Effects of Increased Mechanical Work by Isolated Perfused Rat Heart during Production or Uptake of Ketone Bodies

ASSESSMENT OF MITOCHONDRIAL OXIDIZED TO REDUCED FREE NICOTINAMIDE-ADENINE DINUCLEOTIDE RATIOS AND OXALOACETATE CONCENTRATIONS

By LIONEL H. OPIE* and PATRICIA OWEN

Ischaemic Heart Disease Laboratory, Department of Medicine, Groote Schuur Hospital and University of Cape Town, Cape Town, South Africa

(Received 11 November 1974)

1. Metabolic effects of increased mechanical work were studied by comparing isolated pumping rat hearts perfused by the atrial-filling technique with aortic-perfused non-pumping hearts perfused by the technique of Langendorff. The initial medium usually contained glucose (11 mM) and palmitate (0.6 mM bound to 0.1 mM albumin). 2. During increased heart work (comparing pumping with non-pumping hearts) the uptake of oxygen and glucose increased threefold, but that of free fatty acids was unchanged. Tissue contents of α -oxoglutarate, NH_4^+ , malate, lactate, pyruvate and P_i rose with increased heart work, but contents of ATP, phosphocreatine and citrate fell. 3. Ketone bodies were produced with a ratio of β -hydroxybutyrate/acetoacetate of about 3:1 in both pumping and non-pumping hearts but with higher net production rates in non-pumping hearts. 4. When ketone bodies were added in relatively high concentrations (total 4 mM) to a glucose (11 mM) medium the medium ratios of β -hydroxybutyrate/acetoacetate were not steady even after 60 min of perfusion. 5. The validity of calculating mitochondrial free NAD^+/NADH ratios from the tissue contents of the reactants of the glutamate dehydrogenase system or the β -hydroxybutyrate dehydrogenase system is assessed. The activities of these enzymes are considerably less in the rat heart than in the rat liver, introducing reservations into the application to the heart of the principles used by Williamson *et al.* (1967) for calculation of mitochondrial free NAD^+/NADH ratios of liver mitochondria. A further reservation for the β -hydroxybutyrate dehydrogenase system is that intracellular ketone-body concentrations could not be measured by the techniques used; hence changes in the ratios of extracellular ketone bodies were assumed to have a relation to the intracellular ratios. 6. The β -hydroxybutyrate dehydrogenase system was not in equilibrium in our experimental conditions when extracellular ketone-body concentrations were high, although the very low amounts of ketone bodies produced by pumping hearts perfused with palmitate could more easily have reached equilibrium. 7. There was also no firm evidence for equilibrium or near-equilibrium of the glutamate dehydrogenase system; nevertheless, the reactants of both systems (deduced by using the ratios of produced ketone bodies or tissue contents of glutamate, NH_4^+ and α -oxoglutarate) moved in the direction of an increased NAD^+/NADH ratio during increased heart work. Collateral evidence also suggests that the mitochondrial NAD^+/NADH ratio changes to NAD^+ with increased heart work. Provided that the mitochondrial malate dehydrogenase system is not too far displaced from equilibrium, the mitochondrial oxaloacetate concentration (calculated from the tissue malate content) would rise during increased heart work. 8. During anoxia, uptake of added β -hydroxybutyrate ceased within 1–2 min and uptake of acetoacetate fell and could be accounted for by formation of β -hydroxybutyrate. The glutamate/ α -oxoglutarate- NH_4^+ ratio also rose in anoxia (Lai & Scheuer, 1975). These data suggest that the mitochondrial NAD^+/NADH ratio changes to NADH during anoxia.

The mechanical activity of the isolated perfused rat heart can be increased abruptly by the transition from a Langendorff aortic-perfused non-pumping heart

* To whom reprint requests should be addressed at the Groote Schuur Hospital, Cape Town, South Africa.

to an atrially perfused pumping preparation, with a threefold increase in the rate of O_2 consumption (Opie *et al.*, 1971a). Hence there is an approximately similar increase in the rate of flux through citrate synthase (La Noue *et al.*, 1972), an enzyme which

appears to have an important role in the regulation of the rate of the tricarboxylic acid cycle (Krebs, 1970) in the span acetyl-CoA plus oxaloacetate to α -oxoglutarate (Randle *et al.*, 1970).

Two main proposals for the regulation of citrate synthase (EC 4.1.3.7) activity are: first, regulation by allosteric interaction with modifiers such as ATP, acyl-CoA and NADH (Wieland & Weiss, 1963; Kosicki & Lee, 1966; Atkinson, 1968; Garland, 1968; Wright & Sanwal, 1971; Srere & Matsuoka, 1972); and secondly, regulation by the substrate supply (Randle *et al.*, 1970; Newsholme & Start, 1973), the effects of which may be modified by competitive inhibition with other metabolites (Olson & Williamson, 1971; La Noue *et al.*, 1972). Because citrate synthase appears to operate well below the $V_{\max}$ (Garland *et al.*, 1969; Randle *et al.*, 1970; Smith & Williamson, 1971), important factors should be the concentrations of acetyl-CoA and oxaloacetate; the latter may be regulated in part by the mitochondrial NAD^+/NADH ratio (La Noue *et al.*, 1972), which may also help regulate the production of α -oxoglutarate by citrate synthase and aspartate aminotransferase (La Noue *et al.*, 1973). We have attempted to assess to what extent the principles elucidated by Williamson *et al.* (1967) for the calculation of the mitochondrial ratio of free NAD^+/NADH and the mitochondrial oxaloacetate concentration in the liver, might be applicable to the heart. Measurements were made of the reactants of the glutamate dehydrogenase system or the ratios of β -hydroxybutyrate/acetoacetate, and of tissue malate.

In the present experiments, the myocardial content of ketone bodies was very low; hence ketone bodies were added to the medium indirectly by production during perfusions with long-chain free fatty acids (Evans *et al.*, 1963). Experiments with added ketone bodies were also undertaken to assess circumstances in which changes in added extracellular ketone bodies could reflect changes in mitochondrial NAD^+/NADH ratios during increased heart work or anoxia. Provisional conclusions are drawn about the directions of change of the mitochondrial ratio of free NAD^+/NADH and of the mitochondrial oxaloacetate concentration with increased heart work, but several important reservations are involved.

Materials and Methods

Materials

Rats. Male rats (280–320 g) of a local strain, originally Sprague–Dawley, were fed *ad lib*.

Chemicals. Unless otherwise stated, all chemicals and enzymes used in the analyses of tissue and perfusion fluid were obtained from C. F. Boehringer und Soehne G.m.b.H., Mannheim, Germany. Glucose, sorbitol and EDTA were obtained from British Drug Houses, Poole, Dorset, U.K. The

sources of heparin and [^3H]sorbitol were as described by Chain *et al.* (1969). Albumin-bound palmitate (0.6 mm) or linoleate was prepared by adding the potassium salt to bovine serum albumin (fraction V) obtained from Nutritional Biochemical Co., Cleveland, Ohio, U.S.A. or from Pentax, Kankakee, Ill., U.S.A. DL- β -Hydroxybutyrate came from Sigma Chemical Co., St. Louis, Mo., U.S.A. Acetoacetate was prepared from ethyl acetoacetate (British Drug Houses) which was redistilled and converted into sodium acetoacetate by reflux with NaOH; the sodium compound was evaporated down, neutralized and stored at -20°C .

Methods

Perfusion techniques. Rats were anaesthetized by ether and given 200 i.u. of heparin into the femoral vein. The heart was rapidly removed and plunged into ice-cold perfusion medium before being mounted on an aortic cannula for isolation perfusion.

(i) Pre-perfusion system. In all experiments the arrested heart (approximate fresh wt. 1.0 g) was restarted by perfusion by the method of Langendorff (1895). An initial non-recirculation washout pre-perfusion of 15 min (usually at 65 cm H_2O perfusion pressure) preceded a recirculation perfusion.

(ii) Langendorff aortic-perfused non-pumping recirculation system. This refers to hearts perfused by the Langendorff technique as modified by Morgan *et al.* (1961) to allow recirculation; the volume of perfusion fluid was about 40 ml and the aortic perfusion pressure was 65 cm H_2O .

(iii) Working recirculation system. This refers to hearts perfused via the left atrium, by the technique of Neely *et al.* (1967), at an atrial perfusion pressure of 20 cm H_2O , with a recirculation volume of about 50 ml. The left ventricle spontaneously pumped 40–60 ml of perfusate/min, against a hydrostatic pressure of 100 cm H_2O .

