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Metabolic compartmentation of pyruvate in the isolated perfused rat heart

Keijo J. PEUHKURINEN, J. Kalervo HILTUNEN and Ilmo E. HASSINEN
Department of Medical Biochemistry, University of Oulu, SF-90220 Oulu 22, Finland

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1. Prompted by the finding of markedly differing specific radioactivities of tissue alanine and lactate in isolated rat hearts perfused with [1-^{14}C]pyruvate, a more detailed study on the cytosolic subcompartmentalization of pyruvate was undertaken. Isolated rat hearts were perfused by the once-through Langendorff technique under metabolic and isotopic steady-state conditions but with various routes of radioactive label influx, and the specific radioactivities of pyruvate, lactate and alanine were determined. An enzymic method was devised to determine the specific radioactivity of C-1 of pyruvate. 2. Label introduction as [1-^{14}C]pyruvate resulted in a higher specific radioactivity of tissue alanine and mitochondrial pyruvate than of lactate, and a higher specific radioactivity of perfusate lactate than of tissue lactate. Label introduction as [1-^{14}C]lactate resulted in a roughly similar isotope dilution into the tissue and perfusate pyruvate and the tissue alanine. Label introduction as [3,4-^{14}C]glucose resulted in the same specific radioactivity of tissue lactate and alanine and a roughly similar specific radioactivity of mitochondrial pyruvate. 3. The results can be reconciled with a metabolic model containing two cytosolic functional pyruvate pools. One pool (I) communicates more closely with the glycolytic system, whereas the other (II) communicates with extracellular pyruvate and intracellular alanine. Pool II is in close connection with intramitochondrial pyruvate. The physical identity of the cytosolic subcompartments of pyruvate is discussed.

It is generally assumed that, as far as intermediary metabolism is concerned, the cytosolic space can be treated as a homogeneous space in which the soluble enzymes and substrates are evenly distributed. Some fragmentary data from various sources have nevertheless given repeated evidence for cytosolic subcompartmentation. It has been documented that the tissue lactate/pyruvate ratio does not always follow that measured from the venous fluid of the perfused rat heart preparation, suggesting that either these two metabolites have different characteristics of penetration through the cardiac cell membrane or that several metabolic pools exist in the cytoplasm (Garland *et al.*, 1964). In isolated rat hepatocytes, also, certain substrate pairs expected to be in near-equilibrium with the NAD(H) pool by appropriate high-activity dehydrogenases behaved as if there existed several cytosolic NAD(H) pools (Berry, 1980). Mowbray & Ottaway (1973*b*), when perfusing rat hearts with radioactive lactate, found that, although tissue lactate equilibrates with perfusate lactate, tissue pyruvate does not, suggesting that more than one pyruvate pool exists. It was also found more recently with rat hearts perfused with [1-^{14}C]pyruvate that the tissue specific radioactivity of alanine was 2–3 times that of tissue lactate (Peuhkurinen & Hassinen, 1982). This indicates that several metabolic pools of pyruvate exist.

To study the problem further, we perfused rat hearts by the open-circuit Langendorff technique under metabolic and isotopic steady-state conditions using various metabolic routes of label influx. The specific radioactivities of pyruvate, lactate and alanine were measured in different metabolic situations. The results indicate the existence of at least two functional pyruvate pools in the cytosol of the rat heart cells, one closely connected with glycolysis and the other communicating with the mitochondrial and perfusate pyruvate.

Materials and methods

Reagents

The enzymes were from Sigma Chemical Co., St. Louis, MO, U.S.A., and Boehringer, Mannheim,

Germany. Standard chemicals were obtained from E. Merck A.G., Darmstadt, Germany, and the nucleotides and coenzymes from Boehringer. Sodium [1-^{14}C]pyruvate was purchased from The Radiochemical Centre, Amersham, Bucks., U.K., and sodium [1-^{14}C]lactate from International Chemical and Nuclear Corp., Irvine, CA, U.S.A. The ion-exchange resins were purchased from Fluka A.G., Buchs, Switzerland, and the monocomponent pig insulin was obtained from Novo Industri A/S, Copenhagen, Denmark.

