl (1970). CoA and acetyl-CoA were measured with 2-oxoglutarate dehydrogenase and phosphotransacetylase (Tubbs & Garland, 1969).

After addition of 1μmol of the metabolites as a carrier, 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 eluted in an exponential gradient of formic acid and subsequently with ammonium formate. A 1–2ml sample of the fractions was used for radioactivity determination by liquid-scintillation counting in Bray's (1960) solution. Fractions containing glutamate, aspartate, β-hydroxybutyrate and lactate, which could not be properly resolved in the Dowex-1 column, were further analysed by ion-exchange chromatography on an automatic amino acid analyser. The specific radioactivity of alanine, which was eluted as a single peak from the Dowex-1 (formate form) column, was ascertained by its enzymic interconversion to lactate by alanine aminotransferase, lactate dehydrogenase and an excess of 2-oxoglutarate and NADH. The lactate formed was further purified on a Dowex-1 (formate form) column and its radioactivity determined. The results of the enzymic interconversion demonstrated that 98% of the radioactivity of the first alanine peak was due to alanine. The recovery of the compounds studied was tested by adding known amounts of the radioactive compounds to the $HClO_4$ before homogenizing the freeze-clamped heart. Recovery through

the complete analytical procedure was 90% for malate, 98% for citrate, 80% for glutamate, 74% for aspartate, and 98% for alanine. Corrections for recovery were applied when calculating the specific radioactivities.

In determining the specific radioactivity of citrate, the citrate/isocitrate fraction was used without further separation into components. This is justifiable, since the isocitrate concentration is only one-tenth of that of citrate (Hiltunen & Hassinen, 1977) and the aconitase reaction is considered to be in equilibrium (England *et al.*, 1967).

On the Dowex-1 (formate form) column 92% of the radioactivity of the pyruvate substrate was eluted as a single peak, and the elution point of the remainder did not coincide with that of any of the other compounds measured in the present study.

The rate of oxidation of external pyruvate was determined by measuring the specific radioactivity of perfusate bicarbonate. The perfusion effluent was collected in 1 min fractions under heptane. $^{14}CO_2$ was liberated from the samples by acidification with 2 ml of 10% (w/v) trichloroacetic acid in special vials allowing the collection of CO_2 into NCS solution (The Radiochemical Centre), which was effected by an incubation for 1 h with constant shaking. Samples of the influent perfusate were treated similarly and used as controls for the spontaneous decarboxylation of pyruvate, which under these conditions was very slow.

Specific radioactivity of C-6 of citrate

Citrate was degraded to 2-oxoglutarate and CO_2 by coupled enzymic reactions using aconitase (EC 4.2.1.3) and isocitrate dehydrogenase (EC 1.1.1.42) (Williamson & Corkey, 1969). The reaction was carried out in special vessels equipped with a side arm with an injection port for acidification of the sample with trichloroacetic acid (see below) and with a device for the collection of CO_2 on a filter-paper strip impregnated with 0.2 ml of NCS solution. The incubation temperature was 37°C. The reaction was started by adding 1.0 ml of the sample (neutralized $HClO_4$ extract) to 2.9 ml of a reaction mixture containing 130 mM-Tris/HCl, pH 7.4, 197 μM-$NADP^+$, 0.69 mM-$MgSO_4$, 0.2 mM-$Fe(NH_4)_2(SO_4)_2$, 2 mM-L-cysteine and aconitase (180 munits/ml) (Morrison, 1954). This mixture was first incubated for 1 h to allow the activation of aconitase with the concomitant colour change to run into completion, so that the subsequent isocitrate dehydrogenase reaction could be monitored reliably. Thereafter isocitrate dehydrogenase (1.8 units) was added in 0.5 ml of 20 mM-Tris/HCl (pH 7.4)/0.02% serum albumin, and the mixture was further incubated for 3 h. Next 0.5 ml of 20% (w/v) trichloroacetic acid was added and the CO_2 liberated was collected in 0.2 ml of NCS solution during a further incubation for 2 h with continuous shaking. The radioactivity of the NCS solution was counted in toluene-based scintillant.

The validity of the determinations of the specific radioactivity was tested with 6-^{14}C-labelled citrate. The recovery of the label from [6-^{14}C]citrate was 90–92% and the aconitase plus isocitrate dehydrogenase reactions were complete as observed by spectrophotometric monitoring of the NADPH absorbance.

Results and discussion

Rationale of the experiment

The label of [1-^{14}C]pyruvate can enter the tricarboxylic acid cycle directly by the synthesis of C_4 such as malate or oxaloacetate (via the decarboxylating malate dehydrogenase reaction or pyruvate carboxylase). Since the oxaloacetate concentration is so low that its specific radioactivity could not be determined with sufficient accuracy, only that of malate was determined here. This was justified, since the malate dehydrogenase reaction is considered to be in equilibrium in the heart (Opie & Owen, 1975).