(iv) Variation in heart work. When the aortic perfusion pressure of Langendorff-perfused hearts is increased, the left ventricular pressure increases (Opie, 1965) and the peak left-ventricular pressure equals the aortic-perfusion pressure (Neely *et al.*, 1967). Thus an aortic-perfusion pressure of about 65 cm H_2O represents a left ventricular peak systolic pressure of about 65 cm H_2O . A major increase in left-ventricular pressure can be obtained by filling the left ventricle from the left atrium, with peak left-ventricular pressures of over 200 cm H_2O (Kannengiesser *et al.*, 1975).

The term 'non-working' has been applied to Langendorff-perfused hearts because no external mechanical work is performed, and the term 'working' to atrially perfused hearts performing external work. Nevertheless, all contracting hearts are 'working' in the thermodynamic sense. Because the transition from the Langendorff to the atrial perfusion systems

involved transition from a non-pumping to a pumping heart, the Langendorff and atrially perfused systems will be referred to as 'non-pumping' or 'pumping' systems respectively.

Oxygen uptake. This was measured by passing samples of the perfusate entering and leaving the heart through jacketed glass holders containing Beckmann macroelectrodes (see Chain *et al.*, 1969), or by using a syringe with a blunt needle to withdraw venous perfusate from the right ventricle for assay in an Astrup micro-electrode system. The calculated oxygen content of the perfusate was related to the coronary flow rate.

Perfusion medium. The perfusion medium was a Krebs-Henseleit bicarbonate buffer medium (Krebs & Henseleit, 1932) at 37°C, usually equilibrated with O₂+CO₂ (95:5). Glucose (11.1mM) and palmitate (0.6mM) (bound to 0.1mM-albumin, i.e. 7g/l) or glucose and equimolar ketone bodies (2mM-acetoacetate and 4mM-DL-β-hydroxybutyrate) were the substrates for all perfusion systems, unless otherwise stated. Deionized distilled water was used when NH₄⁺ measurements were made. Concentrations of acetoacetate, β-hydroxybutyrate and glucose were unchanged after mock perfusion for 1 h without any heart. [³H]Sorbitol (5.5mM) was used as an extracellular marker (Morgan *et al.*, 1961) and to determine the exact volume of circulating perfusion medium. Usually the initial medium samples (0.5–2.0ml) were taken 2 min after the onset of recirculation perfusion.

Anoxia. In anoxic experiments, the perfusion medium was equilibrated with N₂+CO₂ (95:5) by enclosure of flexible tubing in larger tubes containing N₂+CO₂ (95:5) to avoid exchange of gas between the anoxic perfusion medium and atmospheric oxygen. This procedure gave an incoming O₂ tension of below 666 Pa (5 mmHg).

Tissue analyses. At the end of the perfusion period hearts were freeze-clamped in aluminium tongs chilled in liquid N₂ (Wollenberger *et al.*, 1960). To obtain consistent dry/wet weight ratios and sorbitol spaces, it was essential to remove carefully (i) the frozen perfusate around the edges of the clamped heart, and (ii) the major part of the atria. This was done by placing the heart on a block of dry ice and gently prising off the unwanted parts with a sharp tip of chilled scissors. The remainder of the heart was rapidly weighed on a torsion balance, powdered in a percussion mortar, and deproteinized in 6% (w/v) HClO₄. After neutralization of the supernatant, ADP and AMP were measured immediately by the method of Adam (1963). ATP and creatine phosphate were measured the next morning (after the tissue extract had been stored at –20°C overnight, which did not change ATP or creatine phosphate values); the method of Lamprecht & Trautshold (1963) was used for ATP, creatine phosphate was then assayed in the same cuvette by addition of ADP and excess of

creatine phosphate kinase (5mg of enzyme/ml of distilled water). These assays were continuously recorded to ensure that there was no 'creep' which would give falsely high values. P_i was measured at the same time by a modification of the method of Seraydarian *et al.* (1961) instead of the method of Berenblum & Chain (1938) which was previously used.

The methods used for spectrophotometric determination of metabolites were: lactate, pyruvate, malate, α-glycerophosphate, dihydroxyacetone phosphate and α-oxoglutarate by the methods of Hohorst *et al.* (1959); acetoacetate and D-β-hydroxybutyrate by the method of Williamson *et al.* (1962); citrate by the citrate lyase method of Moellering & Gruber (1966); NH₄⁺ by the method of Kirsten *et al.* (1963); glutamate by the method of Bernt & Bergmeyer (1963). A separate sample of powdered extract was used for glycogen assay by the method of Good *et al.* (1933), with assay of glucose by the use of glucose 6-phosphate dehydrogenase. Pyruvate, acetoacetate, α-oxoglutarate and dihydroxyacetone phosphate were measured immediately after the neutralization procedure and NH₄⁺, glutamate, lactate, β-hydroxybutyrate, α-glycerophosphate, citrate and malate after storage at –20°C for a few days.

Perfusion-fluid analyses. Methods for assay of glucose, lactate, pyruvate and [³H]sorbitol were as described elsewhere (Chain *et al.*, 1969; Opie & Mansford, 1971). Acetoacetate and β-hydroxybutyrate were measured by the method of Williamson *et al.* (1962) and β-hydroxybutyrate dehydrogenase by a modification of the same method. In some experiments perfusate α-oxoglutarate, glutamate and NH₄⁺ and malate were assayed immediately after sampling by methods similar to those used on tissue.

Calculation of mitochondrial oxaloacetate concentration. The mitochondrial oxaloacetate concentration can be calculated by using the following three assumptions (each of which needs critical assessment; see the Discussion section): that the malate dehydrogenase system is at equilibrium; that malate is evenly distributed between cytoplasm and mitochondria; and that the pool of free NAD⁺/NADH reacting with malate dehydrogenase is in equilibrium with the pool of NAD⁺/NADH reacting with glutamate dehydrogenase and/or β-hydroxybutyrate dehydrogenase. Under these conditions the following equations hold:

$$\frac{[\text{Oxaloacetate}][\text{NADH}]}{[\text{Malate}][\text{NAD}^+]} = K_{\text{malate dehydrogenase}}$$

$$[\text{Oxalacetate}] =$$

$$[\text{malate}] \times \frac{[\alpha\text{-oxoglutarate}][\text{NH}_4^+]}{[\text{glutamate}]} \times \frac{K_{\text{MDH}}}{K_{\text{GDH}}}$$

[Oxaloacetate] =

$$[\text{malate}] \times \frac{[\text{acetoacetate}]}{[\beta\text{-hydroxybutyrate}]} \times \frac{K_{\text{MDH}}}{K_{\beta\text{HBH}}}$$

where

$$K_{\text{MDH}} = K_{\text{malate dehydrogenase}} = 2.78 \times 10^{-5}$$

$$K_{\text{GDH}} = K_{\text{glutamate dehydrogenase}} = 3.87 \times 10^{-3}$$

$$K_{\beta\text{HBH}} = K_{\beta\text{-hydroxybutyrate dehydrogenase}} = 4.93 \times 10^{-2}$$

These are all at 38°C and ionic strength 0.25 (see Williamson *et al.*, 1967). The important question of equilibrium of the glutamate and β -hydroxybutyrate dehydrogenase systems is discussed below.

Calculation of mitochondrial and cytoplasmic free NAD⁺/NADH ratios. Mitochondrial free NAD⁺/NADH ratios can be calculated from measurement of the reactants of either the glutamate dehydrogenase or the β -hydroxybutyrate dehydrogenase system, provided that equilibrium is reached (see the Discussion section). Cytoplasmic free NAD⁺/NADH can be calculated from measurements of the reactants for the lactate dehydrogenase or the α -glycerophosphate dehydrogenase systems, although in some circumstances there is evidence for a dissociation between the behaviour of these two systems and for compartmentation of two cytoplasmic pools of NAD⁺/NADH as has already been given (Opie & Mansford, 1971).

Calculations of NAD⁺/NADH ratios from substrate couples require a knowledge of intracellular [H⁺] which we did not measure. An unchanged tissue

lactate content after increased heart work is presumptive evidence against an altered tissue [H⁺], whereas Saborowski *et al.* (1973) used 5,5-di[¹⁴C]-methyl-2,4-oxazolinedione to show that increased mechanical load on the rat heart *in situ* only slightly changed the intracellular pH from 6.95 to 7.07. A larger change in the mitochondrial pH cannot be excluded.

