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Elimination and replenishment of tricarboxylic acid-cycle intermediates in myocardium

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1. The contribution of CO_2 fixation to the anaplerotic mechanisms in the myocardium was investigated in isolated perfused rat hearts. 2. K^+-induced arrest of the heart was used to elicit a transition in the concentrations of the intermediates of the tricarboxylic acid cycle. 3. Incorporation of ^{14}C from [^{14}C]bicarbonate into tricarboxylic acid-cycle intermediates was measured and the rates of the reactions of the cycle were estimated by means of a linear optimization program which solves the differential equations describing a simulation model of the tricarboxylic acid cycle and related reactions. 4. The results showed that the rate of CO_2 fixation is dependent on the metabolic state of the myocardium. Upon a sudden diminution of cellular ATP consumption, the pool size of the tricarboxylic acid-cycle metabolites increased and the rate of label incorporation from [^{14}C]bicarbonate into the cycle metabolites increased simultaneously. The computer model was necessary to separate the rapid equilibration between bicarbonate and some metabolites from the potentially anaplerotic reactions. The main route of anaplerosis during metabolite accumulation was through malate + oxaloacetate. Under steady-state conditions there was a constant net outward flow from the tricarboxylic acid cycle via the malate + oxaloacetate pool, with a concomitant anaplerotic flow from metabolites forming succinyl-CoA (3-carboxypropionyl-CoA).

Intermediates of the tricarboxylic acid cycle are continuously being used in biosynthetic reactions and must be continuously regenerated by 'anaplerotic' mechanisms (Kornberg, 1966). Moreover, the combined pool of the tricarboxylic acid-cycle intermediates in the muscle undergoes rapid changes in metabolic perturbation for various reasons, the pool size increasing within minutes after the addition of glucose, acetate, ketone bodies or fatty acids to the medium perfusing an isolated heart (Garland & Randle, 1964; Randle *et al.*, 1970; Davis & Bremer, 1973; Safer & Williamson, 1973).

The mutual significance of the various anaplerotic mechanisms that have been claimed to be active in muscle tissue are still in dispute. The carbon skeleton of aspartate can be recovered as oxaloacetate by coupled transaminations and the 2-oxoglutarate used can be regenerated at the expense of pyruvate (Davis & Bremer, 1973; Safer & Williamson, 1973), or as fumarate by means of the purine nucleotide cycle (Lowenstein, 1972).

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Glutamate can be converted into tricarboxylic acid-cycle intermediates by transamination with pyruvate or by oxidative deamination, but the activity of glutamate dehydrogenase (EC 1.4.1.2) is low in muscle tissue (Pette *et al.*, 1962). In contrast with gluconeogenic tissues, the activity of pyruvate carboxylase (EC 6.4.1.1) in muscle is low (Scrutton & Utter, 1968), although fixation of labelled CO_2 has been demonstrated in skeletal-muscle mitochondria, and the label is incorporated into malate. It has been suggested that this is due to the reversal of decarboxylating malate dehydrogenase (EC 1.1.1.39) (Spydevold *et al.*, 1976).

Cardiac muscle has a high capacity for propionate oxidation (Davis *et al.*, 1972), which occurs with a concomitant accumulation of tricarboxylic acid-cycle intermediates, indicating that propionyl-CoA carboxylase (EC 6.4.1.3) is active in this tissue. The presence of propionyl-CoA carboxylase in cardiac muscle also allows for the 2-oxo acids derived from isoleucine, valine and methionine functioning as precursors of the tricarboxylic acid-cycle intermediates (Davis & Bremer, 1973). Valine

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and isoleucine, however, participate in transamination at the expense of 2-oxoglutarate, an intermediate of the tricarboxylic acid cycle, so that anaplerosis by these two amino acids would necessitate a system for 2-oxoglutarate regeneration by coupled transaminations.

Some evidence has been obtained for the carboxylation of pyruvate in anaplerosis in skeletal muscle (Spydevold *et al.*, 1976), but no evidence exists of a comparable role in cardiac muscle.

We have previously demonstrated that the metabolism of pent-4-enoate and pentanoate in the isolated perfused rat heart is rapid and leads to an accumulation of tricarboxylic acid-cycle intermediates (Hiltunen, 1978; Hiltunen *et al.*, 1978), accompanied by incorporation of $H^{14}CO_3^-$ into these intermediates (Hiltunen *et al.*, 1977). Moreover, the rate of CO_2 fixation was appreciable even in the absence of odd-carbon fatty acids. It has more recently been shown that isolated rat heart mitochondria are also able to metabolize pent-4-enoate and pentanoate to tricarboxylic acid-cycle metabolites by a mechanism involving CO_2 fixation (Hiltunen *et al.*, 1980). Arresting of the heart with KCl, which is used clinically as a protective agent in heart surgery (Kirklin *et al.*, 1979), can be employed to elicit a rapid transition in energy consumption and concomitant changes in the tricarboxylic acid-cycle-metabolite concentrations in the myocardium (Hassinen & Hiltunen, 1975; Hiltunen & Hassinen, 1977).