The carbon atoms of oxaloacetate and malate are retained in C-1, -2, -3 and -6 of citrate. When the label enters through the carboxylation of [1-^{14}C]-pyruvate and randomization of the label occurs in the fumarate hydratase reaction, one-half of the label should be in C-6 of the citrate and one-half in the remainder of the molecule. The label of C-6 of citrate is lost in the isocitrate dehydrogenase reaction and its reversal through the mediation of the near-equilibrium of the aconitase reaction. If the label entered the citrate through the prior formation of labelled CO_2 at the pyruvate dehydrogenase step, the label would be found only in C-6 of citrate, owing to the reversal of the isocitrate dehydrogenase and aconitase reactions. In heart muscle the rate of formation of oxaloacetate from citrate via citrate lyase is extremely low (Srere, 1959; Nuutinen *et al.*, 1981*a*), so that appreciable labelling of malate should not occur by this mechanism. The specific radioactivity of CO_2 was found to be so low that its contribution in the direct carboxylation of [1-^{14}C]-pyruvate need not be taken into account.

Lactate and alanine

External [1-^{14}C]pyruvate was used as the source of the label, but for quantitative determination of the rate of incorporation of pyruvate carbon into the metabolites the specific radioactivity of the intracellular pyruvate must be known. The specific radioactivity of lactate was determined for use as an estimate of the specific radioactivity of the intracellular pyruvate pool, but was too low. The specific

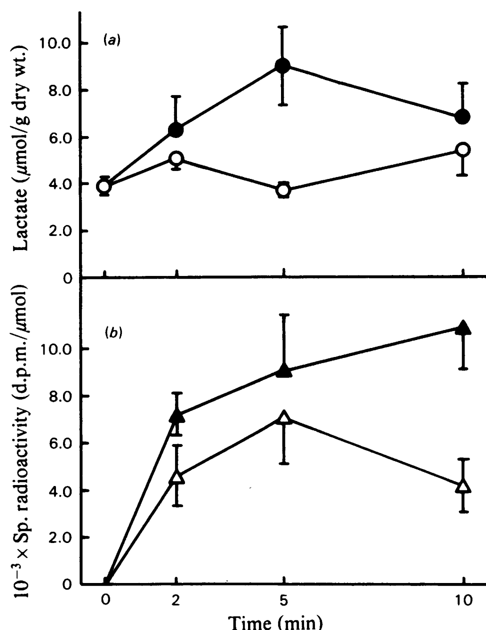

Fig. 1. *Label incorporation from [1-^{14}C]pyruvate into lactate and concentration of lactate in isolated perfused rat hearts*

The conditions were explained in the Materials and methods section. (*a*) Lactate concentration in control (○) and acetate-perfused (●) hearts; (*b*) specific radioactivity of lactate in control (△) and acetate-perfused (▲) hearts. The values are means ± S.E.M. from four to eight independent experiments.

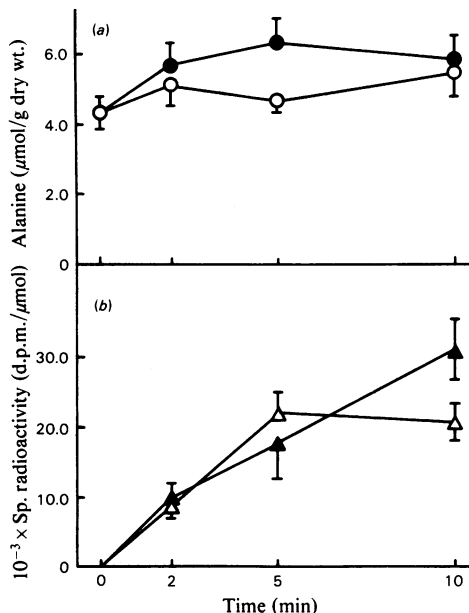

Fig. 2. *Label incorporation from [1-^{14}C]pyruvate into alanine and concentration of alanine in isolated perfused rat hearts*

The conditions were explained in the Materials and methods section. (*a*) Alanine concentration in control (○) and acetate-perfused (●) hearts; (*b*) specific radioactivity of alanine in control (△) and acetate-perfused (▲) hearts. The values are means ± S.E.M. from seven to ten independent experiments.

radioactivity of alanine was 3 times that of lactate (Figs. 1 and 2) and reached 31% of that of pyruvate in the influent perfusion fluid. It has previously been thought that lactate can act as an indicator of intracellular pyruvate labelling because of the high activity of the cytosolic lactate dehydrogenase (Scholz *et al.*, 1978). The present results are not compatible with a near-equilibrium between total myocardial lactate and external pyruvate. Because both lactate and alanine become labelled via intracellular pyruvate, a difference between the specific radioactivities of lactate and alanine is not compatible with one cellular pyruvate pool only. The data rather suggest that two cytosolic pyruvate pools exist.

To test further the principle of two intracellular pyruvate pools, the hearts were also perfused with [1-^{14}C]lactate. In this case the specific radioactivity of tissue alanine reached in 10 min 70% of that of tissue pyruvate labelled from [1-^{14}C]lactate. Under these conditions the specific radioactivity of tissue pyruvate was 6% of that of external lactate.