Animals and perfusion methods

Male Sprague–Dawley rats from the Department's own stocks were used, with no starvation period before the experiments. These were anaesthetized with sodium pentobarbital (80–100 mg/kg body weight, intraperitoneally) and injected intravenously with 500 i.u. of heparin 1 min before excision of the heart. The hearts were perfused with Krebs–Henseleit (1932) bicarbonate solution, pH 7.4, containing 2.5 mM-$CaCl_2$, 5 mM-glucose and 10 units of crystallized pig monocomponent insulin/litre in equilibrium with O_2/CO_2 (19:1), by the Langendorff procedure without recirculation at a hydrostatic pressure of 7.84 kPa (80 cm of water). The glass components of the perfusion apparatus (Takala, 1981) were equipped with water jackets and maintained at 37°C thermostatically with a recirculating water bath. Six lines of experiments were conducted. In the first series an initial perfusion of 15 min was followed by a further 10 min perfusion with a solution containing [3,4-^{14}C]-glucose. In another series of experiments additional exogenous 0.2 mM-pyruvate was present throughout the perfusion. In the third and fourth series the experimental design was the same, except that exogenous [1-^{14}C]pyruvate or [1-^{14}C]lactate was used as the source of label instead of glucose. In the fifth and sixth lines of perfusions 1 mM-pyruvate + 5 mM-glucose + insulin were used, with either [3,4-^{14}C]glucose or [1-^{14}C]pyruvate serving as the label source. The specific radioactivity of the substrates were 127000–202000, 350000–420000 and 23000–36000 d.p.m./μmol for pyruvate, lactate and glucose respectively. The results of the label-incorporation experiments were then normalized to a substrate specific radioactivity of 100000 d.p.m./μmol.

The [1-^{14}C]pyruvate was purified on a Dowex-1 (Cl^- form) column (Korff, 1969) just before the experiments. The effluent was neutralized with NaOH and the osmolarity increase was taken into consideration when preparing the perfusion medium. Thereafter the pyruvate concentration in the perfusion medium was measured enzymically and adjusted accordingly by addition of Krebs–Ringer bicarbonate solution.

Heart and perfusate sampling

Ventricular samples from the heart were obtained at the end of the perfusion by using aluminium tongs cooled with liquid N_2 (Wollenberger *et al.*, 1960). The frozen pulverized sample was extracted with 8% (v/v) $HClO_4$ in 40% (v/v) ethanol precooled to −20°C (Williamson & Corkey, 1969) and the extraction was repeated with 6% $HClO_4$. The effluent perfusion fluid was collected during the last 2 min of the perfusion, and 5 ml of the perfusate was quenched by addition of 0.5 ml of 60% $HClO_4$. The $HClO_4$ extracts were bubbled with air for 5 min to eliminate $^{14}CO_2$ and thereafter neutralized with 3.75 M-K_2CO_3 containing 0.5 M-triethanolamine/HCl, the $KClO_4$ precipitate was separated by centrifugation and the supernatant used for analysis.

Metabolites and specific-radioactivity determination

The metabolites were determined by enzymic methods by measuring the appearance or disappearance of NADH in an Aminco DW-2 dual-wavelength spectrophotometer, by using an $\varepsilon_{340}-\varepsilon_{385}$ value of 5.33×10^3 litre·mol^{-1}·cm^{-1}. Pyruvate was measured as described by Bücher *et al.* (1963), lactate as described by Hohorst (1963) and alanine by the method of Grassl (1970).

To determine the specific radioactivities of the compounds, the metabolites were isolated from the tissue and perfusate extracts by ion-exchange chromatography on a Dowex-1 (formate form) column after an addition of 1 μmol of the metabolites as a carrier and eluted in an exponential gradient of formic acid and subsequently with ammonium formate (LaNoue *et al.*, 1970; Nuutinen *et al.*, 1981). A 1 ml sample of each fraction collected was used for radioactivity determination with water-miscible scintillation solution (Bray, 1960). The purity of the alanine peak has been verified (Peuhkurinen & Hassinen, 1982). Aspartate and glutamate, which were eluted from the column together with lactate, accounted for about 10% of the total radioactivity of the combined peak when [1-^{14}C]pyruvate was used (Peuhkurinen & Hassinen, 1982). The remainder of the combined lactate, aspartate and glutamate peak was therefore pooled and processed further to separate the individual metabolites. The pH of the pooled fraction was adjusted to 2.2, after which 2.0 ml of tightly packed Dowex-50 cation-exchange resin (H^+ form) was added and mixed for 5 min. The mixture was then filtered and the resin washed twice with a small amount of distilled water. The pooled filtrates were extracted with fresh ion-exchange material. When tested beforehand with radioactive aspartate, glutamate and lactate, it was found that over 99% of the amino acids became attached to the resin in the treatment. The recovery of lactate in the pooled

filtrate was $85 \pm 1\%$ ($n = 5$). A correction was therefore applied when calculating lactate radioactivity.