The present study evaluates the significance of CO_2 fixation and decarboxylation reactions under conditions of an energy-dependent regulation of the pool size of the tricarboxylic acid-cycle intermediates by resolving the rates of the various carboxylation and decarboxylation reactions by means of a linear optimization program based on a simulation model for the tricarboxylic acid cycle and related reactions.

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 G.m.b.H. $NaH^{14}CO_3$ was purchased from The Radiochemical Centre, Amersham, Bucks., U.K. T.l.c. plates pre-coated with cellulose (without fluorescent indicator) were purchased from Merck.

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 an intraperitoneal injection of Nembutal (80–100mg/kg body wt.) 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-$CaCl_2$ and 10mM-glucose in equilibrium with O_2/CO_2 (19:1), by the Langendorff (1895) procedure without recirculation, at a hydrostatic pressure of 7.84kPa (80cm of water).

For study of the arrested heart, the KCl concentration in the perfusion fluid was increased to 16mM and the NaCl concentration lowered accordingly.

After an initial perfusion with ordinary Krebs–Ringer bicarbonate solution for 15min, the perfusion was continued with Krebs–Ringer [^{14}C]-bicarbonate solution. A sufficient amount of the Krebs–Ringer bicarbonate solution was equilibrated with the gas mixture. High-radioactivity stock solution of $NaH^{14}CO_3$ was added to the solution in a volume of 0.1% of the total, sealed with liquid paraffin and mixed. No gassing occurred during the perfusion. The final specific radioactivity of [^{14}C]bicarbonate, determined for each experiment, was in the range 36000–167000d.p.m./μmol. The results of the label-incorporation experiments were then normalized to a [^{14}C]bicarbonate specific radioactivity of 41667d.p.m./μmol.

The equilibration of extracellular and intracellular bicarbonate pools was tested by measuring the intracellular water space in perfusions with inulin-[^{14}C]carboxylic acid and assuming that the specific radioactivities of the interstitial and intravascular bicarbonate were the same. The intracellular bicarbonate reached the same specific radioactivity as in the perfusion medium in less than 2min.

Heart extracts

Samples were obtained from the heart by using aluminium tongs cooled with liquid N_2 (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 pH6 with 3.75M-K_2CO_3 containing 0.5M-triethanolamine hydrochloride.

Metabolites

The metabolites were determined by enzymic methods, 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

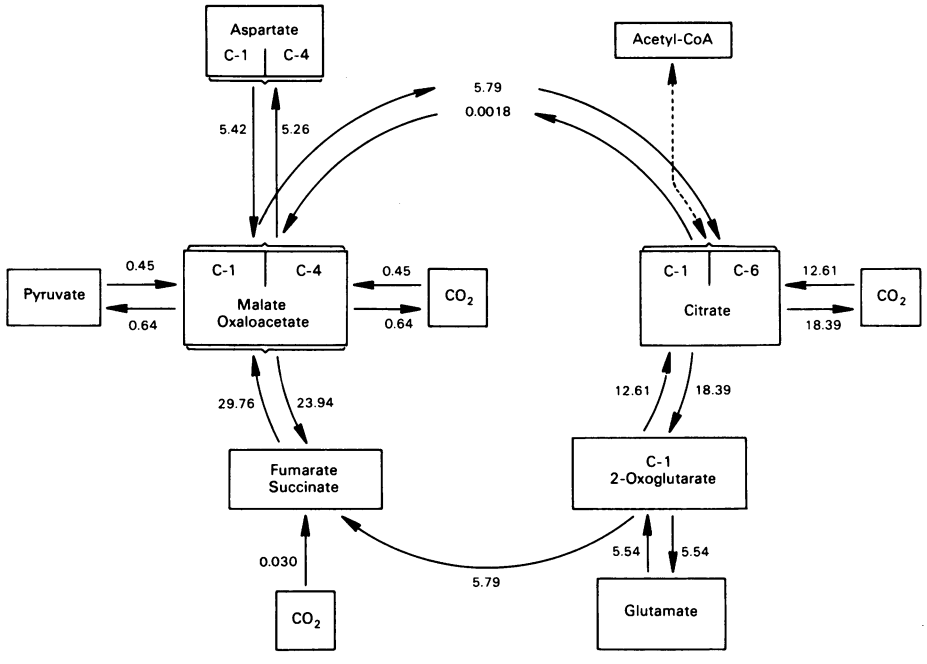
of Williamson & Corkey (1969), glutamate with glutamate dehydrogenase (EC 1.4.1.3) (Bernt & Bergmeyer, 1970), and aspartate with aspartate aminotransferase (EC 2.6.1.1) and malate dehydrogenase (EC 1.1.1.37) (Bergmeyer *et al.*, 1970). The radioactive bicarbonate in the $HClO_4$ extract was eliminated by bubbling the acidified solution with nitrogen. After addition of 1 μmol 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 a linear gradient of formic acid and subsequently with ammonium formate. A sample of the fractions collected was used for radioactivity determination and the remainder evaporated to dryness in a vacuum.

