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Control of the Tricarboxylate Cycle and its Interactions with Glycolysis during Acetate Utilization in Rat Heart

By P. J. RANDLE, P. J. ENGLAND AND R. M. DENTON
Department of Biochemistry, University of Bristol, Bristol BS8 1TD, U.K.

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1. Transient and steady-state changes caused by acetate utilization were studied in perfused rat heart. The transient period occupied 6min and steady-state changes were followed in a further 6min of perfusion. 2. In control perfusions glucose oxidation accounted for 75% of oxygen utilization; the remaining 25% was assumed to represent oxidation of glyceride fatty acids. With acetate in the steady state, acetate oxidation accounted for 80% of oxygen utilization, which increased by 20%; glucose oxidation was almost totally suppressed. The rate of tricarboxylate-cycle turnover increased by 67% with acetate perfusion. The net yield of ATP in the steady state was not altered by acetate. 3. Acetate oxidation increased muscle concentrations of acetyl-CoA, citrate, isocitrate, 2-oxoglutarate, glutamate, alanine, AMP and glucose 6-phosphate, and lowered those of CoA and aspartate; the concentrations of pyruvate, ATP and ADP showed no detectable change. The times for maximum changes were 1 min, acetyl-CoA, CoA, alanine and AMP; 6min, citrate, isocitrate, glutamate and aspartate; 2–4min, 2-oxoglutarate. Malate concentration fell in the first minute and rose to a value somewhat greater than in the control by 6min. There was a transient and rapid rise in glucose 6-phosphate concentration in the first minute superimposed on the slower rise over 6min. 4. Acetate perfusion decreased the output of lactate, the muscle concentration of lactate and the [lactate]/[pyruvate] ratio in perfusion medium and muscle in the first minute; these returned to control values by 6min. 5. During the first minute acetate decreased oxygen consumption and lowered the net yield of ATP by 30% without any significant change in muscle ATP or ADP concentrations. 6. The specific radioactivities of cycle metabolites were measured during and after a 1 min pulse of [1-^{14}C]acetate delivered in the first and twelfth minutes of acetate perfusion. A model based on the known flow rates and concentrations of cycle metabolites was analysed by computer simulation. The model, which assumed single pools of cycle metabolites, fitted the data well with the inclusion of an isotope-exchange reaction between isocitrate and 2-oxoglutarate + bicarbonate. The exchange was verified by perfusions with [^{14}C]bicarbonate. There was no evidence for isotope exchange between citrate and acetyl-CoA or between 2-oxoglutarate and malate. There was rapid isotope equilibration between 2-oxoglutarate and glutamate, but relatively poor isotope equilibration between malate and aspartate. 7. It is concluded that the citrate synthase reaction is displaced from equilibrium in rat heart, that isocitrate dehydrogenase and aconitate hydratase may approximate to equilibrium, that alanine aminotransferase is close to equilibrium, but that aspartate transamination is slow for reasons that have yet to be investigated. 8. The slow rise in citrate concentration as compared with the rapid rise in that of acetyl-CoA is attributed to the slow generation of oxaloacetate by aspartate aminotransferase. 9. It is proposed that the tricarboxylate cycle may operate as two spans: acetyl-CoA → 2-oxoglutarate, controlled by citrate synthase, and 2-oxoglutarate → oxaloacetate, controlled by 2-oxoglutarate dehydrogenase; a scheme for cycle control during acetate oxidation is outlined. The initiating factors are considered to be changes in acetyl-CoA, CoA and AMP concentrations brought about by acetyl-CoA synthetase. 10. Evidence is presented for a transient inhibition of phosphofructokinase during the first minute of acetate perfusion that was not due to a rise in whole-tissue citrate concentration. The probable importance of metabolite compartmentation is stressed.

Newsholme, Randle & Manchester (1962) showed that the oxidation of fatty acids and ketone bodies in the perfused rat heart leads to inhibition of phosphofructokinase and hexokinase. Garland, Randle & Newsholme (1963) and Parmeggiani & Bowman (1963) found that oxidation of fatty acids and ketone bodies may inhibit phosphofructokinase by elevating cell citrate concentrations. England & Randle (1967) observed that inhibition of hexokinase may be explained by the rise in glucose 6-phosphate concentration that is a consequence of phosphofructokinase inhibition. Garland & Randle (1964) showed that the rise in cell citrate concentration during the oxidation of fatty acids and ketone bodies may be a consequence of their metabolism to acetyl-CoA, which leads to an increase in the [acetyl-CoA]/[CoA] ratio. Bowman (1966) concluded that pyruvate may not be utilized for oxaloacetate synthesis in rat heart and that the oxaloacetate required for citrate accumulation during oxidation of fatty acids and ketone bodies may be formed from aspartate by transamination, since he found that ^{14}C from [^{14}C]bicarbonate was not incorporated into malate whereas ^{14}C from [U-^{14}C]aspartate was incorporated.

A study of the changes in metabolite concentrations during the onset of DL-β-hydroxybutyrate oxidation in rat heart showed that the rise in cell citrate concentration takes 3min, whereas acetyl-CoA and CoA concentrations show maximum changes within 1min (Randle *et al.* 1966). Inhibition of phosphofructokinase and hexokinase develops over 6min (England & Randle, 1967). This discrepancy raised questions about the mechanisms that may control cell citrate concentrations in rat heart muscle. Two explanations were conceived for this delayed rise in cell citrate concentration, namely a delay in the provision of oxaloacetate for citrate accumulation or delay in the passage of citrate from mitochondrial (the site of citrate synthesis) to cytoplasmic compartments. This has led us to investigate the changes in metabolite concentrations and the incorporation of ^{14}C from [1-^{14}C]acetate into intermediates after the onset of acetate oxidation in rat heart. Acetate has been used in preference to DL-β-hydroxybutyrate since it is converted into acetyl-CoA without prior oxidation. A preliminary account of some of these findings has been published (Randle, Denton & England, 1968).

EXPERIMENTAL

Materials

Glycolytic intermediates, enzymes, adenine nucleotides, coenzymes, oxaloacetate, 2-oxoglutarate and triethanolamine hydrochloride were from Boehringer (London) Corp., London W.5, U.K., except for glutaminase from Sigma Chemical Co., St Louis, Mo., U.S.A. Amino acids and other chemicals (purest grade available) were from British Drug Houses Ltd., Poole, Dorset, U.K. Insulin was given by Boots Pure Drug Co. Ltd., Nottingham, U.K., or Burroughs Wellcome, Beckenham, Kent, U.K.; a stock solution of 20i.u./ml in 3.3mM-HCl was added to perfusion media. Heparin was from Evans Medical Ltd., Speke, Liverpool, U.K., and veterinary Nembutal from Abbott Laboratories Ltd., Queenborough, Kent, U.K. Ion-exchange resins Dowex 1 (AG1-X4; formate form; 200–400 mesh) and Dowex 50 (AG50W-X2; H^+ form; 200–400 mesh) were from Bio-Rad Laboratories, Richmond, Calif., U.S.A. Acetyl-CoA was synthesized by the method of Simon & Shemin (1953); 2-oxoglutarate dehydrogenase was purified from pig heart by the method of Sanadi, Littlefield & Bock (1952).

Perfusion

Hearts, for perfusion, were removed from male albino Wistar rats, fed on diet 41B (Short & Parkes, 1949) *ad libitum*, after injection with Nembutal and heparin (Newsholme & Randle, 1964). The perfusion medium was bicarbonate-buffered salt solution (Krebs & Henseleit, 1932) gassed with O_2+CO_2 (95:5) containing glucose (5.5mM) and insulin (0.05i.u./ml) with or without sodium acetate (5mM). Details of labelling and specific radioactivities of radiochemicals are given in the Results and Discussion section.

It has been shown in earlier studies that steady-state rates of glucose uptake and glycolysis and steady-state concentrations of a number of metabolites are established after 5min of perfusion. This has been confirmed for glucose uptake and for the concentrations of most of the metabolites assayed during the present study. The oxygen consumption of the heart does, however, fall by about 10% from 5 to 17min of perfusion. In all experiments steady-state rates of glucose uptake and glycolysis and steady-state metabolite concentrations were established by a 5min period of preperfusion by drip-through with medium containing glucose and insulin. Perfusion was then begun either with the same medium or with medium containing glucose, insulin and acetate (introduced from a second chamber via a two-way tap). In short perfusions (up to 2min) the connecting tubes were flushed out immediately before switch-over to ensure that the medium was at 37°C.

Glucose uptake was measured in the micro recirculation perfusion apparatus (England & Randle, 1967). All other measurements were made by using perfusion by drip-through (Newsholme & Randle, 1964). Oxygen consumption and output of $^{14}CO_2$ were measured with the apparatus described by Randle, Newsholme & Garland (1964) except that glass tubing was substituted for plastic tubing because of the permeability of the latter to oxygen. In perfusions with [^{14}C]bicarbonate constant specific radioactivity was achieved as follows. The medium containing glucose, insulin and acetate was saturated at 37°C with O_2+CO_2 in a 1-litre round-bottomed flask and covered with liquid paraffin. The [^{14}C]bicarbonate was then added through the liquid paraffin and dispersed with a magnetic stirrer. The flask was immersed in a water bath at 37°C and the medium perfused by drip-through with replacement by liquid paraffin. Control

experiments showed that radioactivity in the medium remained constant for at least 150 min.

Heart extracts

For metabolite assays hearts were rapidly frozen on the cannula with a tissue clamp at −70°C and powdered in a percussion mortar. A weighed amount of heart powder was extracted with 5% (w/v) $HClO_4$ (approx. 2.5 ml/g of powder) and centrifuged. A measured volume of supernatant was neutralized with saturated $KHCO_3$ (pH 7, indicator paper). For assay of CoA and radioactive metabolites extracts were adjusted to pH 6.5 in the presence of 0.1 M-triethanolamine with KOH by using a glass electrode. $KClO_4$ was centrifuged off at 0°C.

Glucose, lactate and pyruvate in the medium

Glucose was assayed spectrophotometrically by the method of Slein (1963) in 25 μl samples of medium (without deproteinization). Lactate and pyruvate were assayed spectrophotometrically after deproteinization with $HClO_4$ by the methods of Hohorst (1963*b*) and Bücher, Czok, Lamprecht & Latzko (1963).