Calculation of cytoplasmic oxaloacetate concentration. Calculations suggest that total liver oxaloacetate largely resides in the cytoplasm (Williamson *et al.*, 1967). Assuming that total cardiac oxaloacetate and malate are largely cytoplasmic and in equilibrium (Randle *et al.*, 1970) there could be an equilibrium between cytoplasmic oxaloacetate, malate, NAD⁺ and NADH in which case total cytoplasmic oxaloacetate could be calculated from the equation:

[Cytoplasmic oxaloacetate] =

$$\frac{[\text{malate}][\text{pyruvate}]}{[\text{lactate}]} \times \frac{K_{\text{MDH}}}{K_{\text{lactate dehydrogenase}}}$$

Adler-Kastner *et al.* (1972) found a good correlation between measured total cardiac oxaloacetate and calculated cytoplasmic values in non-diabetic rats.

Expression of results. A portion of the powdered frozen tissue was dried to constant weight. The weight of the frozen tissue was corrected by the appropriate factor to express results in terms of fresh weight (dry weight $\times 5$ = fresh weight) which closely corresponded to the weight of the heart in the rat. Medium metabolite changes were expressed as $\mu\text{mol}/\text{perfusion}$

Table 1. Rates of ketone body production during perfusion of isolated rat heart with glucose (11 mM) and palmitate (0.6 mM), bound to 0.1 mM-albumin

Units are $\mu\text{mol}/30\text{min}$ perfusion per g fresh wt. (fresh wt. = dry wt. $\times 5$); values are means $\pm$ S.E.M., with the numbers of determinations in parentheses. Recirculation volume was 50 ml. For definition of non-pumping and pumping, see the text.

	Non-pumping		Pumping		P
Uptake of:					
Oxygen	100 $\pm$ 4	(7)	300 $\pm$ 23	(10)	<0.001
Glucose	15.3 $\pm$ 2.4	(21)	47.2 $\pm$ 5.1	(16)	<0.001
Free fatty acid	5.8 $\pm$ 0.7	(14)	6.6 $\pm$ 0.8	(14)	N.S.
Production of:					
β -Hydroxybutyrate	1.93 $\pm$ 0.19	(20)	0.42 $\pm$ 0.07	(18)	<0.001
Acetoacetate	0.73 $\pm$ 0.07	(20)	0.20 $\pm$ 0.03	(18)	<0.001
Medium ratio β-hydroxybutyrate/acetoacetate					
5 min perfusion	2.15 $\pm$ 0.32	(20)	1.07 $\pm$ 0.23	(13)	<0.01
30 min perfusion	3.61 $\pm$ 0.47	(24)	2.71 $\pm$ 0.38	(17)	N.S.
Medium ratio lactate/pyruvate					
30 min perfusion	11.1 $\pm$ 0.9	(12)	6.5 $\pm$ 0.2	(6)	<0.001
Final concentration ($\mu\text{mol}/\text{g}$ fresh wt.) of:					
β -Hydroxybutyrate	0.034 $\pm$ 0.004	(16)	0.005 $\pm$ 0.00004*	(18)	<0.001
Acetoacetate	0.018 $\pm$ 0.005	(20)	0.003 $\pm$ 0.00004*	(18)	<0.01

* 2 ml medium used in 3 ml cuvettes; results near lower limit of spectrophotometric detection.

period per g fresh weight. Values are given as means $\pm$ S.E.M. *P* values were calculated by the Student's *t* test, by the use of two-tailed values; *P* < 0.05 was deemed to be significant.

Results

Changes in the perfusion medium (Table 1)

During increased heart work of hearts perfused with glucose (11 mM), palmitate (0.6 mM bound to 0.1 mM-albumin) the following changes occurred. The coronary flow rate increased by 2.5 times, which indicates an increased O₂ uptake of about the same order of magnitude (Opie, 1965). Glucose uptake increased; the medium lactate/pyruvate ratio fell; and free fatty acid uptake did not change. Rates of production of β -hydroxybutyrate and acetoacetate were: β -hydroxybutyrate, 1.93 and 0.42 μ mol/30 min per g fresh wt. in non-pumping and pumping hearts respectively; acetoacetate, 0.73 and 0.20 μ mol/30 min per g fresh wt. in non-pumping and pumping hearts respectively. Peak rates of β -hydroxybutyrate production were 2.24 μ mol/30 min per g reached in the first 5 min of non-pumping heart perfusions. The ratio of β -hydroxybutyrate/acetoacetate was about 2.0 at 5 min in non-pumping hearts and 1.0 in pumping hearts. At 30 min, ratios in non-pumping and pumping hearts were similar. After perfusion for 60 min with albumin-bound linoleate as substrate, the β -hydroxybutyrate/acetoacetate ratio was 2.83 $\pm$ 0.27 (*n* = 5) in working hearts and 4.69 $\pm$ 0.88 (*n* = 5) in non-pumping hearts. In non-pumping hearts perfused with albumin-bound palmitate or

linoleate for 30 min, the ratios and concentrations of ketone bodies produced did not differ whether glucose was present in the medium or not.

Uptake of ketone bodies at low concentrations

When ketone bodies were produced from fatty acids both uptake and production of ketone bodies were possible. This was shown in hearts perfused with albumin-bound linoleate for 60 min, either non-pumping throughout or else non-pumping for 30 min and then pumping for 30 min. The mean β -hydroxybutyrate and acetoacetate concentrations after 30 min perfusions of non-pumping hearts were 0.024 $\pm$ 0.004 mM (*n* = 9) and 0.014 $\pm$ 0.002 mM (*n* = 10) respectively (similar to values with palmitate-glucose-albumin) but thereafter the concentrations tended to decline, indicating uptake of previously formed ketone bodies. Decomposition of ketone bodies in the period 30–60 min seemed unlikely because in one heart with low medium values at 30 min (0.005 mM of each ketone body), production of both acetoacetate and β -hydroxybutyrate continued in the second 30 min period. When ketones were added in low concentrations (0.03 mM-acetoacetate, 0.1 mM- β -hydroxybutyrate) even in the presence of glucose and linoleate or palmitate as alternate fuels, ketone bodies were still taken up by both pumping and non-pumping hearts.

Effect of increased heart work on calculated mitochondrial oxaloacetate concentrations and on free NAD⁺/NADH ratios (Table 2)

Because the ketone-body space did not exceed the

Table 2. *Effect of increased heart work on calculated mitochondrial oxaloacetate concentrations and free NAD⁺/NADH ratios during perfusion of isolated rat heart with glucose (11 mM) and palmitate (0.6 mM, bound to 0.1 mM-albumin)*

All units (except for oxaloacetate) are μ mol/g fresh wt. (where fresh wt. = dry wt. $\times$ 5) and expressed as means $\pm$ S.E.M. with the number of determinations in parentheses. Oxaloacetate units: pmol/g fresh wt. For definition of non-pumping and pumping hearts, see the text. Perfusion period, 30 min. For critical evaluation of assumptions made in calculation of oxaloacetate concentrations and NAD⁺/NADH ratios, see the text. The malate concentration was calculated from the overall tissue content which included extracellular fluid, by allowing for the extracellular malate (which could not be detected) and the sorbitol space. N.S., Not significant.

	Non-pumping		Pumping		<i>P</i>
Heart content of:					
α -Oxoglutarate	0.085 $\pm$ 0.010	(7)	0.177 $\pm$ 0.020	(7)	<0.005
Glutamate	4.69 $\pm$ 0.26	(7)	3.63 $\pm$ 0.19	(7)	<0.005
NH ₄ ⁺	0.266 $\pm$ 0.022	(7)	0.508 $\pm$ 0.048	(7)	<0.001
Malate	0.244 $\pm$ 0.016	(31)	0.310 $\pm$ 0.018	(25)	<0.01
Heart concentration of:					
Malate	0.456 $\pm$ 0.030	(31)	0.579 $\pm$ 0.034	(25)	<0.01
Calculated oxaloacetate concentration from:					
(1) Glutamate dehydrogenase system	28 $\pm$ 11	(7)	105 $\pm$ 13	(7)	<0.001
(2) β -Hydroxybutyrate dehydrogenase	80 $\pm$ 7	(24)	159 $\pm$ 76	(18)	<0.005
Calculated free NAD ⁺ /NADH from (1)	1.4 $\pm$ 0.3	(7)	6.4 $\pm$ 0.8	(7)	<0.001
Calculated free NAD ⁺ /NADH from (2)					
1. 5 min perfusion	9.5 $\pm$ 1.4	(20)	19.0 $\pm$ 4.1	(13)	<0.01
2. 30 min perfusion	5.7 $\pm$ 0.7	(24)	7.5 $\pm$ 1.0	(17)	N.S.

