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The Effects of Increased Heart Work on the Tricarboxylate Cycle and its Interactions with Glycolysis in the Perfused Rat Heart

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1. The work of the perfused rat heart was acutely increased by raising the aortic pressure in the Langendorff preparation from 50 to 120mmHg; within 1min in perfusions with media containing glucose or glucose+acetate, rates of oxygen consumption and tricarboxylate-cycle turnover increased 2.5-fold, glycolysis rate doubled and oxidation of triglyceride fatty acid was strikingly enhanced. 2. Increased cardiac work had no significant effects on the heart concentrations of creatine phosphate, ATP, ADP or 5'-AMP. The only significant changes in tricarboxylate-cycle intermediates were a decrease in malate in perfusions with glucose and decreases in acetyl-CoA and citrate and an increase in aspartate in perfusions with glucose+acetate. 3. Measurements of intracellular concentrations of hexose phosphates, glucose and glycogen indicated that work accelerated glycolysis by activation of phosphofructokinase and subsequently hexokinase; the activation could not be accounted for by changes in the known effectors of phosphofructokinase. 4. Acetate at either perfusion pressure increased heart concentrations of acetyl-CoA, citrate, glutamate and malate and decreased that of aspartate; acetate increased tricarboxylate-cycle turnover by 50–60% and inhibited glycolysis and pyruvate oxidation. 5. In view of the markedly different effects of acetate and of cardiac work on the concentrations of cycle intermediates the changes that accompany acetate utilization may be specifically concerned with the regulatory functions of the cycle in control of glycolysis and pyruvate oxidation and not with the associated increase in cycle turnover. It is suggested that the concentrations of key metabolites controlling the rate of cycle turnover may fluctuate with each heart beat and that this may explain why no significant changes (for example, in adenine nucleotide concentrations) have been detected with increased work in the present study.

The onset of acetate utilization in rat hearts perfused with medium containing glucose and insulin increases the rate of tricarboxylate-cycle turnover, impairs glycolysis and suppresses the oxidation of pyruvate formed by glycolysis (Randle *et al.*, 1970). The increased rate of cycle turnover with acetate can be accounted for by the lower P/O ratio of acetate oxidation (as compared with glucose oxidation) and by the elimination of extra-cycle oxidations, which account for one-third of the oxygen consumption during the oxidation of glucose. Inhibition of glycolysis was attributed to inhibition of phosphofructokinase by citrate, which accumulates during acetate utilization in rat heart. Evidence was presented that this accumulation of citrate is brought about by the metabolism of acetate through acetyl-CoA synthase, which elevates the tissue concentration of acetyl-CoA and lowers that of CoA. This appears to result in an increase in flow in the cycle span acetyl-CoA → 2-oxoglutarate (and glutamate), which is transiently greater than that in the span 2-oxoglutarate → oxaloacetate (and aspartate), and, as a consequence, the concentrations of citrate, isocitrate, 2-oxoglutarate and glutamate are markedly increased. The oxaloacetate for these accumulations is formed by transaminations between 2-oxoglutarate and aspartate, and glutamate and pyruvate. It was suggested that the citrate synthase reaction is rate-limiting in the span acetyl-CoA → 2-oxoglutarate and that 2-oxoglutarate dehydrogenase may be the controlling enzyme in the span 2-oxoglutarate → oxaloacetate. The suppressed oxidation of pyruvate in the presence of acetate has been attributed to inhibition of pyruvate dehydrogenase by an increased [acetyl-CoA]/[CoA] ratio (Garland & Randle, 1964) and/or to inactivation of pyruvate dehydrogenase by phosphorylation through an unknown control mechanism (Wieland *et al.*, 1971).

The control mechanisms that increased cycle turnover during perfusions with acetate were not fully

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defined. Evidence was obtained that the utilization of acetate lowers the adenine nucleotide phosphate potential in rat heart. This has suggested as possibilities stimulation of respiratory-chain and substrate-level phosphorylations and acceleration of those cycle enzymes that may be sensitive to this potential: citrate synthase (Kosicki & Srere, 1961; Kosicki & Lee, 1966); NAD^+–isocitrate dehydrogenase (Chen & Plaut, 1963; Goebell & Klingenberg, 1964). A further possibility was that the increased concentration of the cycle substrates, acetyl-CoA, citrate, isocitrate, 2-oxoglutarate and malate, might in some way accelerate cycle turnover. In this connexion it was of particular interest to know whether the alterations in the concentrations of these cycle substrates during acetate utilization are concerned with the control of cycle turnover or with those regulatory functions of the cycle that control the rates of glycolysis or pyruvate oxidation or both. It therefore seemed important to repeat these measurements by using other means of acutely accelerating tricarboxylate-cycle turnover. A study of the effects of increased cardiac work seemed appropriate.

Neely *et al.* (1967) have shown that cardiac work in the perfused rat heart may be changed by altering the aortic pressure either in the Langendorff preparation (aortic perfusion alone) or in a newer preparation in which perfusate is introduced into the left atrium and pumped by the left ventricle against a variable pressure head; because of its simplicity the Langendorff preparation was chosen for the present study. A rise in aortic pressure from 50 to 120mmHg increased oxygen consumption approx. 2.5-fold in perfusions with medium containing glucose (Neely *et al.*, 1967) and it is to be expected that this pressure change would increase the rate of tricarboxylate-cycle turnover by a similar amount. These pressures were utilized in the present study. Earlier studies of the effects of increasing cardiac work on metabolism in the rat heart showed that, in perfusions without substrate, glycogen breakdown and glycolysis were accelerated; with glucose as substrate but in the absence of insulin, glucose transport and glycolysis were accelerated (Neely *et al.*, 1969, 1970). Palmitate, which like acetate suppresses glucose uptake, glycolysis and glucose oxidation at low aortic pressure (Randle *et al.*, 1964), had similar effects at high pressure (Neely *et al.*, 1970). In the present study we have investigated the effects of a rapid increase in cardiac work induced by the sudden increase in perfusion pressure on the rates of tricarboxylate-cycle turnover and glycolysis and on the concentrations of metabolic intermediates during perfusion with media containing glucose+insulin or glucose+acetate+insulin. Insulin was included in perfusion media to maximize rates of glucose transport and thus to simplify interpretation of altered rates of glycolysis. Measurements were made with the two different perfusion media because it was expected that perfusions with media containing glucose or glucose+acetate would enable the effects of cardiac work on cycle turnover to be compared at very different concentrations of cycle intermediates.