When [3,4-^{14}C]glucose was used, however, the total amount of radioactivity in the tissue pyruvate was so low that reliable determination by the chromatographic method was difficult. The C-3 and C-4 atoms of glucose enter the C-1 of pyruvate, where they can be detected as $^{14}CO_2$ after enzymic decarboxylation. Pyruvate decarboxylase from yeast was used for this purpose. The reaction was conducted in special vessels designed for $^{14}CO_2$ collection (Hiltunen & Hassinen, 1976) in the presence of 167mM-citrate buffer (pH 6.0), 67mM-$MgCl_2$, 1.34mM-dithiothreitol and 1.35mM-thiamin pyrophosphate; 2.5ml of tissue extract or 1.5ml of perfusate extract was added to the incubation medium and the pyruvate concentration measured. The reaction was started by the addition of 2.4 units of pyruvate decarboxylase (EC 4.1.1.1) and continued overnight. A small tube containing 2.24mmol of semicarbazide hydrochloride was placed in the incubation bottle to trap the acetaldehyde formed in the reaction. The reaction was stopped by adding 2.0ml of 10% $HClO_4$, after which the bottles were shaken for 1h to collect the $^{14}CO_2$ in NCS solution in the centre well of the reaction vial. After neutralization, the pyruvate concentration in the reaction mixture was measured again to determine the extent of the progress of the reaction, which was 80–90%. The specific radioactivity of C-1 of pyruvate was calculated from the $^{14}CO_2$ radioactivity and the change in pyruvate concentration.

Alanine radioactivity could not be determined by Dowex-1 column chromatography because it was not retarded in the anion-exchange resin under the conditions used. Therefore the alanine that was eluted in the void volume together with glucose was enzymically converted into pyruvate in the presence of excess 2-oxoglutarate and alanine aminotransferase (EC 2.6.1.2). The radioactivity of the pyruvate thus formed was analyzed by re-chromatography on the Dowex-1 (formate form) column without any extra carrier (Nuutinen *et al.*, 1981). The total alanine radioactivity from the samples was calculated and the specific radioactivity of the sample determined on the assumption that the specific radioactivity of alanine was the same as that of the pyruvate formed.

Measurement of $^{14}CO_2$ production

To determine the liberation of $^{14}CO_2$ from the radioactive substrates, the effluent perfusion fluid was collected under heptane during minutes 8–10 of the perfusion. The effluent was acidified with 2.0ml of 10% trichloroacetic acid and shaken for 1h in special flasks for collecting $^{14}CO_2$ into an NCS solution, and the radioactivity was measured (Hiltunen & Hassinen, 1976).

Measurement of the carbon flux through the pyruvate dehydrogenase reaction and the specific radioactivity of intramitochondrial pyruvate in the perfused heart

The specific radioactivity of mitochondrial pyruvate can be calculated if the flux through pyruvate dehydrogenase and the rate of appearance of ^{14}C in the CO_2 from [1-^{14}C]pyruvate are known. It has been verified that over 95% of the $^{14}CO_2$ evolution in the perfused heart under conditions of high pyruvate dehydrogenase activity is due to the pyruvate dehydrogenase reaction and the remainder to the activity of the tricarboxylic acid cycle, whose C_4 intermediates become labelled by pyruvate carboxylation (Peuhkurinen *et al.*, 1982).

It has been shown that in the presence of appropriate substrate concentrations all of the oxygen consumption of the isolated perfused rat heart can be accounted for by the oxidation of external glucose (Hiltunen & Hassinen, 1976). By the same token, in the presence of external glucose plus pyruvate the heart oxidizes only external substrates. The label fluxes between tissue glycogen and tissue glucose are quite slow (Takala & Hassinen, 1981), about 2% of the glycolytic flux, and can be neglected in the present case. In the presence of pyruvate and glucose the pyruvate dehydrogenase flux can be treated as the flux of (external) pyruvate carbon together with that of glycolytic pyruvate carbon from (external) glucose. Separate experiments with labelled glucose + non-labelled pyruvate and with non-labelled glucose + labelled pyruvate enable these pyruvate- and glucose-specific carbon fluxes to be measured in terms of the appearance of the label in CO_2. The total pyruvate dehydrogenase flux is the sum of these fluxes. In any experiment where the pyruvate dehydrogenase flux is known, the specific radioactivity of mitochondrial pyruvate (d.p.m./μmol) can be obtained by dividing the label flux (d.p.m./min) by the pyruvate dehydrogenase flux (μmol/min).