These findings suggest two compartments of pyruvate, lactate and alanine: a 'peripheral' pool, which communicates with extracellular lactate, and a 'glycolytic' pool, which receives most of the lactate from glycolysis. The 'glycolytic' pyruvate pool has a through-flow from glycolysis, and the lactate dehydrogenase reaction is mainly confined to this compartment. If we also bear in mind that the cardiac lactate dehydrogenase reaction is not necessarily in equilibrium (Kaplan & Everse, 1972; Nuutinen *et al.*, 1981*b*), the model would fit the experimental data, i.e. label from external pyruvate equilibrates better with alanine than with lactate, as the 'glycolytic' pyruvate pool is continuously being diluted by glycolytic pyruvate production. When the transfer of label from [^{14}C]lactate is studied, the labelling of alanine shows some lag compared with that of pyruvate. Some earlier reports also indicate cytosolic compartmentation of pyruvate (Mowbray & Ottaway, 1973), and there is evidence of cytosolic subcompartmentation of glycolytic enzymes in rat heart (Jarvie & Ottaway, 1975).

Aspartate

The myocardial aspartate content (Fig. 3) did not change significantly during the first 5 min after the start of the acetate infusion. During acetate perfusion the specific radioactivity of aspartate approached that of malate, but, in hearts perfused with pyruvate only, the specific radioactivity of aspartate was only half of that of malate. The reason might be that under these conditions the oxaloacetate concentration is very low (Hiltunen & Hassinen, 1977) compared with the K_m of aspartate aminotransferase for oxaloacetate (Henson & Cleland, 1964). The data are in accord with the findings of Randle *et al.* (1970), indicating that the label equilibration between malate and aspartate is not complete. Anaplerosis by means of a coupled reaction of the aspartate aminotransferase and alanine aminotransferase reactions (Davis & Bremer, 1973) should result in a decrease in tissue content of aspartate and a stoichiometric increase in the alanine content. The large size of the aspartate pool and its small percentage changes (Fig. 3) made estimation of stoichiometric relations difficult, but the alanine concentration increased slightly during the first 2 min. The labelling of aspartate, however, indicates that pyruvate carboxylation, with the formation of C_4 compounds, does occur.

Malate

During acetate perfusion the malate concentration increased in 10 min by 35% (Fig. 4). The labelling of total tissue malate was rapid, reaching a maximum at 2 min, after which a slow decline in the specific radioactivity occurred simultaneously with an increase in the concentration. Four different possibilities exist for the labelling of malate. The label may be incorporated by means of the pyruvate carboxylase reaction, the 1-^{14}C label of pyruvate appearing in C-1 of the oxaloacetate. Label can also be transferred by means of the decarboxylating malate dehydrogenase (malic enzyme) to C-1 of the malate. Pyruvate dehydrogenase liberates $^{14}CO_2$ from [1-^{14}C]pyruvate, and CO_2 fixation by means of pyruvate carboxylase or malic enzyme would then

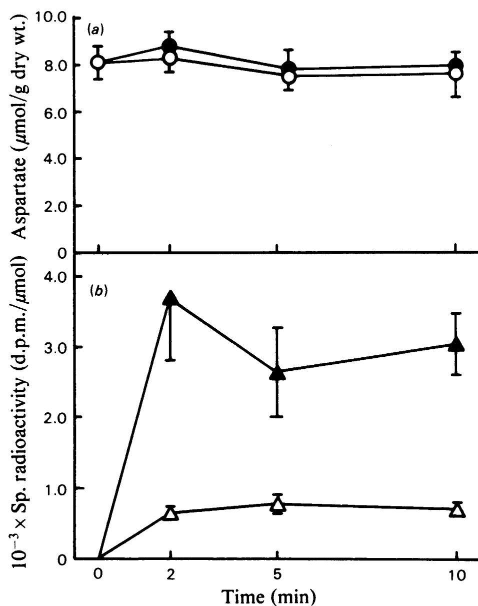

Fig. 3. *Label incorporation from [1-^{14}C]pyruvate into aspartate and concentration of aspartate in isolated perfused rat hearts*

The conditions were explained in the Materials and methods section. (*a*) Aspartate concentration in control (○) and acetate-perfused (●) hearts; (*b*) specific radioactivity of aspartate in control (△) and acetate-perfused (▲) hearts. The values are means ± S.E.M. from eight to twelve independent experiments.