The fractions containing glutamate, aspartate and β-hydroxybutyrate, which could not be resolved on the Dowex-1 column, were pooled, applied to a cellulose-coated t.l.c. plate and analysed by two-dimensional chromatography (Myers & Huang, 1969). The amino acids were located with ninhydrin and the carboxylic acids with Bromocresol Green. The spots were scraped into scintillation-counting vials and their radioactivity was determined in a Wallac liquid-scintillation spectrometer. The counting efficiency, which was controlled by internal standards, was higher and more reproducible when the material in the bottle was extracted with 200 μl of 50mM-H_2SO_4 before the addition of water-miscible scintillation solution (Bray, 1960).

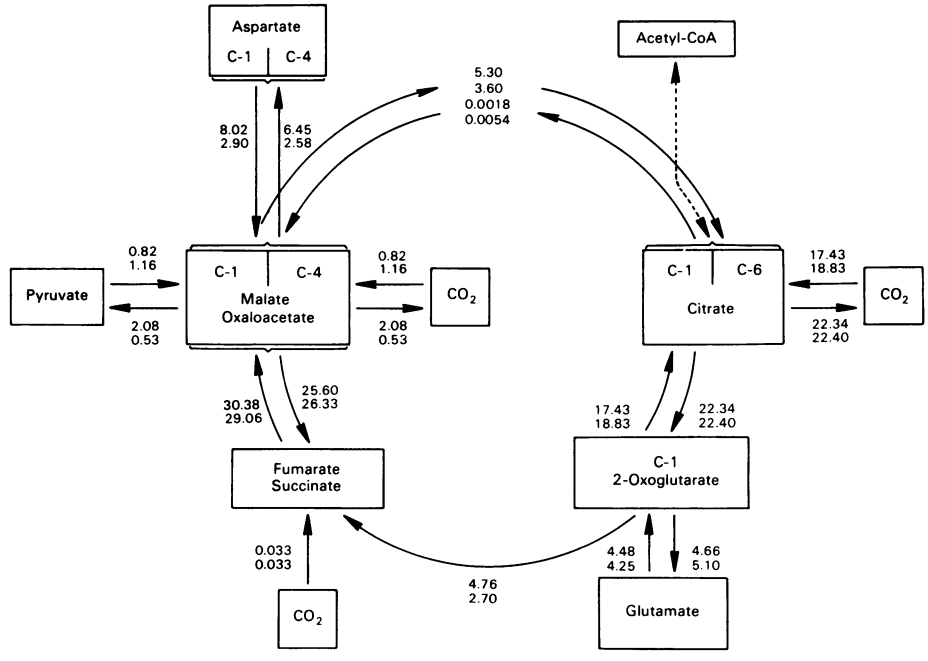
The recovery of the compounds studied was tested by adding known amounts of the radioactive compounds to the $HClO_4$ before homogenization of the freeze-clamped heart. Recovery through the complete analytical procedure varied between 80 and 110%. Corrections for recovery were applied when calculating the specific radioactivities.

Calculations

The model used for the simulation of the tricarboxylic acid cycle is depicted in Schemes 1 and 2. Malate and oxaloacetate were treated as one pool, since the lability and low concentration of oxaloacetate did not allow reliable determination of its specific radioactivity. Also, citrate and isocitrate were treated as one unit, as they were not resolved by the chromatographic procedure. The aconitase reaction was therefore also assumed to be in near-equilibrium (England *et al.*, 1967). Since the individual carbon atoms of oxaloacetate have different fates, C-1 and C-4 were treated separately. C-6 of isocitrate is lost in the isocitrate dehydrogenase reaction and was therefore treated as a separate pool. The remainder of the ^{14}C label incorporated as C-1 and C-4 of oxaloacetate is lost at



Scheme 1. *Calculated fluxes of the tricarboxylic acid cycle in the beating isolated perfused rat heart*
The values are in μmol/min per g dry wt. and were calculated by the linear optimization program described in the Appendix.