Heart metabolites

These were assayed spectrophotometrically or fluorimetrically by the following methods: glucose 6-phosphate (Hohorst, 1963*a*); lactate and pyruvate (see the preceding section); citrate (Moellering & Gruber, 1966); isocitrate (Siebert, 1963); 2-oxoglutarate (Bergmeyer & Bernt, 1963); malate (Holzer & Soling, 1963); acetyl-CoA (Chase, 1967); CoA (Garland, Shepherd & Yates, 1963); acetylcarnitine (Pearson & Tubbs, 1967); ATP (Lamprecht & Trautschold, 1963); ADP and AMP (Adam, 1963); aspartate and asparagine (Pfleiderer, 1963); glutamate (Bernt & Bergmeyer, 1963); glutamine was assayed as glutamate after hydrolysis with glutaminase. Alanine was assayed colorimetrically with ninhydrin after separation in an EEL amino acid analyser on a 50 cm × 1 cm column by the method of Spackman, Stein & Moore (1958).

Oxygen

Oxygen concentration in the perfusion medium was measured at 37°C with a Clark oxygen electrode calibrated with medium saturated with O_2+CO_2 (95:5) at 37°C and with medium freed of oxygen with dithionite.

Radioactivity measurements

Assay of radioactivity. This was done in a Nuclear-Chicago Mark 1 liquid-scintillation system utilizing either dioxan-based scintillator (Butler, 1961) or toluene-based scintillator (Synder, 1961). Quenching corrections were based on channels ratio by using an external standard.

Glycogen. Heart muscle was digested in 30% KOH, and the crude glycogen was precipitated with ethanol–Na_2SO_4 as described by Walaas & Walaas (1950), washed with 70% (v/v) ethanol at 0°C and dissolved in water. Residual salt and radioactive glucose were removed by dialysis against water, volume changes being ascertained by weighing. Radioactivity was assayed with dioxan scintillator.

Carbon dioxide. $^{14}CO_2$ formed during perfusions with radioactive acetate was collected in Hyamine after acidification of samples of perfusion medium, and radioactivity assayed in toluene scintillator. Blank measurements made on initial perfusion medium were applied as a correction since some acetic acid distils into Hyamine under these conditions.

Total radioactivity in metabolites from [1-^{14}C]*acetate.* $HClO_4$ extracts of heart (4 ml) were applied to a combined column of Dowex 1 (formate form) (10 cm × 1 cm) on top of Dowex 50 (H^+ form) (10 cm × 1 cm). After a water wash (100 ml) acetate was specifically eluted with an exponential gradient of M-formic acid into 250 ml of water. This elutes acetate, glutamate and aspartate from Dowex 1 (formate form), and glutamate, aspartate and acetylcarnitine are removed by the Dowex 50 (H^+ form). Radioactivity in metabolites of acetate was calculated by difference: (radioactivity in extract − radioactivity in acetate).

Measurement of specific radioactivities

[1-^{14}C]*Glucose 6-phosphate.* The $HClO_4$ extract of hearts perfused with [1-^{14}C]glucose was heated to 100°C to remove traces of $^{14}CO_2$ and to destroy ATP, and neutralized with KOH in the presence of triethanolamine as described above. Glucose 6-phosphate was then converted quantitatively into 6-phosphogluconate by adding 1 unit of glucose 6-phosphate dehydrogenase to a mixture containing 1 ml of heart extract and 2 ml of 0.1 M-triethanolamine containing (final concentrations) 30 mM-glycylglycine, 10 mM-$MgCl_2$, 0.5 mM-EDTA and 0.2 mM-$NADP^+$, final pH 7.7. This took 1–2 min as shown by the change in extinction at 340 nm. The reaction mixture was then heated to 100°C to inactivate glucose 6-phosphate dehydrogenase and thus avoid possible reduction of $NADP^+$ by glucose in the subsequent longer incubation with 6-phosphogluconate dehydrogenase. Conversion of 6-phospho[1-^{14}C]gluconate into ribulose 5-phosphate and [^{14}C]bicarbonate was effected by addition (after cooling) of 0.25 unit of 6-phosphogluconate dehydrogenase. The extent of conversion, which was approx. 80% over 20 min, was followed by the change in extinction at 340 nm. The contents of the cuvette were transferred to a Marie flask, and $^{14}CO_2$ was collected in Hyamine, by addition of 200 μmol of $NaHCO_3$ and 4 mmol of H_2SO_4, and assayed for radioactivity in toluene scintillator. The recovery of $^{14}CO_2$ was shown in experiments with standard [1-^{14}C]-glucose 6-phosphate solutions to be 90% of that expected from the change in E_{340} during the 6-phosphogluconate dehydrogenase reaction.

[6-^{14}C]*Isocitrate.* $HClO_4$ extracts of hearts perfused with [^{14}C]bicarbonate were evacuated for 15 min over solid KOH, adjusted to pH 4.0 with saturated $KHCO_3$ and left *in vacuo* over solid KOH for 18 h to remove [^{14}C]-bicarbonate and CO_2. The extracts were neutralized with saturated $KHCO_3$, and isocitrate was assayed spectrophotometrically by the change in E_{340} after the addition of 1 unit of isocitrate dehydrogenase (NADP) (EC 1.1.1.42) to a 4 cm-light-path cuvette containing 1.5 ml of neutralized $HClO_4$ extract and 1.5 ml of 0.2 M-triethanolamine

with 10mM-$MgSO_4$ and 0.2mM-$NADP^+$, pH8.0. The reaction was complete within 2min. The contents of the cuvette were quantitatively transferred with 10mM-KOH washes to a Marie flask. $^{14}CO_2$ was then liberated by acidification and collected in Hyamine and assayed for radioactivity in toluene scintillator. Blank corrections were applied through incubations without isocitrate dehydrogenase. Standard [6-^{14}C]isocitrate was not available and recoveries of $^{14}CO_2$ were assumed to be 100% in view of the short period of incubation.

Acetyl-CoA and citrate. The specific radioactivities of acetyl-CoA and of the acetate and oxaloacetate moieties of citrate were determined by separation on Dowex 1 (formate form) by using the principle of enzymic conversion to effect chromatographic resolution as originally described for L-glycerol 3-phosphate and glucose 6-phosphate by Denton & Randle (1967*b*). Crude fractions containing (*a*) acetyl-CoA and (*b*) citrate were separated on Dowex 1 (formate form). Acetyl-CoA in extract (*a*) was specifically converted into citrate with citrate synthase and the citrate was separated by further ion-exchange chromatography. Citrate in extract (*b*) was converted into malate and acetate with citrate oxaloacetate-lyase and malate dehydrogenase, and malate and acetate were separated by further ion-exchange chromatography. The details were as follows.

After assay of acetyl-CoA and citrate approx. 2.5μmol of carrier acetyl-CoA and 10μmol of carrier citrate were added to 8–10ml of $HClO_4$ extract. Acetyl-CoA and citrate were then reassayed. The extract was applied to a 20cm×1cm column of Dowex 1 (formate form) and washed with 150ml of 0.63M-formic acid. Fractions containing citrate (*b*) were eluted with an exponential gradient of 1M-ammonium formate into 250ml of 0.63M-formic acid (elution occurred between 160 and 200ml). The gradient was continued to a volume of 250–300ml and acetyl-CoA was then eluted with a 1M-ammonium formate step.

Fractions containing acetyl-CoA (*a*) were immediately combined and adjusted to pH7.0 (glass electrode) with KOH after the addition of 0.3ml of 2M-triethanolamine hydrochloride. Approx. 4μmol of oxaloacetate was then added and acetyl-CoA was converted into citrate with 14 units of citrate synthase. The reaction was followed by spectrophotometric assay of free thiol in 3ml samples with 5,5′-dithiobis-2-nitrobenzoate and was complete within 10min. The reaction mixture was then passed through Dowex 50 (H^+ form) to remove NH_4^+, and formic acid was removed by freeze-drying. The residue was dissolved in 10ml of 20mM-triethanolamine–HCl buffer, pH7.6, applied to an 8cm×1cm column of Dowex 1 (formate form) and washed with 100ml of 0.63M-formic acid, and citrate was eluted with an exponential gradient of 1M-ammonium formate into 100ml of 0.63M-formic acid. The peak tube of the citrate fraction was freed of ammonium formate (see above) and assayed for citrate and for radioactivity.

Fractions containing citrate (*b*) were freed of ammonium formate and taken up in buffer as described above. Citrate was then converted into acetate and malate by the addition of 20μmol of NADH, 36 units of malate dehydrogenase and 4 units of citrate oxaloacetate-lyase. The reaction (followed at 340nm) was completed in 15min. The mixture was then applied to a 12cm×1cm column of Dowex 1 (formate form) and washed with 50ml of water, and acetate and malate were eluted as separate peaks with an exponential gradient of 2M-formic acid into 250ml of water (elution volumes: acetate, 30–50ml; malate, 120–140ml). The combined fractions were assayed for radioactivity in each case. Malate was assayed spectrophotometrically (Holzer & Soling, 1963). The amount of acetate was assumed to be the same as that of malate.

The recoveries of acetyl-CoA and citrate from the first ion-exchange chromatography were 80% or better. The recovery of acetyl-CoA as citrate after enzymic conversion and chromatography was approx. 75%. The recovery of citrate as malate after enzymic conversion and chromatography was greater than 90%.

Malate, 2-oxoglutarate, citrate and succinate. $HClO_4$ extracts were assayed for malate, 2-oxoglutarate and citrate, after which 10μmol each of carrier malate, 2-oxoglutarate, citrate and succinate were added per 7ml of extract. The extract was then applied to a 15cm×1cm column of Dowex 1 (formate form) and washed with 100ml of water, and metabolites were eluted with exponential gradients of 250ml of 1M-formic acid into 250ml of water followed by 125ml of 2M-formic acid followed by 1M-ammonium formate. The elution volumes for the major radioactive metabolites from the start of the 1M-formic acid gradient were: acetate, 60–90ml; succinate, 175–210ml; malate, 260–290ml; citrate, 430–500ml; 2-oxoglutarate, 510–530ml. The peaks of malate, 2-oxoglutarate and citrate were separately pooled and freeze-dried [after treatment, in the case of citrate and 2-oxoglutarate, with Dowex-50 (H^+ form) to remove NH_4^+]. Malate and 2-oxoglutarate were subjected to descending chromatography on Whatman no. 1 paper with diethyl ether–acetic acid–water (13:3:1, by vol.) (Denison & Phares, 1952) with markers of malate, 2-oxoglutarate, fumarate, succinate and citrate. The markers were located by spraying with Chlorophenol Red and strips of paper (two or three for each spot) cut in corresponding positions from the unknown samples. The malate and succinate fractions from Dowex 1 (formate form) were apparently pure. The 2-oxoglutarate fraction was contaminated with citrate, which was well separated on paper. Hence the specific radioactivity of malate was calculated by assays of radioactivity and malate in the Dowex 1 (formate form) eluate. The specific radioactivity of 2-oxoglutarate was calculated from assays made after elution from the paper chromatogram. Citrate specific radioactivity was measured on eluates from Dowex 1 (formate form). It was assumed that isocitrate is eluted together with citrate and had the same specific radioactivity as citrate; the calculated isocitrate radioactivity was subtracted from the total radioactivity measured in the citrate fraction (the correction was approx. 8%).