Experimental

Materials

Enzymes, coenzymes, adenine nucleotides, 2-oxoglutarate and triethanolamine hydrochloride were from Boehringer (London) Corp., London W.5, U.K. Other chemicals (purest grade available) were from British Drug Houses Ltd., Poole, Dorset, U.K., or from Mallinckrodt Chemical Works, St. Louis, Mo., U.S.A. Insulin was a gift of Boots Pure Drug Co. Ltd., Nottingham, U.K. or of Eli Lilly and Co., Indianapolis, Ind., U.S.A. A stock solution of 20i.u./ml in 3mM-HCl was added to perfusion media. Heparin was from Evans Medical Ltd., Speke, Liverpool, U.K. or from Sigma Chemical Co., St. Louis, Mo., U.S.A. Ion exchange resins, Dowex 1 (AG 1; X4; formate form; 200–400 mesh) and Dowex 50 (AG 50W; X2; H^+ form; 200–400 mesh) were from Bio-Rad Laboratories, Richmond, Calif., U.S.A. Radioactive chemicals, [1-^{14}C]acetate, [^{3}H]-water, [5-^{3}H]glucose and [1-^{3}H]sorbitol, were from The Radiochemical Centre, Amersham, Bucks., U.K., or from New England Nuclear Corp., Boston, Mass., U.S.A.

Perfusion

Hearts were from male albino Wistar rats with free access to diet 41B (Oxoid Ltd., London E.C.4, U.K.) or Sprague–Dawley rats with free access to standard Purina Lab Chow. The rats were injected intraperitoneally with 5mg of heparin 15–30min before excision of the heart and anaesthetized with Nembutal injected intraperitoneally 5min before excision. The perfusion medium was bicarbonate-buffered salt solution (Krebs & Henseleit, 1932), modified by decreasing [$CaCl_2$] to one-half and by adding 0.6mM-EDTA (calcium salt). This medium was gassed with O_2+CO_2 (95:5) and contained glucose (5.5mM) and insulin (0.01i.u./ml) with or without sodium acetate (5mM). Other additions are given in the text, figures or tables.

Hearts were perfused by drip-through by a cannula inserted in the aorta. A previous study (Randle *et al.*, 1970) showed that steady-state rates of metabolism and metabolite concentrations with perfusion media containing glucose and insulin or glucose, acetate and insulin were achieved within 8min of commencing perfusion. In studying the effects of increased cardiac work [which was achieved by raising the perfusion pressure from 50mmHg (6.7kN/m^2) to 120mmHg (16.0kN/m^2)] hearts were pre-perfused

for 8min at 50mmHg. The perfusion pressure was then increased abruptly (within 2s) to 120mmHg. Perfusion pressures were achieved by separate reservoirs for high and low pressure and monitored with a mercury manometer. All reservoirs, connecting tubes and bubble traps were jacketed and maintained at 37°C. For measurements of glycolysis rate and outputs of lactate and pyruvate, effluent medium was allowed to flow from the heart into graduated cylinders cooled to 0°C. For measurement of oxygen consumption, medium was collected under heptane at 0°C into tared tubes from a cannula inserted into the pulmonary artery and the volume ascertained by weighing. The small amount of effluent medium that escaped by other routes was also collected and weighed.

Heart extracts

For metabolite assays hearts were rapidly frozen on the cannula with a tissue clamp at the temperature of liquid N_2, trimmed of fat, and powdered in a percussion mortar at −70° to −150°C. A weighed amount of powder was extracted with 5% (w/v) $HClO_4$ (approx. 2.5ml/g of powder) and centrifuged at 0°C. A measured volume of supernatant was neutralized with saturated $KHCO_3$ at 0°C, and $KClO_4$ removed by centrifugation at the same temperature. For assay of adenine nucleotides and creatine phosphate, extracts were made at −10° to −15°C in 5% (w/v) $HClO_4$ in 15% (w/v) acetone and assayed within 4h. Glycerides were extracted from ventricular muscle as described previously (Denton & Randle, 1967).

Determinations

Glucose, lactate and pyruvate in the medium. Glucose was assayed spectrophotometrically by the method of Slein (1963). Lactate and pyruvate were assayed (after deproteinization with $HClO_4$) spectrophotometrically or fluorimetrically by the methods of Hohorst (1963*b*) and Bücher *et al.* (1963).

Metabolites from heart. These were assayed spectrophotometrically or fluorimetrically by the following methods: glycogen (Randle *et al.*, 1970); glucose 6-phosphate (Hohorst, 1963*a*); citrate (Moellering & Gruber, 1966); 2-oxoglutarate (Bergmeyer & Bernt, 1963); malate (Holzer & Soling, 1963); acetyl-CoA (Chase, 1967); ATP (Lamprecht & Trautschold, 1963); ADP and AMP (Adam, 1963); aspartate (Pfleiderer, 1963); glutamate (Bernt & Bergmeyer, 1963); creatine phosphate (Lamprecht & Stein, 1963). For assay of ADP and AMP, NADH was freed of ADP and AMP by incubation for 2–4h at room temperature with alkaline phosphatase coupled to a water-insoluble polymer, which was completely removed by centrifugation. The latter was prepared as described by Zingaro & Uziel (1970). Assays of creatine phosphate showed variable drift for which correction was applied by extrapolation to zero time.

Oxygen. Oxygen concentrations were measured in the perfusion medium 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.

Total glyceride content. Ventricular glyceride content was assayed as glycerol after saponification as described by Denton & Randle (1967).

Radioactivity. This was assayed in a Nuclear-Chicago mark 1 liquid-scintillation spectrometer or a Beckman model LS 250 liquid-scintillation spectrometer with either methoxyethanol–toluene (2:3, v/v) containing butyl-PBD [5-(4-biphenylyl)-2-(4-t-butylphenyl)-1-oxa-3,4-diazole] and naphthalene or dioxan-based scintillator (Butler, 1961). Quenching corrections were determined by the channels-ratio method by an external standard.

Acetate oxidation. Rates of acetate oxidation were measured by using [1-^{14}C]acetate as described by Randle *et al.* (1970).

Tissue spaces. Total water space, sorbitol space and glucose space were measured by methods described by Morgan *et al.* (1961) and by Randle *et al.* (1970).