Scheme 2. *Calculated fluxes of the tricarboxylic acid cycle in the K^+-arrested isolated perfused rat heart*
The values are in μmol/min per g dry wt. and were calculated by the linear optimization program described in the Appendix. The upper values are mean fluxes for the period 0–2 min and the lower values for the period 2–10 min after cardiac arrest.

the 2-oxoglutarate dehydrogenase step. The fumarate hydratase reaction is taken into consideration, since the extent of label randomization between C-1 and C-4 of malate determines the labelling of 2-oxoglutarate (Potter & Heidelberger, 1949).

Data on the pool size and specific radioactivities of the metabolites were used to calculate the fluxes between the metabolite pools. Some of the fluxes could be measured by independent methods: the acetyl flux has been calculated previously from the oxygen consumption in identical experiments in which the substrate class oxidized was also determined (Hiltunen & Hassinen, 1976), giving results of 5.8 μmol/min per g dry wt. in the beating heart and 5.3 μmol/min per g dry wt. during the first 2 min and 3.6 μmol/min per g dry wt. during the next 8 min after arresting the heart. The flux through the propionate pathway was determined in experiments on amino acid catabolism in isolated perfused hearts, a full acount of which has been published separately (Takala *et al.*, 1980). It was assumed that methionine, valine and isoleucine were the substrates for the propionate pathway, and the catabolism of these compounds yielded a flux of 0.03 μmol/min per g dry wt. in both beating and arrested hearts. The sets of differential equations describing the labelling of the metabolite pools were solved by means of a computer program written in standard ASCII FORTRAN for a Univac 1100 computer. The program accepts a general case of a linear network which can be varied in the search for a model that fits the experimental data.

Under experimental conditions, when the pool sizes of the metabolites and metabolic fluxes were not constant during the observation time, the fluxes were calculated separately for consecutive time periods. For the arrested heart only two sets of flux values were calculated, a value for the time period 0–2 min and another for the period 2–10 min. This is justifiable, as the initial rates of the changes in pool size are the highest.

A more detailed description of the principles of the computer program is given in the Appendix.

Results and discussion

Heart arrest by means of 16 mM-KCl was used to achieve a transition in the pool size of the tricarboxylic acid-cycle intermediates. When fixation of CO_2 was studied as a possible anaplerotic mechanism, it was soon found that the temporal pattern of $^{14}CO_2$ incorporation was so complex that a variety of flow constants had to be calculated in

order to estimate the contribution of certain carboxylation reactions.

As far as the anaplerotic mechanisms are concerned, interpretation of the data on label incorporation into tricarboxylic acid-cycle intermediates is rendered difficult, as (1) the fluxes between the pools of the intermediates are quite high compared with those to and from the cycle, and (2) the complex compartmentation of the metabolites and the shortcomings in the currently available methodology do not allow detailed mathematical treatment of the data. The approach adopted here, however, does permit some deductions to be made about the significance of carboxylation and decarboxylation reactions in the synthesis or elimination of tricarboxylic acid-cycle intermediates in the myocardium.

Total incorporation of $^{14}CO_2$ into acid-stable metabolites

The total amount of CO_2 incorporated was dependent on the metabolic state of the tissue, being 2.60 μmol/g dry wt. in the beating heart and 5.67 μmol/g dry wt. for 10 min in the K^+-arrested heart. The time course suggested that there are reactions with widely differing rates, one with a half-time of 0.64–0.80 min, dependent on the metabolic conditions, and another, which is almost linear with time throughout the experimental period. Most of the rapid phase of CO_2 incorporation in the arrested heart can be accounted for by incorporation into citrate, which in turn closely follows the changes in the pool size of this metabolite. The total incorporation into acid-stable metabolites is approximately equal to the sum of the incorporation into aspartate, glutamate, malate and citrate.

Citrate. The initial rate of label incorporation was only slightly dependent on the energy state of the cell or the changes in the pool size of this metabolite (Fig. 1). The steady-state specific radioactivity of citrate was approximately the same in the beating and arrested heart.

Solution of the simulation model on the basis of experimental data showed that most of the label in citrate originates from the reversal of the isocitrate dehydrogenase reaction, the rate of this backward reaction being 69% of the forward reaction in the beating heart and 84% in the K^+-arrested heart.

Only very few previous reports exist on the incorporation of a radioactivity label from bicarbonate into tricarboxylic acid-cycle intermediates. Randle *et al.* (1970) report that the specific radioactivity of C-6 of isocitrate in perfused rat hearts oxidizing acetate reached 46% of that of the bicarbonate in the medium. Computer solution of the present results demonstrates that the specific radioactivity of C-6 of citrate reaches 70% and 86% of that of the bicarbonate in the medium in perfusions of beating and arrested hearts respectively. The higher specific radioactivities observed here are probably due to the slower forward reactions under conditions of a lower tricarboxylic acid-cycle flux.

