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Respiratory control and substrate effects in the working rat heart

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^{31}P n.m.r. spectroscopy was used to measure the concentration of phosphates commonly proposed to control oxidative phosphorylation. The effect of loading conditions, β-adrenergic stimulation and different substrates (acetate, pyruvate or glucose) was examined under steady-state conditions in the isolated working rat heart. Oxygen consumption and haemodynamic variables were monitored continuously. In response to a 2-fold increase in afterload, there were no significant changes in [ADP], [ATP]/[ADP], or [ATP]/[ADP][P_i]. In the presence of isoprenaline, these variables also tended not to change from afterload. However, isoprenaline, at identical perfusion pressures, consistently decreased the phosphorylation potential and [ATP]/[ADP], but had little effect on [ADP]. Substrates altered the phosphate metabolites in a manner independent of oxygen consumption, and had only minor effects on the relationship between phosphates and work, in contrast with other studies. Thus, metabolites of ATP synthesis are not normally involved in respiratory control. The ^{31}P n.m.r. spectrum can vary greatly, but does not predict oxygen consumption in this preparation. Substrates have no effect on the mechanism of respiratory control. Thus the normal control of respiration in the heart at steady state cannot occur at the level of its substrates. Rather, there must be concerted regulation of the numerous pathways, involving allostery and covalent modification. The attention of future research should be shifted away from the metabolites of ATP and towards identifying the effectors of such regulation.

INTRODUCTION

The control of respiration is concerned with the following question: what factors in the cytosol relay to mitochondria the need for greater ATP synthesis during periods of increased work? This involves the complex interaction of several processes: glycolysis, β-oxidation, tricarboxylic acid cycle, electron transport, oxidative phosphorylation and numerous ion and metabolite transport steps. Although the net flux through all involved pathways must be controlled simultaneously, most discussions focus on the regulation of oxidative phosphorylation only. The study of respiratory control in intact tissues is therefore dominated by three common hypotheses: that respiration is controlled by ADP availability, [ATP]/[ADP] (the 'translocase hypothesis'), or [ATP]/[ADP][P_i], the phosphorylation potential ('near-equilibrium hypothesis'). Hence, there are many who believe that respiration is controlled by the high-energy phosphates in one manner or another [1–6]. Recently, however, a contrary view has become popular, largely as a result of the application of ^{31}P n.m.r. In general, studies *in vivo* have demonstrated the absence of changes in the high-energy phosphate metabolites with work [7–10]; see [11] for a contrast. However, *in vitro* the situation is less clear. For example, studies with the perfused heart have found that the relationship is sensitive to different substrates. Thus, although ^{31}P n.m.r. spectra do not vary with glucose-perfused hearts, they are sensitive to work in hearts supplied with pyruvate and glucose [12,13] or octanoate and palmitate [14].

Since a mixture of substrates is available to the heart *in vivo*, and fatty acids are thought to be the substrate of choice, these results suggest that the phosphate spectrum will change with work. Since this is the opposite of what is found *in vivo*, the effect of different substrates on respiratory control needs further examination. It is also important that the change in oxygen consumption achieved is large enough to maximize the likelihood of detecting a change in metabolic concentrations. This is difficult to achieve, and several interventions are often applied simultaneously (see, e.g., [12,14]). This indiscriminate use of different methods may confuse the final outcome.

The objectives of this study were as follows: to examine respiratory control by using a model of the isolated heart which is the most physiological of those at present available (the working heart), to verify the effects of different substrates on respiratory control with this model, and to distinguish between the effects of different methods of modifying oxygen consumption. The validity of considering respiration as a substrate-level-controlled process will be discussed.

EXPERIMENTAL

Materials

Standard reagents were obtained from Sigma. Isoprenaline (isoproterenol) was supplied by Winthrop–Breon (New York, NY, U.S.A.). Animals used were male Sprague–Dawley rats weighing 350–400 g.

Heart perfusions

Rats were injected with heparin (200 units, intraperitoneally) and pentobarbital (15 mg/kg, intraperitoneally). The working rat heart model of Neely *et al.* [15] was used with an apparatus adapted for n.m.r. similar to that previously described [16]. The perfusate was a modified phosphate-free Krebs–Henseleit bicarbonate buffer [16] with a free Ca^{2+} concentration of 1.2 mM. The supplied substrate was acetate, glucose with 5 units of insulin/l, or pyruvate. Each was supplied at a concentration of 10 mM, thought to be saturating [17].

