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The Protective Action of Pyruvate on Recovery of Ischemic Rat Heart: Comparison with Other Oxidizable Substrates

Lucia Cavallini, Marina Valente and Maria Pia Rigobello

Dipartimento di Chimica Biologica and Centro Studio Fisiologia Mitocondriale, Universitá di Padova, via Trieste, 75. Padova, Italy

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L. Cavallini, M. Valente and M. P. Rigobello. The Protective Action of Pyruvate on Recovery of Ischemic Rat Heart. Comparison with other Oxidizable Substrates. *Journal of Molecular and Cellular Cardiology* (1990) **22**, 143–154. The recovery of both contractile performance and metabolic response of rat heart following 1 h of ischemia after equilibration with glucose + insulin (glucose-ischemia) or with pyruvate (pyruvate-ischemia), was tested in normoxic reperfusion in the presence of glucose + insulin, pyruvate, lactate or acetate. In glucose-ischemia only the reperfusion with pyruvate results in a complete recovery of the contractile force (left ventricular pressure, LVP) (170%) and good recovery of high energy phosphate compounds. Lower LVP and tissue energy charge were found in glucose reperfusion and even less in lactate and acetate reperfusion. Disappearance of the IMP accumulated during ischemia is evident only in the pyruvate reperfusion indicating a higher metabolic recovery. On the contrary in pyruvate-ischemia all types of reperfusion tested were effective in reactivating the contractile force (although acetate to a lesser extent); the contractile activity was accompanied by a good recovery of phosphocreatine, ATP, energy charge and by the decrease of IMP. Large decreases of adenine nucleotides and NADP and lower decreases of NAD are observed during ischemia/reperfusion in both systems.

Pyruvate-ischemia is quite similar to, if not worse than glucose-ischemia, for all the metabolic parameters considered, but not worse for the possibility of recovery. Some specific effect of pyruvate should be exerted during the ischemic phase.

The mechanism of pyruvate protection is discussed in relationship to: (i) the possible activation of pyruvate dehydrogenase, (ii) the activation of NADPH-dependent peroxide scavenging systems, (iii) the direct scavenging action of pyruvate on H_2O_2.

Key Words: Pyridine nucleotides; GSH; Oxidative stress; Adenine nucleotides; Pyruvate; Heart perfusion.

Introduction

Several papers have reported the beneficial effect of pyruvate alone or in the presence of glucose on contractile performance and other physiological parameters (Liedtke and Nellis, 1978; Mochizuki and Neely, 1980; Bünger *et al.*, 1986; Zweier and Jacobus, 1987; Camacho *et al.*, 1988) of normoxic perfused hearts (Liedtke and Nellis, 1978; Zweier and Jacobus, 1987), as well as in various kinds of ischemic or anoxic conditions in different animals (Liedtke and Nellis, 1978; Mochizuki and Neely, 1980; Bünger *et al.*, 1986; Camacho *et al.*, 1988). Such an effect was correlated to several parameters, i.e. the increase of ATP and phosphocreatine (PCr) (Mochizuki and Neely, 1980; Bünger *et al.*, 1986; Zweier and Jacobus, 1987) the lowering of the ischemic acidosis (Liedtke and Nellis, 1978; Camacho *et al.*, 1988) and the stimulation of the tricarboxylic acid cycle on reperfusion (Liedtke and Nellis, 1978).

However it has to be pointed out that in some experimental conditions pyruvate failed to protect myocardium from ischemic damage (Hearse and Chain, 1972) and in some cases it even resulted in a higher incidence of arrhythmias (Bricknell and Opie, 1978) without altering the ATP or PCr content.

The capability of pyruvate to stimulate oxidative metabolism could be related to its efficiency in reoxidizing cytosolic NADH; such

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an effect is inferred by Bünger *et al.* (1986) but direct measurements are not available. It is debated if this effect is dependent on lactate dehydrogenase (LDH) since the latter is reported to be inhibited by high pyruvate concentration (Kaplan and Goodfriend, 1964; Peuhkurinen and Hassinen, 1982). Furthermore a modulation of tricarboxylic acid (TCA) cycle has to be considered since during ischemia an inhibition of the active complex of pyruvate dehydrogenase could result by means of the coupled stimulation of PDH kinase and the inhibition of PDH phosphatase accompanied by a decrease of NAD^+/NADH (Pettit *et al.*, 1975; Vary and Randle, 1984).

Another mechanism responsible for pyruvate protection has been proposed by Camacho *et al.* (1988) who demonstrated a lower decrease of pH during ischemia together with a limitation of the loss of adenosine nucleotide (AdN) derivatives in the reperfusion.

We used the model of total ischemia *in vitro* (no flow at 37°C) followed by normoxic reperfusion for its similarity to *in vivo* conditions where essentially no washout of metabolite occurs from the ischemic zone as reported by Jennings *et al.* (1981). The present study was carried out to elucidate the parameters and the phases of the ischemia–reperfusion in which pyruvate exerts its maximum effect compared to glucose + insulin, lactate or acetate. The parameters considered, all primarily involved in the recovery process, were: PCr, ATP, ADP, AMP, IMP, pyridine nucleotides (amount and redox state), glutathione (content and redox state). Glutathione and LDH effluxes were measured as markers of oxidative stress and membrane damage. The aim of our study was also to provide some insight for the possible utilization of physiological substrates in the reperfusion period as modulators of heart metabolism and as agents which counteract the oxidative stress, hence increasing the possibility of recovery of ischemic hearts.

Materials and Methods

Reagents

The enzymes and coenzymes were from Sigma Chemical Co., (St. Louis, MO). Substrates were from Boehringer GmbH, (Mannheim, W. Germany). The routine chemicals and glucose were obtained from E. Merck AG (Darmstadt, W. Germany). Insulin was from Eli Lilly and Co. (Indianapolis).