Measurement of glycolysis rate

Principle. To measure glycolysis during drip-through perfusion, a new method was developed with [5-^{3}H]glucose. Metabolism of [5-^{3}H]glucose through glycolysis leads to loss of ^{3}H into water at the triose phosphate isomerase reaction; if this reaction is incomplete any remaining ^{3}H is lost into water at the enolase reaction. Exchange between labelled glucose in glucose 6-phosphate and unlabelled glucose in glycogen occurs (Randle *et al.*, 1970), leading to incomplete equilibration of label between medium glucose and intracellular glucose 6-phosphate. Accordingly it was necessary to measure the specific radioactivity of glucose 6-phosphate to convert rates of production of ^3H_2O into absolute rates of glycolysis of glucose. Separation of ^3H_2O in perfusion medium was achieved by distillation under reduced pressure. Under these conditions of near-infinite dilution the concentration of ^3H_2O in the distillate is only 90% of that in the distilland because the relative vapour pressures are inversely related to relative molecular weights (18:20). This method would not detect glycolysis of unlabelled glucose residues in glycogen. However, measurements of tissue glycogen concentrations showed no net change over the period of perfusion in these studies.

Procedure. Hearts were pre-perfused for 8min at 50mmHg with medium containing insulin, [5-^{3}H]-glucose (5.5mM, 0.03μCi/μmol) ± acetate (5mM). Perfusion was then continued either at the same pressure

or at 120mmHg. Medium was collected at the times given in the text or the figures, its volume recorded and a sample taken and stored in stoppered tubes at 0°C. For measurement of ^3H_2O, water was distilled *in vacuo* from 8ml of medium spread in a 250ml flask with Kleenex tissue and warmed to 60°C; collections (of 2–3ml) were made in 15ml tubes cooled in liquid N_2. Samples of distillate were counted for radioactivity by liquid-scintillation spectrometry.

The combined specific radioactivities of glucose 6-phosphate and fructose 6-phosphate were measured in $HClO_4$ extracts of heart as follows. The extract (1ml) was diluted with 4ml of 0.2M-triethanolamine chloride, pH7.5, containing 5mM-$MgSO_4$. Triose phosphates and fructose 1,6-diphosphate were freed of ^3H through isotope exchange with water by incubation for 1h at room temperature with 1 unit of aldolase and 50 units of triose phosphate isomerase, followed by freeze-drying, which was repeated once after addition of 10ml of water. Glucose 6-phosphate and fructose 6-phosphate were then converted into glycerol phosphate and ^3H_2O was liberated by addition of water (4ml) and ATP (8μmol), and phosphofructokinase (13.5 units), aldolase (4 units), triose phosphate isomerase (50 units), glycerol phosphate dehydrogenase (8 units) and glucose phosphate isomerase (20 units). The extent of conversion was followed by the change in extinction at 340nm caused by oxidation of NADH; ^3H_2O formed was then separated by distillation and assayed for radioactivity. The rate of glycolysis (g) in μmol of glucose/min per g dry wt. of heart was calculated from the measured rate of production of ^3H_2O in d.p.m./min per g dry wt. of heart (A), and the specific radioactivity of glucose 6-phosphate and fructose 6-phosphate in d.p.m./μmol (F), by the formula $g = (20/18)\cdot(A/F)$.

Expression of results

Flow rates in metabolic pathways are given as μmol/min per g dry wt. of heart, metabolite concentrations as μmol/g dry wt. of heart, and tissue spaces as ml/g dry wt. of heart. Since it has been found that hearts from albino Wistar rats (Bristol) and from Sprague–Dawley rats (Hershey) give indistinguishable rates of metabolism and metabolite concentrations under identical conditions of perfusion, results with the two strains have not been shown separately. In detail, rates of glucose uptake, glycolysis and lactate and pyruvate output and heart concentrations of glucose sorbitol, water and glycogen were measured with hearts from Sprague–Dawley rats. Rates of oxygen consumption and heart concentrations of acetyl-CoA, glyceride glycerol, creatine phosphate and adenine nucleotides were measured with hearts from Wistar rats. Other metabolite concentrations are derived by pooling results in control and experimental series for each of the two strains.

Results and Discussion

Measurement of glycolysis rate with [5-^3H]glucose

The rate of equilibration of intracellular and extracellular ^3H_2O was measured by perfusing hearts at 120mmHg for 1 or 2min with medium containing ^3H_2O after a period of pre-perfusion (without ^3H_2O) for 3min at 50mmHg. The ^3H_2O space and total water space (measured by drying) were then compared. The ^3H_2O space in the heart was 102.5% of the total water space after 1min and also after 2min. The rate of equilibration of ^3H_2O thus appears to be sufficiently rapid to enable minute-by-minute measurements of ^3H_2O production by glycolysis of [5-^3H]-

Table 1. *Specific radioactivities of glucose in perfusion medium and heart glucose 6-phosphate and fructose 6-phosphate*

Hearts were pre-perfused for 8min at 50mmHg pressure with medium containing [5-^3H]glucose (5.5mM)± acetate (5.0mM) and then perfused as shown. Hearts were frozen, powdered and extracted and specific radioactivities determined (see the Experimental section). There were five observations in each group.

Perfusion			Specific radioactivity (d.p.m./μmol)	
Pressure (mmHg)	Time (min)	Substrates	Medium (glucose)	Heart muscle (glucose 6-phosphate+fructose 6-phosphate) (mean±S.E.M.)
50	0	Glucose	70154	53457±2174
50	12			52335±1894
120	12			51984±1964
50	0	Glucose+acetate	78434	66982±3059
50	12			73728±1647
120	12			69728±3529

Table 2. *Effect of work on steady-state rates of metabolism in perfused rat heart*

For details of perfusion see the Experimental section and the legend to Fig. 1. Theoretical oxygen equivalents (mol/mol): acetate, 2 (in the tricarboxylate cycle); glucose, 6 (4 in the cycle+1 at pyruvate dehydrogenase+1 in oxidation of glycolytic NADH). Palmitate oxidation is assumed to account for that part of the oxygen consumption not utilized in the oxidation of glucose or in the oxidation of acetate. 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 for lactate output was assumed to be 2 μmol of ATP/μmol of glucose; for pyruvate output the value was assumed to be 8 μmol of ATP/μmol of glucose (2 for glycolysis and 6 for oxidation of cytoplasmic NADH). Observed rates are shown as means ± S.E.M. for at least five observations. The rates of acetate oxidation at 50 mmHg were taken from Randle *et al.* (1970). Rates of glycogen synthesis were calculated from tissue glycogen concentrations at the beginning and end of perfusion.