The pre-load for most experiments was set at 12 cmH_2O. The lowest afterload studied was 60 cmH_2O, for which the coronary flow rate was adequate to maintain function. An intermediate level of 100 cmH_2O was chosen. The highest afterload studied depended on the substrate. Both glucose- and pyruvate-perfused hearts were stable for at least 30 min at an afterload of

Abbreviation used: PCr, phosphocreatine.

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150 cmH_2O. Acetate-perfused hearts were not able to maintain this pressure, and so were studied at afterloads of 60 and 100 cmH_2O only.

The effects of isoprenaline (3 μM) were studied at pressures of 100 cmH_2O and 150 cmH_2O. Isoprenaline was not added at the lowest afterload, since the coronary flow rate was probably too low to support the increase in oxygen demand. Since this gave only one level of afterload with isoprenaline in acetate-perfused hearts, an additional group of acetate-perfused hearts was studied with a pre-load of 6 cmH_2O. This decreased the work output enough to allow the addition of isoprenaline at an afterload of 60 cmH_2O.

Peak aortic systolic pressure and the heart rate were measured by using a pressure transducer attached to the aortic outflow line by means of a T-piece. The rate-pressure product was calculated by multiplying peak systolic pressure and heart rate. Cardiac output was measured with a graduated cylinder and stop-watch. Oxygen partial pressure was measured continuously by mounting two miniature Clark-type electrodes (Diamond Electro-Tech, Ann Arbor, MI. U.S.A.) 15 cm above the heart inside the magnet. Perfusate was pumped past each electrode at identical rates: one source was from the chamber supplying fluid to the left atrium, and the other was from a cannula placed in the pulmonary artery. Oxygen consumption was calculated from the difference in oxygen partial pressure and the coronary flow rate.

N.m.r. spectroscopy

^{31}P n.m.r. spectra were obtained at 122 MHz with a Nicolet NT300 spectrometer. For quantification, a fully relaxed spectrum (140 acquisitions, 45° pulses applied every 5 s) was obtained before collecting spectra under the conditions used throughout the remainder of the experiment (240 acquisitions, 45° pulses, 1 s delay). The conditions used gave a signal-to-noise ratio for the β-phosphate of ATP of 10 or above. Spectra were collected by using 4K data points, baseline-corrected to remove d.c. offsets, zero-filled to improve digital resolution, multiplied by an exponential function to improve signal-to-noise, and Fourier-transformed. The data were transferred to a Digital VAX 11/750 computer for non-linear least-squares curve-fitting of the peaks, assuming a Lorenzian line shape. Measurements of peak areas and pH [18] were made. Estimates of concentrations were made from peak areas by comparison with measurements of ATP from hearts studied under the same conditions. The values (μmol/g dry wt., $\pm$S.D.) obtained were 15.2 ± 1.3 ($n = 8$), 12.6 ± 2.1 ($n = 15$) and 13.7 ± 2.6 ($n = 11$) for acetate-, glucose- and pyruvate-perfused hearts respectively. The cytosolic concentration of free ADP was estimated by using the equilibrium constant (1.66×10^9 mol^{-1}) for creatine kinase [19], assuming 1 mM free Mg^{2+} and measurements of [PCr], [ATP] and pH. In some hearts perfused in the absence of isoprenaline, however, P_i, and therefore pH, could not be measured. Nevertheless, there was little variation in pH among those situations where it could be determined. Hence, in instances where it was not possible to measure pH, an average value was used to estimate [ADP]. The average pH values (mean $\pm$ S.D.) for each substrate group were 7.08 ± 0.03 ($n = 27$), 7.05 ± 0.06 ($n = 17$) and 7.04 ± 0.05 ($n = 16$) for acetate-, glucose- and pyruvate-perfused hearts respectively. The concentration for creatine was obtained after subtracting the value for [PCr] from that for total tissue creatine. Total creatine was measured in separate perfusions under identical conditions. The values (μmol/g dry wt.) obtained were 48.8 ± 5.8 ($n = 11$), 42.0 ± 5.1 ($n = 15$) and 49.2 ± 6.2 ($n = 4$) for acetate-, glucose- and pyruvate-perfused hearts respectively.