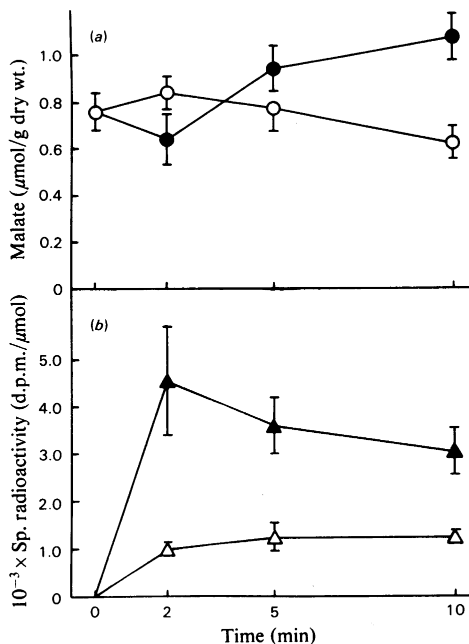

Fig. 4. *Label incorporation from [1-^{14}C]pyruvate into malate and concentration of malate in isolated perfused rat hearts*

The conditions were explained in the Materials and methods section. (*a*) Malate concentration in control (○) and acetate-perfused (●) hearts; (*b*) specific radioactivity of malate in control (△) and acetate-perfused (▲) hearts. The values are means ± S.E.M. from four to eight independent experiments.

lead to the labelling of C-1 and C-4 of oxaloacetate and malate. A further possibility is the citrate lyase reaction, by which label incorporated from bicarbonate to C-6 of isocitrate and citrate by reversal of the isocitrate dehydrogenase (and aconitase) reaction can reach the malate. The activity of citrate lyase is low in the heart, however (Srere, 1959).

The specific radioactivity of malate reaches a maximum at 2min (Fig. 4). This rapid label incorporation could be explained by a short initial burst of pyruvate carboxylation, which is known to be regulated by acetyl-CoA (Utter & Scrutton, 1969). Measurements of the myocardial CoA and acetyl-CoA concentrations indicate that the changes in the concentrations of these effectors are sufficiently fast to explain a rapid activation of pyruvate carboxylase. The tissue acetyl-CoA concentration shows a rapid but transient increase, the maximum occurring at 2min after the onset of perfusion with acetate plus pyruvate. The changes in the CoA concentration are reciprocal to those in acetyl-CoA (Fig. 5). These changes are reminiscent of those observed after acetate addition only (Randle *et al.*, 1970), but here they are accentuated, probably owing to the initially high acetyl-CoA production by pyruvate oxidation and a rapid acetate-induced inhibition of pyruvate dehydrogenase, which was evident when the $^{14}CO_2$ production from [1-^{14}C]-pyruvate was measured. The rate of pyruvate decarboxylation was inhibited by 88% 2min after the addition of acetate, and 1min later the inhibition was 90% (Fig. 6). In addition to the possible decline in the pyruvate carboxylase activity after an initial burst suggested by the changes in the acetyl-CoA concentration, another mechanism could contribute to the decline in the specific radioactivity of malate after the maximum at 2min, i.e. the slow increase in the size of the malate pool (Fig. 4), possibly owing to reshuffling of carbon between the tricarboxylic acid-cycle intermediates.

When [1-^{14}C]pyruvate was added to the perfusate 10min after the beginning of the perfusion with pyruvate + acetate, the labelling of malate over the next 10min reached about 60% of that observed (Fig. 4) 10min after the start of the acetate perfusion. The data indicate that considerable pyruvate carboxylation also occurs under conditions in which the tricarboxylic acid-cycle metabolite concentrations do not change, and that higher steady-state concentrations of the cycle intermediates necessitate higher anaplerotic fluxes, probably owing to their higher rates of disposal.

Citrate

The increase in the citrate concentration continued for 10min after the addition of acetate to the perfusion medium. Simultaneously, ^{14}C-labelling of citrate occurred, which, compared with the perfusions without acetate, was faster during the initial 2min (Fig. 7). In hearts perfused with acetate, the specific radioactivity of C-6 was lower than that of the remainder of the molecule (C-1–C-5), which can be treated as a unit (Fig. 8). After an initial maximum, the specific radioactivity of C-6 declined,

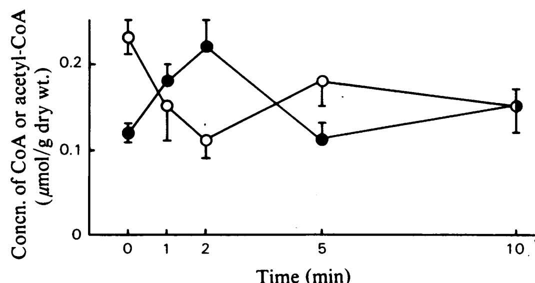

Fig. 5. *Time-course of changes in tissue CoA* (○) *and acetyl-CoA* (●) *after the onset of acetate perfusion*

The conditions were as described in the Materials and methods section. The values are means ± S.E.M. from four independent experiments.