Table 3. *Effect of increased heart work on tissue contents of glycogen, adenine nucleotides, phosphocreatine, P_i, citrate, lactate, pyruvate, α -glycerophosphate and dihydroxyacetone phosphate in isolated perfused rat heart*

Units are $\mu\text{mol/g}$ fresh wt. (fresh wt. $\times 5$) expressed as means $\pm$ s.e.m.; 30 min recirculation perfusion. Initial perfusate concentrations given. Palmitate bound to 0.1 mM-albumin. Ketone bodies are β -hydroxybutyrate (2 mM) and acetoacetate (2 mM). N.S., Not significant.

Substrate	...	Glucose (11 mM) + palmitate (0.6 mM)			Glucose (11 mM) + ketone bodies (4 mM)		
		Non-pumping	Pumping	P	Non-pumping	Pumping	P
ATP		3.87 $\pm$ 0.27 (12)*	2.40 $\pm$ 0.15 (12)†	<0.001	4.58 $\pm$ 0.16 (18)*	3.65 $\pm$ 0.09 (24)†	<0.001
ADP		1.11 $\pm$ 0.06 (4)	1.04 $\pm$ 0.07 (4)	N.S.	1.12 $\pm$ 0.03 (18)	1.03 $\pm$ 0.04 (17)	N.S.
AMP		0.27 $\pm$ 0.045 (4)	0.29 $\pm$ 0.035 (4)	N.S.	0.29 $\pm$ 0.03 (18)	0.25 $\pm$ 0.01 (15)	N.S.
P _i		3.94 $\pm$ 0.24 (4)	5.68 $\pm$ 0.81 (4)	N.S.	3.75 $\pm$ 0.56 (6)	6.94 $\pm$ 0.53 (13)	<0.001
Creatine phosphate		5.14 $\pm$ 0.22 (12)	3.64 $\pm$ 0.30 (12)*	<0.001	6.15 $\pm$ 0.43 (18)	4.80 $\pm$ 0.24 (24)*	<0.01
Citrate		0.389 $\pm$ 0.068 (3)†	0.134 $\pm$ 0.052 (3)†	<0.05	1.095 $\pm$ 0.063 (22)†	0.723 $\pm$ 0.047 (14)†	<0.005
Pyruvate		0.027 $\pm$ 0.004 (12)†	0.084 $\pm$ 0.009 (8)	<0.001	0.065 $\pm$ 0.010 (8)†	0.089 $\pm$ 0.007 (14)	N.S.
Lactate		0.527 $\pm$ 0.065 (15)*	1.412 $\pm$ 0.098 (11)	<0.001	1.95 $\pm$ 0.56 (14)*	1.62 $\pm$ 0.25 (21)	N.S.
Dihydroxyacetone phosphate		0.013 $\pm$ 0.004 (4)*	0.017 $\pm$ 0.004 (4)	N.S.	0.021 $\pm$ 0.001 (5)†*	0.025 $\pm$ 0.004 (6)†	N.S.
α -Glycerophosphate		0.121 $\pm$ 0.106 (4)	0.184 $\pm$ 0.058 (4)	N.S.	0.268 $\pm$ 0.086 (3)†	0.225 $\pm$ 0.044 (6)†	N.S.
Ratio of contents of:							
(1) Lactate/pyruvate		19 $\pm$ 5 (8)†	18 $\pm$ 3 (8)	N.S.	47 $\pm$ 5 (8)†	24 $\pm$ 2 (14)	<0.001
(2) α -Glycerophosphate/dihydroxyacetone phosphate		6 $\pm$ 4 (8)	9 $\pm$ 2 (8)	N.S.	13 $\pm$ 3 (3)†	15 $\pm$ 4 (6)	N.S.
Calculated cytoplasmic free NAD ⁺ /NADH ratio							
From system (1)		476 $\pm$ 118 (12)	502 $\pm$ 84 (8)	N.S.	190 $\pm$ 20 (8)	378 $\pm$ 27 (14)	<0.001
From system (2)		1913 $\pm$ 1275 (8)	1275 $\pm$ 283 (8)	N.S.	885 $\pm$ 195 (3)†	765 $\pm$ 187 (6)†	N.S.

* $P < 0.05$ for comparison of glucose + palmitate with glucose + ketone bodies.

† $P < 0.005$ for comparison of glucose + palmitate with glucose + ketone bodies.

‡ Values from 60 min perfusions.

sorbitol spaces the intracellular concentrations of ketone bodies were very low (see Morgan *et al.*, 1961), and extracellular ketone-body ratios were used in the calculation on the assumption that these ratios reflected those available to the mitochondrial enzyme. Calculated mitochondrial oxaloacetate values during production of ketone bodies from fatty acid-perfused hearts were about twice as high in pumping as in non-pumping hearts (Table 1); the changes in calculated oxaloacetate concentrations were chiefly related to changes in the concentration of malate (Table 2). The possibility of altered rates of pyruvate carboxylation, as may occur in liver, is rendered unlikely because CO₂ fixation and entry of pyruvate into the citrate cycle by routes other than decarboxylation is not an important pathway in the perfused rat heart (Evans *et al.*, 1963).

As calculated from the tissue contents of reactants of the glutamate dehydrogenase system, the mitochondrial oxaloacetate concentration rose three to four times during increased heart work (Table 1). In pumping hearts similar oxaloacetate values were obtained by the use of either of the dehydrogenase systems (glutamate or β -hydroxybutyrate), but in non-pumping hearts use of the β -hydroxybutyrate dehydrogenase system gave oxaloacetate concentrations which were two to three times higher (probably owing to failure to reach equilibrium, see below).

When the mitochondrial free NAD⁺/NADH ratios were calculated from the glutamate dehydrogenase system, the ratio was 6.4 in pumping and 1.4 in non-pumping hearts. These values do not allow for dilution of tissue water by extracellular fluid in the whole-heart biopsy. However, the extracellular values of α -oxoglutarate, glutamate and NH₄⁺ were all negligibly low and the sorbitol space did not change with heart work. Hence equal dilution factors would apply in calculating the true tissue concentration of each compound and the final NAD⁺/NADH ratio would be unchanged. Values calculated from the medium β -hydroxybutyrate/acetoacetate ratio only corresponded to the above values in pumping hearts and after 30 min perfusion; other values suggest equilibrium was not reached, in agreement with the findings of Lai & Scheuer (1975).

Effect of increased heart work on high-energy phosphate compounds (Table 3)

During increased heart work in hearts perfused with glucose and palmitate or ketone bodies, tissue contents of creatine phosphate and ATP fell; ATP contents were lower with palmitate-containing perfusates, in keeping with unpublished results showing that palmitate decreases tissue ATP values in both non-pumping and pumping hearts. There were no changes in the contents of ADP and AMP.