Experimental protocol

Glucose- or pyruvate-perfused hearts at a pre-load of 12 cmH_2O were studied in the following order: (1) afterload 60 cmH_2O; (2) afterload 150 cmH_2O; (3) afterload 100 cmH_2O; (4) afterload 100 cmH_2O + isoprenaline; and (5) afterload 150 cmH_2O + isoprenaline. Acetate-perfused hearts at a pre-load of 12 cmH_2O were studied in a similar fashion, except that the stages involving an afterload of 150 cmH_2O were not performed. Acetate-perfused hearts at a pre-load of 6 cmH_2O were studied as follows: (1) 100 cmH_2O; (2) 60 cmH_2O; (3) 60 cmH_2O + isoprenaline; and (4) 100 cmH_2O + isoprenaline. The order of study was chosen to decrease experimental time. It was confirmed that the order in which the different afterloads were applied had no effect on mechanical performance; it was not determined whether the effect of isoprenaline was reversible. In each case, a fully relaxed spectrum and two rapid-pulse spectra were collected during the first stage; thereafter, only rapid-acquisition spectra were collected. The spectra were collected during a period in which heart function was constant (steady state).

Statistical analysis

The data are shown as means $\pm$ S.D. (number in each group). A three-way analysis of variance was performed as a split-plot design with experimental samples (individual hearts) nested under substrate [20]. Significance of difference between experimental groups was determined by using the Tukey 'honestly significant difference test' with Kramer's adjustment for different sample sizes [21]. Significant difference was considered to be reached at the 0.05 level. The analysis was performed by the SAS general linear models (GLM) procedure (SAS Institute, Cary, NC, U.S.A.).

RESULTS

Haemodynamic response

Table 1 gives various measures of cardiac performance. The values reported are from the same period as for spectral data collection, during which heart performance was stable. As expected, both increasing afterload and addition of isoprenaline caused significant increases in oxygen consumption. Similarly, both interventions caused significant increases in the rate-pressure product (increasing afterload raising peak systolic pressure, and isoprenaline causing an increased heart rate). Cardiac output showed statistically significantly different changes with isoprenaline in pyruvate-perfused hearts only; isoprenaline increased cardiac output ($P < 0.001$ at afterloads of 100 and 150 cmH_2O).

No significant differences were found in the haemodynamic variables for hearts perfused with different substrates. There was borderline significance for oxygen consumption, however ($P < 0.06$). Inspection of the means for each substrate suggests a trend, with acetate-perfused hearts having the highest oxygen consumption, and glucose-perfused hearts the lowest.

Biochemical response

Fig. 1 shows spectra from three different experiments (one for each substrate, all at a pre-load of 12 cmH_2O) collected under several of the loading conditions used. For all three substrates, there were no differences in the spectra when the lowest and highest afterloads were compared. However, the addition of isoprenaline had a marked effect, increasing P_i. Isoprenaline also caused an increase in the resonances found in the phospho-monoester region (5–7 p.p.m.), probably as a result of the well-known effect on glycolysis and glycogenolysis.

Various metabolic variables measured from spectra such as found in Fig. 1 are presented in Fig. 2 and Table 2. The changes in pH with work load do not show any trend. There were no

Table 1. Haemodynamic measurements from working rat hearts perfused under various conditions

Abbreviations and units: MVO_2, oxygen consumption (μmol/min per g dry wt.); RPP, rate-pressure product (10^3 mmHg/min); CO, cardiac output (ml/min per g dry wt.); L, M, H, low (60), medium (100) and high (150) afterload (cmH_2O); −, +, absence or presence of isoprenaline (3 μM). Data are means ± S.D. (number in each group). Significant differences: * effect of isoprenaline at constant afterload; † effect of afterload in absence or presence of isoprenaline. Preloads are in cmH_2O.