Aspartate, asparagine, glutamate, glutamine and alanine. These were separated without addition of carrier by an EEL amino acid analyser (see section above on heart metabolites), which failed to resolve aspartate and asparagine. The latter were separated by subsequent electrophoresis on cellulose acetate strips by the method of Cook & Luscombe (1960). The analyser was initially calibrated for the position of peaks by runs in which ninhydrin was used. For assay of specific radioactivity the ninhydrin system was disconnected and the effluent run into a fraction collector. The peak tubes were taken

for assay of radioactivity and of individual amino acids by methods given above.

Calculations

Uptakes and outputs were calculated as μmol of substrate or product/min per g dry wt. of heart. Intracellular metabolite concentrations were calculated as μmol/g dry wt. of heart. Dry weight of heart was determined by freeze-drying (13–15% of wet wt. of heart).

RESULTS AND DISCUSSION

Acetate utilization and acetate oxidation

The time-course of acetate utilization and of acetate oxidation was followed by measuring the incorporation of ^{14}C from [1-^{14}C]acetate into its metabolic products. Radioactivity was measured in medium bicarbonate and carbon dioxide and in perchloric acid-soluble metabolites in the heart. Control experiments showed that there was negligible incorporation of ^{14}C into perchloric acid-insoluble material (less than 0.1% of the calculated rate of acetate utilization). Recovery experiments showed that virtually all (80–100%) of the radioactivity in perchloric acid-soluble metabolites could be accounted for by the radioactivities of tricarboxylate-cycle intermediates together with acetylcarnitine, glutamate and aspartate. No radioactivity was detected in lactate or alanine and hence (by inference) there was no detectable isotope exchange between acetate and pyruvate. The utilization of acetate carbon in this tissue may thus be largely confined to the formation of carbon dioxide, cycle intermediates and acetylcarnitine, glutamate and aspartate.

Acetate utilization (μmol/g dry wt. of heart) at each time-interval studied (see Fig. 1) was calculated from the incorporations of ^{14}C (d.p.m./g dry wt. of heart) and the specific radioactivity of acetate in the medium (d.p.m./μmol). Acetate oxidation (μmol/g dry wt. of heart) was calculated from the acetate utilization by subtracting the increases in concentrations of intermediates formed from acetate (μmol/g dry wt. of heart). Since one carbon is lost as carbon dioxide in the isocitrate dehydrogenase reaction only one-half of the increase in the concentration of 2-oxoglutarate + glutamate was subtracted. Acetate oxidation was therefore calculated as the difference [(acetate utilization) − (increase in concentration of acetyl-CoA + acetylcarnitine + citrate + isocitrate) − 0.5 × (increase in concentration of 2-oxoglutarate + glutamate)].

Fig. 1 shows the time-course of acetate utilization (A), acetate oxidation (B) and the conversion of acetate carbon into carbon dioxide (C) calculated from (^{14}C in carbon dioxide)/(specific radioactivity of acetate in the medium). The difference $A-B$ thus represents the increase in concentrations of intermediates formed from acetate; and the difference $A-C$ represents the incorporation of acetate carbon into intermediates of acetate metabolism. As curves for B and C are not parallel even after 12 min of perfusion with [1-^{14}C]acetate it is apparent that isotope equilibration between C-1 of acetate and the carbon atoms of metabolites formed from it is not complete after 12 min; the specific radioactivities of acetate metabolites were still increasing at the twelfth minute. The curve for B shows that the steady-state rate of acetate utilization was 12 μmol/min per g dry wt. of heart and this was essentially attained by the end of the second minute. A steady-state rate of acetate oxidation of 12 μmol/min per g dry wt. of heart was achieved after approx. 2 min. In the first minute of acetate perfusion the rate of acetate utilization was 11.1 μmol/min per g dry wt. of heart and the rate of acetate oxidation was 6.35 μmol/min per g dry wt. of heart.

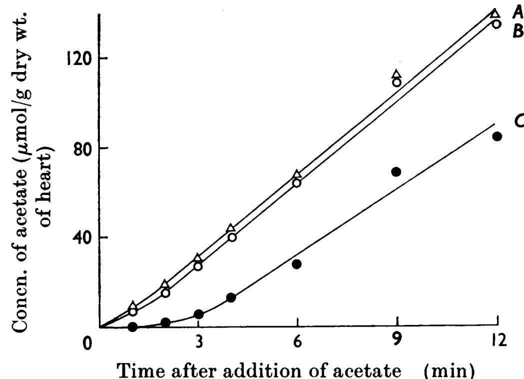


Fig. 1. Time-course of acetate utilization (△), acetate oxidation (○) and conversion of acetate carbon into CO_2 calculated from incorporation of ^{14}C from [1-^{14}C]acetate into CO_2 (●). Hearts were preperfused for 5 min with medium containing glucose (1 mg/ml) and insulin (0.05 i.u./ml) and switched at zero time to medium that contained in addition 5 mM-[1-^{14}C]acetate (0.07 μCi/ml). Two hearts were perfused to provide the radioactivity results for each time-period. For details of calculations see the text.

The radioactive-acetate space of the heart frozen with the tissue clamp attained a constant value of 0.5 ml/g wet wt. of heart within 1 min. This is comparable with the extracellular space of the heart measured with sorbitol and frozen with the tissue clamp (P. J. Randle, P. J. England & R. M. Denton, unpublished work).

Oxygen consumption

Fig. 2 shows the time-course of oxygen consumption in control and acetate perfusions. In control perfusions the oxygen consumption fell steadily

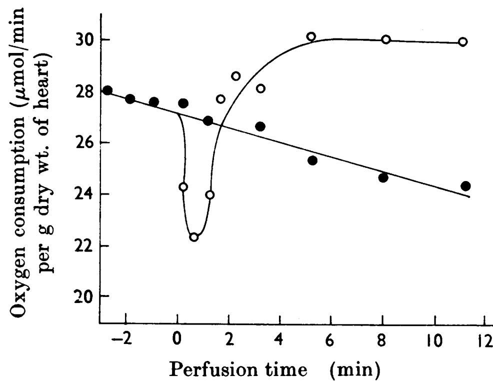


Fig. 2. Time-course of oxygen consumption in control (●) and acetate (○) perfusions. Mean values are shown for three hearts in each group after 7 min of preperfusion. For composition of media see the Experimental section.

from 27.2 μmol/min per g dry wt. of heart at zero time to 24 μmol/min per g dry wt. of heart after 12 min.

Acetate perfusion led to an approx. 20% fall in oxygen consumption in the first minute followed by a rise to a new steady-state rate of 30 μmol/min per g dry wt. of heart by the end of the fifth minute. In the steady state acetate oxidation accounted for 80% of the oxygen consumption.

Tricarboxylate cycle: metabolite concentrations

Fig. 3 shows muscle concentrations of acetyl-CoA and CoA, citrate, isocitrate and the [citrate]/[isocitrate] ratio, and Fig. 4 muscle concentrations of glutamate, aspartate, 2-oxoglutarate and malate. In control perfusions the concentrations of these metabolites showed little if any change except those for malate and aspartate, which showed a slow fall in concentration. With acetate marked changes were seen in all these parameters. Acetyl-CoA concentration increased and that of CoA decreased markedly within 0.5 min of acetate perfusion, and apart from some indication of an overshoot showed little change thereafter. Acetylcarnitine concentrations (not shown) increased rapidly to a steady-state value 20 times that of acetyl-CoA (within 0.5–1 min). The concentration of malate fell rapidly during the first minute of perfusion with acetate and increased thereafter to reach a new steady-state value, somewhat greater than controls, after 6 min. The concentrations of citrate and isocitrate increased steadily with acetate perfusion to reach a new steady-state concentration after 6 min. Over the same time-period the concentration of glutamate rose and that of aspartate fell. The concentration of 2-oxoglutarate rose in the first 3 min and then fell slightly.

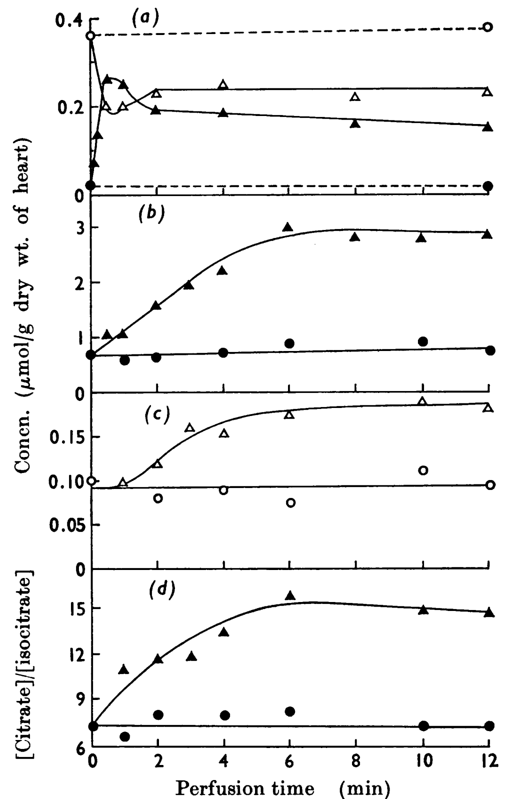


Fig. 3. Time-course of changes in whole-tissue concentration of metabolites in rat heart after onset of acetate perfusion. (*a*) CoA concentration during control perfusion (○) and acetate perfusion (△); acetyl-CoA concentration during control perfusion (●) and acetate perfusion (▲). (*b*) Citrate concentration during control perfusion (●) and acetate perfusion (▲). (*c*) Isocitrate concentration during control perfusion (○) and acetate perfusion (△). (*d*) [Citrate]/[isocitrate] ratio during control perfusion (●) and acetate perfusion (▲). Pooled values are shown for six to 20 hearts preperfused for 5 min and perfused thereafter for the time shown. For composition of media see the Experimental section.

These findings might be interpreted as follows: acetate, through acetyl-CoA synthetase, rapidly alters concentrations of acetyl-CoA and CoA and these lead to increases in the concentrations of citrate, isocitrate, 2-oxoglutarate and glutamate with consumption of oxaloacetate. Oxaloacetate is initially furnished from the malate pool, which may be subsequently slowly replenished, over 6 min, from oxaloacetate formed by transamination between aspartate and 2-oxoglutarate.