Perfusion medium ...	Glucose		Glucose+acetate	
Perfusion pressure (mmHg)	50	120	50	120
Rate (μmol of glucose equivalent/min per g dry wt. of heart)				
Glycolysis	6.5±0.30	11.2±0.91	2.0±0.41	4.6±0.40
Glycogen synthesis	1.7±1.15*	0.3±1.08*	0.2±0.82*	0.3±0.70*
Output of lactate	2.3±0.24	3.5±0.35	1.8±0.21	2.6±0.26
Output of pyruvate	0.5±0.06	1.1±0.11	0.8±0.09	1.3±0.13
Glucose oxidation	3.7	6.6	−0.6	0.7
Rate (μmol of O_2 or O_2 equivalent/min per g dry wt. of heart)				
Oxygen consumption	25 ±2.0	61 ±2.0	29 ±3.7	76 ±6.0
Oxidation of pyruvate+glycolytic NADH	22.7	40.7	0.8	5.5
Palmitate oxidation	2.3	20.3	4.2	41.1
Acetate oxidation	—	—	24	29.4
Tricarboxylate cycle	16.4	40.5	26.9	60.8
Pyruvate dehydrogenase	3.7	6.6	0	0.7
Glycolytic NADH	4.2	7.7	0.8	2.0
β-Oxidation of palmitate	0.7	6.2	1.3	12.5
Rate (μmol of acetyl-CoA/min per g dry wt. of heart)				
Tricarboxylate-cycle turnover	8.2	20.2	13.5	30.4
Rate (μmol of ATP/min per g dry wt. of heart)				
ATP net yield	163	380	154	422
Percentage of O_2 used in oxidation of:				
Glucose	90	67	3	7
Acetate	—	—	83	39
Palmitate	10	33	14	54

* Values are not significantly different from zero and neglected in subsequent calculations.

glucose to be made in the perfusion medium. The specific radioactivity of glucose 6-phosphate and fructose 6-phosphate in both glucose and glucose+acetate perfusions was approx. 80% of that of medium glucose. No significant alteration in specific radioactivity of these hexose monophosphates was detected during the period of perfusion used in measurements of glycolytic rate (Table 1).

The measured rates of glycolysis with [5-^{3}H]-glucose perfused by drip-through at 50 mmHg pressure (in μmol/min per g dry wt. of heart) were 6.5 with glucose and 2.0 with glucose+acetate (Table 2). These are comparable with values of 6.6 and 3.7 obtained previously in recirculation experiments from measurements of rates of glucose uptake and glycogen synthesis (Randle *et al.*, 1970).

Effects of work on rates of glycolysis and tricarboxylate-cycle turnover in perfused rat heart

These results are shown in Figs. 1 and 2 and Table 2. Increasing perfusion pressure from 50 to 120 mmHg

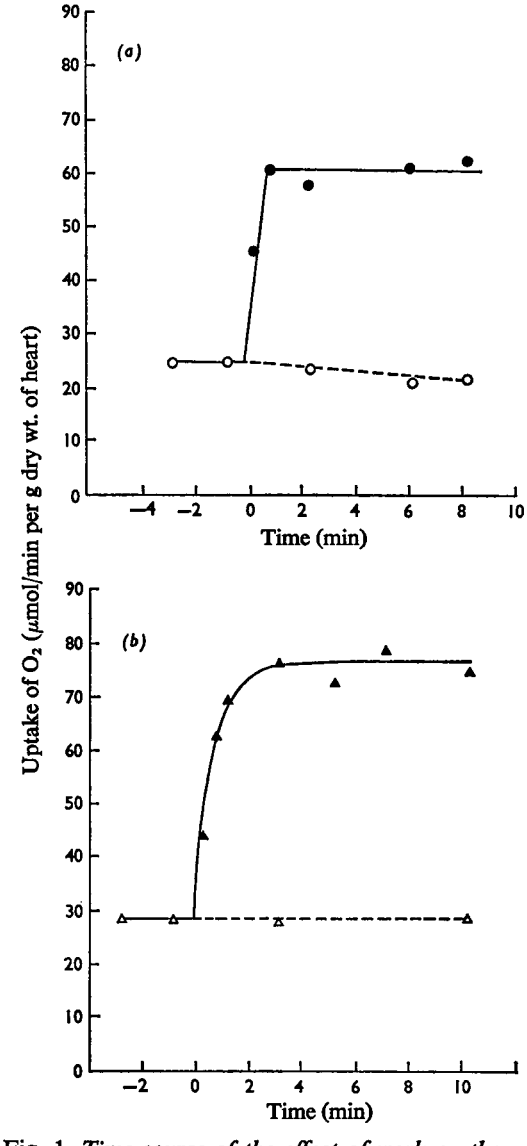

Fig. 1. *Time-course of the effect of work on the rate of oxygen uptake by perfused rat hearts*

All hearts were pre-perfused at 50 mmHg pressure for 8 min with medium containing (*a*) glucose (○, ●) or (*b*) glucose+acetate (△, ▲). At zero time perfusion pressure was either increased to 120 mmHg (●, ▲) or, in control hearts, was unaltered (○, △). Values shown are the means of observations on at least five hearts.

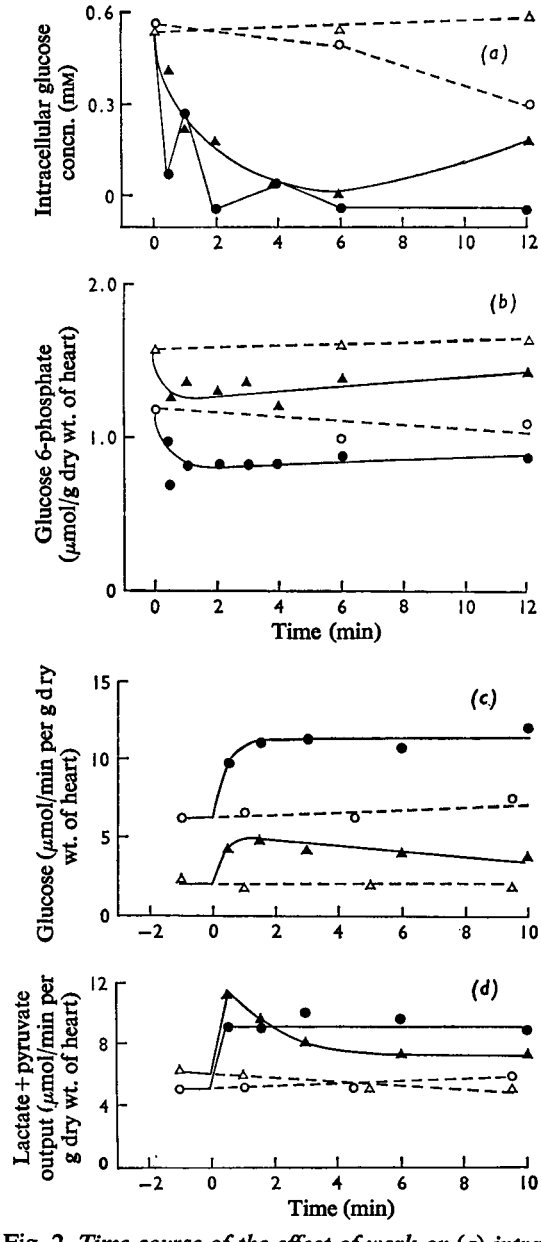

Fig. 2. *Time-course of the effect of work on (a) intracellular glucose concentration, (b) whole tissue concentration of glucose 6-phosphate, (c) glycolysis rate and (d) output of lactate plus pyruvate in perfused rat hearts*

