Cardiac High-Energy Phosphates Adapt Faster Than Oxygen Consumption to Changes in Heart Rate

M.H.J. Eijgelshoven, J.H.G.M. van Beek, I. Mottet, M.G.J. Nederhoff, C.J.A. van Echteld, N. Westerhof

Abstract  To investigate the dynamic control of cardiac ATP synthesis, we simultaneously determined the time course of mitochondrial oxygen consumption with the time course of changes in high-energy phosphates following steps in cardiac energy demand. Isolated isovolumically contracting rabbit hearts were perfused with Tyrode’s solution at 28°C (n = 7) or at 37°C (n = 7). Coronary arterial and venous oxygen tensions were monitored with fast-responding oxygen electrodes. A cyclic pacing protocol in which we applied 64 step changes between two different heart rates was used. This enabled nuclear magnetic resonance measurement of the phosphate metabolites with a time resolution of ~2 seconds. Oxygen consumption changed after heart-rate steps with time constants of 14 ± 1 (mean ± SEM) seconds at 28°C and 11 ± 1 seconds at 37°C, which are already corrected for diffusion and vascular transport delays. Doubling of the heart rate resulted in a significant decrease in phosphocreatine (PCr) content (11% at 28°C, 8% at 37°C), which was matched by an increase in inorganic phosphate (Pi) content, although oxygen supply was shown to be nonlimiting. The time constants for the change of both Pi and PCr content, ~5 seconds at 28°C and 2.5 seconds at 37°C, are significantly smaller than the respective time constants for oxygen consumption. The changes in phosphate metabolites during changes in oxygen consumption suggest that regulation of oxidative phosphorylation could occur partly via products of ATP hydrolysis, but the unequal time constants of PCr and oxygen consumption suggest that other regulatory mechanisms also play a role. These dissimilar time constants further suggest that there might be an appreciable transient contribution of nonaerobic, presumably glycolytic, ATP synthesis to buffer the high-energy phosphates during fast transitions in cardiac work. (Circ Res. 1994;75: 751-759.)

Key Words  • myocardial energy metabolism • rabbit hearts • mitochondrial control • 31P nuclear magnetic resonance spectroscopy • oxidative phosphorylation

When cardiac work load increases suddenly, a concomitant increase in the hydrolysis of ATP occurs. Since myocardial reserves of high-energy phosphates are limited, only brief deficits in ATP synthesis can be tolerated without deterioration of contractile performance. Hence, ATP production must adapt fast to match ATP hydrolysis.

ATP can be generated in the myocardium by two metabolic pathways: glycolysis and oxidative phosphorylation. The majority of ATP is produced by the latter pathway.1 The first explanations for the regulation of mitochondrial ATP production emphasized the role of ADP and inorganic phosphate (Pi).2,3 These phosphate metabolites would serve as a feedback signal and thereby adequately regulate mitochondrial ATP production. Later, several authors reported that they were not able to detect changes in high-energy phosphates or in the products of ATP hydrolysis after a change in cardiac work load in vivo.4,7 The lack of detectable changes in high-energy phosphates and Pi despite increases in myocardial oxygen consumption suggested that the phosphate metabolites may not be the primary regulators of cardiac mitochondrial respiration.

The absence of a change in ATP or phosphocreatine (PCr) after a change in metabolic demand would also mean that mitochondrial ATP production and hence oxygen consumption must adapt immediately to a change in cytosolic ATP hydrolysis. In the isolated perfused rabbit heart, this is not the case, because oxygen consumption adapts to a new work load with a mean response time of at least 6 seconds.8-10 This mean response time can be viewed as the average delay between the change in cytosolic ATP hydrolysis and mitochondrial ATP synthesis. In view of the measured delay in mitochondrial ATP synthesis, we predict some depletion of high-energy phosphate stores following an increase in metabolic demand,8,10 unless glycolytic ATP production fills the gap. Thus, a large response time would indicate slow mitochondrial adaptation and extensive depletion of PCr. The accompanying accumulation of Pi and ADP might then play a role in the regulation of oxidative phosphorylation. Dissimilar time courses of changes in oxygen consumption and phosphate metabolites would indicate contributions by glycolytic ATP synthesis.11,12

Only limited data on the transient behavior of the phosphate metabolites and oxygen consumption immediately after a change in cardiac work load are available,9 since most of the studies either intentionally focused on periods of stable myocardial metabolism or...
lacked sufficient time resolution to detect possible changes. Therefore, the aim of the present study was to combine time-resolved 31P nuclear magnetic resonance (NMR) spectroscopy and continuous oxygen tension measurements to simultaneously determine the time course of adaptation of cardiac mitochondrial oxygen consumption and the time course of possible changes in high- and low-energy phosphates during changes in cardiac energy demand.