	Acetate (pre-load = 6)	Acetate (pre-load = 12)	Glucose (pre-load = 12)	Pyruvate (pre-load = 12)
MVO_2				
L−	44.4 ± 5.3 (5)	45.9 ± 4.7 (5)	30.9 ± 3.3 (4)	39.9 ± 10.3 (8)
M−	66.4 ± 10.1 (5)†	65.9 ± 3.5 (5)†	40.4 ± 6.2 (4)†	64.5 ± 13.6 (7)†
H−			59.0 ± 11.0 (4)†	77.5 ± 21.2 (6)†
L+	66.3 ± 10.5 (4)*			
M+	99.1 ± 21.5 (4)*†	95.3 ± 13.3 (4)*	78.9 ± 10.8 (4)*	93.9 ± 22.0 (7)*
H+			99.2 ± 12.0 (4)*†	116 ± 18.1 (5)*†
RPP				
L−	12.0 ± 1.7 (5)	12.0 ± 0.8 (5)	11.6 ± 1.8 (4)	10.1 ± 1.8 (8)
M−	20.8 ± 2.3 (5)†	19.9 ± 1.8 (5)†	17.6 ± 2.5 (4)†	17.8 ± 2.8 (7)†
H−			25.6 ± 4.5 (4)†	26.0 ± 4.4 (6)†
L+	13.9 ± 1.8 (4)			
M+	25.2 ± 2.7 (4)*†	21.8 ± 3.6 (4)	26.3 ± 1.0 (4)*	21.7 ± 4.7 (7)*
H+			41.3 ± 1.7 (4)*†	38.9 ± 6.2 (5)*†
CO				
L−	255 ± 54.9 (5)	335 ± 42.8 (5)	273 ± 80.1 (4)	291 ± 85.6 (8)
M−	227 ± 56.8 (5)	284 ± 39.4 (5)	215 ± 86.5 (4)	250 ± 75.1 (7)
H−			189 ± 92.0 (4)	210 ± 69.7 (6)
L+	249 ± 55.3 (4)			
M+	206 ± 51.7 (4)	264 ± 46.7 (4)	277 ± 78.9 (4)	336 ± 131 (7)*
H+			234 ± 68.6 (4)	280 ± 46.6 (5)*

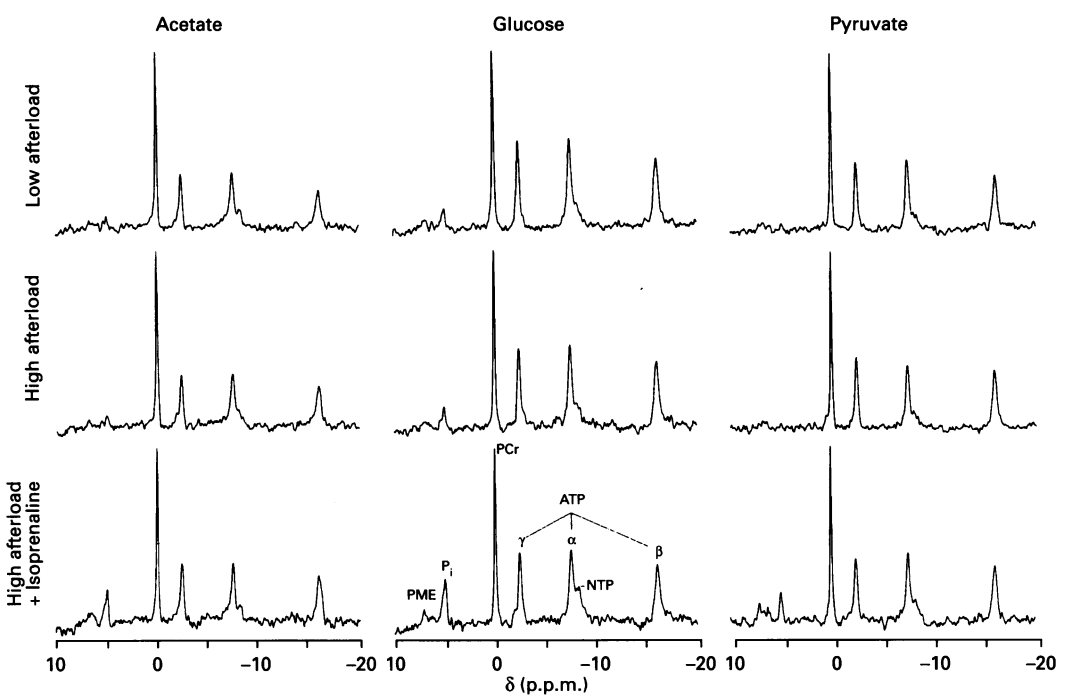

Fig. 1. Effect of afterload and isoprenaline on ^{31}P n.m.r. spectra of working rat hearts perfused with different substrates

Spectra from three hearts perfused with different substrates are shown: acetate (left column), glucose with 5 units of insulin/l (middle column) and pyruvate (right column), each at a concentration of 10 mM. In each case, the pre-load was 12 cmH_2O. The afterload was 60 cmH_2O (upper row), 100 cmH_2O (acetate-perfused heart, bottom two rows) or 150 cmH_2O (glucose- and pyruvate-perfused hearts, bottom two rows). Isoprenaline was present when the spectra shown on the bottom row were collected. Spectra are 240 acquisitions (45° pulses) collected every 1.0 s. Abbreviation: PME, phosphomonoesters.

statistically significant differences for [ADP] as a result of increasing afterloads in the presence or absence of isoprenaline. The addition of isoprenaline had no effect on [ADP] in hearts perfused with glucose or pyruvate. In acetate-perfused hearts, [ADP] was increased in both the 6 cmH_2O ($P < 0.001$) and 12 cmH_2O ($P < 0.01$) pre-load groups at medium afterload.