Hearts were pre-perfused at 50 mmHg for 8 min with medium containing glucose (○, ●) or glucose+acetate (△, ▲). At zero time the perfusion pressure was either increased to 120 mmHg (●, ▲) or, in control hearts, was unaltered (○, △). Values shown are the means of observations on at least five hearts.

increased O_2 consumption to a new steady-state rate of approx. 250% of the control rate within 1 min (Fig. 1). Rates with glucose+acetate at both perfusion pressures were 15–25% greater than with glucose alone and this change, together with the absolute rates at 50 mmHg, corresponds closely to those observed previously (Randle *et al.*, 1970). The rate of glycolysis rose to a new steady-state value within 1 min of increasing the perfusion pressure. The new rate was approx. 170% of the control with glucose alone and approx. 230% of the control with glucose+acetate. These changes in rate were associated with diminutions in the intracellular concentrations of glucose 6-phosphate and glucose, thus suggesting activation of phosphofructokinase and hexokinase. Acetate inhibited glucose uptake and glycolysis at both perfusion pressures and increased the intracellular concentration of glucose 6-phosphate, suggesting inhibition of phosphofructokinase and hexokinase (Fig. 2). No overall change in tissue glycogen concentration was detected under any of the conditions of perfusion used (Table 2). No significant net synthesis or breakdown of glycogen was detected (Table 2). Increasing the perfusion pressure led to an abrupt rise in the output of lactate, which reached a maximum within 1 min. In perfusions with glucose alone outputs of lactate and pyruvate accounted for some 40% of glycolysis; in acetate perfusions lactate and pyruvate output accounted for virtually the whole of glucose metabolism (Fig. 2).

With glucose alone, oxidation of glucose accounted for 90% of oxygen consumption at low pressure but only for 67% at high pressure. Previously we have assigned the oxygen uptake not accounted for by oxidation of glucose to oxidation of endogenous glyceride fatty acids (shown in Table 2 as palmitate; see Denton & Randle, 1967). On this basis increased perfusion pressure led to a tenfold rise in the rate of oxidation of glyceride fatty acids. With acetate+glucose, oxidation of glucose was largely suppressed and the rate of oxidation of acetate was very similar at both perfusion pressures; the O_2 uptake not accounted for by glucose and acetate oxidation was 4.2 μmol of O_2/min per g dry wt. of heart at 50 mmHg perfusion pressure, and this was increased to 41.1 μmol of O_2 at 120 mmHg (Table 2). Thus, after a 9 min perfusion period, hearts perfused at the higher pressure would have utilized an additional 332 μmol of O_2 equivalents of glyceride fatty acid/g dry wt. of heart than at the lower pressure. If it is assumed that the fatty acid is palmitate, the expected decrease in triglyceride content would be $332/(3\times 23) = 4.8$ μmol/g dry wt. of heart over the 9 min perfusion period. The observed concentrations of glyceride glycerol were (in μmol/g dry wt.) 10.30 ± 0.84 (six hearts) after 9 min at low pressure and 5.34 ± 0.42 (five hearts) after 9 min at high pressure.

Table 2 shows that the rate of tricarboxylate-cycle turnover was increased by approx. 250% at the high perfusion pressure with either glucose alone or with glucose+acetate. The presence of acetate increased the rate of tricarboxylate-cycle turnover by about 50–60% at both perfusion pressures. The rate of flow through pyruvate dehydrogenase and the rate of mitochondrial oxidation of cytoplasmic NADH were also greater at the higher perfusion pressure. Also given in Table 2 are the net yields of ATP; the yield was increased substantially on raising the perfusion pressure but was not affected appreciably by acetate.

Effects of work on concentrations of metabolites in perfused rat heart

Adenine nucleotides and creatine phosphate. These results are shown in Table 3. Although increased cardiac work led to substantial increases in the rates of oxygen consumption and glycolysis there was no significant decrease in heart concentrations of creatine phosphate and ATP and no significant increase in concentrations of ADP and 5′-AMP with any period of increased cardiac work from 5 s to 5 min with either glucose or glucose+acetate. The results may perhaps show some suggestion of a fall in creatine phosphate concentration with periods of cardiac work of 1–5 min with glucose but this was not significant. There was no suggestion of any change in creatine phosphate and adenine nucleotides in the first minute of increased work, during which the increased rates of oxygen consumption, tricarboxylate-cycle flow and glycolysis became established. Addition of acetate at either perfusion pressure led to the increase in the heart concentration of 5′-AMP shown previously (Randle *et al.*, 1970).

Previous studies of the effects of cardiac work on concentrations of creatine phosphate and adenine nucleotides have given conflicting results. Neely *et al.* (1970) observed no change in these concentrations provided that an adequate supply of oxidizable substrate was maintained. Some evidence for a fall in the concentration of creatine phosphate and perhaps ATP and for a rise in 5′-AMP with increased cardiac work has been obtained in other studies (Boerth *et al.*, 1969; Hochrein & Döring, 1960; Opie *et al.*, 1971). Opie *et al.* (1971) have suggested that such changes in adenine nucleotides may provide a basis for the increased rates of oxygen consumption and glycolysis. The present study has failed to show any significant effects of cardiac work on adenine nucleotide and creatine phosphate concentrations. We would suggest that such changes where they have been detected may have been the result of an inadequate supply of oxygen at high perfusion pressures. In this regard the inclusion of insulin in perfusions with glucose in the present study could be important in that the hormone facilitates glycolysis and may therefore minimize potential effects of

Table 3. *Effects of work on rat heart concentrations of creatine phosphate and adenine nucleotides*

Hearts were pre-perfused for 8 min at 50 mmHg pressure with medium containing glucose (5.5 mM) or glucose (5.5 mM)+acetate (5 mM) and perfusion was then continued as shown. For details of methods of analysis see the Experimental section. Results are means±S.E.M. for the numbers of hearts shown in parentheses. *$P<0.02$ versus zero time, 50 mmHg (glucose). **$P<0.01$ versus zero time, 50 mmHg (glucose). ***$P<0.05$ versus 5 min, 50 mmHg (glucose). ****$P<0.02$ versus zero time, 50 mmHg (glucose+acetate).