Table 4. *Effect of increased heart work on medium concentrations of ketone bodies, glucose, lactate and pyruvate and on ratios of β -hydroxybutyrate/acetoacetate in tissue and in perfusion medium of isolated rat heart perfused with glucose (11 mM) and ketone bodies (4 mM)*

	Non-pumping hearts			Pumping hearts		
	0	30	60	0	30	60
Duration of perfusion (min)	...	...	...	...	...	...
Acetoacetate (mM)	1.89 ± 0.09 (22)	1.25 ± 0.10 (22)	1.05 ± 0.19 (10)	1.84 ± 0.08 (23)	1.05 ± 0.09 (22)	0.61 ± 0.10 (10)
β -Hydroxybutyrate (mM)	1.73 ± 0.06 (22)	1.87 ± 0.07 (22)	2.16 ± 0.10 (10)	1.74 ± 0.04 (23)	1.65 ± 0.05 (23)	1.49 ± 0.13 (10)
Glucose (mM)	11.06 ± 0.05 (10)	10.62 ± 0.07 (10)	10.26 ± 0.16 (10)	10.97 ± 0.05 (10)	10.28 ± 0.11 (10)	9.57 ± 0.22 (10)
Lactate (mM) (C3)	0.10 ± 0.02 (10)	0.82 ± 0.15 (10)	1.58 ± 0.27 (27)	0.25 ± 0.04 (10)	1.03 ± 0.19 (10)	1.50 ± 0.22 (10)
Pyruvate (μ M) (C3)	13 ± 2 (10)	60 ± 7 (10)	87 ± 16 (10)	26 ± 2 (10)	79 ± 6 (10)	97 ± 11 (10)
Medium ratios (β/A)	1.0 ± 0.03 (18)	1.7 ± 0.08 (17)	2.8 ± 0.3 (9)	1.0 ± 0.03 (18)	1.6 ± 0.06 (19)	2.6 ± 0.2 (10)
Tissue ratio (β/A)	1.0 ± 0.05 (10)	2.3 ± 0.2 (12)	2.1 ± 0.3 (7)	1.1 ± 0.11 (10)	2.0 ± 0.1 (12)	2.7 ± 0.5 (6)

Hearts from rats fed *ad lib.* were pre-perfused for 15 min in a washout system and then recirculated in a Langendorff non-pumping (perfusion pressure 65 cmH₂O), or perfused in a pumping system (atrial-perfusion pressure 20 cmH₂O) for 30–60 min. Initial perfusion medium contained 11 mM-glucose, 2 mM- β -hydroxybutyrate and 2 mM-acetoacetate. At the end of the perfusion period hearts were freeze-clamped and analysed for metabolites (Table 3). Values are means ± S.E.M. with the numbers of determinations in parentheses. β/A , Ratio of β -hydroxybutyrate/acetoacetate.

Table 5. *Sizes of tissue compartments at 60 min in isolated rat hearts perfused with glucose (11 mM) and ketone bodies (2 mM- β -hydroxybutyrate, 2 mM-acetoacetate)*

For procedure see the text and legend to Table 1. The values are μg /g fresh wt. and are means $\pm$ S.E.M. with the number of observations in parentheses. For definition and procedure see Morgan *et al.* (1961).

Tissue compartment	Non-pumping	Pumping
Total water space	849 $\pm$ 3 (11)	862 $\pm$ 3 (14)
Sorbitol space	534 $\pm$ 26 (8)	536 $\pm$ 20 (9)
Glucose space	502 $\pm$ 12 (4)	556 $\pm$ 20 (10)
Lactate space	1583 $\pm$ 269 (10)	1499 $\pm$ 233 (10)
Pyruvate space	850 $\pm$ 124 (10)	787 $\pm$ 119 (10)
Acetoacetate space	564 $\pm$ 42 (7)	498 $\pm$ 44 (6)
β -Hydroxybutyrate space	479 $\pm$ 32 (6)	668 $\pm$ 54 (7)

There was loss of total adenine nucleotides in pumping hearts, concentrations in hearts perfused with glucose and ketone bodies being ($\mu\text{mol/g}$ fresh wt.): 15.5 ± 0.8 (6) after pre-perfusion, 15.3 ± 1.3 (11) for non-pumping hearts after 1 h perfusion and 10.6 ± 0.1 (13) for pumping hearts ($P < 0.005$).

Tissue ratios of ketone bodies (Tables 4 and 5)

In experiments with added ketone bodies (4 mM), the tissue concentrations and ratios were similar to the medium values, which was not surprising because all the ketone bodies apparently measured in the tissue were extracellular (as shown in Table 5 by comparison of the ketone-body space and the sorbitol spaces). Presumably tissue ketone bodies could have been detected by use of much more sensitive methods but other problems were that sorbitol might not always accurately reflect the extracellular space, and that there might have been permeability barriers between ketone bodies in various compartments of the heart cell, as in skeletal muscle (Owen *et al.*, 1973). The possibility that the extracellular ratio could be altered by release of β -hydroxybutyrate dehydrogenase and NAD(H) into the perfusate was eliminated by the measured absence of enzyme from the medium. Tissue ketone bodies could also not be detected in hearts perfused with glucose and palmitate.

Effects of anoxia on medium ratios of β -hydroxybutyrate/acetoacetate (Table 6)

When the Langendorff-perfused heart was changed from a medium equilibrated with O_2 to one equilibrated with N_2 , the uptake of acetoacetate rose 0.5–1 min after the change, and then fell after 10–15 min. These changes were the result of changes in the coronary flow rate, because the concentration of acetoacetate leaving the heart rose from the start of the anoxic period, i.e. the arteriovenous difference decreased. At the same time, the concentration of β -hydroxy-

butyrate in the effluent rose, and the small uptake of β -hydroxybutyrate found in the well-oxygenated hearts changed to output in anoxic hearts. From 2 to 15 min of anoxia, the uptake of acetoacetate was recovered as output of β -hydroxybutyrate. These changes are in accordance with the concept that the reaction catalysed by the activities of the mitochondrial enzyme D- β -hydroxybutyrate dehydrogenase can respond to the redox state of the mitochondrial pool of nicotinamide-adenine dinucleotide. It appears that the equilibrium changes in favour of β -hydroxybutyrate with increased mitochondrial formation of NADH during anoxia. Nevertheless, the ratio β -hydroxybutyrate/acetoacetate in the effluent medium only rose from 1.09 ± 0.02 (23) in well-oxygenated hearts to 1.18 ± 0.2 (25) in anoxic hearts, a highly significant ($P < 0.005$) but only small increase of only 8%. The effluent lactate/pyruvate ratio in anoxic hearts greatly exceeded the value in well-oxygenated hearts. Similar results were obtained in hearts perfused with higher ketone-body concentrations (2.1 mM-acetoacetate, 1.8 mM- β -hydroxybutyrate) with the added presence of glucose (11 mM).

In palmitate-perfused hearts producing ketone bodies, the rate of production of β -hydroxybutyrate was lower in anoxic than in normoxic hearts, but acetoacetate production fell even more. Consequently medium ratios of β -hydroxybutyrate/acetoacetate rose as did lactate/pyruvate ratios in medium and in tissue. Changes in the medium ratios of β -hydroxybutyrate/acetoacetate in response to anoxia occurred only with the very low concentrations of endogenously produced ketone bodies, with only minor changes in the ratio occurring during anoxia in the presence of higher concentrations of added ketone bodies.

Effect of increased heart work on cytoplasmic NAD^+ /NADH ratio and on calculated cytoplasmic oxaloacetate concentration

After 30 and 60 min the ratio lactate/pyruvate in the tissue was lower in pumping than in non-pumping hearts perfused with glucose and ketone bodies, but the ratio α -glycerophosphate/dihydroxyacetone phosphate was the same in pumping and non-pumping hearts at 60 min (ratio not measured at 30 min). However, contents of ATP and creatine phosphate were higher in non-pumping hearts, suggesting that the fall in lactate/pyruvate ratio in such hearts was not caused by hypoxia. Increased heart work might have been expected to change the lactate/pyruvate ratio towards lactate by virtue of a greater flow through glycolysis and also by increased ketone-body utilization (Garland *et al.*, 1964); the changes observed in the present study are unexpected but argue against tissue hypoxia in the pumping heart. Although the lactate dehydrogenase system changed

Table 6. *Effect of anoxia on uptake (+) or output (-) on medium ratios of β -hydroxybutyrate/acetate and lactate/pyruvate in effluent medium on non-pumping perfused rat heart*

Hearts perfused at a low work load (perfusion per 65 cmH₂O hydrostatic pressure); added substrates: D- β -hydroxybutyrate (0.46 mM), acetoacetate (0.49 mM), no glucose. All hearts were first perfused for 5 min with medium equilibrated with O₂ + CO₂ (95:5), followed by either further 15 min perfusion with the same medium or with medium equilibrated with N₂ + CO₂ (95:5). Mean values $\pm$ s.e.m. (numbers of observations). Units for uptake/output: mmol/min per g wet wt.