Wistar albino rats (300 g) from the department's own stock were used. Hearts excised from the rats anaesthetized with ether and decapitated were perfused at 37°C by a non-recirculating Langendorff procedure (Lagendorff, 1885) with a Krebs–Henseleit medium composed of 115 mM NaCl, 25 mM $NaHCO_3$, 4 mM KCl, 0.9 mM KH_2PO_4, 1.1 mM $MgCl_2$, 1.5 mM $CaCl_2$, at pH 7.4. The substrates added were 5.5 mM glucose + 5 U/l insulin, and all the other substrates were 5 mM. The medium was kept at 37°C and saturated with 95% O_2 and 5% CO_2. Ventricular pressure (LVP) was measured by introducing into the left ventricular cavity a liquid-filled balloon connected to a pressure transducer. During normoxic perfusion the coronary flow rate was 12 ml/min, while in the ischemia the flow was completely abolished. Hearts were maintained at 37°C during ischemia in a thermostated chamber. On starting reperfusion, the flow was increased to 12 ml/min in about 30 s. The above conditions of perfusion and ischemia were utilized to perform two experimental models: *glucose-ischemia* and *pyruvate-ischemia*. In glucose-ischemia hearts were equilibrated with glucose + insulin for 20 to 30 min, in order to obtain haemodynamic and metabolic stabilization and then made ischemic for 1 h: when reperfused only one substrate was present and reperfusion started 30 min after the maximum of resting pressure was reached, i.e. after 50 to 60 min of total ischemia. Reperfusion was performed for 30 min. In the pyruvate-ischemia the only difference was the presence of pyruvate instead of glucose + insulin during the equilibration phase.

Fractions of the coronary effluent were utilized for the determination of the glutathione and LDH efflux. At the end of the perfusion, the hearts were quickly-frozen with aluminum clamps pre-cooled with liquid N_2 and immersed into liquid N_2. If not used immediately, frozen samples were maintained at −70°C. One weighed portion was homogenized with an Ultra Turrax in 5 ml of cold 125 mM NaCl medium, buffered at pH 7.4 with 15 mM HEPES and 15 mM Tris, containing 1 mM

EDTA + 5 mM EGTA, and used for the determination of tissue glutathione and protein; 2-ml aliquots were centrifuged at 105000 **g** for 30 min and the supernatant was used for the determination of the LDH activity.

The second portion of the tissue was subjected to alkaline extraction for the determination of adenine and pyridine nucleotides (by means of this extraction reduced forms of NAD(P) could also be recovered): the portion was ground in a porcelain mortar immersed in liquid N_2 and the powders underwent an alkaline extraction essentially as described by Stocchi *et al.* (1985). Modifications include the treatment of the frozen powders with 4 ml of 0.5 M KOH and, after vigorous mixing, a centrifugation at 15000 **g** for 3 min; the clear supernatant neutralized with 1.4 ml of 0.1 M KH_2PO_4 was then placed on a CF 50 A Amicon membrane and centrifuged at 2500 **g** for 20 min in a refrigerated Sorvall centrifuge. Aliquots of the neutralized filtrate were quickly-frozen in liquid N_2 and rigorously maintained at −70°C until used for the HPLC determination. One aliquot of each sample was utilized for PCr determination (as ATP formed) after incubation for 10 min at 30°C in the presence of 5 U of creatine phosphokinase, 1.5 mM $MgCl_2$ and 1 mM ADP, at pH 6.5 where the reverse reaction does not occur. At the end of the incubation the sample was filtered through a 0.22 μm Millipore filter and quickly frozen. Adenine and pyridine nucleotides were determined at 260 nm essentially by following the HPLC procedure described by Stocchi *et al.* (1985) after separation on a Perkin Elmer C-18 10 μm × 25 cm × 4.6 mm i.d. column at 1 ml/min flow. The gradient program used for the separation of the various nucleotides was: 6 min in isocratic condition with 0.1 M KPi pH 5.5 followed by a 5 min linear gradient to 100% of 0.1 M KPi pH 6.0 containing 10% methanol; separation was accomplished in about 30 min. Retention times and peak areas were obtained by a Perkin Elmer LC-100 laboratory computing integrator. NADH and NADPH were fluorimetrically determined at 470 nm using an excitation wavelength at 340 nm in a parallel separation by means of a Shimadzu R.F. 530 fluorescence HPLC monitor. Samples were defrosted immediately before the injection since NADH and NADPH are prone to disappear also after brief intervals at 0°C at neutral pH.

Protein content of the homogenate was determined on the 15% (w/v) TCA precipitated protein by the biuret method (Gornall *et al.*, 1949). Glutathione was measured essentially with the procedure of Tietze (1969) modified for the determination of GSSG as described by Xia *et al.* (1985), in which GSH is enzymatically trapped by methylglyoxal in the presence of glyoxalase I. LDH was determined at 25°C by following the NADH oxidation sparked by 5 mM pyruvate on 5 μl of the 105000 **g** supernatant fraction of the homogenized tissue diluted to 2.5 ml with 0.05 M KPi pH 7.4, or in 2.5 ml of the coronary effluent fraction; when lactate (5 mM) was used as the exogenous substrate 0.5 ml aliquots of coronary effluent were used in order to avoid the reverse reaction.

All parameters reported in the various tables are referred to the total heart. The average weight of hearts used in our experiments was 1.2 ± 0.1 g. The weight remained constant in controls but it varied in other conditions: it decreased during ischemia down to 0.8 g and it increased variously after reperfusion due to water retention. The highest increases (up to 1.8 g) were observed when the recoveries were the lowest.