Some support for this view is provided by the balance data shown in Table 1. In control and acetate perfusions losses of [aspartate+malate]

were somewhat greater than increases in [citrate + isocitrate + 2-oxoglutarate + glutamate]. However, the discrepancies were less than 10% of the total initial concentrations of these metabolites. The loss of these metabolites into the perfusion medium over a 6min period was measured in some experiments and amounted to less than 10% of the concentrations in the heart. Glutamine and asparagine did not appear to contribute carbon to the tricarboxylate cycle. Thus the concentration of glutamine was 3.6 μmol/g dry wt. of heart at the beginning and 2.7 μmol/g dry wt. of heart after 6min of control or acetate perfusion. The concentration of asparagine was 3.4 μmol/g dry wt. of heart throughout perfusion.

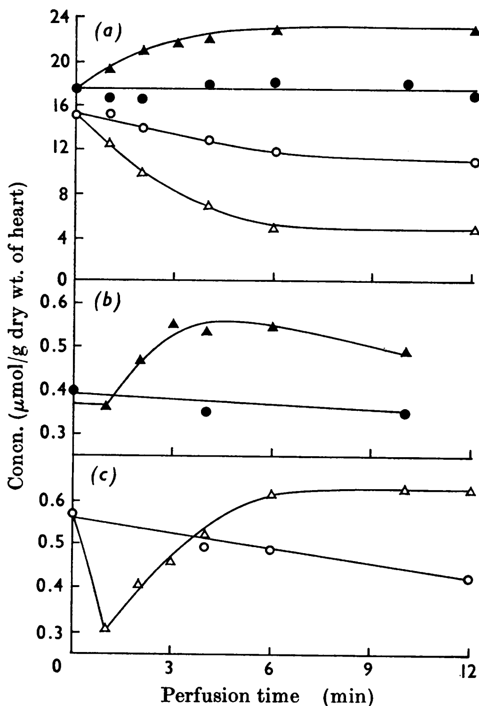


Fig. 4. Time-course of changes in whole-tissue concentrations of metabolites in rat heart after onset of acetate perfusion. (*a*) Glutamate concentration during control perfusion (●) and acetate perfusion (▲); aspartate concentration during control perfusion (○) and acetate perfusion (△). (*b*) 2-Oxoglutarate concentration during control perfusion (●) and acetate perfusion (▲). (*c*) Malate concentration during control perfusion (○) and acetate perfusion (△). Pooled values are shown for six to 20 hearts preperfused for 5min and perfused thereafter for the time shown. For composition of media see the Experimental section.

If the accumulations of citrate, isocitrate, 2-oxoglutarate and glutamate occur at the expense of aspartate carbon then there must be an equivalent loss of aspartate nitrogen. The nitrogen equivalent to the accumulations of citrate, isocitrate and 2-oxoglutarate could be incorporated into other amino acids, especially alanine by aminotransferase reactions or released as ammonia through reactions catalysed by aspartate aminotransferase and glutamate dehydrogenase. As shown in Fig. 7 acetate perfusion increased the concentration of alanine in the heart. Table 2 shows the changes in aspartate, glutamate and alanine concentrations during control and acetate perfusions. In most instances the loss of aspartate was somewhat greater than the increases in glutamate and alanine, although the discrepancies were small (less than 7%) in relation to the concentrations of these metabolites.

The [citrate]/[isocitrate] ratio increased steadily with acetate to a new steady-state value, which was reached after 6min of perfusion (Fig. 3).

Tricarboxylate cycle: flow rates from balance data

The rate of tricarboxylate-cycle turnover (i.e. the rate of complete oxidation of acetyl-CoA) was calculated in control perfusions from the rate of oxygen consumption. Balance data showed in control perfusions (see Table 3) that glucose oxidation accounted for approx. 75% of the oxygen consumption, as compared with 82% in an earlier

Table 1. *Effect of acetate perfusion of rat heart on the distribution of oxaloacetate carbon between [aspartate + malate] and [citrate + isocitrate + 2-oxoglutarate + glutamate]*

		Change in concn. during perfusion at time shown (μmol/g dry wt. of heart)					
		Control perfusions at			Acetate perfusions at		
Metabolite	Initial concn. (μmol/g dry wt. of heart)	1min	3min	6min	1min	3min	6min
Citrate	0.85	0	0	0	0.4	1.1	2.2
Isocitrate	0.10	0	0	0	0	0.07	0.08
2-Oxoglutarate	0.38	0	−0.02	−0.04	0	0.15	0.17
Glutamate	17.50	0	0	0	1.7	4.1	5.7
Aspartate	15.0	−0.6	−2.2	−3.2	−2.5	−6.6	−10.0
Malate	0.55	0	−0.03	−0.07	−0.26	−0.10	0.07
Total	34.38	−0.6	−2.25	−3.31	−0.66	−1.28	−1.78

Table 2. *Effect of acetate perfusion of rat heart on the distribution of nitrogen between aspartate, glutamate and alanine*

		Change in concn. during perfusion at time shown (μmol/g dry wt. of heart)					
		Control perfusions at			Acetate perfusions at		
Amino acid	Initial concn. (μmol/g dry wt. of heart)	1 min	3 min	6 min	1 min	3 min	6 min
Aspartate	15.0	−0.6	−2.2	−3.2	−2.5	−6.6	−10.0
Glutamate	17.5	0	0	0	1.7	4.1	5.7
Alanine	4.75	0	0.2	0.5	1.4	1.9	2.1
Total	37.25	−0.6	−2.0	−2.7	0.6	−0.6	−2.2

Table 3. *Effect of acetate on steady-state and transient rates of metabolism in perfused rat heart*

For details of perfusion see the Experimental section and legend to Fig. 1. Theoretical oxygen equivalents (mol/mol): acetate, 2(tricarboxylate cycle); glucose, 6 (4 cycle+1 pyruvate dehydrogenase+1 glycolytic NADH); palmitate, 23 (16 cycle + 7 β-oxidation). The partition of oxygen for cycle/non-cycle oxidations is 2/1 for glucose and 16/7 for palmitate. Palmitate oxidation is assumed to account for oxygen not utilized for glucose or acetate oxidation. In calculating ATP yield the following P/O ratios were assumed: glucose, 3.17; acetate, 2.5; palmitate, 2.8. The net yield of ATP from lactate and pyruvate output was taken as 2 μmol of ATP/μmol of glucose equivalent. Glycolysis rate was computed as (glucose uptake − glycogen synthesis) and glucose oxidation as (glycolysis − lactate output − pyruvate output). Mitochondrial oxidation of glycolytic NADH was computed from (glucose oxidation + pyruvate output + alanine synthesis).

	Perfusion (0–1 min)		Steady state (8–12 min)	
	Control	Acetate	Control	Acetate
Rate (μmol of glucose or glucose equiv./min per g dry wt. of heart)				
Glucose uptake	6.6	2.8	6.6	3.7
Glycogen synthesis	Zero	Not known	Zero*	1.3
Lactate + pyruvate output	3.6	2†	3.6	3.6
Glycolysis	6.6	2.8	6.6	2.4
Glucose oxidation	3.0	0.3‡	3.0	Zero
Rate (μmol of O_2 or O_2 equiv./min per g dry wt. of heart)				
Oxygen consumption	27.8	22.2	25.3	30.25
Oxidation (pyruvate + glycolytic NADH)	18.25	2.85	18	0.25
Palmitate oxidation	9.55	6.65	7.05	6
Acetate oxidation	Zero	12.7	Zero	24
Tricarboxylate cycle	18.35	18.45	16.6	28
Pyruvate dehydrogenase	3.0	0.3	3	Zero
Glycolytic NADH	3.25	1.35§	3.25	0.25
β-Oxidation of palmitate	3.2	2.1	2.45	2
Rate (μmol of acetyl-CoA/min per g dry wt. of heart)				
Tricarboxylate-cycle flow:				
Turnover	9.2	8.8	8.4	14
Span acetyl-CoA → 2-oxoglutarate	9.2	10.4	8.4	14
Rate (μmol of ATP/min per g dry wt. of heart)				
ATP net yield	175	122	162	157

* The apparent rate was 0.4, which was assumed to be insignificant. Previous studies with longer periods of control perfusion showed no change in glycogen concentration (Randle *et al.* 1964).

† Calculated from difference (medium lactate and pyruvate − change in heart lactate and pyruvate).

‡ Calculated from difference (glycolysis rate − lactate and pyruvate output − change in muscle alanine).

§ Ignores change in cytoplasmic [NADH]/[NAD^+] ratio.

investigation (Denton & Randle, 1967*a*). The remaining 25% of oxygen consumption is assumed to represent oxidation of muscle glyceride fatty acids (taken as palmitate), for reasons given by Denton & Randle (1967*a*). The oxygen equivalent for glucose is 6 μmol of O_2/μmol of glucose, of which 4 μmol is utilized in the oxidation of acetyl-CoA. The oxygen equivalent for palmitate is 23 μmol of O_2/μmol of palmitate, of which 16 μmol is utilized in the oxidation of acetyl-CoA. In control per-

fusions cycle turnover in oxygen equivalents was calculated from the sum ($0.67\times$oxygen consumed in glucose oxidation + $0.70\times$oxygen consumed in palmitate oxidation). Since the proportions (0.67 and 0.70) are so close, any error in determinations of the relative contributions of glucose and palmitate to oxidative metabolism will have relatively little effect on the calculated rate of cycle turnover. The oxygen equivalent for acetate oxidation is 2μmol of O_2/μmol of acetate. In acetate perfusions cycle turnover in oxygen equivalents was calculated from (oxygen consumed in acetate oxidation + $0.67\times$oxygen consumed in glucose oxidation + $0.70\times$oxygen consumed in palmitate oxidation).

The results are given in Table 3. In control perfusions the rate of tricarboxylate-cycle turnover was 9.2μmol of acetyl-CoA oxidized/min per g dry wt. of heart in the first minute, and 8.4 in the twelfth minute. In acetate perfusions the rate of cycle turnover was 8.8μmol of acetyl-CoA oxidized/min per g dry wt. of heart in the first minute, and 14 in the steady state (from 8 to 12min). Acetate thus increased the rate of cycle turnover in the steady state by approx. 70%. This calculated increase in the steady-state rate of cycle turnover with acetate can be equated with the 20% increase in oxygen consumption and with the diminution in glucose oxidation. In the first minute of acetate perfusion the rate of turnover of the tricarboxylate cycle matched that of the control in spite of a 20% diminution in oxygen consumption. This can be equated with the diminution in glucose and palmitate oxidation. In the absence of accurate measurements of glycogen synthesis during the first minute of acetate perfusion, rates of glycolysis and hence glucose oxidation are uncertain. The calculated rates given in Table 3 assume no net synthesis or breakdown of glycogen. The extreme limits for the rate of glycolysis are 2.5 and 4.2μmol/min per g dry wt. of heart and for the oxidation of glucose 0 and 1.7μmol/min per g dry wt. of heart. Whichever value is taken acetate inhibits glucose oxidation in the first minute. The calculation of cycle turnover is not influenced by this value.