Perfusion			Concn. (μmol/g dry wt. of heart)			
Pressure (mmHg)	Time (min)	Substrates	Creatine phosphate	ATP	ADP	AMP
50	0	Glucose (5.5 mM)	39.4±1.2 (15)	24.9±0.53 (22)	3.8±0.14 (12)	0.52±0.04 (12)
50	5		34.7±2.8 (5)	27.8±0.8 (5)*	4.2±0.10 (5)	0.77±0.03 (5)**
120	0.08		43.2±5.7 (4)	27.4±1.2 (9)	4.0±0.30 (4)	0.41±0.01 (5)
120	0.16		42.0±2.6 (5)	24.5±1.2 (10)	4.1±0.36 (5)	0.38±0.01 (5)
120	0.33		—	25.4±0.9 (6)	4.0±0.25 (6)	0.47±0.03 (5)
120	0.50		36.0±4.6 (5)	24.5±0.7 (11)	4.2±0.14 (6)	0.44±0.05 (6)
120	1.0		35.8±3.3 (5)	24.1±1.1 (5)	3.6±0.15 (5)	0.54±0.06 (5)
120	5.0		30.5±2.4 (5)	25.1±0.6 (5)***	3.7±0.33 (5)	0.95±0.15 (5)
50	0	Glucose (5.5 mM)+acetate (5 mM)	39.1±0.9 (5)	23.2±0.3 (5)	3.5±0.18 (5)	1.41±0.06 (5)
50	5		37.9±2.8 (5)	20.8±1.4 (5)	3.2±0.32 (5)	1.31±0.10 (5)
120	0.08		38.4±1.7 (5)	22.5±0.9 (5)	3.2±0.11 (5)	1.18±0.11 (5)
120	0.50		36.3±0.5 (5)	21.8±0.7 (5)	3.8±0.23 (5)	1.08±0.09 (5)****
120	1.00		37.1±1.0 (5)	20.8±0.6 (5)	3.5±0.25 (5)	1.14±0.06 (5)
120	5.00		38.6±2.1 (4)	21.6±1.1 (4)	3.8±0.25 (4)	1.26±0.26 (4)

hypoxia on concentrations of adenine nucleotides and creatine phosphate.

Tricarboxylate-cycle intermediates. These results are shown in Figs. 3 and 4. When the rate of tricarboxylate-cycle turnover in rat heart is increased by acetate at low perfusion pressure the steady-state concentrations of acetyl-CoA, malate, citrate and glutamate are increased and that of aspartate is decreased (Randle *et al.*, 1970). Similar changes with acetate were observed in the present study at both perfusion pressures. When the rate of tricarboxylate-cycle turnover was increased in the present study by raising the perfusion pressure the pattern of metabolite concentrations was very different. With glucose alone, there were no changes in the concentrations of citrate, aspartate or glutamate whereas the concentration of malate fell. (The concentration of acetyl-CoA, less than 20nmol/g dry wt., was too low for accurate measurements.) With glucose and acetate, the concentrations of acetyl-CoA and citrate fell, the concentration of aspartate increased, whereas that of glutamate was not significantly altered. The concentration of 2-oxoglutarate was unchanged by work with either substrate.

Intracellular glucose concentration. These results are shown in Fig. 2. In perfusions with either glucose alone or with glucose+acetate, the intracellular glucose concentration fell rapidly on switching to the higher perfusion pressure. It is concluded that increased cardiac work accelerated the intracellular phosphorylation of glucose by hexokinase.

General discussion

Effects of cardiac work on the rate of glycolysis and rates of oxidation of glucose, glyceride fatty acid and acetate. Increased cardiac work approximately doubled the rates of glycolysis and glucose phosphorylation in hearts perfused with media containing either glucose or glucose+acetate. Measurements of the tissue concentrations of hexose phosphate and glucose indicated that this resulted from activation

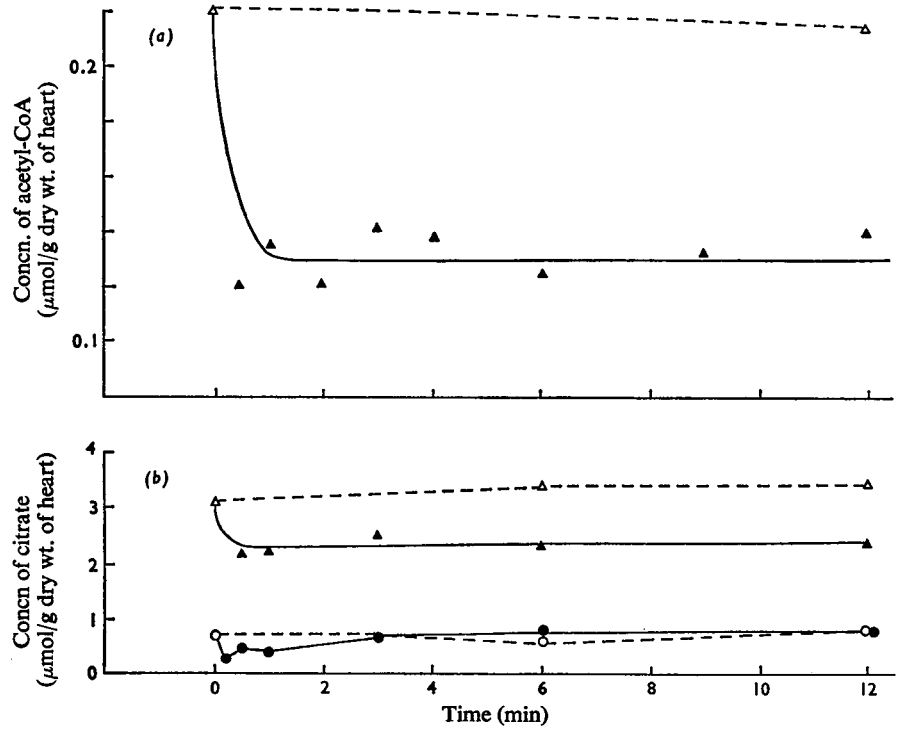

Fig. 3. *Time-course of the effect of work on the whole tissue concentrations of (a) acetyl-CoA and (b) citrate in perfused rat heart*

Hearts were pre-perfused at 50mmHg for 8min with medium containing glucose (○, ●) or glucose + acetate (△, ▲). At zero time the perfusion pressure was either increased to 120mmHg (●, ▲) or, in control hearts, was unaltered (○, △). Values shown are the means of observations on at least five hearts.