Results

Glucose-ischemia

The *in vitro* Langendorff perfusion of rat hearts can be considered a suitable model for the study of heart metabolism due to its stability when appropriate substrates are added to the perfusion medium. Table 1 shows that the total amount of AdN and PCr do not decrease in comparison to the unperfused rat heart, while the value of the tissue energy charge shows a slight increase. The improvement is surely due to the rapid freeze clamping of the beating heart perfused *in vitro*. When ischemia is induced in glucose + insulin perfused hearts by stopping the flow, a sudden decline of the developed pressure occurs and heart arrests in a few minutes [Fig. 1(a)]. As can be seen in the figure, after 20 to 30 min the resting pressure increases to a maximum followed by a slow decrease to a plateau after about 50 to

TABLE 1. Effect of ischemia and reperfusion with various substrates on high energy phosphate compounds in glucose equilibrated heart

Conditions	Phospho-creatine (μmol/heart)	Total AdN (μmol/heart)	IMP (μmol/heart)	Charge
Glucose controls	3.70 ± 0.85 (5)	4.06 ± 0.32 (5)	n.d.	0.84 ± 0.02 (5)
Unperfused controls	3.08 ± 0.8 (3)	3.94 ± 0.7 (3)	n.d.	0.77 ± 0.04 (3)
Glucose-ischemia	0.18 ± 0.07 (5)	1.88 ± 0.42 (5)	0.124 ± 0.083 (6)	0.28 ± 0.05 (5)
Glucose reperfused	0.87 ± 0.19 (5)	1.53 ± 0.18[a] (5)	0.02 ± 0.06 (4)	0.65 ± 0.06[a] (5)
Pyruvate reperfused	1.42 ± 0.34[a] (5)	1.48 ± 0.11[a] (5)	n.d.	0.66 ± 0.04[a] (5)
Lactate reperfused	0.35 ± 0.21[c] (5)	1.10 ± 0.18[a,b,c] (5)	0.02 ± 0.01 (5)	0.42 ± 0.04[a,b,c] (5)
Acetate reperfused	0.94 ± 0.31[a] (5)	1.05 ± 0.22[a,b,c] (5)	0.06 ± 0.07 (5)	0.51 ± 0.06[a,b,c] (5)

n.d., not detected.

Values reported as μmol/whole heart are the mean ± S.D. Number of experiments in parentheses. ATP, ADP, AMP and IMP are reported as % of the corresponding total AdN content. Tissue energy charge (Charge) is defined as {[ATP] + 1/2[ADP]}/[total AdN].

Statistical analysis of data was performed by "one way analysis of variance" (ANOVA). Values significant at confidence level of 95% are indicated: [a] when referred versus ischemia; [b] when versus glucose reperfused; [c] when versus pyruvate reperfused.

60 min ischemia. In a previous work (Bindoli *et al.*, 1988b) we found that reperfusion with glucose without insulin induces a sudden increase of the resting pressure and a very poor contractile recovery. This is associated to a rapid and massive efflux of glutathione (mainly in its reduced form). As shown in Figure 1(b), inclusion of insulin, which is reported (Maroko *et al.*, 1972; Mochizuki and Neely, 1980) to improve the recovery of ischemic hearts, induces a partial recovery of contractility (average recovery 42% of the pressure developed before ischemia). If pyruvate is used on reperfusion instead of glucose + insulin a more than complete recovery of the contractile force is obtained. Lactate or acetate are far less effective. The resumption of contractility on reperfusion was observed in 100% of the experiments with pyruvate, in 75% of those with glucose + insulin (three over four), in 40% of those with lactate and acetate (two over five). The metabolic data reported in Table 1 are consistent with the effect shown on LVP: PCr which is depleted during ischemia is only partially recovered in lactate reperfusion, slightly more in the glucose + insulin and acetate reperfusion, while the highest increase is observed in pyruvate-reperfused hearts. Higher tissue energy charge are observed both in glucose + insulin and pyruvate reperfusion (0.62 and 0.67, respectively) compared to lactate and acetate reperfusion (0.43 and 0.52, respectively). AdN catabolism during ischemia is tested by the presence of IMP (about 6% of the total AdN) together with hypoxanthine (data not reported). On reperfusion a recovery of AdN metabolism is evident only with pyruvate, as tested by the

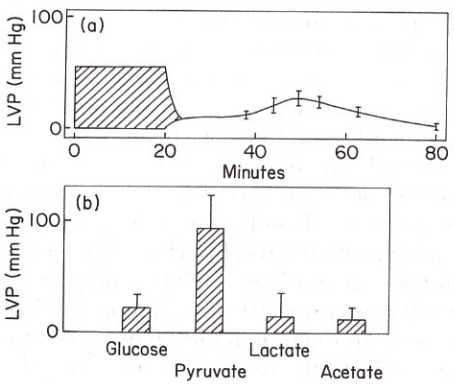

FIGURE 1. Behaviour of the left ventricular pressure (LVP) in the glucose-ischemia model (a) and recovery on reperfusion with different substrates (b). LVP recorded as described in Materials and Methods is reported during the equilibration and the ischemic phase. The recoveries on reperfusion with the different substrates are reported as bars where the standard deviation is also indicated and represents the mean of at least three experiments for each condition.

disappearance of IMP. A loss of total AdN is generally observed on reperfusion. This is less evident with pyruvate or glucose + insulin, and much more significant with lactate and acetate. The AdN related parameters do not account, however, for the much better performance of the hearts reperfused with pyruvate in which a more than complete LVP recovery is obtained compared to the hearts reperfused with glucose. In Table 2 the amount of glutathione released in the reperfusion medium and that retained in the tissue after reperfusion are reported together with the released LDH. Glutathione is continuously lost to the perfusion medium. The rate of glutathione release is about 2.5 nmoles/min. A large release of glutathione is observed in the first minutes of the reperfusion after ischemia followed by a drop to the basal release value. The peak release on reperfusion was respectively 16 and 18% of the initial total glutathione with pyruvate and glucose + insulin.