The rate of flow in the limited cycle span acetyl-CoA $\rightarrow$ 2-oxoglutarate was increased in the first minute of acetate perfusion from 9.2 to 10.4μmol of acetyl-CoA/min per g dry wt. of heart (Table 3). Flow in this span of the cycle is greater than cycle turnover during each of the first 6min of acetate perfusion because of accumulations of citrate, isocitrate, 2-oxoglutarate and glutamate.

Tricarboxylate cycle: isotope data

Table 4 shows the specific radioactivities of a number of tricarboxylate-cycle metabolites after pulse perfusions with [1-^{14}C]acetate delivered during the first or twelfth minute of acetate perfusion. Duplicate experiments performed with 60s pulses at either period of perfusion showed that the separate determinations of acetyl-CoA, citrate (acetate moiety) and citrate (oxaloacetate moiety) specific radioactivities agreed to within 5%.

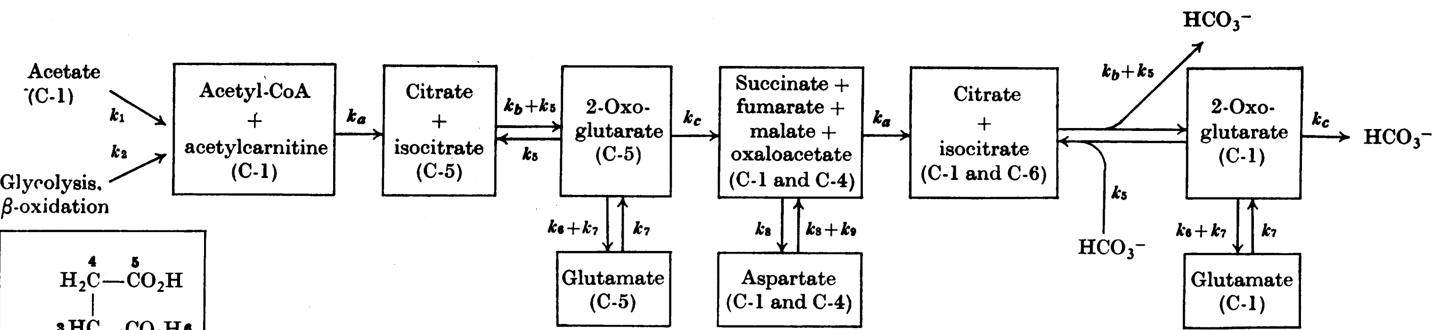
The following pattern of labelling of tricarboxylate-cycle intermediates is assumed to apply. ^{14}C from [1-^{14}C]acetate enters C-5 of citrate, isocitrate, 2-oxoglutarate and glutamate and becomes randomized in the carboxyl carbon atoms of succinate, fumarate, malate, oxaloacetate and aspartate; the carboxyl carbon atoms of oxaloacetate enter citrate and isocitrate at C-1 and C-6 respectively and 2-oxoglutarate and glutamate at C-1. With this pattern of labelling isocitrate dehydrogenase removes C-6 of isocitrate as carbon dioxide and 2-oxoglutarate dehydrogenase removes C-1 of 2-oxoglutarate.

In perfusion experiments with [^{14}C]bicarbonate added to medium containing glucose, insulin and acetate, radioactive medium was introduced into the heart after 11min of acetate perfusion. It was found that ^{14}C is incorporated into C-6 of isocitrate in the heart, presumably by the reverse reaction of isocitrate dehydrogenase. A constant specific radioactivity of [6-^{14}C]isocitrate was reached within 4min and was found to be 46($\pm$0.5)% (mean $\pm$ S.E.M., six hearts) of that of the bicarbonate in the medium.

Tricarboxylate cycle: interpretation of ^{14}C incorporations

A number of factors may determine the rate of incorporation of ^{14}C from [1-^{14}C]acetate into intermediates of the tricarboxylate cycle. Factors essential to any scheme are the pool sizes of cycle intermediates and the rate of cycle turnover. Additional factors, which may have to be taken into account, are isotope exchange between adjacent intermediates of the cycle and between cycle intermediates and related metabolites (e.g. 2-oxoglutarate and glutamate, and oxaloacetate and aspartate). Compartmentation of metabolites (e.g. between mitochondrion and cytoplasm) may be a further factor if the rate of exchange between compartments is low in relation to the rate of cycle turnover. The importance of these various factors has been tested by computer analysis of a number of different models. Scheme 1 shows the simplest model that is, with certain assumptions, consistent with the observed specific radioactivities.

The model is based on single pools of tricarboxylate-cycle intermediates and treats as single pools [acetyl-CoA + acetylcarnitine], [citrate + isocitrate] and [succinyl-CoA + succinate + fumarate + oxaloacetate + malate]. The model assumes therefore



Scheme 1. Arrangement of metabolite pools used in the simulation of radioactive labelling of the tricarboxylate cycle in rat heart perfused with [1-^{14}C]acetate. The numbers in parentheses refer to the carbon atoms labelled.

Definitions and values of rate constants. All rates are quoted as μmol/min per g dry wt. of heart.

Constant	Definition	Values after [^{12}C]acetate perfusion for 0 min	11 min
k_1	[1-^{14}C]Acetate utilization from the medium	11.1	12.3
k_2	[^{12}C]Acetyl units from glycolysis and β-oxidation	1.5	1.7
k_3	Increase in the acetyl-CoA and acetylcarnitine pool	8.0	0
k_4	Increase in the citrate and isocitrate pool	0.3	0
k_5	Exchange rate across isocitrate dehydrogenase	11.9	11.9
k_6	Increase in the glutamate pool	1.5	0
k_7	Exchange rate between 2-oxoglutarate and glutamate	180.0	180.0
k_8	Exchange rate between aspartate and malate	20.0	10.0
k_9	Portion of decrease in aspartate pool flowing into oxaloacetate	1.5	0
k_{10}	Portion of decrease in aspartate pool flowing into medium	1.0	0
k_a	$t<0.5$: $k_a=k_1+k_2-k_3$; $t>0.5$: $k_a=k_1+k_2$		
k_b	$=k_a-k_4$		
k_c	$=k_b-k_6$		

t is the simulated time (min).
The value of k_5 is calculated from the expression

$$k_5=k_b/\left(\frac{\text{sp. radioactivity of }HCO_3^-}{\text{sp. radioactivity of isocitrate}}-1\right)$$

where the specific radioactivity of isocitrate is that attained after perfusion with [^{14}C]bicarbonate, glucose, insulin and acetate.

Values of pool sizes

Pool	Values (μmol/g dry wt. of heart) after [^{12}C]acetate perfusion for: 0 min	11 min
(1) Acetyl-CoA+acetylcarnitine	0.4	*
(2) Citrate+isocitrate	0.89	3.0
(3) 2-Oxoglutarate	0.4	0.6
(4) Glutamate	17.5	23.0
(5) Succinate+fumarate+malate+oxaloacetate	1.5	2.0
(6) Aspartate	15.0	5.0

* Not applicable as the experimental acetyl-CoA specific radioactivities were used as in the input for the rest of the model.
Pool 1: [acetyl-CoA]/[acetylcarnitine] ratio 1:20.
Pool 2: [citrate]/[isocitrate] ratio is given in Fig. 3.
Pool 3: [succinate]:[fumarate]:[malate]:[oxaloacetate] proportions were calculated from relative radioactivities and published oxaloacetate concentrations (Williamson, 1965) to be of the order of 1.0:0.02:0.5:0.005.

that isotope equilibration is complete in these combined pools. The relative contributions of individual components to combined pools are shown at the foot of Scheme 1. The model includes an isotope-exchange reaction between [citrate+isocitrate] and [2-oxoglutarate+bicarbonate]. Initial calculations using a simpler model omitting this isotope exchange gave values for the relative specific radioactivities of (citrate+isocitrate)/2-oxoglutarate that were too high. Subsequent perfusion experiments with [^{14}C]bicarbonate showed that isotope exchange may occur at a rate of 11.9μmol/min per g dry wt. of heart (for details of calculation see foot of Scheme 1). The model also includes isotope-exchange reactions between glutamate and 2-oxoglutarate and between aspartate and the combined pool containing oxaloacetate. The rates of these exchanges were computed as 180μmol/min per g dry wt. of heart for 2-oxoglutarate and glutamate, and 10–20μmol/min per g dry wt. of heart for malate and aspartate (the specific radioactivity of oxaloacetate could not be measured).

Some uncertainties are introduced into analysis of the model by limitations in the perfusion technique that make it difficult to measure inputs and outputs over short time-periods. When [1-^{14}C]-acetate is introduced in the twelfth minute of acetate perfusion, metabolite concentrations and rates of metabolism are steady state, but the rate of isotope equilibration in pool(s) of acetate in the heart is not known. This uncertainty has led us to use the observed specific radioactivity of acetyl-CoA as the input of radioactivity over this time-period. The situation in the first minute of perfusion with acetate is more complex. The combined effects of dead space in the cannula and extracellular volume in the heart may impose a lag of up to 2s for one replacement of extracellular water. It has been computed that the effects of such a lag on metabolite specific radioactivities would be 10% at 10s and less than 1% by 60s. The utilization of acetate in the first minute of acetate perfusion was 11.1μmol/min per g dry wt. of heart as compared with a steady-state uptake of 12μmol/min per g dry wt. of heart. In analysing the model it has been assumed that acetate uptake of 11.1μ moles/min per g dry wt. of heart is achieved instantaneously at the beginning of the first minute.

Table 4. *Experimental and computed specific radioactivities of tricarboxylate cycle metabolites after perfusion of rat heart with* [1-^{14}C]*acetate*

The specific radioactivity of [1-^{14}C]acetate used for perfusions was 0.06μCi/μmol. The experimentally determined values of specific radioactivities were measured in pooled $HClO_4$ extracts of four hearts (eight in the case of acetyl-CoA and citrate at 60s). The computed values are those obtained from the model, indicated in Scheme 1, as described in the text.

Period of [1-^{14}C]-acetate pulse (s)	Sp. radioactivity (% of acetate sp. radioactivity)						
	Acetyl-CoA	Citrate (acetate)	2-Oxoglutarate	Glutamate	Malate	Aspartate	Citrate (oxaloacetate)
(A) Experimental results:							
(i) 0min of preceding [^{12}C]acetate perfusion							
10	97	20					
20	88	34					
60	88	64	35	30	17.5	7	8.0
(ii) 11min of preceding [^{12}C]acetate perfusion							
10	28	3					
20	55	18					
60	63	43	23	20	13.5	6	4.7
(B) Computed results:							
(i) 0min of preceding [^{12}C]acetate perfusion							
10	79	21					
20	85	28					
60	88	61	35	30	14	6	7
(ii) 11min of preceding [^{12}C]acetate perfusion							
10	28*	6					
20	55*	22					
60	63*	42	23	20	13.5	7	7

* These specific radioactivities are those obtained experimentally, which have been used as the input of radioactivity in analysis of the model.