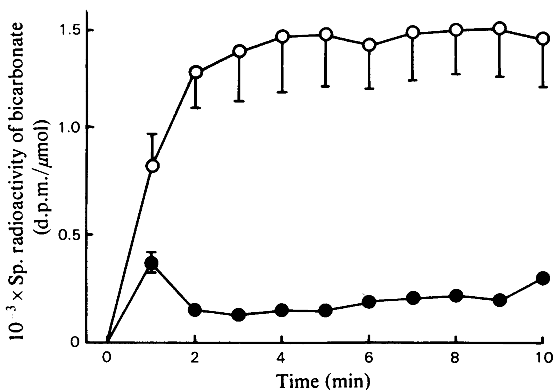

Fig. 6. *Specific radioactivity of perfusate bicarbonate during [1-^{14}C]pyruvate metabolism in the absence and presence of acetate*

The hearts were perfused and the specific radioactivity of bicarbonate was measured as explained in the Materials and methods section. At zero time perfusion was continued with [1-^{14}C]pyruvate alone (○) or together with acetate (●). The specific radioactivity of the [1-^{14}C]pyruvate in the influent perfusate varied from 346988 to 414825d.p.m./μmol. The values are means ± S.E.M. from three independent experiments, normalized to a [1-^{14}C]-pyruvate specific radioactivity of 100000d.p.m./μmol.

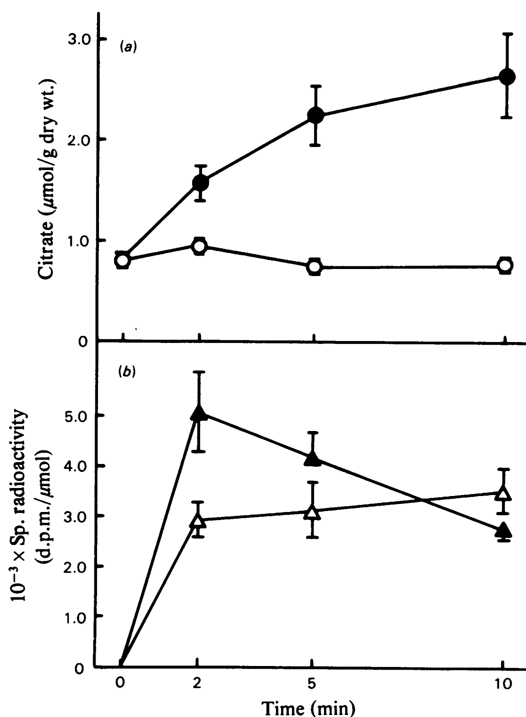

Fig. 7. *Label incorporation from [1-^{14}C]pyruvate into citrate and concentration of citrate in isolated perfused rat hearts*

The conditions were explained in the Materials and methods section. (*a*) Citrate concentration in control (○) and acetate-perfused (●) hearts; (*b*) specific radioactivity of citrate in control (△) and acetate-perfused (▲) hearts. The values are means ± S.E.M. from 8 to 12 independent experiments.

probably because of inhibition of pyruvate dehydrogenase and a decrease of the radioactivity of bicarbonate. Because of the high rates of isocitrate dehydrogenase and its reversal and the near-equilibrium of the aconitase reaction, the radioactivity of C-6 of citrate should follow that of bicarbonate, reaching 50–80% of the specific radioactivity of the latter (Randle *et al.*, 1970; Nuutinen *et al.*, 1981*a*). In the absence of acetate, the specific radioactivity of C-6 of citrate was approximately the same as that of bicarbonate (Fig. 6). In its presence the specific radioactivity of C-6 of citrate was about three times that of bicarbonate. This indicates that under conditions of a high anaplerotic rate the equilibration of label through the aconitase and isocitrate dehydrogenase reactions is not complete. In the remainder of the citrate molecule the label is higher in the acetate-perfused hearts (Fig. 8*b*). The label content of C-1–C-5 of citrate is more than half of that of malate, which could be taken as an indication of a compartmentation of malate, the synthesis of citrate being from a pool of higher specific radioactivity.

One factor contributing to the labelling of oxaloacetate, malate and citrate is the tricarboxylic acid-cycle flux, the rate of which shows no fixed relationship to anaplerosis (Nuutinen *et al.*, 1981*a*). Any decrease in this flux should result in an increase in the specific radioactivity of the C_4 intermediates, and also in an increased loss of label on the reversal of the isocitrate dehydrogenase and aconitase reaction. Oxygen consumption decreases transiently by 17% on commencement of the acetate perfusion and returns to the control values 3 min after the beginning of acetate perfusion. These findings are reminiscent of those of Randle *et al.* (1970) obtained in the absence of pyruvate (Fig. 9). Changes in the same direction as in the oxygen consumption must occur in the tricarboxylic acid-cycle flux, which are then in accord with the data on the labelling of malate and C-1–C-5 of citrate.

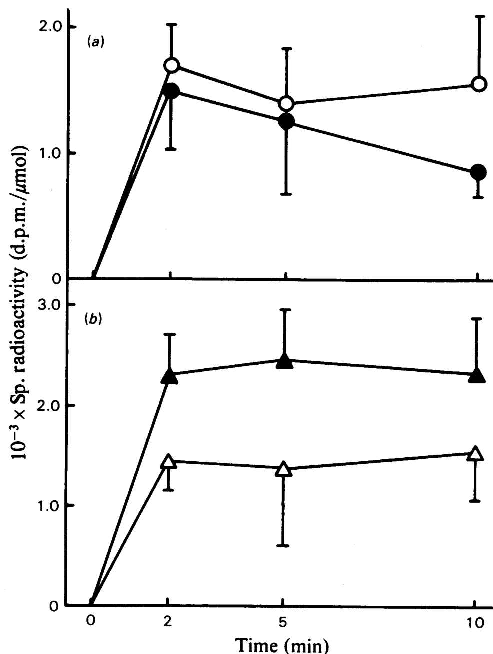

Fig. 8. *Specific radioactivity of C-6 and C-1–C-5 of citrate*

The conditions were as explained in the Materials and methods section. (*a*) Specific radioactivity of C-6 in control (○) and acetate-perfused (●) hearts; (*b*) specific radioactivity of C-1–C-5 in control (△) and acetate-perfused (▲) hearts. The values are means ± S.E.M. from four to five independent experiments.