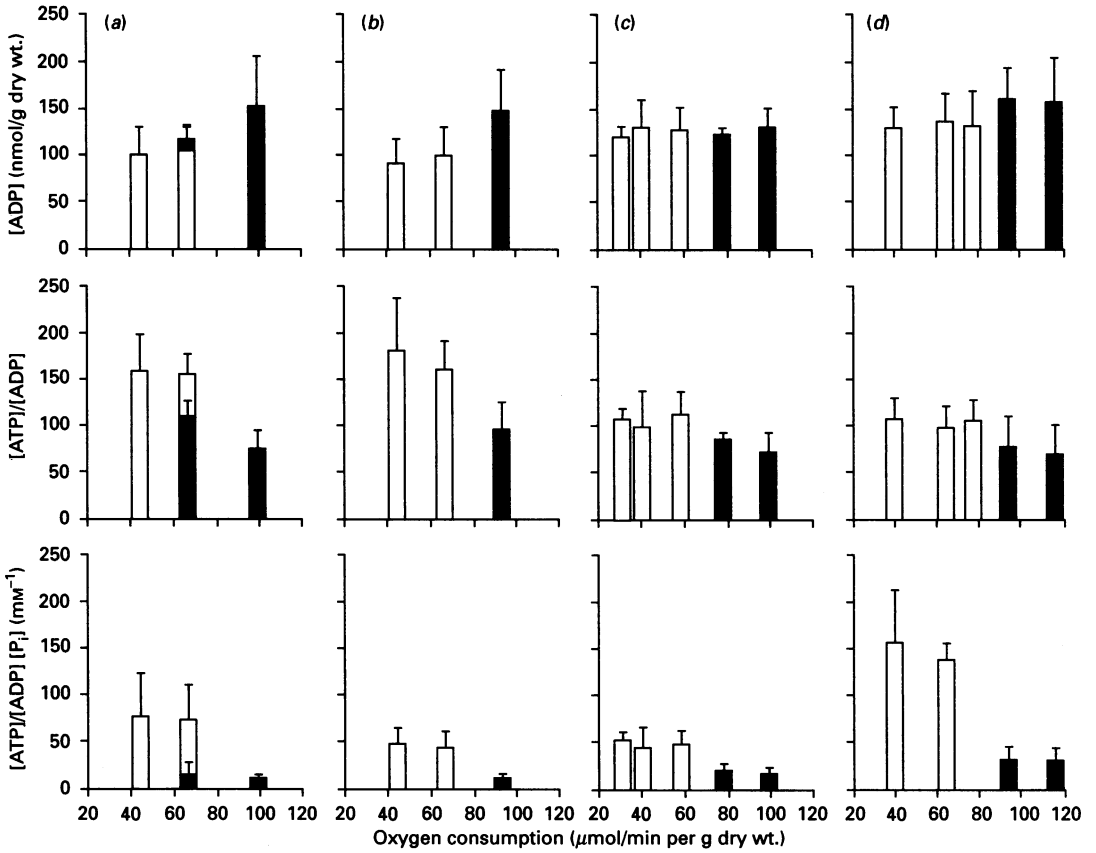

Fig. 2. Relationship between oxygen consumption and various phosphate metabolites in working rat hearts perfused with different substrates

The metabolic variables [ADP] (upper row), [ATP]/[ADP] (middle row) and [ATP]/[ADP][P_i] (lowest row) are shown plotted against the average oxygen consumption for each experimental group studied. The substrate groups shown are: acetate (*a* and *b*), glucose (*c*) and pyruvate (*d*). The pre-load was either 6 cmH_2O (*a*) or 12 cmH_2O (*b–d*). □, Hearts perfused in the absence of isoprenaline; ■, hearts perfused with 3 μM-isoprenaline. Error bars shown are 1 S.D.