LDH is slowly released from perfused hearts. Also in this case a peak release is observed in the first minutes of reperfusion after ischemia. With glucose + insulin and pyruvate the release of LDH was 10 and 7%. Higher releases of both glutathione and LDH were observed in lactate and acetate.

In general, pyruvate appears to be the most efficient substrate followed by glucose, lactate and acetate for all the considered metabolic parameters although the differences, particularly relative to glucose, are only marginal.

The redox state of pyridine nucleotides could also reflect the tissue metabolic state. As shown in Table 3 the NAD^+/NADH ratio, which is mainly shifted towards NAD^+ in the control, shifts towards NADH during ischemia (where a ratio of 1.61 is found). When hearts are reperfused some reoxidation of NADH is observed, although the steady state achieved is always significantly more reduced than in the pre-ischemic situation. For technical reasons (residual lactate dehydrogenase activity present in the samples containing pyruvate) an exact determination of the NAD^+/NADH ratio in the pyruvate perfused heart cannot be obtained. A fraction of the NAD pool is lost after the ischemic phase. This is in the order of 25% in the glucose + insulin, pyruvate and lactate reperfusion; it is significantly higher with acetate; in this latter situation the functional recovery is also the least, and the residual NAD pool is also more highly oxidized.

The behaviour of NADP has also been considered. The $NADP^+$/NADPH ratio which is 0.68 in the control drops to very low values on reperfusion after ischemia (except in acetate reperfusion where a higher oxidation state is observed). The variations in the redox state are accompanied by a large loss (over 50%) of the total NADP pool. A possible explanation for these observation is that a large fraction of the (more oxidized) cytosolic pool is lost after ischemia and that the higher reduction measured in reperfused heart represent the contribution of the mitochondrial fraction. The highest NADP reduction is observed in the reperfusion with pyruvate.

Pyruvate-ischemia

When 5 mM pyruvate is used as the only substrate [Fig. 2(a)] the increment of the resting pressure induced by ischemia still occurs (it is even more pronounced than in the glucose-ischemia, as shown by the maximum of the resting pressure which is almost doubled). On reperfusion with normoxic media after pyruvate-ischemia [Fig. 2(b)] the functional performance is significantly better than after glucose-ischemia. Resumption of contractility was obtained with all four substrates (with the exception of only two cases in the acetate group). Reperfusion with pyruvate shows a more than complete recovery with a mean of 120% of the pressure developed during the equilibration period. Essentially the same result is obtained with glucose (125%), while hearts tend to recover only partially with lactate (40%) and the lowest recovery (27%) is observed with acetate.

Metabolic parameters regarding the model of the pyruvate-ischemia and reperfusion are reported in Table 4. During ischemia after equilibration with pyruvate the PCr and AdN levels decrease to a level comparable to that observed in glucose-ischemia, the loss of AdN is even higher (cf. Table I) and IMP accumulates. Upon reperfusion however a general improvement is observed: the PCr level increases again in all cases over the corresponding values in the glucose-ischemia model. The effect is particularly evident with glucose and

TABLE 2. Effect of various substrates on glutathione redox state and effluxes of glutathione and LDH in the reperfusion phase following glucose-ischemia

Conditions	Glutathione tissue		Glutathione peak efflux[a]		LDH peak efflux[a]	
	GSH/GSH + GSSG	nmol/heart	nmol	%total	mU LDH	% total LDH
Glucose controls	0.84 ± 0.01 (5)	1450 ± 290 (5)				
Glucose-ischemia	0.81 ± 0.05 (6)	1438 ± 260 (5)				
Glucose reperfused	0.76 ± 0.11 (4)	1138 ± 231 (4)	270 ± 157 (4)	18	11.8 ± 8.7 (4)	9.4
Pyruvate reperfused	0.78 ± 0.11 (3)	1190 ± 330 (3)	238 ± 107 (4)	16	9.0 ± 8.0 (4)	7.2
Lactate reperfused	0.76 ± 0.12 (5)	1006 ± 213 (5)	428 ± 303 (5)	28	29.9 ± 16.4 (4)	16.0
Acetate reperfused	0.72 ± 0.06 (5)	893 ± 160 (5)	444 ± 167 (5)	29	29.1 ± 15.5 (5)	23.2

Values are reported as mean ± s.D. and indicate the total content of the various parameters in the tissue or in the perfusate. Number of experiments in parentheses.
[a]The efflux in the first 10 min of reperfusion.

TABLE 3. Effect of various substrates on pyridine nucleotides redox state in the reperfusion phase following glucose-ischemia

Conditions	$NADP^+$ (nmol/heart)	NADPH (nmol/heart)	Ratio	% AdN	NAD^+ (nmol/heart)	NADH (nmol/heart)	Ratio	% AdN
Glucose controls	39	58	0.68 ± 0.30 (3)	2.40 ± 0.1 (3)	565	57	9.92 ± 2.90 (3)	15 ± 0.1 (3)
Glucose-ischemia	n.d.	19 ± 0.007 (7)		1.31 ± 0.29 (7)	408	259	1.61 ± 0.80 (5)	37 ± 15.0 (5)
Glucose reperfused	3	39	0.08 ± 0.04 (3)	2.60 ± 0.3 (3)	331	158	2.09 ± 0.24 (3)	32 ± 9.0 (4)
Pyruvate reperfused	1	51	0.02 ± 0.002 (3)	3.50 ± 0.3 (3)	464 ± 81 total (3)			31 ± 3.5 (3)
Lactate reperfused	1	30	0.04 ± 0.04 (3)	2.80 ± 1.5 (3)	230	135	1.70 ± 0.50 (3)	33 ± 11.0 (3)
Acetate reperfused	28	14	1.93 ± 1.30 (3)	4.00 ± 0.8 (3)	275	50	5.44 ± 2.70 (3)	31 ± 6.0 (5)

n.d., not detectable.
Values are expressed as mean ± s.D. Number of experiments in parentheses. % values are referred to the corresponding total AdN values.