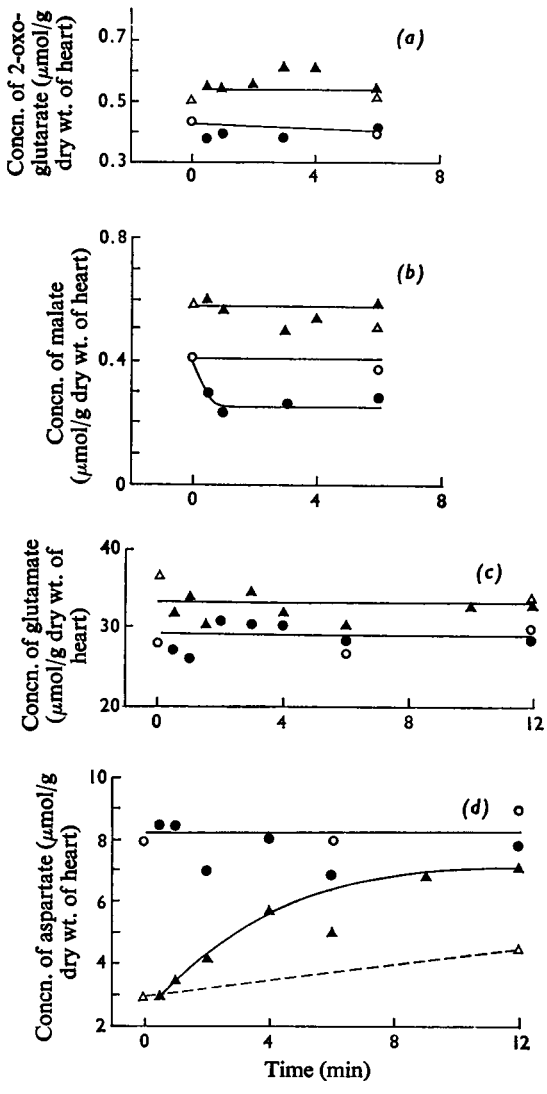

Fig. 4. *Time-course of the effect of work on the whole tissue concentrations of* (*a*) *2-oxoglutarate*, (*b*) *malate*, (*c*) *glutamate and* (*d*) *aspartate in perfused rat hearts*

Further details and symbols are as given in Fig. 3.

of phosphofructokinase and hexokinase. The mechanism of these changes in phosphofructokinase activity is not entirely clear. Previous studies of the regulation of glycolysis in rat heart by anoxia, by diabetes and by the oxidation of fatty acids and ketone bodies have provided evidence for regulation of the enzyme by intracellular concentrations of ATP and citrate (inhibitors) and of ADP, 5′-AMP and P_i (activators) (Regen *et al.*, 1964; Newsholme & Randle, 1964; Parmeggiani & Bowman, 1963; Garland *et al.*, 1963; Pogson & Randle, 1966). In perfusions with glucose+acetate increased cardiac work lowered the intracellular citrate concentration and this could conceivably account for the activation of phosphofructokinase. However, in perfusions with glucose alone there were no detectable changes in the concentrations of citrate, ATP, ADP or 5′-AMP. Other possibilities that have not been investigated include increases in the concentrations of P_i and of SO_4^{2-}, which are activators of the enzyme, and transient changes in adenine nucleotides associated with individual ventricular systoles. Acetate utilization was found to inhibit glycolysis by about 70% at low pressure and by about 60% at high pressure, i.e. cardiac work had little, if any, effect on the inhibition of glycolysis and phosphofructokinase by acetate utilization.

Increased cardiac work doubled the rate of oxidation of pyruvate in perfusions with media containing glucose. With media containing glucose+acetate pyruvate oxidation was almost totally suppressed by acetate at both perfusion pressures. The rate of oxidation of acetate was only slightly increased by work and, since the concentration of acetyl-CoA fell, it would seem reasonable to conclude that the acetyl-CoA synthase reaction is near saturation at the lower perfusion pressure. The most dramatic change in respiratory fuel with increased cardiac work was a tenfold increase in the rate of oxidation of fatty acids derived from muscle glycerides. This change was seen in perfusions with media containing either glucose alone or glucose+acetate. Cardiac work thus presumably activated lipolysis in heart muscle. It should be noted that *in vivo* the heart may additionally utilize fatty acids from the plasma free fatty acid and triglyceride pools for respiration. If our observations are applicable to the situation *in vivo* the circulating fatty acids may be of particular importance in increased cardiac work under physiological conditions. It is known that some factors that increase cardiac work *in vivo* (e.g. exercise) lead to an increased turnover of plasma free fatty acids and to an increase in plasma glycerol, suggesting accelerated adipose-tissue lipolysis (Carlson *et al.*, 1963; Havel *et al.*, 1963).

Effects of cardiac work on tricarboxylate-cycle turnover and on the concentrations of metabolites associated with the cycle. As in an earlier study (Randle *et al.*, 1970), acetate utilization at the lower perfusion pressure increased the rate of tricarboxylate-cycle turnover by about 50% and produced marked changes in the concentrations of acetyl-CoA, citrate, 2-oxoglutarate, glutamate and malate (increased) and of aspartate (decreased); essentially similar changes with acetate have now been shown at the higher perfusion pressure. In marked contrast, increased cardiac work more than doubled tricarboxylate-cycle

turnover in perfusions with glucose or with glucose+acetate without causing the changes in the concentrations of cycle metabolites observed when cycle turnover is stimulated by acetate utilization. The fact that the effects of work on cycle turnover were independent of the external substrate supplied leads to the conclusion that the effects of work are not dependent on the concentrations of those cycle metabolites that were altered by acetate utilization. Conversely the changes in metabolite concentrations that accompany acetate utilization would appear to be specifically concerned with the regulatory function of the cycle in the control of glycolysis and pyruvate oxidation and not with the associated increase in the rate of cycle turnover.