N.m.r. measures the concentration of metabolites that are free in the cytosol. The concentration of P_i in hearts is normally low, as found in this study during isoprenaline-free perfusion. Hence it was not possible to measure [P_i] accurately in all cases. The concentration in pyruvate-perfused hearts was such that [P_i] was measurable in only a few of the eight hearts studied. More success was achieved with acetate-perfused hearts, where [P_i] could be measured in approx. 80% of hearts studied at each afterload. [P_i] was higher in glucose-perfused hearts, but its measurement was still difficult. This problem did not occur when isoprenaline was used, because the P_i resonance was much larger in all substrate groups. Nevertheless, analysis of these data found no changes with afterload alone, but significant changes were found with isoprenaline addition at all afterloads and pre-loads studied in acetate-perfused hearts ($P < 0.001$), at high afterload in glucose-perfused hearts ($P < 0.05$), and at medium afterload in pyruvate-perfused hearts ($P < 0.05$).

Changes in afterload or addition of isoprenaline had no effect on [PCr]/[ATP]. Afterload was found to affect [ATP]/[ADP] in only one instance: the ratio was decreased on going from low to medium afterload in the 6 cmH_2O-pre-load acetate-perfused hearts in the presence of isoprenaline ($P < 0.05$). Isoprenaline caused a decrease in [ATP]/[ADP] at all afterloads examined in the acetate-perfused heart groups ($P < 0.0001$). Significant decreases were also found with isoprenaline at high afterload in pyruvate-perfused hearts ($P < 0.01$). No effect was detected in glucose-perfused hearts.

Because of its dependence on [P_i], measurement of the phosphorylation potential was also affected by the low concentration of P_i. Thus estimation of the phosphorylation potential in hearts perfused without isoprenaline was associated with a large degree of error. These estimations probably represent the lower limit for this variable. Multiple comparison tests of the data for this variable found only three differences of interest that were significant. Isoprenaline caused a decrease in phosphorylation potential at both low ($P < 0.05$) and medium ($P < 0.001$) afterloads in acetate-perfused hearts at a pre-load of 6 cmH_2O. Isoprenaline also caused a significant decrease at medium afterload in pyruvate-perfused hearts ($P < 0.001$).

DISCUSSION

Oxygen consumption

The ranges in oxygen consumption (μmol/min per g dry wt.) achieved during this study were 44.4–99.1 for acetate, 30.9–99.2 for glucose, and 39.9–116.1 for pyruvate. These ranges are larger than those found in most of the heart studies addressing the problem of respiratory control [12,22–27]. If any of the high-energy phosphates are sensitive to work rate, these ranges should have been adequate for detection.

Effect of changes in work on phosphate metabolites

The two methods used to cause a change in oxygen consumption were increases in afterload and the additon of isoprenaline. Of all the measurements made, afterload had an effect on the phosphorus metabolites in only one case: a decline

Table 2. Metabolite measurements by n.m.r. in perfused working rat hearts

Abbreviations: PP, phosphorylation potential ([ATP]/[ADP][P_i]); L, M, H, low (60), medium (100) and high (150) afterload (cmH_2O); −, +: absence or presence of isoprenaline (3 μM). Units: [P_i], μmol/g dry wt.; [ADP], nmol/g dry wt.; PP, mM^{-1}. Data are means ± S.D. (number in each group). Significant differences: * effect of isoprenaline at constant afterload; † effect of afterload in presence of isoprenaline.