Conditions	Equilibrating gas	Coronary flow rate (ml/min per g)	Concentrations in effluent medium (mM)		Ratio β -hydroxybutyrate/acetate in effluent medium	Acetoacetate uptake	β -Hydroxybutyrate uptake or output(-)
			Acetoacetate	β -Hydroxybutyrate			
No heart		—	492 $\pm$ 2 (19)	456 $\pm$ 2 (11)	0.93 $\pm$ 0.002 (11)	0	0
Duration of further perfusion (min):							
0.5-1	O ₂	5.5 $\pm$ 0.1 (3)	403 $\pm$ 3 (3)	436 $\pm$ 23 (3)	1.08 $\pm$ 0.07 (3)	+485 $\pm$ 25 (3)	+111 $\pm$ 133 (4)
2-2.5	O ₂	5.6 $\pm$ 0.3 (4)	402 $\pm$ 7 (5)	440 $\pm$ 15 (5)	1.09 $\pm$ 0.03 (5)	+475 $\pm$ 50 (4)	+25 $\pm$ 81 (4)
5	O ₂	5.6 $\pm$ 0.2 (4)	397 $\pm$ 10 (5)	437 $\pm$ 16 (5)	1.08 $\pm$ 0.05 (5)	+498 $\pm$ 71 (4)	+85 $\pm$ 119 (4)
10	O ₂	5.5 $\pm$ 0.1 (5)	395 $\pm$ 11 (5)	429 $\pm$ 13 (5)	1.09 $\pm$ 0.04 (4)	+518 $\pm$ 81 (4)	+199 $\pm$ 78 (4)
15	O ₂	6.1 $\pm$ 0.2 (4)	393 $\pm$ 7 (5)	423 $\pm$ 19 (5)	1.07 $\pm$ 0.03 (5)	+589 $\pm$ 65 (4)	+231 $\pm$ 147 (4)
0.5-1	N ₂	9.4 $\pm$ 0.4 (5)*	418 $\pm$ 1 (5)†	471 $\pm$ 14 (5)	1.13 $\pm$ 0.04 (5)	+692 $\pm$ 102 (5)	-121 $\pm$ 123 (5)
2-2.5	N ₂	9.7 $\pm$ 0.7 (5)*	429 $\pm$ 6 (5)†	511 $\pm$ 13 (5)†	1.19 $\pm$ 0.03 (5)†	+602 $\pm$ 61 (5)	-545 $\pm$ 139 (5)†
5	N ₂	7.1 $\pm$ 0.9 (5)	435 $\pm$ 5 (5)*	524 $\pm$ 12 (5)†	1.20 $\pm$ 0.02 (5)†	+408 $\pm$ 68 (5)	-440 $\pm$ 35 (5)†
10	N ₂	3.7 $\pm$ 1.2 (5)	441 $\pm$ 8 (5)†	419 $\pm$ 16 (5)†	1.18 $\pm$ 0.05 (5)	+151 $\pm$ 22 (5)	-172 $\pm$ 46 (5)†
15	N ₂	3.0 $\pm$ 0.5 (5)†	449 $\pm$ 8 (5)†	512 $\pm$ 5 (5)†	1.18 $\pm$ 0.04 (5)	+112 $\pm$ 21 (5)†	-199 $\pm$ 90 (5)

* For difference between hearts equilibrated in O₂ and N₂, $P < 0.001$.

† For difference between hearts equilibrated in O₂ and N₂, $P < 0.01$.

‡ For difference between hearts equilibrated in O₂ and N₂, $P < 0.05$.

For all the other values, $P > 0.05$.

towards pyruvate formation, the glycerophosphate dehydrogenase system did not change towards dihydroxyacetone phosphate formation. In hearts perfused with glucose and palmitate, cytoplasmic free NAD^+/NADH ratios were similar whichever system of reactants was used. Thus the addition of ketone bodies to the perfusate, as in the diabetic state, caused dissociation of the cytoplasmic pools of NAD^+/NADH associated with the lactate dehydrogenase and glycerophosphate dehydrogenase systems. Calculated cytoplasmic oxaloacetate concentrations exceeded mitochondrial values by 20–40 times in accordance with the oxaloacetate gradient postulated for liver by Williamson *et al.* (1967).

Discussion

Problems in the assessment of the mitochondrial NAD^+/NADH ratio from β -hydroxybutyrate dehydrogenase system in the perfused rat heart

Considering the principles derived by Williamson *et al.* (1967) for rat liver, we have investigated the use of the ratios of β -hydroxybutyrate/acetoacetate as an index of the mitochondrial ratios of free NAD^+/NADH .

The activity of β -hydroxybutyrate dehydrogenase is relatively low in rat heart (about $50 \mu\text{mol}$ of NADH formed/min per g at 20°C) being about one-half that of rat liver (Lehninger *et al.*, 1960). An estimate of the activity *in situ* can be obtained from rates of β -hydroxybutyrate production during anoxia (Table 6, peak value, $15 \mu\text{mol}/30\text{min}$ per g at 37°C), during perfusion of non-pumping hearts with acetoacetate (5mm) and insulin, or with 2mm- β -hydroxybutyrate ($26\text{--}33 \mu\text{mol}/30\text{min}$ per g fresh wt. at 37°C ; Williamson & Krebs, 1961). β -Hydroxybutyrate uptake in pumping hearts (perfused with acetoacetate and β -hydroxybutyrate, each 2mm), was about $8 \mu\text{mol}/30\text{min}$ per g. A deviation from NAD^+/NADH equilibrium at the enzyme site could exist if the rate of flow through the enzyme were significant in relation to the enzyme activity (Bücher & Rüssman, 1964). Experiments involving β -hydroxybutyrate production during palmitate perfusions were less open to criticism because the average rate of production from 5 to 30min of perfusion was only $1.0 \mu\text{mol}$ in non-pumping hearts and even less in pumping hearts.

The heart concentrations of ketone bodies are not readily measured (Table 5; Adler-Kastner *et al.*, 1972; Fath & Kako, 1973). We used the extracellular (perfusate) ratios of ketone bodies taken up or produced on the assumption that such ratios are related to the heart tissue ratios, but there may be concentration gradients in skeletal muscle (Owen *et al.*, 1973). It should be noted that: (1) the production rate of β -hydroxybutyrate fell and the uptake rate of β -hydroxybutyrate rose during increased heart work,

when other evidence also suggests a change in the mitochondrial NAD^+/NADH towards NAD^+ (see below); (2) the uptake of β -hydroxybutyrate by the non-pumping heart changed to output during anoxia (Table 6) when there is a simultaneous change in flavin and pyridine fluorescence towards the reduced forms (Scholz *et al.*, 1969; Chance *et al.*, 1972). These directional changes suggest that the metabolism of extracellular ketone bodies may in specified conditions reflect the direction of changes in the mitochondrial NAD^+/NADH ratio in spite of the low activity of β -hydroxybutyrate dehydrogenase.

In pumping hearts the close correspondence between mitochondrial ratios of free NAD^+/NADH calculated from either the β -hydroxybutyrate dehydrogenase or the glutamate dehydrogenase system could be interpreted to suggest that equilibrium has been reached during ketone-body production, which may be ascribed to the very low extracellular ketone-body concentrations in pumping hearts (total, $8 \mu\text{M}$, Table 1), compared with non-pumping hearts (total, 0.05mM). Equilibrium conditions could not hold during perfusions with added ketone bodies in concentrations such as 4mm; mitochondrial oxaloacetate concentrations calculated from data obtained from hearts perfused in such conditions (Krebs, 1970; Opie *et al.*, 1971b) are probably misleading.

Problems in the use of the glutamate dehydrogenase system

Lai & Scheuer (1975) also used the values of the reactants of the glutamate dehydrogenase system for calculation of mitochondrial free NAD^+/NADH ratios. In rat heart homogenates the reactants of the glutamate dehydrogenase system reached equilibrium as rapidly as did those of the lactate dehydrogenase reaction, whereas the β -hydroxybutyrate dehydrogenase system failed to reach equilibrium even after 30min. In addition, in isolated rat heart mitochondria, the ratio glutamate/ α -oxoglutarate $\times \text{NH}_4^+$ doubled as the oxygen content of the equilibrating gas decreased from 100 to 20%, and rapidly increased further when the oxygen content was decreased from 5 to 0% (Lai & Scheuer, 1975).