Results

Label introduction as [1-^{14}C]pyruvate

When the influent perfusate contained 0.2mM-labelled pyruvate, the specific radioactivity of tissue alanine reached 26% of that of the influent external pyruvate (Table 1). The specific radioactivity of the effluent perfusate lactate was 16%, but that of the tissue lactate was only 9% of that of the influent pyruvate (Table 1). Under these conditions the calculated specific radioactivity of intramitochondrial pyruvate reached 27% of that of the influent external pyruvate (Table 2).

Table 1. *Specific radioactivities of pyruvate and lactate in tissue and venous perfusion fluid and that of tissue alanine in isolated rat hearts*

The hearts were perfused with Krebs–Ringer bicarbonate solution and the ^{14}C-labelled and non-labelled substrates indicated in the column 'Conditions'. Experimental details are as described in the Materials and methods section. The values represent the means ± S.E.M. for the numbers of perfusions indicated in parentheses. Perfusion time was 10 min, and the perfusate analyses are from samples collected during the last 2 min of the perfusion. The specific radioactivities are normalized to a C_3-unit specific radioactivity of 100000 d.p.m./μmol in the labelled substrate used.

	10^{-3} × Specific radioactivity (d.p.m./μmol)				
	Pyruvate		Lactate		
Conditions	Tissue	Perfusate	Tissue	Perfusate	Alanine
0.2 mM-[1-^{14}C]pyruvate + 5 mM-glucose	—	—	9.3 ± 2.0 (8)	15.5 ± 3.2 (8)	26.4 ± 6.9 (6)
0.2 mM-[1-^{14}C]lactate + 5 mM-glucose	10.8 ± 1.4 (5)	11.1 ± 2.0 (5)	—	—	7.3 ± 0.8 (5)
0.2 mM-pyruvate + 5 mM-[3,4-^{14}C]glucose	—	—	72.1 ± 9.2 (6)	93.6 ± 13.8 (6)	72.1 ± 5.3 (5)
5 mM-[3,4-^{14}C]glucose	88.5 ± 19.2 (6)	86.9 ± 24.5 (7)	83.3 ± 23.3 (7)	87.0 ± 16.4 (7)	81.8 ± 2.5 (3)
1 mM-[1-^{14}C]pyruvate + 5 mM-glucose	—	—	31.4 ± 8.9 (6)	53.9 ± 12.5 (4)	64.4 ± 11.4 (6)
1 mM-pyruvate + 5 mM-[3,4-^{14}C]glucose	—	—	47.4 ± 9.6 (3)	55.3 ± 3.2 (3)	30.6 ± 1.0 (3)
2 mM-[1-^{14}C]pyruvate + 5 mM-glucose	—	—	54.2 ± 12.6 (3)	79.7 ± 9.2 (3)	91.4 ± 11.7 (3)

Table 2. *Appearance of ^{14}C radioactivity in CO_2 and the specific radioactivity of intramitochondrial pyruvate in isolated perfused rat hearts*

The hearts were perfused with Krebs–Ringer bicarbonate solution and the ^{14}C-labelled and non-labelled substrates indicated in the column 'Conditions'. Experimental details are as described in the Materials and methods section. The venous effluent perfusion fluid was collected during the last 2 min of the 10 min perfusion. The ^{14}C-label-appearance data were normalized to a precursor specific radioactivity of the C_3 moieties of glucose and pyruvate of 100000 d.p.m./μmol. The results are the means ± S.E.M. for the numbers of perfusions given in parentheses. The specific radioactivity of intramitochondrial pyruvate was based on the calculated pyruvate dehydrogenase flux of 8.83 μmol/min per g dry wt. taken as the sum of the C_3-unit fluxes in the presence of 1 mM-pyruvate and 5 mM-glucose (last two lines of the middle column of this Table).