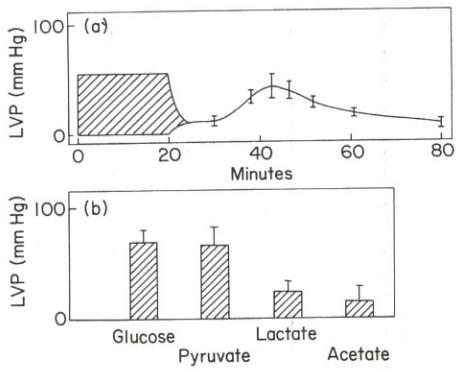

FIGURE 2. Behaviour of the left ventricular pressure (LVP) in the pyruvate-ischemia model (a) and recovery on reperfusion with different substrates (b). The conditions are as described in Figure 1.

pyruvate. The energy charge on reperfusion after pyruvate-ischemia is significantly higher than after glucose-ischemia. The general improvement of the recovery after pyruvate-ischemia is also documented by the disappearance of the IMP on reperfusion (except in the case of acetate). Parameters referring to the cellular glutathione redox state and to glutathione and LDH effluxes of the corresponding groups are reported in Table 5. Glutathione, 83% of which is in the reduced form in the control, does not decrease during ischemia; it generally becomes slightly more oxidized on reperfusion. As for the glucose-ischemia some glutathione is lost to the perfusion medium in the control with pyruvate at a rate of less than 2 nmoles/min. A peak release is always observed on reperfusion. However such a peak is smaller after pyruvate-ischemia than after glucose-ischemia. As expected a decrease of total tissue glutathione is observed after reperfusion. However such a loss is not observed in reperfusion with pyruvate: this may be due to a resynthesis or to a release of GSH from the mixed disulfide pool. On reperfusion after pyruvate-ischemia, the release of LDH is similar to that observed in reperfusion following glucose-ischemia, although generally less pronounced.

The pyridine nucleotide content and redox state relative to the condition of pyruvate-ischemia are reported in Table 6. They compare rather closely to those shown in Table 3 for the glucose-ischemia. It is clear from the result of Figures 1 and 2 that pyruvate protects significantly the functional performance of perfused hearts against the ischemic damage. The best protection is obtained when the hearts are perfused with pyruvate prior to the onset of the ischemic conditions, although pyruvate also exerts a clear effect when used in the reperfusion of a glucose-ischemic heart. Rather surprisingly, only marginal effects of pyruvate could be observed on a series of biochemical parameters, except for PCr. The content of PCr was generally higher in the conditions when the functional recovery was more complete.

It is remarkable that an apparently complete functional recovery is obtained on reperfusion after pyruvate-ischemia, in spite of the large loss of cellular adenine and pyridine nucleotides.

TABLE 4. Effect of ischemia and reperfusion with various substrates on high energy phosphate compounds in pyruvate equilibrated heart

Conditions	Phospho-creatine (μmol/heart)	Total AdN (μmol/heart)	IMP (μmol/heart)	Charge
Pyruvate controls	5.49 ± 0.74 (4)	3.68 ± 0.62 (4)	n.d.	0.89 ± 0.04 (4)
Pyruvate-ischemia	0.15 ± 0.11 (5)	1.16 ± 0.10 (5)	0.49 ± 0.31 (5)	0.28 ± 0.03 (5)
Glucose reperfused	3.39 ± 0.44[a] (4)	1.24 ± 0.15 (4)	n.d.	0.73 ± 0.04[a] (4)
Pyruvate reperfused	3.64 ± 0.65[a] (4)	1.53 ± 0.24 (4)	n.d.	0.77 ± 0.04[a] (4)
Lactate reperfused	2.10 ± 0.88[a,b,c] (4)	1.25 ± 0.10 (4)	0.01 ± 0.01 (3)	0.65 ± 0.10[a,c] (4)
Acetate reperfused	0.63 ± 0.36[a,b,c] (7)	0.60 ± 0.27[a,b,c] (7)	0.12 ± 0.08 (7)	0.50 ± 0.08[a,b,c,] (7)

n.d., not detected.
Conditions as in Table 1.

TABLE 5. Effect of various substrates on glutathione redox state and effluxes of glutathione and LDH in the reperfusion phase following pyruvate-ischemia

	Glutathione tissue		Glutathione peak efflux[a]		LDH peak efflux[a]	
Conditions	GSH/GSH + GSSG	nmol/heart	nmol	total	mU LDH	% total LDH
Pyruvate controls	0.84 ± 0.005 (5)	1495 ± 440 (5)				
Pyruvate-ischemia	0.84 ± 0.003 (6)	1469 ± 59 (4)				
Glucose reperfused	0.65 ± 0.16 (3)	1067 ± 240 (3)	265 ± 50 (3)	17	9.0 ± 4.6 (3)	7.2
Pyruvate reperfused	0.74 ± 0.11 (4)	1513 ± 243 (4)	200 ± 100 (4)	13	6.8 ± 6.0 (3)	5.4
Lactate reperfused	0.72 ± 0.03 (3)	1006 ± 208 (3)	285 ± 27 (3)	19	9.5 ± 5.5 (3)	7.6
Acetate reperfused	0.72 ± 0.025 (3)	1109 ± 180 (5)	355 ± 228 (4)	24	18.8 ± 14.0 (4)	15.0

Conditions as in Table 2.