Details of the computer methods used in calculations based on the model are given in the following paper (England, 1970).

Isotope incorporation into acetyl-CoA. In the first minute of acetate perfusion approx. 80% of acetyl-CoA was formed from acetate (calculated from μmol/min per g dry wt. of heart: for acetate utilization, 11.1; acetyl-CoA utilization in tricarboxylate cycle, 10.4; acetyl-CoA and acetylcarnitine accumulation, 3.6).

The observed values of acetyl-CoA specific radioactivity were 97% of that of the acetate at 10s and 88% at 20 and 60s (Table 4). This may represent good agreement, since estimates of acetyl-CoA formation over short periods of perfusion are not accurate (see the preceding section). A rapid increase in acetyl-CoA specific radioactivity is only to be expected since the [acetyl-CoA+acetylcarnitine] pool is initially very small and increases fourfold within 10s and sevenfold within 20s. This rate of labelling of acetyl-CoA suggests that there is little, if any, isotope exchange between acetyl-CoA and either citrate or pyruvate. It was not possible to obtain accurate estimates of the specific radioactivity of acetyl-CoA with perfusion times below 10s and thus the extent of any lag in the incorporation of label into acetyl-CoA could not be measured.

In the twelfth minute of acetate perfusion approx. 85% of acetyl-CoA was formed from acetate (calculated from steady-state rates in μmol/min per g dry wt. of heart: for acetate utilization 12; for tricarboxylate-cycle turnover, 14). In experiments in which [1-^{14}C]acetate was introduced after 11min of acetate perfusion the maximum specific radioactivity of acetyl-CoA should therefore be 85% of that of acetate. The incorporation of radioactivity into acetyl-CoA at this period of perfusion is presumably the result of turnover alone, since metabolite concentrations are constant.

If the pool of unlabelled acetate in the heart is ignored then it may be calculated that the specific radioactivity of acetyl-CoA should reach 40% of that of acetate at 10s, 62% at 20s and 84% at 60s. The observed values were appreciably lower (Table 4). Thus after 60s the specific radioactivity of acetyl-CoA was still increasing and was only 63% of that of acetate. This might be explained by some delay in isotope equilibration in pools of acetate in the heart. This delay cannot be explained by treating the acetate space of the heart (3ml/g dry wt. of heart) as a single extracellular pool turning over at the rate of perfusion flow (approx. 100ml/g dry wt. of heart). Current perfusion techniques do not permit accurate investigation of the kinetics of acetate space(s) in the heart. In testing the model during this period of perfusion, a smooth curve of acetyl-CoA specific radioactivities was generated from the observed values and used as the input of radioactivity into the cycle.

Isotope incorporation into other tricarboxylate-cycle intermediates. These results are shown in Table 4. At both time-periods the observed incorporations of radioactivity into citrate (acetate and oxaloacetate moieties), 2-oxoglutarate and malate were very close to those predicted by the model. This suggests that isotope equilibration between different pools of these metabolites (e.g. cytoplasmic and mitochondrial) is rapid in the heart and that there is very little isotope exchange between 2-oxoglutarate and malate.

Isotope incorporation into glutamate and aspartate. With either period of perfusion the specific radioactivity of glutamate was very close to that of 2-

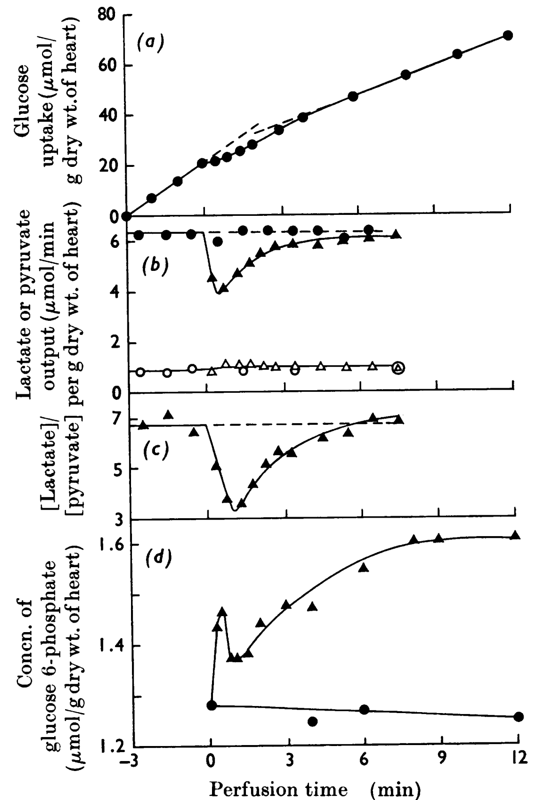


Fig. 5. Time-courses of (*a*) glucose uptake, (*b*) lactate output (●, control; ▲, acetate) and pyruvate output (○, control; △, acetate), (*c*) [lactate]/[pyruvate] ratio in the medium (control values after zero time are shown by a broken line) and (*d*) muscle glucose 6-phosphate concentration (●, control; ▲, acetate). Hearts were preperfused for 8min and switched to control or acetate perfusion at zero time. Each point is the mean of three to six observations. For composition of media see the Experimental section.

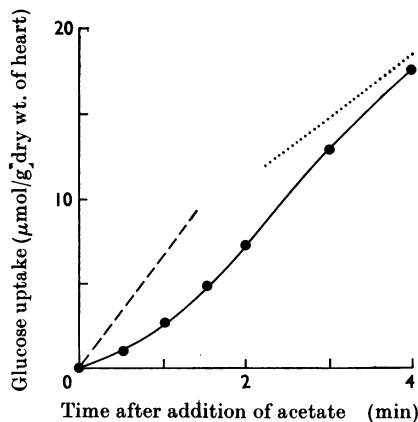


Fig. 6. Enlarged version of part of Fig. 5(*a*), showing transient changes in glucose uptake on addition of acetate. ——, Glucose uptake during period shown; ----, steady-state glucose uptake in control perfusions; ······, steady-state glucose uptake in acetate perfusions.

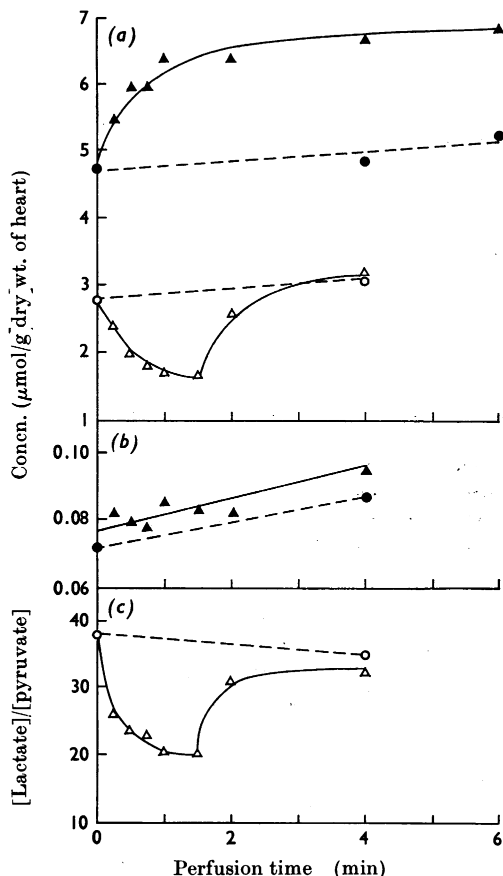


Fig. 7. Time-courses of (*a*) muscle alanine concentration (●, control; ▲, acetate) and muscle lactate concentration (○, control; △, acetate); (*b*) muscle pyruvate concentration (●, control; ▲, acetate); (*c*) muscle [lactate]/[pyruvate] ratio (○, control; △, acetate). Pooled values are shown for four hearts preperfused for 5min and perfused thereafter for time shown. For composition of media see the Experimental section.

oxoglutarate, indicating rapid isotopic equilibration (calculated rate was 180 μmol/min per g dry wt. of heart). This equilibrium is presumably catalysed by transaminases and glutamate dehydrogenase (EC 1.4.1.2). The specific radioactivity of aspartate was appreciably less than that of malate, suggesting poor isotope equilibration between oxaloacetate and aspartate (calculated rate was 20 μmol/min per g dry wt. of heart in the first minute and 10 μmol/min per g dry wt. of heart in the twelfth minute). This is consistent with the apparently low rate of aspartate transamination that was suggested by the rate of the change in the concentrations of malate, aspartate, glutamate and citrate.

Radioactivity could not be detected in glutamine or asparagine at either time-period, indicating that these compounds do not exchange carbon with glutamate or aspartate. This could be consistent with the relatively constant concentrations of glutamine and asparagine noted above.

Associated changes in glucose metabolism

These data are shown in Figs. 5, 6 and 7 and Table 3.

Steady-state changes. After the introduction of acetate into the heart new steady-state rates of glucose uptake and lactate output, and muscle lactate and alanine concentrations, and [lactate]/[pyruvate] ratios in muscle and medium were achieved within 6min. Acetate decreased the steady-state glucose uptake from 6.6 to 3.7 μmol/min per g dry wt. of heart. The steady-state outputs of lactate and pyruvate, the muscle concentrations of lactate and pyruvate and the [lactate]/[pyruvate] ratios in medium and muscle were not changed by acetate. The muscle concentration of glucose 6-phosphate increased from 1.25 to 1.62 μmol/g dry wt. of heart, and the concentration of alanine rose from 4.7 to 6.8 μmol/g dry wt. of heart.

The rate of net glycogen synthesis was calculated from the incorporation of radioactivity from [1-^{14}C]glucose into glycogen from the fourth to the sixth minute of control or acetate perfusions as follows:

$$\text{Net glycogen synthesis } (\mu\text{mol/min per g dry wt. of heart}) = \frac{P}{g_2} - G\left(1 - \frac{g_2}{g_1}\right)$$

where P is the radioactivity in d.p.m. incorporated/min per g dry wt. of heart into glycogen at 4–6min of perfusion, G is the glucose uptake (μmol/min per g dry wt. of heart), g_1 is the specific radioactivity of glucose in the medium (d.p.m./μmol) and g_2 is the specific radioactivity of muscle glucose 6-phosphate (d.p.m./μmol).