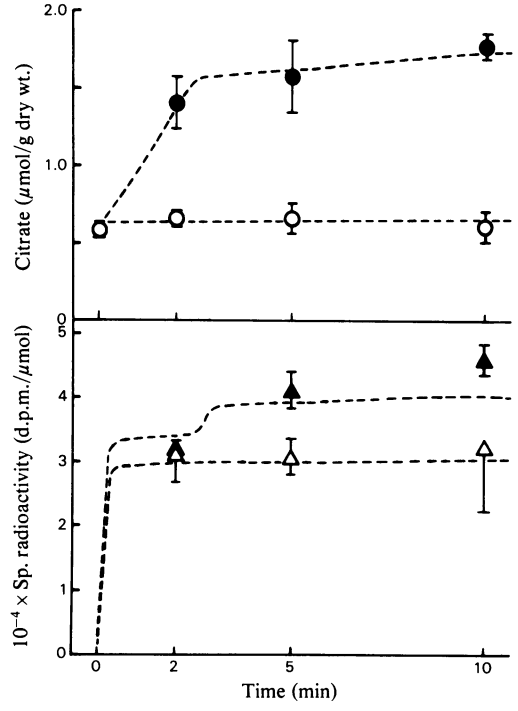


Fig. 1. *Label incorporation from [^{14}C]bicarbonate into citrate and concentration of citrate in isolated perfused rat hearts*

The conditions were as explained in the Materials and methods section. Citrate concentration in control (○) and K^+-arrested (●) hearts and specific radioactivity of citrate in control (△) and K^+-arrested (▲) hearts were measured. The values are means ± S.E.M. from four to eight independent experiments. The curves were fitted by the simulation program described in the Appendix.

Malate. The time course of the label incorporation into malate and its concentration changes are shown in Fig. 2. The computer solution shows that there was a net outward flow of 0.19 μmol of C/min per g dry wt. from the malate (+oxaloacetate) pool in the beating heart. It has been shown (Takala *et al.*, 1980) that there is a steady anaplerotic flux from amino acids in the isolated perfused heart, and that its rate is dependent on the oxygen consumption. The data necessitate an average elimination rate for the cycle intermediates of 0.13 μmol/min per g dry wt. in the beating heart and 0.04 μmol/min per g dry wt. in the arrested heart in 1 h experiments. The present data are in accord-

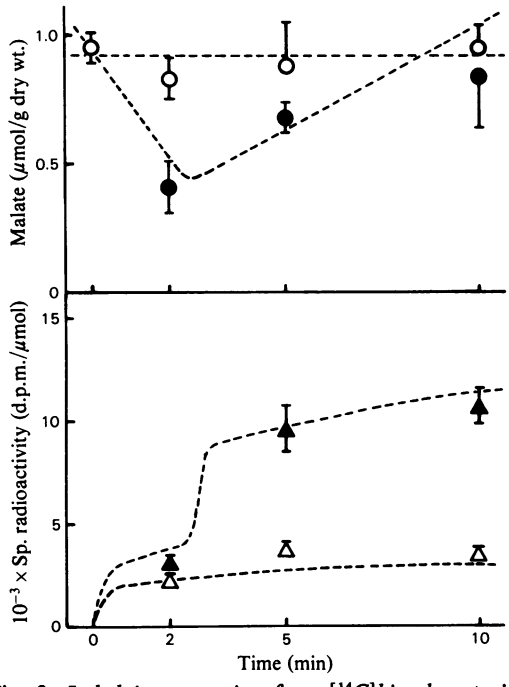


Fig. 2. *Label incorporation from [^{14}C]bicarbonate into malate and concentration of malate in isolated perfused rat hearts*

The values are means ± S.E.M. from four to six independent experiments. Experimental conditions and symbols were as in Fig. 1.