Conditions	^{14}C label appearance in CO_2 (μmol of perfusate C_3 units/min per g tissue dry wt.)	10^{-3} × Specific radioactivity of intramitochondrial pyruvate (d.p.m./μmol)
0.2 mM-[1-^{14}C]pyruvate + 5 mM-glucose	2.37 ± 0.76 (8)	26.8
0.2 mM-pyruvate + 5 mM-[3,4-^{14}C]glucose	4.62 ± 1.18 (6)	52.3
1 mM-[1-^{14}C]pyruvate + 5 mM-glucose	8.15 ± 1.82 (3)	92.3
1 mM-pyruvate + 5 mM-[3,4-^{14}C]glucose	0.68 ± 0.10 (3)	7.7

When the labelled pyruvate concentration was increased to 1 mM, the specific radioactivities of tissue lactate, alanine and calculated mitochondrial pyruvate reached 31%, 64% and 92% of that of the perfusate pyruvate respectively (Tables 1 and 2).

Label introduction as [3,4-^{14}C]glucose

The specific radioactivities of the C_3 compounds would approach that of external glucose even in the presence of multiple subcellular pools of pyruvate and lactate, since the heart muscle is known under almost similar conditions to rely on the oxidation of external pyruvate only (Hiltunen & Hassinen, 1976).

In this case the specific radioactivities of perfusate and tissue lactate, pyruvate and alanine were almost similar, about 85% of that of the C_3 moieties of perfusate glucose (Table 1).

Label introduction as [1-^{14}C]lactate

In the presence of 0.2 mM labelled lactate in the influent perfusion medium the specific radioactivity

of tissue pyruvate reached 11% of that of influent lactate, and that of alanine 7% (Table 1). The $^{14}CO_2$ production was 0.62 ± 0.14 μmol/min per g dry wt. of tissue, when expressed in terms of the precursor C_3 units processed. This suggests that the specific radioactivity of the mitochondrial pyruvate is 7% of that of the influent lactate, but the same as that of the tissue alanine, when calculated in the manner detailed in the legend of Table 2, i.e. the isotope dilution of pyruvate in the perfusion with labelled lactate was higher than that of lactate in the perfusions with equimolar labelled pyruvate.

Label introduction as [3,4-^{14}C]glucose in the presence of non-labelled pyruvate

The perfusions with labelled glucose and non-labelled pyruvate were performed to estimate, in combination with the results of perfusion with non-labelled glucose and labelled pyruvate, the total pyruvate dehydrogenase flux from the production of $^{14}CO_2$. When the concentration of non-labelled pyruvate in the perfusate was 0.2 mM the specific radioactivities of tissue lactate and alanine both reached 72% of the C_3-unit specific radioactivity of glucose (Table 1). The specific radioactivity of the intramitochondrial pyruvate reached 52% of that of the C_3 units of perfusate glucose (Table 2). When the concentration of non-labelled pyruvate in the perfusate was raised to 1 mM, the tissue lactate and alanine and calculated intramitochondrial pyruvate reached 47%, 31% and 8% of that of the C_3 units of the glucose respectively (Tables 1 and 2).

Discussion

The results can be interpreted as demonstrating that (1) label introduction to the isolated heart as [1-^{14}C]pyruvate in the presence of glucose resulted in a higher specific radioactivity of tissue alanine and calculated mitochondrial pyruvate than of tissue lactate and that the labelling of extracellular lactate was higher than of intracellular lactate, (2) label introduction as [1-^{14}C]lactate in the presence of glucose resulted in equal isotope dilutions in the tissue alanine and intramitochondrial pyruvate, and (3) label introduction as [3,4-^{14}C]glucose resulted in equal high labelling of the perfusate and tissue lactate and pyruvate and the tissue alanine.

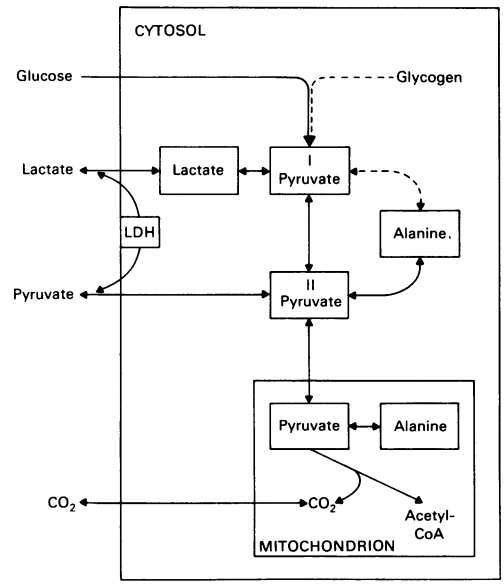
This labelling pattern complicates the use of tracer methodology in studies on the regulation of pyruvate metabolism in the myocardium, and implies that some of the conventions of the indicator metabolite method appropriate in other organs such as the liver may not be applicable to the heart.