A major problem in the use of the glutamate dehydrogenase reaction is that the activity of the transaminase system, involving the malate-aspartate shuttle for transport of reducing equivalents from cytoplasm to mitochondria, is thought to be high (La Noue *et al.*, 1973): the possibility of equilibrium at the level of glutamate dehydrogenase is thereby lessened. The comparative activities of the relevant enzymes are glutamate dehydrogenase, $322 \mu\text{mol}/\text{h}$ per g at 25°C (Williamson *et al.*, 1967), glutamate-oxaloacetate transaminase, $3800 \mu\text{mol}/\text{h}$ per g wet wt. at 25°C , hence the activity of the transaminase system could introduce significant deviations in the

glutamate dehydrogenase system from equilibrium (Cryer & Bartley, 1974). However, measurements of the reactants of the glutamate-oxaloacetate system (Neely *et al.*, 1972) suggest that there is no major change in equilibrium during increased heart work; by analogy with rat liver *in vivo* (Brosnan & Williamson, 1974) the two enzyme systems could be in equilibrium. In the non-pumping rat heart, mitochondrial NAD^+/NADH ratios, calculated by using the glutamate dehydrogenase system, moved in the direction of NADH during impaired oxygenation; the rate of change was, however, slow compared with epicardial NADH fluorescence, suggesting that the glutamate dehydrogenase system did respond to mitochondrial NAD^+/NADH changes but did not accurately reflect rapid changes (Lai & Scheuer, 1975). In addition, the reactants may not be located exclusively in the mitochondria (La Noue *et al.*, 1972), although the force of this argument has been countered in the case of the liver (Williamson *et al.*, 1967).

The mitochondrial free NAD^+/NADH ratios rose during increased heart work when calculated from ratios of ketone-body production at 5 but not at 30 min, whereas when calculated from the reactants of the glutamate dehydrogenase system the mitochondrial NAD^+/NADH of 1.4 in non-pumping hearts rose to 6.4 in pumping hearts after 30 min (Table 2). Owing to the reservations expressed above, these values indicate a direction of change rather than an absolute change. Collateral evidence for a change in the mitochondrial NAD^+/NADH ratio to NAD^+ during increased heart work is that total mitochondrial NAD^+/NADH (i.e. free and bound) also increased towards NAD^+ as assessed by direct measurement and by epicardial fluorescence changes in conditions in which cytoplasmic NAD^+/NADH changes were minimal, when the peak intraventricular pressure was increased by increasing the aortic perfusion pressure (Moravec *et al.*, 1974). In rat heart mitochondria incubated with pyruvate and malate, the measured total NAD^+/NADH ratio varies from 1 to 10, comparing state 4 with state 3 respiration (La Noue *et al.*, 1972); increased heart work is probably associated with a state 4 to state 3 change (Moravec *et al.*, 1974). In a computer-simulated model of metabolic regulation in rat hearts perfused with glucose, M. J. Achs & D. Garfinkel (personal communication) found that the mitochondrial NAD^+/NADH ratio rose threefold within 20 s of the onset of increased heart work induced by atrial perfusion. A change in the mitochondrial NAD^+/NADH towards NAD^+ during increased heart work has also been suggested by Neely *et al.* (1972) on the basis of large decreases in the ratios malate/oxaloacetate, glutamate/ α -oxoglutarate and isocitrate/ α -oxoglutarate; however, their measurements of malate and oxaloacetate were

on the whole heart tissue, and the problem of separate tissue pools of NAD^+/NADH was not fully assessed.

Problems in assessment of mitochondrial oxaloacetate concentration

If the evidence presented argues for a change in the mitochondrial ratio of free NAD^+/NADH towards NAD^+ with increased heart work, could the direction of change of the mitochondrial oxaloacetate concentration also be assessed?

Each of the three assumptions required for the calculation of the mitochondrial oxaloacetate concentration from the mitochondrial free NAD^+/NADH ratio (see under 'Methods') requires careful appraisal. First, the malate dehydrogenase system may not be at equilibrium. Malate dehydrogenase is found both in mitochondrial and cytoplasmic compartments, each of which also contains separate pools of NAD^+/NADH ; hence it is the equilibrium of the reactants of the malate dehydrogenase located in the mitochondria that is relevant. Malate dehydrogenase is not thought to be subject to allosteric control nor to be a site of regulation in the tricarboxylic acid cycle (Newsholme & Start, 1973). A further argument for equilibrium stems from a comparison of the activity of the enzyme with the peak rate of flow through mitochondrial malate dehydrogenase in the pumping heart. The total activity of malate dehydrogenase in the left ventricle of the rat heart is 179 units/g wet wt. at 25°C (Cryer & Bartley, 1974). The calculated flux through mitochondrial malate dehydrogenase in the pumping heart is 154 $\mu\text{mol}/30\text{min}$ per g fresh wt. at 37°C (M. J. Achs & D. Garfinkel, personal communication). If one-half of the activity of malate dehydrogenase is mitochondrial (Bücher & Klingenberg, 1958), then the activity of mitochondrial malate dehydrogenase greatly exceeds (by more than 30 times) the maximum flow through the enzyme in our experimental conditions. Bücher & Rüssmann (1964) showed that the rate of formation of the malate dehydrogenase-NADH complex did not produce any appreciable deviation from equilibrium. From the above points of view, the conditions set by Bücher & Rüssmann (1964) for equilibrium between free NAD^+ and NADH at the enzyme sites appear to be fulfilled. In addition, Randle *et al.* (1970) found isotopic equilibrium between malate and oxaloacetate in the perfused non-pumping rat heart. Thus, although firm evidence for equilibrium of malate dehydrogenase in our conditions is lacking, indirect evidence suggests the existence of near-equilibrium.

Another assumption required to calculate mitochondrial oxaloacetate is that malate be evenly distributed between cytoplasm and mitochondria. Rognstad & Katz (1972) showed that in rat kidney

mitochondria the exchange of radioactively labelled malate was rapid, and suggested that the malate in both cytoplasmic and mitochondrial compartments was close to equilibrium with NADH. Similar data for heart mitochondria are lacking, but the operation of the malate-aspartate shuttle in rat heart mitochondria requires rapid rates of transport of malate through the mitochondrial membranes (La Noue & Williamson, 1971; Digeress & Reddy, 1975).

The third assumption for the calculation of the concentration of mitochondrial oxaloacetate is the absence of compartmentation of mitochondrial NAD^+/NADH pools. Calculated ratios of free NAD^+/NADH from reactants of the glutamate dehydrogenase or β -hydroxybutyrate dehydrogenase systems were similar in rat liver mitochondria (Williamson *et al.*, 1967) and in our pumping hearts but not in non-pumping hearts (Table 2). We suggest that the discrepancies are caused by failure of the β -hydroxybutyrate dehydrogenase reactants to reach equilibrium, but other explanations include imperfect equilibrium of the reactants of the glutamate dehydrogenase system and the existence of more than one mitochondrial pool of NAD^+/NADH in heart.

Direct evidence for a correlation between increased mitochondrial NAD^+/NADH ratios, mitochondrial oxaloacetate concentrations and flux through citrate synthase has been found in isolated rabbit heart mitochondria incubated with high concentrations of malate or succinate (Hull *et al.*, 1973).

Rate control of tricarboxylic acid cycle in pumping heart

The calculated mitochondrial oxaloacetate concentration was three to four times higher during increased heart work (Table 2), and the increase in the O_2 uptake (and hence rates of citrate-cycle turnover) was three times. Owing to the many assumptions involved in the calculations, the present values for mitochondrial oxaloacetate concentrations could only be regarded as provisional. Nevertheless, M. J. Achs & D. Garfinkel (personal communication) used a computer-simulated model to calculate that the mitochondrial oxaloacetate concentration rose about four times within 6 s of the onset of external pumping work in rat hearts perfused with glucose; the computer was provided with data on the rates of increase of glycolytic flux and O_2 uptake, and tissue contents of citrate and malate (Opie *et al.*, 1971a). Their value for the mitochondrial oxaloacetate concentration in pumping hearts was 100 pmol/g fresh wt., corresponding closely to the values in Table 2. The close association between estimated rates of change of mitochondrial oxaloacetate concentrations and citrate-cycle turnover is compatible with a role for changes in the concentrations of oxaloacetate in the control of citrate synthase

activity during increased heart work, especially because of the very low K_m for citrate synthase. Contrary conclusions, suggesting an unchanged or decreased mitochondrial oxaloacetate concentration during increased heart work (Krebs, 1970; Opie *et al.*, 1971b) were based on experiments in which the initial extracellular ketone-body concentration was far too high (4 mM) for equilibrium to be reached in pumping hearts.