	Acetate (pre-load = 6)	Acetate (pre-load = 12)	Glucose (pre-load = 12)	Pyruvate (pre-load = 12)
pH				
L−	7.08 ± 0.01 (4)	7.05 ± 0.04 (4)	7.05 ± 0.06 (2)	6.99 ± 0.04 (4)
M−	7.08 ± 0.05 (3)	7.09 ± 0.02 (4)	7.07 ± 0.08 (3)	7.01 ± 0.01 (2)
H−			7.05 ± 0.04 (4)	7.03 (1)
L+	7.04 ± 0.02 (4)			
M+	7.08 ± 0.01 (5)	7.09 ± 0.01 (3)	7.01 ± 0.01 (4)	7.05 ± 0.02 (5)
H+			7.05 ± 0.07 (4)	7.08 ± 0.02 (4)
[ADP]				
L−	97 ± 26.7 (5)	91 ± 24.1 (5)	120 ± 9.0 (4)	130 ± 22.9 (8)
M−	103 ± 20.4 (4)	100 ± 27.6 (5)	128 ± 30.0 (4)	136 ± 28.3 (7)
H−			125 ± 26.0 (4)	133 ± 36.0 (6)
L+	112 ± 15.2 (4)			
M+	147 ± 51.0 (5)*	148 ± 42.1 (4)*	124 ± 7.1 (4)	159 ± 33.9 (7)
H+			128 ± 22.2 (4)	158 ± 44.5 (5)
[P_i]				
L−	7.6 ± 4.6 (4)	8.7 ± 3.5 (4)	5.6 ± 1.0 (2)	2.2 ± 0.8 (4)
M−	7.6 ± 4.8 (3)	8.5 ± 2.8 (4)	6.1 ± 1.6 (3)	1.8 ± 0.4 (2)
H−			5.9 ± 3.0 (4)	3.2 (1)
L+	18.1 ± 4.5 (4)*			
M+	20.6 ± 6.5 (5)*	16.5 ± 4.2 (3)*	11.4 ± 4.5 (4)	7.3 ± 3.3 (5)*
H+			10.9 ± 3.6 (4)*	5.4 ± 1.6 (4)
$\frac{[PCr]}{[ATP}$				
L−	1.81 ± 0.26 (5)	1.77 ± 0.20 (5)	1.42 ± 0.04 (4)	1.47 ± 0.15 (8)
M−	1.69 ± 0.13 (4)	1.78 ± 0.17 (5)	1.40 ± 0.07 (4)	1.49 ± 0.21 (7)
H−			1.41 ± 0.13 (4)	1.53 ± 0.29 (6)
L+	1.72 ± 0.18 (4)			
M+	1.74 ± 0.57 (5)	1.54 ± 0.26 (4)	1.43 ± 0.08 (4)	1.45 ± 0.20 (7)
H+			1.56 ± 0.10 (4)	1.59 ± 0.22 (5)
$\frac{[ATP]}{[ADP]}$				
L−	157 ± 37.9 (5)	180 ± 53.5 (5)	107 ± 8.1 (4)	110 ± 17.0 (8)
M−	152 ± 22.9 (4)	159 ± 63.5 (5)	100 ± 36.4 (4)	100 ± 21.8 (7)
H−			104 ± 28.5 (4)	107 ± 20.4 (6)
L+	109 ± 14.9 (4)*			
M+	75 ± 17.2 (5)*†	93 ± 31.9 (4)*	80 ± 8.0 (4)	78 ± 32.5 (7)
H+			74 ± 19.8 (4)	71 ± 24.7 (5)*
PP				
L−	74.4 ± 46.9 (4)	48.1 ± 10.7 (4)	53.6 ± 7.0 (2)	155.2 ± 53.3 (4)
M−	73.0 ± 35.3 (3)	43.5 ± 14.1 (4)	45.5 ± 15.9 (3)	136.7 ± 14.1 (2)
H−			47.5 ± 10.9 (4)	59.1 (1)
L+	16.1 ± 5.4 (4)*			
M+	10.3 ± 4.1 (5)*	12.2 ± 0.9 (3)	18.9 ± 4.4 (4)	32.3 ± 10.0 (5)*
H+			17.3 ± 3.8 (4)	32.9 ± 10.7 (4)

in [ATP]/[ADP] in acetate-perfused hearts. Isoprenaline, in contrast, caused an increase in oxygen consumption similar to that found on raising afterload, but had more effects on the high-energy phosphates. It caused significant increases in [ADP] in acetate-perfused hearts at medium afterload, and significant decreases in [ATP]/[ADP] and the phosphorylation potential in acetate- and pyruvate-perfused hearts. Hence the changes in these metabolic variables are probably not related to oxygen consumption. In particular, the acetate-perfused hearts studied at a pre-load of 6 cmH_2O provide examples where the average oxygen consumption was similar in two of the experimental conditions employed, and yet the measured [ATP]/[ADP] and [ATP]/[ADP][P_i] ratios were significantly different (Fig. 2).

It is clear that under these conditions the high-energy phosphates are not necessarily sensitive to increases in oxygen consumption. Isoprenaline did cause significant changes in the phosphate spectrum while also increasing oxygen utilization, but it is not possible to determine whether these changes are related to increased ATP utilization, or to some metabolic effect of isoprenaline. Therefore, care should be taken in studies of respiratory control to distinguish properly between the effects of different interventions.

Substrate differences

The differences in phosphate levels found in hearts perfused with different substrates also do not conform to any of the three main hypotheses. None of the variables [ADP], [ATP]/[ADP] or [ATP]/[ADP][P_i] show any significant differences. Although there

was some evidence that oxygen consumption varies with substrate under the same loading conditions, the effect on oxygen consumption may be contrary to that expected: the phosphorylation potential in pyruvate-perfused hearts tended to be larger than in glucose-perfused hearts, yet the former may have higher, not lower, oxygen consumption rates.