**Materials and Methods**

**Animal Preparations**

Male New Zealand White rabbits (n=14), weighing 2.0±0.4 kg (mean±SD), were anesthetized with intramuscularly administered 10 mg/kg fluanisone and 0.3 mg/kg fentanyl citrate (Hypnorm, Janssen Pharmaceutica) supplemented with pentobarbital sodium (40 mg/kg iv). After intravenous heparinization (250 IU heparin per kilogram), the heart was rapidly excised and cooled in ice-cold perfusate. After cannulation of the aorta, the hearts were perfused according to Langendorff with a constant flow of Tyrode’s solution containing glucose (11 mmol·L⁻¹) as exogenous substrate. Adenosine (10⁻⁴ mol·L⁻¹) was added to the perfusion medium to obtain maximal vasodilatation, which is desirable for our method to determine the mean response time. The right atrium was closed by ligation of the caval veins to ensure that venous effluent leaves the heart via the right ventricle and pulmonary artery. For assessment of contractile function, a water-filled latex balloon was inserted into the left ventricle and was connected to a Statham P23Db pressure transducer (Statham Instruments). The atroventricular node was destroyed by crushing the tissue, resulting in a low spontaneous heart rate. Hearts were then electrically stimulated via two “necklaces” of copper wire and resistors in series, which were attached to the right ventricular outflow tract. This method allowed electrical pacing without the introduction of electromagnetic interference. Hearts were then placed in a 30-mm-diameter NMR tube together with a glass capillary containing a solution of methylenediphosphonate (MDP) for spectral reference. The glass tube was lowered into the magnet, and the heart was positioned in the center of a Helmholtz coil. The effluent was removed from a level above the heart, leaving the heart submerged in perfusate for optimal magnetic susceptibility matching. Myocardial temperature was kept constant by water-jacketed perfusion lines and a continuous stream of heated air around the sample tube.

**Experimental Procedures**

The present study was carried out at 37°C and also at 28°C in a separate group of hearts, because at this latter temperature oxygen supply has been shown to be nonlimiting. The perfusate was filtered using a 0.45-μm filter (Millipore) and was saturated with gas containing 95% O₂/5% CO₂, resulting in a pH of about 7.3. Perfusion flow was kept constant by means of a peristaltic pump and was continuously measured with an electromagnetic flow probe (MDL 503 system, Skalar). Coronary perfusion pressure was measured with a Statham P23Db pressure transducer and was initially adjusted to a pressure of about 85 mm Hg. Coronary oxygen tensions in arterial inflow and venous outflow were continuously monitored with fast-responding Clark-type oxygen electrodes, which did not interfere with the NMR measurement and which were located 1 cm above the top of the heart. The electrodes were calibrated before and after the experiment. Oxygen concentrations were calculated with an oxygen solubility of 1.53 μmol O₂ · L⁻¹ · Tyrode · mm Hg⁻¹ (where L_Tyrode indicates 1 L Tyrode’s solution) at 28°C and 1.33 μmol O₂ · L⁻¹ · Tyrode · mm Hg⁻¹ at 37°C. Data were recorded on a thermal recorder (Gould 4000) and simultaneously digitized and stored on a personal computer (PCP 90, Olivetti).

**Calculation of the Mean Response Time of Mitochondrial Oxygen Consumption**

The speed at which mitochondrial ATP production adapts to a change in metabolic demand can be described by the mean response time, which is equivalent to the time constant if the time course is monoeponential but which is also generally applicable to nonmonoeponential responses. From the time course of the oxygen tension transient in the venous effluent, a measured response time (tₘ) can be determined. Because of diffusion of oxygen between capillaries and mitochondria and transport of oxygen in the coronary blood vessels toward the oxygen electrode, we have to correct tₘ, measured in the coronary venous effluent, for the transport time (t_transport) to obtain the mean response time of mitochondrial oxygen consumption (tₘᵣᵢₜ) according to the following equation:

\[ t_{\text{mit}} = t_{\text{m}} - t_{\text{transport}} \]

Thus, the true response time of oxidative phosphorylation at the level of the mitochondria is obtained. The derivation of this equation and the precise mathematical definitions tₘ, tᵣᵢₜ, and t_transport have been described before, but a brief explanation of their use follows for the reader’s convenience.