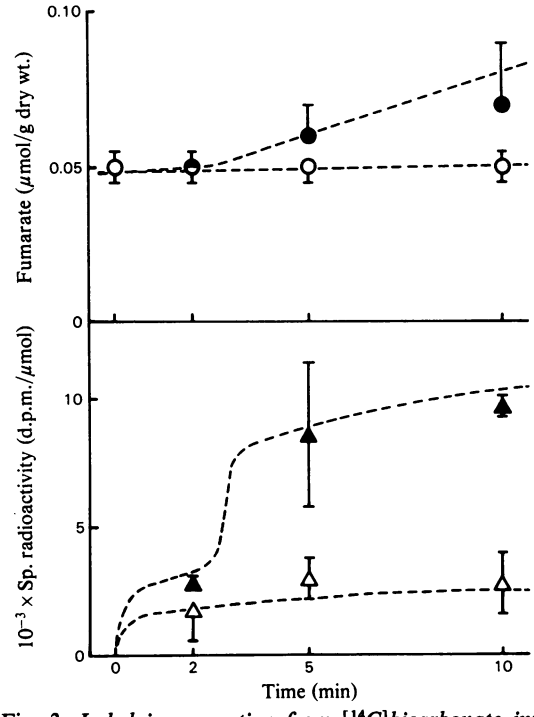


Fig. 3. *Label incorporation from [^{14}C]bicarbonate into fumarate and concentration of fumarate in isolated perfused rat hearts*

Experimental conditions and symbols were as in Fig. 1.

ance with these observations. The solution of the model gave an outward flow from malate during the initial 2 min after cardiac arrest and demonstrates that the initial feed-in of carbon skeletons is from aspartate, which showed an initial decrease in concentration. At 2 min after cardiac arrest the carbon flow reverses and shows a net carboxylation of 0.63 μmol/min per g dry wt., forming malate. If the malate decarboxylation is made into a CO_2-exchange reaction by restraining the inward and outward fluxes to the same values, the fit deteriorates, and a model in which all label incorporation occurs via the propionate pathway is not compatible with the experimental data. The calculated fluxes are in accordance with the metabolite measurements, which show a statistically significant increase in the total pool size of tricarboxylic acid-cycle intermediates plus aspartate and glutamate from 39.2 ± 1.3 ($n = 4$) to 46.6 ± 2.5 ($n = 4$) μmol/g dry wt. (means ± S.E.M.). Therefore anaplerosis cannot occur solely at the expense of aspartate plus glutamate. The ATP citrate lyase reaction (EC 4.1.3.8) offers the possibility that malate (+oxaloacetate) may be labelled by the isocitrate dehydrogenase reaction, but this route is known to be slow in heart muscle. From the concentration and kinetic properties of the enzyme as determined by Srere (1959), it may be calculated that the rate of this reaction does not exceed 1.8 nmol/min per g dry wt. in the beating heart and 5.4 nmol/min per g dry wt. in the arrested heart. These fluxes were used in the simulation program.

Fumarate. The concentration of fumarate (Fig. 3) was about one-tenth of that of malate. The specific radioactivity of fumarate after 10 min of perfusion was 80% of that of malate in the beating heart and 90% of that of malate in the arrested heart. The concentration is lower than that calculated from the fumarate hydratase equilibrium (Hill & Bradshaw, 1969) and could indicate that the subcellular compartmentations of malate and fumarate are different. The fumarate hydratase reaction determines the randomization of radioactivity between C-1 and C-4 of malate and the extent of 2-oxoglutarate labelling (see below). The solution of this model indicated that the reversed fumarate hydratase reaction was equivalent to 80% of the forward reaction in the beating heart and 84% during the first 2 min after cardiac arrest.

Succinate. This metabolite was well resolved in

ion-exchange chromatography, but no significant incorporation of label could be detected. The estimated extremely low rate of the propionate pathway (Takala *et al.*, 1980) is also consistent with these data. Since label was found in the fumarate, the succinate dehydrogenase reaction cannot be in equilibrium.

2-Oxoglutarate. The specific radioactivity of 2-oxoglutarate (Fig. 4) at 10min was 39% and 40% of that of the malate in beating and arrested hearts respectively. These values indicate that label randomization occurs on reversal of the fumarate hydratase reaction. Solution of the simulation model gave a specific radioactivity for 2-oxoglutarate which was 47% and 39% of that of malate at 10min in beating and arrested hearts respectively. This is in accordance with the known randomization of the label of malate in the fumarate hydratase reaction.

Glutamate and aspartate. The net incorporation of label into aspartate and glutamate was greatly influenced by the metabolic state of the heart (Figs. 5 and 6). Although the aspartate content of the tissue diminished on arrest, the total label content of the aspartate pool in the arrested heart at 10min was three times that found in the beating heart. The label content of the glutamate pool in the arrested heart was five times that in the beating heart.

If cytosolic NADH is mainly reoxidized by means of the malate–aspartate shuttle (Safer & Williamson, 1973; Borst, 1963), the exchange rates between the 2-oxoglutarate and glutamate pools and malate and aspartate pools, respectively, should be equal to or higher than the acetyl-CoA flux into the cycle during glucose oxidation, which is the situation under these experimental conditions (Hiltunen & Hassinen, 1976).