Although no differences were found on increasing afterload, the response to isoprenaline varied with substrate. Thus, substrates do not appear to change the mechanism of respiratory control. This is in contrast with other studies, where phosphate metabolites were found to be sensitive to work in pyruvate-perfused hearts [12,13] and in the presence of fatty acids [14]. One possible explanation for the different findings may be a result of neglecting to separate the effects of different methods of altering work. In this study, for example, no change in the phosphate metabolites was found in glucose-perfused hearts whether comparing afterload or isoprenaline. However, with acetate and pyruvate as substrates, isoprenaline caused significant changes, whereas afterload did not. Thus, if the methods of altering work had not been distinguished, a similar conclusion would have been drawn: that respiratory control involves the substrates of ATP synthesis in acetate- and pyruvate-perfused hearts, but not in glucose-perfused hearts. Another possible explanation for the differing results is that different perfusion models were used. There is evidence that the isovolumic heart, the model used in the above studies [12–14], is ischaemic at high work loads [28].

The absence of any change in the phosphate spectrum with increases in work mediated by changing afterload found in this study is similar to the results found *in vivo*. In the intact animal, a variety of fuels are available to the heart, and so the different results obtained *in vivo* as opposed to *in vitro* (where the phosphates are often found to be sensitive to oxygen consumption) may be a result of different substrates. However, the fact that different substrates did not change the relationship between phosphate metabolites and work in this study *in vitro* makes this explanation unlikely.

Respiratory control in the heart

This study demonstrates that ATP metabolite concentrations do not change with work over a wide range of oxygen consumption, irrespective of the perfusion substrate provided. Thus this investigation *in vitro* provides support for the growing evidence *in vivo* that respiratory control does not occur at the level of pathway substrate concentration.

Most discussions of respiratory control are limited to the concept of regulation by pathway substrate concentration [2–6]. Thus it is concluded that regulation involves changes in the degree of saturation with substrate. In this case, large changes in [ADP] and [P_i] would be expected [29], but are not found. Furthermore, an understanding of respiratory control involves consideration of the simultaneous regulation of multiple pathways. Unfortunately, many discussions focus on the regulation of a single stage, implying that all others are at equilibrium. For example, in the absence of any evidence that the metabolites of ATP synthesis change in concentration with work, regulation by changes in [NADH] is frequently implicated. However, the evidence that [NADH] correlates with work is controversial [13,30–32]. Nevertheless, even if [NADH] is important, this still does not explain how the other stages are regulated. Finally, the third substrate of ATP synthesis, the electrochemical gradient, may control respiration. However, although unique relationships have been found between the rate of ATP synthesis and the protonmotive force in isolated mitochondria [33], no evidence for a regulatory role has been found in an intact system.

There is one hypothesis, based on control by substrate concentration, which the data of this study support. This is the hypothesis of Newsholme & Leech [34], which proposes that all components of ATP synthesis are at equilibrium. Therefore, all enzyme activities are high, and only a small change in concentration will cause a substantial change in pathway flux. These concentration changes will be smaller than that detectable by methods available at present. Respiratory control is envisioned to be caused by small changes in [ADP], resulting from changes in ATP consumption. However, given the evidence from ^{31}P n.m.r. magnetization-transfer studies of intact hearts [35], and isotope-kinetic studies of mitochondria [36], it is unlikely that oxidative phosphorylation is at equilibrium.

Therefore, simultaneous regulation of several pathways must be considered. Studies based on the control theory of Kacser & Burns and of Heinrich & Rapoport have demonstrated that control is distributed over several steps (see, e.g., [37]). Such a form of regulation is a candidate for allostery and/or covalent modification, and a number of individual components of respiration are under such control. For example, the activity of several dehydrogenases are sensitive to Ca^{2+} [38,39], and electron-transport chain activity has been shown to affect the rate of ATP release from the synthase [40]. Recently, it has been demonstrated that ATP synthase activity is increased by electrical stimulation or addition of isoprenaline in myocytes [41]. Since this activation survived sonication and dilution in assay buffers, it must have occurred by a non-allosteric mechanism. The challenge of future research is to find a mechanism of control common to all pathways involved, and to demonstrate their role *in vivo*. A strong candidate for such a mechanism involves Ca^{2+}. In addition to its presently accepted role in stimulating contraction and many metabolic pathways, it has been suggested that Ca^{2+} manipulates ATP synthase activity through the recently discovered Ca^{2+}-dependent inhibitor protein [42].

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