The data are not compatible with only one cytosolic functional pyruvate pool, as at least two such pools are required to reconcile the model with the experimental results. One pool (I) can be identified as 'glycolytic', being associated more closely with glycolysis and tissue lactate, while the other 'peripheral' pool (II) communicates more closely with extracellular pyruvate than with cellular lactate. The results also indicate that pool II has a closer connection with mitochondrial pyruvate than does pool I (Scheme 1).

The radioactivity of the mitochondrial pyruvate could only be assessed by indirect means, for which purpose the pyruvate dehydrogenase flux must first be estimated. This is simplified by the fact that under these conditions all of the oxygen consumption can be accounted for by pyruvate oxidation (Hiltunen & Hassinen, 1976). The pyruvate dehydrogenase flux as measured by the method described in the present paper and based on the estimation of mutual isotope dilutions of pyruvate and glucose is close to the flux observed in independent experiments by other methods (Hiltunen & Hassinen, 1976), thus validating the present method.

Alanine appears to be more closely connected with mitochondrial pyruvate than with tissue lactate, and consequently an uneven distribution of cytosolic alanine or alanine aminotransferase should also exist. This is also supported by the finding that the isotope dilution in the mitochondrial pyruvate is lower than that in the tissue alanine in perfusions with labelled glucose and non-labelled pyruvate.

A mitochondrial isoenzyme of alanine aminotransferase has been demonstrated in myocardium (Katunuma *et al.*, 1965), but compart-



Scheme 1. *Apparent functional compartmentation of pyruvate in the cardiac myocyte*

Abbreviation: LDH, lactate dehydrogenase.

mentalization involved in the mitochondrial transmembrane transport of alanine in heart muscle has not been determined experimentally.

The data also suggest that lactate dehydrogenase is unevenly distributed in the cytosol. Part of it appears to be superficially located or outwardly oriented, operating with a subsarcolemmal substrate pool different from pool II (Scheme 1), since the labelling of perfusate lactate is higher than that of tissue lactate in perfusions with labelled pyruvate. These data are in accord with those of Mowbray & Ottaway (1973*a*).

The physical identity behind the apparent multiplicity of pools or non-homogeneity of distribution of cytosolic pyruvate is difficult to determine. The paradoxically closer connection between the extracellular substrates and the mitochondria than between the latter and the glycolytic metabolite pool could be partially explained by the morphology of the heart myocyte, in which mitochondria are gathered in rows between the myofibrils and in the subsarcolemmal space. Hence, the interfibrillar plus subsarcolemmal space could comprise the 'peripheral' pool (II), whereas the interfilamentous (intrafibrillar) space would contain the glycolytic pool (I), so that the contractile elements form the relative diffusion barriers.

Other explanations have been put forward previously to cope with the observation of apparent cytosolic metabolic subcompartmentalization in cardiac myocytes: certain glycolytic enzymes have been found to be preferentially located in the I zone of the sarcomere (Jarvie & Ottaway, 1975), and the T-tubulus system also shows periodicity. The longitudinal periodicity of the enzymes and extensions of the plasma membrane nevertheless does not explain the correlation between the peripheral and mitochondrial pyruvate pools, since the mitochondrial population density does not show periodicity in the longitudinal dimension of striated muscle. Non-muscular cells constitute more than half of the cell population in the adult myocardium (Grove *et al.*, 1969), but their total cell mass is too low to explain the present data.

The importance of metabolite compartmentation in the regulation of intermediary metabolism is increasingly being appreciated, and measurements of metabolite concentrations in the myocardial cytosol and mitochondria *in situ* (Soboll & Bünger, 1982; Kauppinen *et al.*, 1980, 1982) will probably contribute to our understanding of metabolic regulation and reveal the causes of the apparent functional compartmentation observed in the present study.

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