When the fluxes of aspartate and glutamate exchange are of the same magnitude as the tricarboxylic acid-cycle flux, the fit of the calculated labelling kinetics to the experimental values is relatively good (Figs. 5 and 6). At still higher exchange rates, simulating equilibrium conditions, the fit deteriorates, necessitating several compartments for these metabolites. Since no experimental data exist for the subcellular distribution of metabolites in perfused hearts, these were treated here as whole tissue pools.

Conclusions

The present results show that carboxylation and

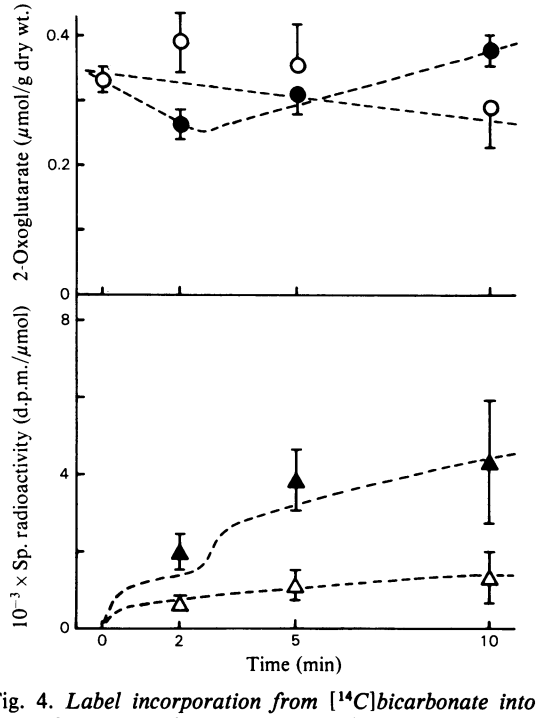


Fig. 4. *Label incorporation from [^{14}C]bicarbonate into 2-oxoglutarate and concentration of 2-oxoglutarate in isolated perfused rat hearts*

Experimental conditions and symbols were as in Fig. 1.

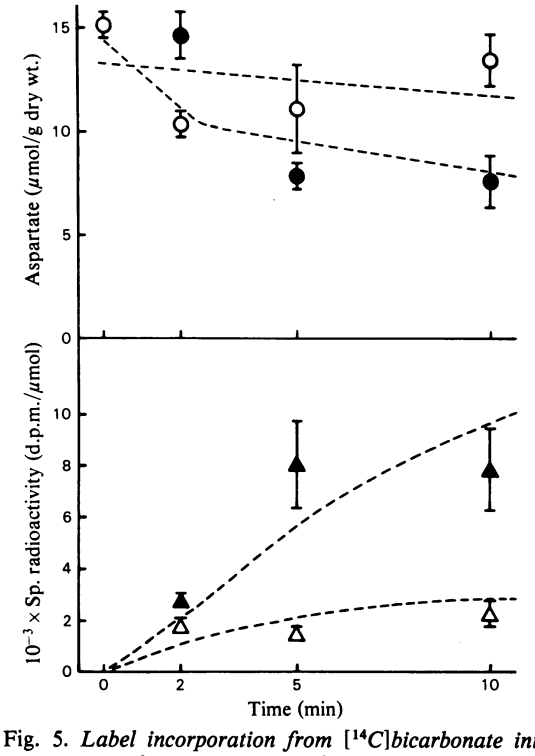


Fig. 5. *Label incorporation from [^{14}C]bicarbonate into aspartate and concentration of aspartate in isolated perfused rat hearts*

Experimental conditions and symbols were as in Fig. 1.

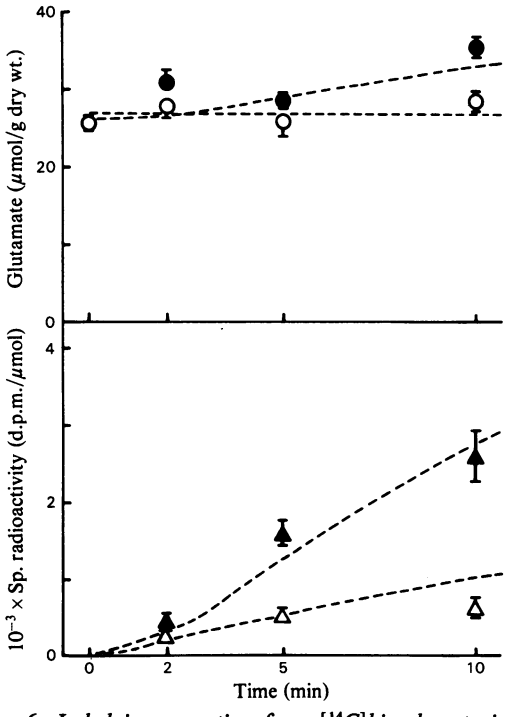


Fig. 6. *Label incorporation from [^{14}C]bicarbonate into glutamate and concentration of glutamate in isolated perfused rat hearts*