Mechanisms controlling tricarboxylate-cycle turnover during cardiac work. When cardiac work was increased by raising the perfusion pressure the rates of tricarboxylate-cycle turnover and of ATP turnover were increased approx. 2.5-fold in perfusions with either glucose alone or glucose+acetate, yet no meaningful changes were detected in the concentrations of metabolites that might provide a mechanism for the increases. It is to be expected that increased cardiac work will be associated with accelerated conversion of ATP into ADP+P_i, and we have assumed that this will lead to a rise in the concentration of ADP, stimulation of respiration, oxidation of NADH and acceleration of cycle dehydrogenase reactions. There were, however, no detectable changes in the whole-tissue concentrations of ATP, ADP, 5′-AMP or creatine phosphate with increased cardiac work. There were, moreover, no detectable increases in the concentrations of a number of substrates for reactions in the tricarboxylate cycle. The only changes detected were falls in the concentrations of malate (perfusions with glucose) and acetyl-CoA, citrate and aspartate (in perfusions with glucose+acetate) and it is difficult to see how these changes might contribute to an increased rate of cycle turnover. We must conclude that the particular approach utilized in this study is inadequate for the purpose of investigating mechanisms controlling cycle turnover, and some discussion of the possible basis of this inadequacy may be useful.

In past studies with skeletal muscle, considerable difficulty was experienced in demonstrating breakdown of ATP or of creatine phosphate in single contractions. Refinement of analytical methods led eventually to the demonstration of creatine phosphate breakdown, but ATP breakdown could not be detected. This was only achieved by treatment of muscle with 1-fluoro-2,4-dinitrobenzene to inhibit creatine phosphokinase. Using this technique, Cain & Davies (1962) were able to demonstrate stoicheiometric conversion of ATP into ADP, AMP and P_i with a single contraction. The question therefore arises whether similar considerations have prevented us from detecting the changes in ADP concentration in particular, which we believe must be associated with alterations in cardiac work. The perfused rat

Table 4. *Comparison of whole tissue concentrations of metabolites with the change in their turnover per heart beat induced by raising the perfusion pressure from 50 to 120mmHg in perfusions with media containing insulin and either glucose or glucose+acetate*

Whole tissue concentrations for steady state at 50mmHg are taken from Figs. 3 and 4 and Table 3. Isocitrate concentrations were calculated from those of citrate (England *et al.*, 1968). Turnover values were calculated from results in Table 2, a rate of beating of 240/min being assumed. All values are in μmol/g dry wt. of heart.

Perfusion medium ...	Glucose			Glucose+acetate		
Metabolite	Concn.		Δ (Turnover/beat)	Concn.		Δ (Turnover/beat)
Creatine phosphate	39.4			39.1		
ATP	25	69	0.9	23	68	1.1
ADP	4			4		
AMP	0.5			1.4		
NADH	—		0.25	—		0.35
Acetyl-CoA	0.01	0.16	0.05	0.12	2.52	0.07
Acetylcarnitine	0.15			2.4		
Citrate	0.6	0.7	0.05	2.4	2.6	0.07
Isocitrate	0.1			0.2		
2-Oxoglutarate	0.4	25.4	0.05	0.6	33.6	0.07
Glutamate	25			33		
Malate	0.25	8.25	0.05	0.6	7.6	0.07
Aspartate	8			7		
Oxygen	—		0.15	—		0.19

heart shows spontaneous and regular contractions at a rate of approx. 240/min under the conditions of our experiments. Each cardiac cycle (systole and diastole) therefore lasts 250ms and the electrocardiogram shows that approx. 40% of this time is occupied by contraction (ventricular systole) and 60% by relaxation (ventricular diastole). There is thus the possibility that the turnover of ATP and of the metabolic reactions associated with it are intermittent and associated predominantly with each contraction. Consequently, if there are metabolite changes associated with increased work during each contraction that are reversed during diastole, such changes might be largely obliterated by the random nature of freeze-clamping in relation to the cardiac cycle and by the time required for cooling with current techniques (approx. 100ms; Wollenberger *et al.*, 1960). It is thus possible that greater resolution may be achieved by freezing hearts more rapidly at fixed points in the cardiac cycle.

Table 4 shows the overall pool sizes of adenine nucleotides and creatine phosphate and of tricarboxylate-cycle metabolites, and the calculated increase in turnover of these pools with each beat when cardiac work is increased by raising the perfusion pressure from 50 to 120mmHg. Presumably only those metabolites for which the change in turnover per beat is an appreciable fraction of the pool size may be expected to show detectable concentration changes in each contraction. The change in turnover of ADP is approx. 25% of the overall pool size. However, much of the ADP may be firmly bound to actin (Perry, 1954) and hence the fraction of ADP that turns over may be small in relation to its overall pool size. Moreover, the change in turnover of ADP is very small (<1.5%) in relation to the size of the overall pool of adenine nucleotides and creatine phosphate. The operation of the creatine phosphokinase reaction could thus interfere with the detection of changes in the ADP concentrations. It is possible that the action of adenylate kinase (EC 2.7.4.3) might generate detectable changes in AMP concentrations. The AMP pool is relatively small and, if adenylate kinase is operating at near equilibrium and at a fixed ATP concentration, then any change in [ADP] will lead to much larger changes in [AMP].

In the tricarboxylate cycle, the pool size of acetyl-CoA is very small in relation to the change in turnover per beat. In perfusions with glucose the change in turnover per beat is at least five times the pool size and with glucose+acetate it is of the same order as the pool size. The change in turnover of acetyl-CoA per beat is also appreciable in relation to the size of the combined pool of acetyl-CoA+acetylcarnitine. The possible importance of acetylcarnitine and of carnitine acetyltransferase as a buffer mechanism for the acetyl-CoA pool during each beat can readily be appreciated from these results. The changes in turnover of isocitrate and 2-oxoglutarate are also appreciable in relation to their overall pool sizes. However, radioisotope measurements show that citrate and isocitrate, and 2-oxoglutarate and glutamate, may be well equilibrated in rat heart (Randle *et al.*, 1970) and the change in tricarboxylate-cycle turnover per beat is small in relation to the size of the combined pools of (citrate+isocitrate) and of (2-oxoglutarate+glutamate). This may suggest further buffer mechanisms in relation to the operation of the tricarboxylate cycle involving aconitase and glutamate–alanine and glutamate–aspartate aminotransferases. The change in turnover of malate is also appreciable in relation to its pool size. The pool size of oxaloacetate in rat heart is not accurately known but published measurements suggest concentrations of the order of 5–50nmol/g dry wt. (Garland & Randle, 1964; Williamson, 1965), which are small in relation to turnover. Radioisotope measurements have shown that malate and oxaloacetate may be in close equilibrium but that oxaloacetate and aspartate may not be closely equilibrated (Randle *et al.*, 1970). It is thus possible that detectable fluctuations in the concentrations of oxaloacetate and malate may occur during each contraction. These considerations suggest that it will be necessary to attempt to study transient changes in metabolism that may be associated with each individual cardiac contraction to explore more fully the control of energy metabolism by cardiac contraction. The use of inhibitors of carnitine acetyltransferase and of the aminotransferases may be a useful adjunct to such studies.

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