Steady-state specific radioactivity was achieved by 4min of perfusion. The rates of glycogen synthesis were 0.4μmol of glycogen glucose/min per g dry wt. of heart in controls and 1.3 in acetate perfusions. These correspond to a 13% increase/h in the control (which is unlikely to be significant and assumed to be zero in Table 3) and to a 47% increase/h with acetate. The calculated rate of glycolysis (glucose uptake – glycogen synthesis) was thus decreased by acetate from 6.6 to 2.4μmol of glucose/min per g dry wt. of heart. The calculated rate of glucose oxidation [glycolysis rate – (lactate + pyruvate) output] was decreased by acetate from 2.6μmol of glucose/min per g dry wt. of heart to zero.

These results are for the most part consistent with those of studies of the effects of acetate oxidation on steady-state rates of glucose metabolism in the perfused rat heart by Williamson (1964, 1965). The changes in hexose monophosphates are consistent with inhibition of phosphofructokinase by acetate utilization as postulated for a number of respiratory substrates including acetate (Newsholme *et al.* 1962; Williamson, 1965). Since acetate inhibited glucose oxidation markedly without altering the intracellular pyruvate concentration in the steady state, it may be concluded that acetate oxidation specifically inhibited the oxidation of pyruvate formed from glucose. This is most readily explained by inhibition of pyruvate dehydrogenase as postulated for a number of respiratory substrates including acetate (Williamson, 1964; Garland, Newsholme & Randle, 1964; Garland & Randle, 1964).

Transient changes. During the first 1–2min of acetate perfusion there was a marked decrease in lactate output and muscle lactate concentration. These then increased slowly during the next 4–5min to attain steady-state values after approx. 6min that were identical with the control values. The concentration of alanine in the heart increased over the first 1–2min and then remained constant. The output of pyruvate and the muscle pyruvate concentration did not change. The increase in alanine, if it is formed from pyruvate by transamination, could account for approx. 20% of the decline in lactate output. Most of this diminution in lactate output is presumably due to inhibition of glycolysis (see below). The [lactate]/[pyruvate] ratio shows a transient and marked fall in muscle and medium during the first 1–2min of acetate perfusion and returns to control values by 6min. This presumably reflects a fall in the cytoplasmic [NADH]/[NAD^+] ratio and may indicate relatively greater inhibition of glycolysis as compared with the oxidation of cytoplasmic NADH.

The rate of glucose uptake fell to approx. 30% of the control value in the first 30s of acetate perfusion; between 30s and 4min the rate increased to a maximum approx. 86% of the control, and then declined to a steady-state rate by 6–8min of approx. 56% of the control.

It seems probable that there will be roughly parallel changes in the rate of glycolysis, but it was not possible to obtain accurate estimates of the rate of glycogen synthesis in periods of perfusion below 4min. The concentration of glucose 6-phosphate showed a transient increase over the first 30s that was superimposed on a slower rise over 6–8min. These changes may indicate transient inhibition of phosphofructokinase during the first minute of acetate perfusion superimposed on a more gradually developing inhibition during 6–8min.

Adenine nucleotide concentrations and net yield of ATP

These data are shown in Fig. 8 and Table 3. Acetate perfusion increased the muscle concentration of AMP approximately twofold in the steady

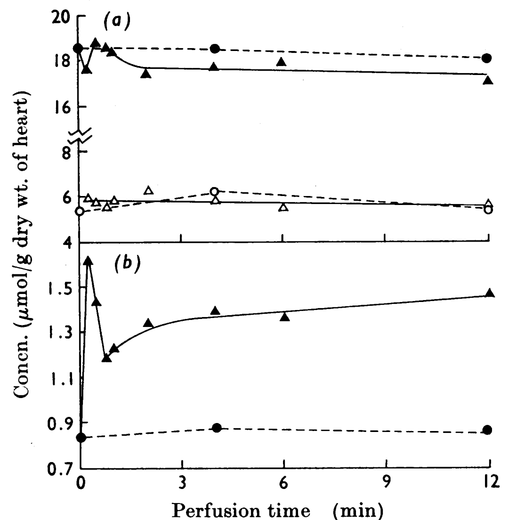


Fig. 8. Time-courses of (*a*) ATP (●, control; ▲, acetate) and ADP (○, control; △, acetate) and (*b*) AMP (●, control; ▲, acetate). Pooled values are shown for four to 12 hearts preperfused for 5min and perfused thereafter for the time shown. For composition of media see the Experimental section.

state, which was reached after approx. 3min. Superimposed on this gradual increase was a transient and rapid rise within 30s. There was no obvious change in the concentrations of ADP or ATP.

The net yield of ATP has been computed from oxygen consumption assuming maximum P/O ratios for acetate, glucose and palmitate oxidation given at the head of Table 3. In the steady state acetate had no effect on the net yield of ATP, although the oxygen consumption increased by 20%. This may be equated with the lower overall P/O ratio for acetate oxidation than for glucose and palmitate oxidation. In the first minute of acetate perfusion the oxygen consumption fell by 20% and the net yield of ATP was decreased by 30%. The oxygen consumption and net yield of ATP reached steady-state values by about the fifth minute.

GENERAL DISCUSSION

Control of tricarboxylate-cycle metabolite concentrations. The utilization of acetate by rat heart leads to maximum changes in the concentrations of acetyl-CoA and CoA within 30s. On the other hand the change in citrate concentration took 6min to reach a maximum and was relatively small in the first minute. This delay in citrate accumulation can now be explained by the relatively slow generation of oxaloacetate by aspartate transamination. There is a constant drain on oxaloacetate during the first 6min of acetate perfusion because of accumulation of the tricarboxylate-cycle metabolites citrate, isocitrate and 2-oxoglutarate and also glutamate. In the first minute this oxaloacetate is formed from malate and aspartate. In the subsequent 5min this oxaloacetate is formed exclusively from aspartate, which may also be used to regenerate the malate pool. It can be deduced from our results that the concentration of oxaloacetate fell in the first minute of acetate perfusion. There was a 50% decrease in malate concentration. The concentrations of alanine and glutamate increased, those of pyruvate and 2-oxoglutarate were unchanged whereas that of aspartate fell. This may be interpreted as a coupled transamination between aspartate and pyruvate catalysed by aspartate aminotransferase and alanine aminotransferase and initiated by a fall in oxaloacetate concentration. Alanine aminotransferase appears to catalyse an equilibrium reaction in perfused rat heart. The mass-action ratio [alanine]-[2-oxoglutarate]/[glutamate] [pyruvate] was approx. 1.5–2.0 in control and acetate perfusions compared with $K_{eq.} = 1.52$ (Krebs, 1953). The rise in 2-oxoglutarate concentration at the end of the first minute of acetate perfusion presumably stops the rise in alanine concentration. Since isotope equilibration between malate and aspartate was incomplete aspartate aminotransferase is presumably displaced from equilibrium. Since malate increases in concentration after the first minute of acetate perfusion some increase in oxaloacetate concentration may also occur. However, Williamson (1965) found a decrease in the steady-state whole-tissue oxaloacetate concentration during acetate perfusion. We have not included the results of oxaloacetate assays; because of the very low concentration and instability of oxaloacetate in heart extracts their accuracy is questionable.

These metabolic changes could be interpreted as showing that the rise in acetyl-CoA concentration is primarily responsible for the accumulation of citrate, isocitrate, 2-oxoglutarate and glutamate and a factor in the increased rate of citrate synthesis, but that the effects of this rise are modified by the availability of oxaloacetate. The total activity of citrate synthase in rat heart was found to be approx. 600 units/g dry wt. of heart at 37°C (P. J. Randle, P. J. England & R. M. Denton, unpublished work) compared with a maximum flow of 14μmol/min per g dry wt. of heart during acetate perfusion. Nevertheless it would appear that this reaction is displaced from equilibrium in the perfused heart, since we were unable to obtain any evidence for isotope exchange between acetyl-CoA and citrate. Either citrate synthase is inhibited in the heart or it is subject to rate control by the concentration of its substrates, or both. If acetyl-CoA is confined to mitochondrial water then it can be calculated that acetate increased the concentration of acetyl-CoA from 40 to 400μM. Pig heart citrate synthase has a K_m for acetyl-CoA of 11μM with competitive inhibition by ATP (K_i 0.5mM) (Kosicki & Srere, 1961; Kosicki & Lee, 1966). The apparent K_m for acetyl-CoA at 10mM-ATP would thus be 231μM. If rat heart citrate synthase has similar properties then the rise in acetyl-CoA with acetate perfusion could be a factor in the increased rate of citrate synthesis and account for the accumulation of citrate and other cycle metabolites. The increased flow from minutes 1–6 of acetate perfusion may also depend on some improvement in oxaloacetate concentration.

These findings also provide some confirmation for the suggestion (Bowman, 1966) that aspartate transamination furnishes the oxaloacetate for steady-state citrate accumulation during acetate oxidation. However, the changes that we have detected in transient and steady-state conditions raise other questions about the control of the tricarboxylate cycle and its metabolites that call for further comment. There is, for example, no reason *a priori* why the additional oxaloacetate required for citrate accumulation should not be formed from the substantial glutamate pool in the heart. Our

isotope results showed very rapid carbon flow between glutamate and 2-oxoglutarate. However, measurements of metabolite concentrations showed that glutamate is not the source of oxaloacetate. The utilization of aspartate (and to a smaller extent of malate) as sources of this additional oxaloacetate must therefore be determined by control of an enzymic step in the cycle span 2-oxoglutarate $\rightarrow$ malate. The most likely control point is 2-oxoglutarate dehydrogenase. The enzyme from pig heart has been found to be subject to regulation by substrates and products in an analogous manner to pyruvate dehydrogenase. The enzyme is inhibited by increased [succinyl-CoA]/[CoA] and [NADH]/[NAD^+] ratios (Garland, 1964). During the first minute of acetate perfusion pyruvate oxidation is inhibited and, although 2-oxoglutarate oxidation is not inhibited, its rate fails to keep pace with that of preceding cycle reactions. If pyruvate dehydrogenase and 2-oxoglutarate dehydrogenase share common pools of CoA, NADH and NAD^+ then our findings might suggest that the concentration of succinyl-CoA falls together with that of CoA.

In its operation at the onset of acetate utilization the tricarboxylate cycle may therefore show phased control at the citrate synthase and 2-oxoglutarate dehydrogenase steps. The sequence of events may be as follows. An increase in the concentration of acetyl-CoA increases the rate of citrate synthesis and the flow in the cycle span citrate $\rightarrow$ 2-oxoglutarate. At the same time oxaloacetate concentration falls, leading to transamination between aspartate and 2-oxoglutarate and between glutamate and pyruvate. This delays a rise in 2-oxoglutarate concentration, the CoA concentration is low, and as a consequence the rate of flow in the cycle span 2-oxoglutarate $\rightarrow$ malate does not keep pace with the rate of flow in the cycle span acetyl-CoA $\rightarrow$ 2-oxoglutarate in the first 6min. It may be predicted that the reverse will occur when acetate perfusion is stopped. This scheme includes some control of the 2-oxoglutarate dehydrogenase step by the 2-oxoglutarate concentration during acetate utilization. The possible role of the respiratory chain and of respiratory-chain phosphorylation in the control of cycle turnover (as opposed to rate adjustments within the cycle) is discussed below.