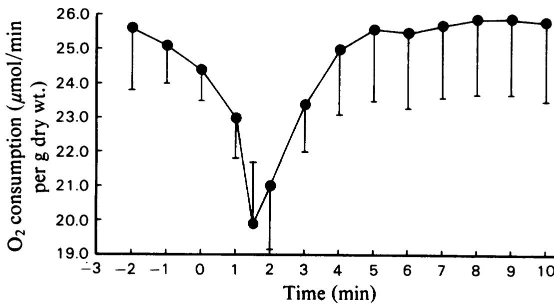

Fig. 9. *Time-course of oxygen consumption during preperfusion and acetate perfusion* (●)

The hearts were preperfused as described in the Materials and methods section. At zero time perfusion was continued with a medium containing additional acetate. The values are means ± S.E.M. from three independent experiments.

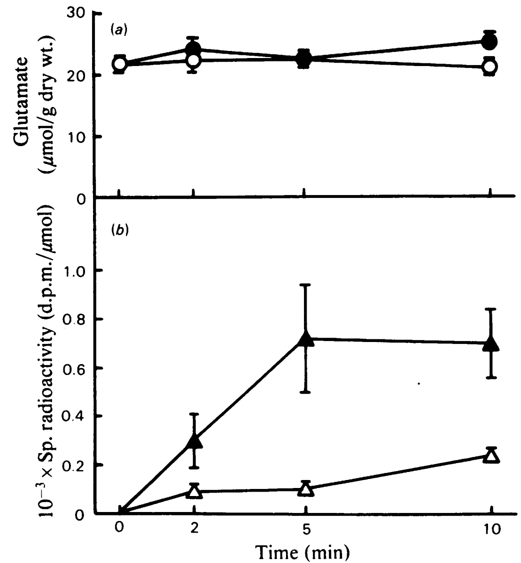

Fig. 10. *Label incorporation from* [*1-*^{14}*C*]*pyruvate into glutamate and concentration of glutamate in isolated perfused rat hearts*

The conditions were explained in the Materials and methods section. (*a*) Glutamate concentration in control (○) and acetate-perfused (●) hearts; (*b*) specific radioactivity of glutamate in control (△) and acetate-perfused (▲) hearts. The values are means ± S.E.M. from 8 to 12 independent experiments.

The reason for the transient decrease in the oxygen consumption remains to be identified. This phenomenon occurs concomitantly with an increase in the coronary flow, and a decrease in the beating frequency and systolic intraventricular pressure. These changes in the mechanical performance and coronary circulation could be linked to a derangement of the energy metabolism because of an accumulation of AMP (Randle *et al.*, 1970), which, owing to the location of acetyl-CoA synthetase, is confined to the intramitochondrial space (Aas, 1971). The mitochondrial AMP is not accessible to the cytosolic adenylate kinase (Brdiczka *et al.*, 1968) and this may lead to an interference with the recycling of ATP. The latter could also influence the cytosolic AMP concentration, and could then increase the coronary flow by a breakdown of AMP to adenosine (Berne, 1963).

2-Oxoglutarate and glutamate

The temporal pattern of changes in the 2-oxoglutarate concentration was reminiscent of those observed in malate. The 2-oxoglutarate concentration decreased temporarily at the onset of the acetate perfusion. This may indicate differential fluxes in the two spans of the tricarboxylic acid cycle, i.e. from oxaloacetate to 2-oxoglutarate and from 2-oxoglutarate to oxaloacetate, probably owing to a perturbation of the near-equilibrium of the aspartate aminotransferase reaction.

The total radioactivity of 2-oxoglutarate isolated from the tissue was so low that its specific radioactivity was not determined. The specific radioactivity of glutamate (Fig. 10) reached a value about one-half of that of the C-1–C-5 moiety of citrate. A similar ratio between the specific radioactivities of 2-oxoglutarate and glutamate has been observed previously (Nuutinen *et al.*, 1981). Label incorporation was greatly enhanced by perfusion with acetate in addition of pyruvate, and this enhancement was greater than the enhancement of labelling of the tricarboxylic acid-cycle intermediates. An explanation of this could be the large pool size of glutamate, which then operates as a sink for the label of the cycle intermediates, thereby decreasing their specific radioactivity.