The mathematical derivation shows that t_transport is obtained by dividing the change in the amount of oxygen in the heart (ΔQ) by the change in oxygen consumption (ΔM) that caused this change in the amount of oxygen:

\[ t_{\text{transport}} = \frac{-\Delta Q}{\Delta M} = V_{\text{d,an}} F \]

ΔQ is obtained from a model based on the Krogh cylinder and is normalized to the change in venous oxygen tension (ΔC), giving V_d,an (ΔQ/ΔC). F is the flow through the heart. To obtain the data for the model estimation of V_d,an, two different interventions (ie, small step changes in perfusion flow or in arterial oxygen tension) are applied. In both cases, the change in amount of oxygen can be measured as the washout of oxygen from the tissue in the venous effluent above a baseline value that corresponds with a constant oxygen consumption. The normalized change in the amount of oxygen after a step change in perfusion flow (V_d) directly gives a good estimate of V_d,an. This implies that the mean response time obtained from the recording of the venous oxygen tension during a step change in perfusion flow can directly be substituted for t_transport in Equation 1, provided the flow after the step is the same as during the change in metabolic demand. The alternative way to determine V_d,an is more complex: after a stepwise change in arterial oxygen tension, the mean transit time for oxygen is obtained from the venous oxygen tension transient. After multiplication of this mean transit time with flow, the volume of distribution of oxygen (V_os) (ie, the change in amount of oxygen normalized to the change in venous oxygen tension) is obtained. V_d,an can then be calculated from V_os, taking the coronary blood vessel volume into account, which has been determined in previous experiments in the same preparation.

Oxygen reversibly bound to myoglobin is explicitly included in the model and is measured during the washout procedures just mentioned. The capacitive action of oxygen binding to myoglobin is thus incorporated in the calculation of t_transport. The effective oxygen solubility we determined from V_os, in our isolated rabbit heart preparation is not much higher than the physical oxygen solubility, which indicates that there was little deoxygenation of myoglobin caused by the 10% decrease in oxygen concentration.

**NMR Measurements**

NMR measurements were performed on a Bruker MSL 200 NMR spectrometer equipped with a 4.7-T vertical bore magnet (diameter, 15 cm) and a single-tuned 30-mm 31P probe. Magnetic field homogeneity was optimized by shimming on water protons, resulting in line widths of about 9 Hz. 31P control spectra were obtained from 64 accumulated free induction decays (FIDs) after 70° pulses repeated at 10-second intervals, with a spectral width of 5000 Hz. Further, an NMR protocol was used; this protocol allowed us to measure changes in...
metabolite contents with a time resolution of 2.0 seconds at 28°C and 1.8 seconds at 37°C during repeated steps back and forth between two heart rates. For these acquisitions a 70 pulse was also used. Both the NMR spectrometer and the cardiac stimulator were triggered by the Bruker pulse programer, which ensured measurement always at the same phase (end diastole) of the heart cycle. This heart-rate–step NMR protocol was repeated 64 times to obtain a sufficient signal-to-noise ratio for data analysis (see “Experimental Protocol”). FIDs were sequentially accumulated in separate memory blocks corresponding with fixed time points in the cyclic pacing protocol. NMR data were quantified by a time-domain fitting routine.16

PCr, P_i, and MDP were corrected for partial saturation. Saturation factors were identical at both heart rates and were 1.3 and 1.3 for PCr, 1.0 and 1.1 for P_i, and 1.5 and 1.8 for MDP at 28°C and 37°C, respectively. These factors were determined by using data of the control spectra with a 10-second repetition time. pH values were calculated from the chemical shift difference (δ, in parts per million) between intracellular P_i and PCr by use of the following relation: pH = 6.75 + 10^(log(δ-3.27)) (5.69-8).17 This relation was used for both temperatures, since there is very little change in pKa between the two temperatures used in our study.18 A value of 0 ppm was assigned to PCr.

**Experimental Protocol**

Hearts were perfused either at 28°C (n = 7) or at 37°C (n = 7). The hearts were paced at two different heart rates: 60 and 120 beats per minute at 28°C or 100 and 200 beats per minute at 37°C. We performed a cyclic heart-rate–step NMR protocol (Fig 1) where we repeatedly switched back and forth between the two heart rates while sequentially recording FIDs in separate memory blocks spanning an entire cycle of the protocol. At 28°C, each cycle of the cyclic heart-rate–step NMR protocol consisted of 6.0 seconds at 60 beats per minute, 62.0 seconds at 120 beats per minute, and 60.0 seconds at 60 beats per minute. At 37°C, the cycles consisted of 3.6 seconds at 100 beats per minute, 39.6 seconds at 200 beats per minute, and 39.6 seconds at 100 beats per minute again. During the NMR data acquisition, oxygen tension was recorded continuously.