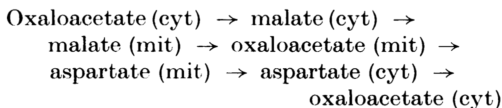
In the transient and steady-state period the rate of flow through the cycle steps catalysed by aconitate hydratase and isocitrate dehydrogenase is also increased by acetate utilization. The total activity of aconitate hydratase in rat heart (100 units/g dry wt. of heart at 37°C) is sufficient to maintain near-equilibrium between citrate and isocitrate at the concentrations of these metabolites in the tissue. Although the [citrate]/[isocitrate] ratio increases during acetate utilization, we have suggested elsewhere (England, Denton & Randle, 1967) that this may result from an alteration of the equilibrium constant of the aconitate hydratase reaction by formation of magnesium citrate (or the citrate salt of another bivalent metal). There was no convincing evidence for control of isocitrate oxidation by factors other than substrate concentrations. Measurement of the rate of isotope exchange between [^{14}C]bicarbonate and isocitrate indicated that the rate of the back reaction was 46% of the rate of isocitrate oxidation. The [isocitrate]/[2-oxoglutarate] ratio in the tissue showed little change from the control at any time during acetate perfusion.

Oxygen consumption and net yield of ATP. Acetate utilization in the steady state increased oxygen consumption without affecting the net yield of ATP. This may apparently be equated with the lower P/O ratio of acetate oxidation. On the other hand oxygen consumption fell during the first minute of acetate perfusion and the net yield of ATP decreased by 30% without any detectable change in ATP or ADP concentrations. This may be explained either by a decrease in ATP utilization (due for example to a transient diminution in cardiac contraction) or by utilization of creatine phosphate. The latter was not measured, but published values (Williamson, 1965) suggest that the muscle concentration may be insufficient to account for the deficit. There was no obvious diminution in cardiac contraction by visual inspection.

Tricarboxylate-cycle turnover. Acetate oxidation increased the rate of tricarboxylate-cycle turnover by 66% in the steady state, which was achieved after 5–6min of acetate perfusion. The increase in cycle turnover begins after approx. 2min of acetate perfusion. It can be calculated that in the steady state 45% of the increase in cycle turnover may be equated with the rise in oxygen consumption and 55% with the elimination of extra-cycle oxidations by the substitution of acetate for glucose and palmitate as respiratory fuel. The increased cycle turnover in the steady state may therefore be maintained by mechanisms that control oxygen consumption and the mitochondrial oxidation of pyruvate and cytoplasmic NADH. In the transient period the control of cycle turnover may be more complex and involve not only mechanisms that adjust the rates of the citrate synthase, pyruvate dehydrogenase and 2-oxoglutarate dehydrogenase reactions, but also other factors controlling oxygen consumption (see above). The rise in oxygen consumption with acetate in the steady state might be explained by the change in adenine nucleotide phosphate potential if the readily detected change in AMP concentration is an indicator of a smaller (and less readily detected) change in that of ADP (Chance, 1959). The marked and rapid 15-fold

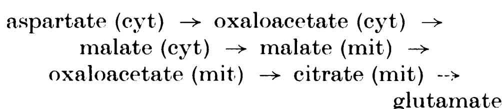
change in the [acetyl-CoA]/[CoA] ratio with acetate could account for the inhibition of pyruvate oxidation (Garland & Randle, 1964; Randle *et al.* 1966).

Mitochondrial oxidation of glycolytic NADH. In the steady state the rate of mitochondrial oxidation of NADH generated by glycolysis was 6.5 μmol of NADH/min per g dry wt. of heart in control perfusions and 0.5 in acetate perfusions (Table 3). The fall with acetate in the steady state appeared to be due mainly to inhibition of pyruvate oxidation, which is accomplished without any change in the steady-state cytoplasmic [NADH]/[NAD^+] ratio (as shown by the [lactate]/[pyruvate] ratio). In the transient state of acetate utilization more complex changes were seen. In the first minute of acetate perfusion the rate of mitochondrial oxidation of cytoplasmic NADH that accompanied pyruvate oxidation fell from 6 to 0.3 μmol of NADH/min per g dry wt. of heart. The overall rate of oxidation with acetate at this time-period is probably in excess of 2.3 μmol of NADH/min per g dry wt. of heart, since there was pyruvate output of 1 μmol/min per g dry wt. of heart, alanine formation of approx. 1 μmol/min per g dry wt. of heart and the muscle [lactate]/[pyruvate] ratio fell substantially. After the first minute alanine synthesis slowed rapidly and the [lactate]/[pyruvate] ratio increased to reach the control value by 4 min. Rat liver mitochondria may oxidize extramitochondrial NADH by the cycle:



Oxaloacetate (cyt) $\rightarrow$ malate (cyt) $\rightarrow$ malate (mit) $\rightarrow$ oxaloacetate (mit) $\rightarrow$ aspartate (mit) $\rightarrow$ aspartate (cyt) $\rightarrow$ oxaloacetate (cyt)

(Chappell, Henderson, McGivan & Robinson, 1968). If this pathway operates in heart mitochondria then the transient fall in the cytoplasmic [NADH]/[NAD^+] ratio might be driven by citrate synthesis through the pathway:



aspartate (cyt) $\rightarrow$ oxaloacetate (cyt) $\rightarrow$ malate (cyt) $\rightarrow$ malate (mit) $\rightarrow$ oxaloacetate (mit) $\rightarrow$ citrate (mit) $\dashrightarrow$ glutamate

Control of phosphofructokinase by citrate. The observed changes in glucose uptake and in muscle glucose 6-phosphate and citrate concentrations from 2 to 6 min of acetate perfusion are consistent with the idea that accumulation of citrate during acetate oxidation may retard glycolysis by inhibiting phosphofructokinase. There is, however, no simple explanation for the transient inhibition of glycolysis and phosphofructokinase during the first minute of acetate perfusion, since there is little, if any, increase in the whole tissue concentration of citrate. If citrate is responsible for this transient inhibition then it must be assumed that there is a concentration gradient of citrate from mitochondrion to cytoplasm (lowest) in control perfusions and that acetate utilization causes a transient efflux of citrate from mitochondrion to cytoplasm.

Acetate utilization increased the concentration of AMP, especially during the first minute, and AMP may be expected to activate phosphofructokinase in rat heart (Pogson & Randle, 1966). Since acetyl-CoA synthetase may be intramitochondrial (Campagnari & Webster, 1963) it is possible that the change in AMP concentration is primarily intramitochondrial and therefore without influence on phosphofructokinase, or for that matter on glycogen phosphorylase *b*. The mass-action ratio for adenylate kinase [ATP][AMP]/$[ADP]^2$ increased in the first minute of acetate perfusion from 0.52 at zero time to 0.94 at 30 s and 0.62 after 1 min. The equilibrium constant of adenylate kinase increases with increasing concentrations of free Mg^{2+} and is 0.52 at a free Mg^{2+} concentration of 0.03 mM (Rose, 1968). Since adenylate kinase may be predominantly extramitochondrial (Pette, 1966), this apparent displacement from equilibrium of an extremely active enzyme may result from mitochondrial sequestration of AMP.

Compartmentation of metabolites. One major difficulty in studies of the tricarboxylate cycle in the perfused heart is lack of knowledge of the distribution of its metabolites between mitochondrial and cytoplasmic compartments. Some of these difficulties have been emphasized in the present discussion. The model used for interpreting isotope data assumes single pools of tricarboxylate-cycle metabolites. The goodness of fit may indicate therefore that mitochondrial anion-transporting systems are sufficiently active to maintain very rapid isotope equilibration between the different compartments. This does not, however, imply concentration equilibration, since one or more of the mitochondrial anion-transporting systems may actively maintain concentration gradients (Chappell *et al.* 1968).

One of the reasons for undertaking the isotope studies was the possibility that the delayed rise in citrate might be due to slow diffusion of citrate formed within the mitochondrion to the cytoplasm. Thus the citrate that accumulates during acetate perfusion might be confined initially to the mitochondrion but equilibrate between mitochondria and cytoplasm in the steady state. Since the volume of mitochondrial water may only be one-quarter of the cytoplasmic volume, such a slow diffusion might lead to a higher relative specific radioactivity of citrate in the first minute of acetate perfusion than in the twelfth. No such difference was detected. When the present studies were begun it had not been possible to detect the citrate-transporting

system in rat heart mitochondria, but this has since been achieved with an isotope method (England & Robinson, 1969).

General conclusions. The scheme that we have outlined for regulation of the tricarboxylate cycle during acetate oxidation in rat heart involves mechanisms that control the rate of cycle turnover, especially in the steady state, and control mechanisms that determine the concentration of cycle metabolites by adjusting relative rates within the cycle especially during the transient period. It is suggested that the cycle functions as two separate spans: acetyl-CoA → oxoglutarate, which is controlled by citrate synthase; 2-oxoglutarate → oxaloacetate, which is controlled by 2-oxoglutarate dehydrogenase (the possibility of further control points in this span has not been excluded). In the transient period a rise in acetyl-CoA concentration may increase flow in the span acetyl-CoA → 2-oxoglutarate; the accompanying fall in CoA concentration may restrain flow in the span 2-oxoglutarate → oxaloacetate. These rate changes lead to accumulations of citrate, isocitrate, 2-oxoglutarate and glutamate and determine the utilization of aspartate for oxaloacetate formation. In the steady state metabolite concentrations attain stability because the rates of the two cycle spans become equal. This may be brought about by the rise in 2-oxoglutarate concentration. The rise in AMP concentration generated by acetyl-CoA synthetase may stimulate respiration through the formation of ADP. This, together with inhibition of pyruvate oxidation, may accelerate dehydrogenase reactions in the cycle and, in conjunction with the raised concentrations of cycle intermediates, increase the rate of cycle turnover.

Acetate perfusion may thus be regarded as a simple method of acutely changing the rate of tricarboxylate-cycle turnover in rat heart, permitting a study of mechanisms of cycle control. This particular experimental procedure is unlikely to be of major physiological significance in the rat, although it may find its counterpart *in vivo* in conditions where fatty acids replace glucose as the major respiratory fuel.

It will, however, provide a basis for a study of a major physiological stimulus for tricarboxylate cycle turnover, namely increased cardiac work.

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