Succinate

No measurable label incorporation into succinate was detected. This is in accordance with the known properties of the stereochemistry and mechanisms of the reactions of the tricarboxylic acid cycle. Since some label was found in fumarate (results not shown), the results indicate that the reversal of the succinate dehydrogenase reaction is negligible.

This can be also reconciled with the observation that the rate of fumarate reduction by isolated mammalian succinate dehydrogenase is only a few per cent of the forward reaction (Singer *et al.*, 1973).

[*1-*^{14}*C*]*Lactate versus* [*1-*^{14}*C*]*pyruvate as a source of labelling of the tricarboxylic acid-cycle intermediates*

In the light of the low specific radioactivity of the cycle intermediates, reaching maximally 5% of that

of [1-^{14}C]pyruvate, and data suggesting cytosolic subcompartmentation, a limited number of experiments was conducted with [1-^{14}C]lactate. The extent of labelling of the tricarboxylic acid-cycle intermediates was comparable with that from [1-^{14}C]-pyruvate. In the presence of lactate the metabolite pool sizes of the tricarboxylic acid cycle increased on addition of acetate in a similar manner to that described above for acetate plus pyruvate.

Conclusions

The labelling kinetics of the tricarboxylic acid-cycle intermediates during acetate-induced anaplerosis indicates that carboxylation of pyruvate is involved, and that surprisingly rapid activation of the pyruvate-carboxylating pathways occurs on the stimulus of anaplerosis, although the present data do not positively identify the enzyme involved. It has been shown, however, that measurable activity of pyruvate carboxylase (2.2 nmol·min^{-1}·mg^{-1} of protein) can be detected in rat heart mitochondria (Davis *et al.*, 1980). This value is equivalent to 1.36 μmol·min^{-1}·g^{-1} dry wt. of heart, with a myocardial mitochondrial protein content of 80 mg·g^{-1} wet wt. (Kinnula & Hassinen, 1977) and a wet-weight/dry-weight ratio of 7.7. This activity is higher than is needed for the observed accumulation of tricarboxylic acid-cycle intermediates plus aspartate and glutamate, which was 0.98 μmol·min^{-1}·g^{-1} dry wt. during the first 2 min. The observed total incorporation of radioactivity into these compounds was 54000 d.p.m.·g^{-1} dry wt. within 2 min, which is equivalent to 0.27 μmol·min^{-1}·g^{-1} dry wt. calculated on the basis of perfusate pyruvate specific radioactivity, but 5.4 μmol·min^{-1}·g^{-1} dry wt. when the mean specific radioactivity of alanine during the first 2 min is taken to represent the intracellular pyruvate specific radioactivity. The [1-^{14}C]lactate-perfusion data indicate that alanine reaches 35% of the specific radioactivity of pyruvate in 2 min, so that a more accurate estimate of pyruvate incorporation would be 1.9 μmol·min^{-1}·g^{-1} dry wt. This value, within experimental error, is in accord with the enzyme activity reported *in vitro* and the observed anaplerotic rate. A more exact estimate of the rate of pyruvate incorporation can only be obtained by computer modelling of the metabolic network involved (Nuutinen *et al.*, 1981*a*). The malic enzymes are able in principle to catalyse the carboxylation of pyruvate. The NAD(P)-linked enzyme will catalyse a reversible reaction, however (Lin & Davis, 1974), and its activity in the rat heart is very low (Nagel *et al.*, 1980). NADP^{+}-dependent malic enzyme exists in rat heart, but the reversal of the reaction proceeds at very high concentrations of bicarbonate (200 mM) and high concentrations of pyruvate (50 mM), and even then the reverse activity is only 6% of the forward reaction. In accordance with this, it has been found that the mitochondrial malic enzyme operates in the disposal of the tricarboxylic acid-cycle intermediates (Hiltunen & Davis, 1981).

Thus the present data corroborate the functioning of pyruvate carboxylation in cardiac muscle, previously suggested to occur on the basis of data on enzyme activities *in vitro*.

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References

Aas, M. (1971) *Biochim. Biophys. Acta* **231**, 32–47

Bergmeyer, H.-U., Bernt, E., Möllering, H. & Pfleiderer, G. (1970) in *Methoden der Enzymatischen Analyse* (Bergmeyer, H.-U., ed.), pp. 1651–1653, Verlag Chemie, Weinheim

Berne, R. M. (1963) *Am. J. Physiol.* **204**, 317–322

Bernt, E. & Bergmeyer, H.-U. (1970) in *Methoden der Enzymatischen Analyse* (Bergmeyer, H.-U., ed.), pp. 1659–1664, Verlag Chemie, Weinheim

Bowman, R. H. (1966) *J. Biol. Chem.* **241**, 3041–3148

Bray, G. A. (1960) *Anal. Biochem.* **1**, 279–285

Brdiczka, D., Pette, D., Brunner, G. & Miller, F. (1968) *Eur. J. Biochem.* **5**, 294–304

Bücher, T., Czok, R., Lamprecht, W. & Latzko, E. (1963) in *Methods of Enzymatic Analysis* (Bergmeyer, H.-U., ed.), pp. 253–259, Academic Press, New York