Experimental conditions and symbols were as in Fig. 1.

decarboxylation reactions involving the malate + oxaloacetate pool also occur in cardiac muscle, in contrast with the common view that carboxylation reactions are not significant in non-gluconeogenic tissue. During steady-state conditions in a beating isolated perfused heart there is an outward flow from the tricarboxylic acid cycle in the malate + oxaloacetate region. The increase in the total amount of tricarboxylic acid-cycle intermediates occurs partly at the expense of aspartate and partly by CO_2 fixation, yielding malate + oxaloacetate. The mechanism of the conversion of aspartate into tricarboxylic acid-cycle intermediates has not been identified. The direction of the changes in alanine concentrations (Hiltunen, 1978) apparently rules out the possibility of any major contribution from coupled transamination with pyruvate, and the direction of the changes in ammonia production rules out the importance of the purine nucleotide cycle (Takala *et al.*, 1980). The present experiments do not identify the carboxylation reaction involved. Another line of recent evidence suggests that some carboxylation of pyruvate also occurs in the myocardium. Radioactivity is incorporated from [1-^{14}C]pyruvate into tricarboxylic acid-cycle intermediates in the isolated perfused heart, indicating that in this tissue, too, pyruvate can enter the cycle at sites other than acetyl-CoA (K. J. Peuhkurinen, E. M. Nuutinen, E. P. Pietiläinen, J. K. Hiltunen & I. E. Hassinen, unpublished work).

Estimation of the contributions of potentially anaplerotic reactions in the regulation of metabolite concentrations under physiological conditions necessitates tracer methodology, and the large differences between the metabolic fluxes involved make the use of a computer indispensable. We describe here a computer model of the tricarboxylic acid cycle intended to solve the label-incorporation kinetics. The model accepts a general case of a linear network easily adaptable according to the detail of the description and the stoichiometries of the reactions, and therefore is of use for any experimentation of intermediary metabolism employing labelled compounds.

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APPENDIX

Given n pools of initial sizes P_i, we consider the specific radioactivity α_i of the pool i. Let k_{ji} be the flux rate from the pool j to the pool i. Since the metabolite concentrations are given as molar units, it is necessary to weight the mass and/or label fluxes by factors m_{ji} and l_{ji} respectively in order to obtain the correct molar stoichiometry for the reactions. Hence the incoming flux k_{ji} increases the label content of the pool i at a rate $l_{ji}k_{ji}\alpha_j$. On the other hand, the label content of the pool i decreased at a rate $k_{ij}\alpha_i$ owing to the outgoing flux k_{ij}. Summing over all incoming and outgoing fluxes, the total change in the label contents is given by

$$\frac{d}{dt}(Q_i\alpha_i) = \sum_{j=0}^{n} l_{ji}k_{ji}\alpha_j - \alpha_i \sum_{j=0}^{n} k_{ij}$$

or

$$Q_i\frac{d\alpha_i}{dt} = \sum_{j=0}^{n} l_{ji}k_{ji}\alpha_j - \alpha_i \sum_{j=0}^{n} k_{ij} - \alpha_i\frac{dQ_i}{dt}$$

where Q_i stands for the pool size at time t and subscript 0 for the source. Replacing Q_i at time t by the expression:

$$Q_i = P_i + \int_0^t \left(\sum_{j=0}^{n} m_{ji}k_{ji} - \sum_{j=0}^{n} k_{ij}\right)dt,$$

the time development of the specific radioactivity is seen to be given by

$$\frac{d\alpha_i}{dt} = \frac{1}{Q_i}\left(\sum_{j=0}^{n} l_{ji}k_{ji}\alpha_j - \alpha_i \sum_{j=0}^{n} m_{ji}k_{ji}\right) \qquad (1)$$

The system (1) cannot be integrated exactly, since in general the coefficients are functions of time. Since they also differ greatly, the equation (1) is stiff in nature (Gear, 1971).

The implicit trapezoidal rule was applied in view of its A-stability and easy control of the truncation error. The linearity of the system (1) was exploited either by solving the resulting equations exactly or by iterating from the previously computed values.

Since only relatively few measurements were available, the number of free parameters had to be kept as low as possible. This was done by imposing constraints on the fluxes and concentrations and choosing the simplest non-trivial parametric form for the fluxes k_{ji}. In the case where the fluxes change continuously with time, these were approximated by step functions taking a prescribed number of values during the observation time. In performing the numerical calculations the Peckham (1970) method was modified to take account of the constraints and to obtain information on the standard deviations of the parameters estimated.

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