TABLE 6. Effect of various substrates on pyridine nucleotides redox state in the reperfusion phase following pyruvate-ischemia

	$NADP^+$	NADPH			NAD^+	NADH		
Conditions	(nmol/heart)		Ratio	% AdN	(nmol/heart)		Ratio	% AdN
Pyruvate controls	23	56	0.42 ± 0.15 (3)	2.00 ± 0.1 (3)	560 ± 5 total (3)			14 ± 0.1 (3)
Pyruvate-ischemia	n.d.	21 ± 0.002 (5)		2.55 ± 0.6 (5)	364	311	1.17 ± 0.6 (3)	65 ± 11 (3)
Glucose reperfused	8	21	0.38 ± 0.04 (3)	2.40 ± 0.3 (3)	338	108	3.14 ± 0.5 (3)	36 ± 3 (3)
Pyruvate reperfused	8	38	0.23 ± 0.31 (3)	3.03 ± 0.8 (3)	420 ± 15 total (3)			28 ± 1 (4)
Lactate reperfused	8	16	0.50 ± 0.28 (3)	2.00 ± 0.15 (3)	260	13	19.6 ± 7.0 (3)	23 ± 0.5 (3)
Acetate reperfused	9	6	1.43 ± 1.10 (7)	2.84 ± 0.80 (7)	378	50	10.9 ± 9.7 (7)	45 ± 16 (7)

n.d., not detectable.
Conditions as in Table 3.

Discussion

After glucose-ischemia reperfusion with pyruvate is the only condition in which a clear functional recovery is obtained. Accordingly higher ATP, PCr and energy charge value are observed in these conditions (see Table 1). Pyruvate appears far more efficient than the other substrates on the contractile recovery [see Fig. 1(b)]. The possibility that an AdN salvage pathway could be stimulated by pyruvate on reperfusion is consistent with the data on IMP (Table 1) which disappears from the pyruvate reperfused hearts, while, to a different extent, it is still present in all the other groups. The lower amounts of glutathione and lactate dehydrogenase release shown in Table 2 on reperfusion with pyruvate, compared to glucose and also to lactate and acetate, may be indicative of an effective prevention by pyruvate of some tissue damage sparked by the oxidative stress in the reperfusion. Similar findings on pyruvate preventing reperfusion failure and in increasing the phosphorylation potential in guinea-pig heart have recently been reported by Bünger *et al.* (1989).

In the second model (pyruvate-ischemia) functional recovery is also generally improved although the behavior of the resting pressure shows a paradoxical increase during the ischemia [cf. Fig. 2(a) and Fig. 1(a)]. Also the metabolic parameters analyzed in this study do not account for the protection afforded by pyruvate during the ischemia. On reperfusion, pyruvate shows about the same efficiency as glucose and, in both cases, the recovery is more than complete. In contrast with glucose-ischemia, a recovery of PCr is evident with all the reperfusion media (except acetate) after pyruvate-ischemia. The loss of AdN is higher in pyruvate-ischemia, but no further loss occurs during reperfusion (on the contrary a slight increase is found). The recovery of AdN metabolism is supported by the absence of IMP. The inhibition of the key enzyme involved in the salvage pathway, i.e. hypoxanthine-guanine phosphoribosyl transferase, in cultured anoxic rat heart cell is relieved by pyruvate (Ravid *et al.*, 1984) and this effect is attributed to cytosolic NADH oxidation. The lower efficacy of lactate in the recovery of the ischemic hearts could be related to the lack of further metabolism due to the high concentration of NADH. This blocks both LDH and pyruvate dehydrogenase and is particularly evident in glucose-ischemic reperfused hearts where the NAD^+/NADH ratio remains very low also after 30 min of reperfusion.

The recovery in acetate reperfused hearts is impaired in both models of ischemia since acetate activation through an ATP-dependent thiokinase (which produces AMP and PP_i) is probably impaired in these ischemic hearts, both due to the very low concentration of ATP after 1 h of ischemia and to the low availability of CoA-SH which is also decreased by the accumulation of acyl-CoA (Vary *et al.*, 1981). Moreover, the increased production of AMP due to acetate activation increases the AdN catabolism as tested by the highest value of IMP found in the acetate reperfusion experiments. Acetate fails to prevent membrane damage as shown by the high release of glutathione and LDH (Tables 2 and 5) and effectively it completely fails to reduce $NADP^+$.

The prevailing effect of pyruvate on reperfusion, shared both in glucose and pyruvate equilibrated ischemic hearts, is probably dependent on the combined effect of pyruvate on $NADP^+$ reduction and on the stimulation of the tricarboxylate pathway, as indicated by the higher phosphorylating capacity of pyruvate-reperfused hearts parallel to the increase of the purine nucleotide salvage pathway tested by IMP disappearance. The amount of AdN recovered on reoxygenation of anaerobic myocytes is also reported to occur primarily at the expense of IMP, which is produced during anaerobiosis (Altschuld *et al.*, 1987).

The efficacy of pyruvate as an anaplerotic substrate at a concentration higher than physiological has been already reported (Peuhkurinen and Hassinen, 1982; Sundqvist *et al.*, 1987), and its effectiveness in raising the tricarboxylate intermediates (particularly malate and citrate) can increase the steady state level of NADPH production in the cytosol through the malic enzyme (Hiltunen and Davis, 1981) or the isocitrate dehydrogenase activity (Pfeifer *et al.*, 1986). Both the latter enzymes are reported to be present at high concentration in the myocardial tissue (Andrés *et al.*, 1980). On the contrary the

glucose-6-phosphate dehydrogenase activity is very low (Andrés *et al.*, 1980) and as a consequence glucose results in a less efficient recovery after the ischemic insult. Via the production of NADPH (which is necessary for the reduction of glutathione oxidized by the action of the glutathione peroxidase) pyruvate might contribute to the removal of active oxygen species arising from the activity of xanthine oxidase which is formed during reperfusion (Bindoli *et al.*, 1988a, b), or by autoxidation of the flavoproteins kept reduced during ischemia by the high NAD cellular redox state (a sort of reductive stress) (Jones, 1985).