Studies with anoxia

In the clinical assessment of hypoxia by extracellular changes, as in coronary sinus blood, changes in rate of uptake of β -hydroxybutyrate are likely to be a more sensitive index of intramitochondrial NAD^+/NADH changes than are changes of the ratio of β -hydroxybutyrate/acetooacetate in the extracellular fluid (Table 6; Whereat & Chan, 1972; Opie *et al.*, 1973). Earlier suggestions that measurement of the extracellular ketone-body ratios could give a good reflexion of the mitochondrial NAD^+/NADH ratio (Borst, 1963; Opie, 1968) do not appear to be justified for the heart.

We thank the Chris Barnard Fund, the Medical Research Council of South Africa and the University of Cape Town for supporting the project. Sir Hans Krebs, F.R.S., Dr. J. Scheuer and Dr. D. H. Williamson are thanked for advice. P. O. was supported by the Chris Barnard Fund.

References

- Adam, A. (1963) in *Methods of Enzymatic Analysis* (Bergmeyer, H., ed.), pp. 573–577, Academic Press, London
- Adler-Kastner, L., Keller, B., Kraupp, O., Stuhlinger, W. & Turnheim, K. (1972) *J. Mol. Cell. Cardiol.* **4**, 391–400
- Atkinson, D. E. (1968) *Biochem. Soc. Symp.* **27**, 23–39
- Berenblum, I. & Chain, E. (1938) *Biochem. J.* **32**, 295–298
- Bernt, E. & Bergmeyer, H.-U. (1963) in *Methods of Enzymatic Analysis* (Bergmeyer, H., ed.), pp. 384–388, Academic Press, London
- Borst, P. (1963) in *Funktionelle und Morphologische Organisation der Zelle*, p. 137, Springer-Verlag, Berlin
- Brosnan, J. T. & Williamson, D. H. (1974) *Biochem. J.* **138**, 453–462
- Bücher, Th. & Klingenberg, M. (1958) *Angew. Chem.* **70**, 552–570
- Bücher, Th. & Rüssmann, W. (1964) *Angew. Chem. Int. Ed. Engl.* **3**, 426–439
- Chain, E. B., Mansford, K. R. L. & Opie, L. H. (1969) *Biochem. J.* **115**, 537–546
- Chance, B., Salkovitz, I. A. & Kovach, A. G. B. (1972) *Am. J. Physiol.* **223**, 207–218
- Cryer, A. & Bartley, W. (1974) *J. Mol. Cell. Cardiol.* **6**, 61–71
- Evans, J. R., Opie, L. H. & Shipp, J. C. (1963) *Am. J. Physiol.* **205**, 766–770

- Fath, P. A. & Kako, K. J. (1973) *J. Mol. Cell. Cardiol.* **5**, 359–373
- Garland, P. B. (1968) *Biochem. Soc. Symp.* **27**, 41–60
- Garland, P. B., Newsholme, E. A. & Randle, P. J. (1964) *Biochem. J.* **93**, 665–678
- Garland, P. B., Shepherd, D., Nicholls, D. G., Yates, D. W. & Light, P. A. (1969) in *Citric Acid Cycle Control and Compartmentation* (Lowenstein, J. M., ed.), pp. 163–212, Marcel Dekker, New York
- Good, C. A., Kramer, H. & Somogyi, M. (1933) *J. Biol. Chem.* **100**, 485–491
- Hohorst, H. J., Kreutz, F. H. & Bücher, T. (1959) *Biochem. Z.* **332**, 18–46
- Hull, F. E., Cheney, H. & Baker, L. (1973) *J. Mol. Cell. Cardiol.* **5**, 319–339
- Kannengiesser, G. J., Lubbe, W. F. & Opie, L. H. (1975) *J. Mol. Cell. Cardiol.* **7**, 135–151
- Kirsten, E., Gerez, C. & Kirsten, R. (1963) *Biochem. Z.* **337**, 312–319
- Kosicki, G. W. & Lee, L. P. K. (1966) *J. Biol. Chem.* **241**, 3571–3574
- Krebs, H. A. (1970) *Advan. Enzyme Regul.* **8**, 335–353
- Krebs, H. A. & Henseleit, K. (1932) *Hoppe-Seyler's Z. Physiol. Chem.* **210**, 33–66
- Lai, F. & Scheuer, J. (1975) *J. Mol. Cell. Cardiol.* in the press
- Lamprecht, W. & Trautschold, I. (1963) in *Methods of Enzymatic Analysis* (Bergmeyer, H., ed.), pp. 543–551, Academic Press, London
- Langendorff, D. (1895) *Pflügers Arch. Gesamte Physiol. Menschen Tiere*, **61**, 291–332
- La Noue, K. F. & Williamson, J. R. (1971) *Metab. Clin. Exp.* **20**, 119–140
- La Noue, K. F., Bryla, J. & Williamson, J. R. (1972) *J. Biol. Chem.* **247**, 667–679
- La Noue, K. F., Walajtys, E. & Williamson, J. R. (1973) *J. Biol. Chem.* **248**, 7171–7183
- Lehninger, A. L., Sudduth, H. C. & Wise, J. B. (1960) *J. Biol. Chem.* **235**, 2450–2455
- Moellering, H. & Gruber, W. (1966) *Anal. Biochem.* **17**, 369–376
- Moravec, J., Corsin, A., Owen, P. & Opie, L. H. (1974) *J. Mol. Cell. Cardiol.* **6**, 187–200
- Morgan, H. E., Henderson, M. J., Regen, D. M. & Park, C. R. (1961) *J. Biol. Chem.* **236**, 253–261
- Neely, J. R., Liebermeister, H., Battersby, E. J. & Morgan, H. E. (1967) *Am. J. Physiol.* **212**, 804–814
- Neely, J. R., Rovetto, M. J. & Oram, J. F. (1972) *Progr. Cardiovasc. Dis.* **15**, 289–321
- Newsholme, E. A. & Start, C. (1973) *Regulation in Metabolism*, Wiley, London
- Olson, M. S. & Williamson, J. R. (1971) *J. Biol. Chem.* **246**, 7794–7803
- Opie, L. H. (1965) *J. Physiol. (London)* **180**, 529–541
- Opie, L. H. (1968) *Am. Heart J.* **76**, 685–698
- Opie, L. H. & Mansford, K. R. L. (1971) *Eur. J. Clin. Invest.* **1**, 295–306
- Opie, L. H., Mansford, K. R. L. & Owen, P. (1971a) *Biochem. J.* **124**, 475–490
- Opie, L. H., Owen, P. & Mansford, K. R. L. (1971b) *Cardiovasc. Res. Suppl.* **1**, 87–95
- Opie, L. H., Owen, P., Thomas, M. & Samson, R. (1973) *Am. J. Cardiol.* **32**, 295–305
- Owen, O. E., Markus, H., Sarshik, S. & Mozzoli, M. (1973) *Biochem. J.* **134**, 499–506
- Randle, P. J., England, P. J. & Denton, R. M. (1970) *Biochem. J.* **117**, 677–695
- Rognstad, R. & Katz, J. (1972) *Biochem. J.* **132**, 349–352
- Saborowski, F., Lang, D., Scholand, C. & Albers, C. (1973) *Annu. Meet. Int. Study Group Res. Cardiac Metab.* 6th in the press
- Seraydarian, K., Mommaerts, W. F. H. M., Wallner, A. & Guillory, R. J. (1961) *J. Biol. Chem.* **236**, 2071–2075
- Scholz, R., Thurman, R. G., Williamson, J. R., Chance, B. & Bücher, T. (1969) *J. Biol. Chem.* **244**, 2317–2324
- Smith, C. M. & Williamson, J. R. (1971) *FEBS Lett.* **18**, 35–38
- Srere, P. A. & Matsuoka, Y. (1972) *Biochem. Med.* **6**, 262–266
- Whereat, A. F. & Chan, A. (1972) *Am. J. Physiol.* **223**, 1398–1406
- Wieland, O. & Weiss, L. (1963) *Biochem. Biophys. Res. Commun.* **13**, 26–31
- Williamson, J. R. & Krebs, H. A. (1961) *Biochem. J.* **80**, 540–547
- Williamson, D. H., Mellanby, J. & Krebs, H. A. (1962) *Biochem. J.* **82**, 90–96
- Williamson, D. H., Lund, P. & Krebs, H. A. (1967) *Biochem. J.* **103**, 514–527
- Wollenberger, A., Ristau, O. & Schoffa, G. (1960) *Pflügers Arch. Gesamte Physiol. Menschen Tiere* **270**, 399–412
- Wright, J. A. & Sanwal, B. D. (1971) *J. Biol. Chem.* **246**, 1689–1699