Davis, E. J. & Bremer, J. (1973) *Eur. J. Biochem.* **38**, 86–97

Davis, E. J. & Quastel, J. H. (1964) *Can. J. Biochem.* **42**, 1605–1621

Davis, E. J., Spydevold, Ø. & Bremer, J. (1980) *Eur. J. Biochem.* **110**, 255–262

England, P. J., Denton, R. M. & Randle, P. J. (1967) *Biochem. J.* **105**, 32c–33c

Folbergrová, J., Ljunggren, B., Norberg, K. & Siesjö, B. K. (1974) *Brain Res.* **80**, 265–279

Garland, P. B. & Randle, P. J. (1964) *Biochem. J.* **93**, 678–687

Gruber, W. & Möllering, H. (1966) *Biochem. Z.* **346**, 78–88

Grassl, M. (1970) in *Methoden der Enzymatischen Analyse* (Bergmeyer, H.-U., ed.), pp. 1637–1640, Verlag Chemie, Weinheim

Henson, C. P. & Cleland, W. W. (1964) *Biochemistry* **3**, 338–345

Hiltunen, J. K. (1978) *Biochem. J.* **170**, 241–247

Hiltunen, J. K. & Davis, E. J. (1981) *Biochim. Biophys. Acta* in the press

Hiltunen, J. K. & Hassinen, I. E. (1977) *Int. J. Biochem.* **8**, 505–509

Hohorst, H.-J. (1963) in *Methods of Enzymatic Analysis* (Bergmeyer, H.-U., ed.), pp. 266–270, Academic Press, New York

Jarvie, D. R. & Ottaway, J. H. (1975) *Histochem. J.* **7**, 165–178

Kaplan, N. O. & Everse, J. (1972) *Adv. Enzyme Regul.* **10**, 323–336

Kinnula, V. & Hassinen, I. E. (1977) *Acta Physiol. Scand.* **99**, 462–466

Kornberg, H. L. (1966) *Essays Biochem.* **2**, 1–31

Krebs, H. A. & Henseleit, K. (1932) *Hoppe-Seyler's Z. Physiol. Chem.* **210**, 33–36

LaNoue, K., Nicklas, W. J. & Williamson, J. R. (1970) *J. Biol. Chem.* **245**, 102–111

Lin, R. C. & Davis, E. J. (1974) *J. Biol. Chem.* **249**, 3867–3875

Lowenstein, J. M. (1972) in *Energy Metabolism and the Regulation of Metabolic Processes in Mitochondria* (Mehlman, M. A. & Hanson, R. W., eds.), pp. 53–61, Academic Press, New York

Morrison, J. F. (1954) *Biochem. J.* **58**, 685–692

Mowbray, J. & Ottaway, J. H. (1973) *Eur. J. Biochem.* **36**, 369–379

Nagel, W. O., Danchy, R. T. & Sauer, L. A. (1980) *J. Biol. Chem.* **255**, 3849–3854

Narins, R. G. & Passonneau, J. V. (1970) in *Methoden der Enzymatischen Analyse* (Bergmeyer, H.-U., ed.), pp. 1540–1542, Verlag Chemie, Weinheim

Nuutinen, E. M., Peuhkurinen, K. J., Pietiläinen, E. P., Hiltunen, J. K. & Hassinen, J. E. (1981*a*) *Biochem. J.* **194**, 867–875

Nuutinen, E. M., Hiltunen, J. K. & Hassinen, I. E. (1981*b*) *FEBS Lett.* **128**, 356–360

Opie, L. H. & Owen, P. (1975) *Biochem. J.* **148**, 403–415

Randle, P. J., England, P. J. & Denton, R. M. (1970) *Biochem. J.* **117**, 677–695

Safer, B. & Williamson, J. R. (1973) *J. Biol. Chem.* **248**, 2570–2579

Scholz, R., Olson, M. S., Schwab, A. J., Schwabe, U., Noell, C. & Braun, W. (1978) *Eur. J. Biochem.* **86**, 519–530

Scrutton, M. C. & Utter, M. F. (1968) *Annu. Rev. Biochem.* **37**, 249–302

Singer, T. P., Kearney, E. B. & Kenney, W. C. (1973) *Adv. Enzymol.* **37**, 189–272

Spydevold, Ø., Davis, E. J. & Bremer, J. (1976) *Eur. J. Biochem.* **71**, 155–165

Srere, P. A. (1959) *J. Biol. Chem.* **234**, 2544–2547

Takala, T. (1981) *Basic Res. Cardiol.* **76**, 44–61

Tubbs, P. K. & Garland, P. B. (1969) *Methods Enzymol.* **13**, 541–544

Utter, M. F. & Scrutton, M. C. (1969) *Curr. Top. Cell. Regul.* **1**, 253–297

Williamson, J. R. & Corkey, B. E. (1969) *Methods Enzymol.* **13**, 434–513

Wollenberger, A., Ristau, O. & Schoffa, G. (1960) *Pflügers Arch. Gesamte Physiol.* **270**, 399–412