However, it is to be pointed out that a large loss of NADP occurs during ischemia/reperfusion in both models, which does not appear to affect the functional performance of the hearts. The low $NADP^+$/NADPH ratio during reperfusion is largely due to a decrease of $NADP^+$. As already pointed out (see Results) this could indicate the loss of cytosolic NADP.

The general effect of pyruvate as a protecting agent shown in the model of pyruvate-ischemia must consider some special effects exerted in the onset of the ischemic phase. In the pyruvate-ischemia the tissue is clearly more protected against reperfusion damage, but no difference in the status of the considered metabolic parameters has been shown. The first minutes of reperfusion are generally considered the more dramatic moment for the onset of tissue injury and this has been linked to the oxygen insult, since it is in this early phase that oxygen could give rise to the damaging active oxygen species (Woodward and Zakaria, 1985; Arroyo *et al.*, 1987; Hearse and Tosaki, 1987; Zweier *et al.*, 1987; Manning, 1988; Zweier, 1988).

Accumulation of H_2O_2 in ischemia is reported by Vandeplassche and Borgers (1988) and its involvement in the ischemic tissue damage is suggested by Myers *et al.* (1985). Several indirect evidences, reviewed by Manning (1988), strongly support the involvement of hydrogen peroxide in the ischemic damage since pre-treatment with agents that prevent H_2O_2 formation or the production of its toxic intermediates (e.g. Fe ion chelators) exert a positive effect on the recovery of ischemic hearts. In this context an alternative mechanism by which pyruvate could exert its protective effect could be related to its ability to promote peroxide breakdown. As reported by Varma and Morris (1988) and references therein, H_2O_2 is decomposed in the presence of pyruvate. In the conditions of our experiments 50% decomposition of 0.4 mM H_2O_2 occurs in 2 min with 5 mM pyruvate and pyruvate also promotes the breakdown of organic hydroperoxides (not shown). In this way pyruvate could exert a direct scavenging activity towards hydrogen peroxide or other peroxide during both the ischemic and the reperfusion phase. In this case when oxygen is readmitted to the cellular compartment pyruvate could help tissue to be more protected against the oxidative injury resulting in a more rapid recovery. This is consistent with the limited peak loss of glutathione and LDH observed on reperfusion after pyruvate-ischemia (cf. Tables 5 and 2).

Another possibility is that pyruvate may activate pyruvate dehydrogenase (by inhibiting its phosphorylation) as reported by Randle *et al.* (1977), increasing the pyruvate dehydrogenase flux as suggested Bünger *et al.* (1989).

Our data support the possibility of utilizing pyruvate in reperfusion both as a protecting agent against the oxidative stress (through the NADPH sustained reduction of glutathione and hence activation of GSH-dependent cellular protecting systems or as a direct scavenger of H_2O_2) and as a modulator of oxidative metabolism (through an efficient stimulation both of the oxidative metabolism and of the AdN salvage pathways).

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References

Altschuld RA, Gamelin LM, Kelley RE, Lambert MR, Apel LE, Brierley GP (1987) Degradation and resynthesis of adenine nucleotides in adult rat heart myocytes. J Biol Chem **262**: 13527–13533.

Andrès A, Satrustegui J, Machado A (1980) Development of NADPH-producing pathways in rat heart. Biochem J **186**: 799–803.

Arroyo CM, Kramer JH, Dickens BF, Weglicki WB (1987) Identification of free radicals in myocardial ischemia/reperfusion by spin trapping with nitrone DMPO. FEBS Lett **221**: 101–104.

Bindoli A, Cavallini L, Rigobello MP, Coassin M, Di Lisa F (1988a) Modification of the xanthine-converting enzyme of perfused rat heart during ischemia and oxidative stress. Free Radical Biol Med **4**: 163–167.

Bindoli A, Cavallini L, Rigobello MP, Coassin M, Di Lisa F (1988b) Conversion of Rat Xanthine Dehydrogenase to Xanthine Oxidase During Oxidative Stress. In: *The Role of Oxygen Radicals in Cardiovascular Diseases*, edited by A L'Abbate, F Ursini. Dordrecht/Boston/London, Kluwer Academic Publishers, pp 51–69.

Bricknell, OL, Opie LH (1978) Effects of substrates on tissue metabolic changes in the isolated rat heart during underperfusion and on release of lactate dehydrogenase and arrhythmias during reperfusion. Circ Res **43**: 102–115.

Bünger R, Mallet, RT, Hartman DA (1989) Pyruvate-enhanced phosphorylation potential and inotropism in normoxic and postischemic isolated working heart. Near-complete prevention of reperfusion contractile failure. Eur J Biochem **180**: 221–233.

Bünger R, Swindall B, Brodie D, Zdunek D, Stiegler H, Walter G (1986) Pyruvate attenuation of hypoxia damage in isolated working guinea-pig heart. J Mol Cell Cardiol **18**: 423–438.

Camacho SA, Parmley WW, James TL, Abe H, Wu ST, Botvinick EH, Watters TA, Schiller N, Sievers R, Wikman-Coffelt J. (1988) Substrate regulation on the nucleotide pool during regional ischaemia and reperfusion in an isolated rat heart preparation: a phosphorus-31 magnetic resonance spectroscopy analysis. Cardiovasc Res **22**: 193–203.

Gornall AG, Bardawill CJ, David MM (1949) Determination of serum proteins by means of the biuret reaction. J Biol Chem **177**: 751–756.

Hearse DJ, Chain EB (1972) The role of glucose in the survival and "recovery" of the anoxic isolated perfused rat heart. Biochem J **128**: 1125–1133.

Hearse DJ, Tosaki A (1987) Reperfusion-induced arrhythmias and free radicals: studies in the rat heart with DMPO. J Cardiovasc Pharmacol **9**: 641–650.

Hiltunen JK, Davis EJ (1981) The disposition of citric acid cycle intermediates by isolated rat heart mitochondria. Biochim Biophys Acta **678**: 115–121.

Jennings RB, Reimer KA, Hill ML, Mayer SE (1981) Total ischemia in dog hearts, *in vitro*. 1. Comparison of high energy phosphate production, utilization, and depletion, and of adenine nucleotide catabolism in total ischemia *in vitro* vs. severe ischemia. Circ Res **49**: 892–900.

Kaplan NO, Goodfriend TL (1964) Role of the two types of lactic dehydrogenase. Adv Enzyme Reg **2**: 203–212.

Langendorff O (1885) Untersuchungen am uberlebenden saugerthierherzen. Pflügers Arch **61**: 291–332.

Liedtke AJ, Nellis SH (1978) Effect of buffered pyruvate on regional cardiac function in moderate, short-term ischemia in swine heart. Circ Res **43**: 189–199.

Jones DP (1985) The Role of Oxygen Concentration in Oxidative Stress: Hypoxic and Hyperoxic Models. In: *Oxidative Stress*, edited by H Sies. London, Academic Press, pp 151–195.

Manning AS (1988) Reperfusion-induced arrhythmias: do free radicals play a critical role? Free Radical Biol Med **4**: 305–316.

Maroko PR, Libby P, Sobel BE, Bloor CM, Sybers HD, Shell WE, Covell JW, Braunwald E (1972) Effect of glucose-insulin-potassium infusion on myocardial infarction following experimental coronary artery occlusion. Circulation **45**: 1160–1175.

Mochizuki S, Neely JR (1980) Energy metabolism during reperfusion following ischemia. J Physiol, Paris **76**: 805–812.

Myers CL, Weiss SJ, Kirsh MM, Shlafer M (1985) Involvement of hydrogen peroxide and hydroxyl radical in the "oxygen paradox": reduction of creatine kinase release by catalase, allopurinol or deferoxamine, but not by superoxide dismutase. J Mol Cell Cardiol **17**: 675–684.

Pettit FH, Pelley JW, Reed LJ (1975) Regulation of pyruvate dehydrogenase kinase and phosphatase by acetyl-CoA/CoA and NADH/NAD ratios. Biochem Biophys Res Commun **65**: 575–582.

Peuhkurinen KJ, Hassinen IE (1982) Pyruvate carboxylation as anaplerotic mechanism in the isolated perfused rat heart. Biochem J **202**: 67–76.

Pfeifer R, Karl G, Scholz R (1986) Does the pentose cycle play a major role for NADPH supply in the heart? Biol Chem Hoppe-Seyler **367**: 1061–1068.

Randle PJ, Huston NJ, Kerbey AL (1977) Metabolism of Pyruvate in Animals. In: *Regulatory Mechanism of Carbohydrate Metabolism, FEBS Symposia*, Vol 42, edited by V. Esmann. Oxford, Pergamon Press, pp 3–12.

Ravid K, Diamant P, Avi-dor Y (1984) Regulation of the salvage pathway of purine nucleotide synthesis by the oxidation state of NAD^+ in rat heart cells. Arch Biochem Biophys **229**: 632–639.

Stocchi V, Cucchiarini L, Magnani M, Chiarantini L, Palma P, Crescentini G (1985) Simultaneous extraction and reverse-phase high-performance liquid chromatographic determination of adenine and pyridine nucleotides in human red blood cells. Anal Biochem **146**: 118–124.
Sundqvist KE, Heikkila J, Hassinen IE, Hiltunen JK (1987) Role of NAD^+-linked malic enzymes as regulators of the pool size of tricarboxylic acid-cycle intermediates in the perfused rat heart. Biochem J **243**: 853–857.
Tietze F (1969) Enzymatic method for quantitative determination of nanograms amounts of total and oxidized glutatione. Anal Biochem **27**: 502–522.
Vandeplassche G, Borgers M (1988) NADH oxidase as a source of hydrogen peroxide (H_2O_2) in ischemic myocardium. J Mol Cell Cardiol **20** [Suppl 5], PT 139.
Varma SD, Morris SM (1988) Peroxide damage to the eye lens *in vitro* prevention by pyruvate. Free Rad Res Commun **4**: 283–290.
Vary TC, Randle PJ (1984) The effect of ischaemia on the activity of pyruvate dehydrogenase complex in rat heart. J Mol Cell Cardiol **16**: 723–733.
Vary TC, Reibel DK, Neely JR (1981) Control of energy metabolism of heart muscle. Ann Rev Physiol **43**: 419–430.
Woodward B, Zakaria NM (1985) Effect of some radical scavengers on reperfusion induced arrhythmias in the isolated rat heart. J Mol Cell Cardiol **17**: 485–493.
Xia, Y, Hill KE, Burk RF (1985) Effect of selenium-deficiency on hydroperoxide-induced glutathione release from the isolated perfused rat heart. J Nutr **115**: 733–742.
Zweier JL (1988) Measurement of superoxide-derived free radicals in the reperfused heart. Evidence for a free radical mechanism of reperfusion injury. J Biol Chem **263**: 1353–1357.
Zweier JL, Flaherty JT, Weisfeldt ML (1987) Direct measurement of free radical generation following reperfusion of ischemic myocardium. Proc Natl Acad Sci USA **84**: 1404–1407.
Zweier JL, Jacobus WE (1987) Substrate-induced alterations of high energy phosphate metabolism and contractile function in the perfused heart. J Biol Chem **262**: 8015–8021.