In addition to the cyclic heart-rate–step NMR protocol, a series of interventions (Fig 1) were carried out at 37°C; these interventions were necessary to determine t_max and t_x, which were, in turn, used to calculate t_min (see “Calculation of the Mean Response Time of Mitochondrial Oxygen Consump-

**Fig 1.** Schematic representation of the experimental protocol at 28°C and 37°C. Several interventions were performed to determine the mean response time of mitochondrial oxygen consumption: step in arterial oxygen concentration (ACS), step in perfusion flow (PPS), and heart-rate step (HRS) (see “Materials and Methods”). Steady-state 31P nuclear magnetic resonance (NMR) control spectra (CS) were obtained with 10-second repetition time at both heart rates (60 and 120 beats per minute at 28°C or 100 and 200 beats per minute at 37°C). During the cyclic heart-rate–step NMR protocol, we switched 64 times each way between two heart rates: 60 and 120 beats per minute at 28°C or 100 and 200 beats per minute at 37°C.

**Data Analysis**

Data are presented as mean±SEM unless indicated otherwise. An ANOVA for repeated measurements19 was used to analyze the effect of various experimental conditions on myocardial oxygen consumption. Phosphate metabolite contents at different heart rates were compared by a paired t test. A test result with a value of P<.05 was considered significant. To estimate the time constants of the changes in phosphate metabolite contents, an exponential curve was fitted to the data by a nonlinear least-squares fitting routine using a finite difference Marquardt-Levenberg algorithm.20 Since the changes in P_i and PCr were relatively small and NMR measurements are relatively insensitive, the data of all hearts at the same temperature were averaged to obtain a sufficient signal-to-noise ratio before fitting the exponential curve. In contrast, t_min Values were calculated for each heart individually. Average t_min values were then compared with time constants of phosphate metabolites by an unpaired t test.19 An ANOVA for repeated measurements19 followed by a post hoc contrast test was used to test for changes in pH over time. Using this post hoc contrast, we compared the pH at 28°C for the first eight points after a step in heart rate with the last eight points at the same heart rate, and pH values at 37°C during the first and last five points were compared.

We have calculated the ADP concentration as follows: a cytosolic water volume of 0.4 mL·g dry wt^{-1} was used;21,22 the apparent equilibrium constant (K_{eq}) of the creatine kinase reaction was 210 at 28°C and 180 at 37°C, according to recently published data;23 and total creatine was assumed to be 60 μmol·g dry wt^{-1} according to previously published data24 and our own high-performance liquid chromatography measurements on isolated rabbit hearts according to published methods.25

**Results**

**General**

Wet weight of the hearts was 7.3±0.6 g (mean±SD) at the end of the experiment. Dry weight, determined after 3 days of storage at 80°C, was 0.9±0.1 g (mean±SD). Average coronary flow, perfusion pressure, and oxygen consumption during the cyclic heart-rate–step NMR protocol are shown in Table 1. Flow and oxygen consumption measured before, during, and after the NMR protocol were not different, indicating a stable preparation. Perfusion pressure increased slightly over time by ≈4% per hour. Left ventricular systolic pressure decreased with heart rate (Fig 2). At 28°C, end-diastolic pressure increased significantly with higher heart rate because of incomplete relaxation at this low temperature.
TABLE 1. Physiological Parameters

<table>
<thead>
<tr>
<th>Temperature</th>
<th>Flow, mL·min⁻¹·g wet wt⁻¹</th>
<th>Perfusion pressure, mm Hg</th>
<th>Oxygen consumption, μmol·min⁻¹·g dry wt⁻¹</th>
</tr>
</thead>
<tbody>
<tr>
<td>28°C</td>
<td>7.7±0.4</td>
<td>85±2</td>
<td>16.5±1</td>
</tr>
<tr>
<td>37°C</td>
<td>8.5±0.4</td>
<td>85±1</td>
<td>8.5±1</td>
</tr>
<tr>
<td></td>
<td></td>
<td>High heart rate</td>
<td>91±2</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Low heart rate</td>
<td>83±2</td>
</tr>
<tr>
<td></td>
<td></td>
<td>High heart rate</td>
<td>20.6±2.0*</td>
</tr>
</tbody>
</table>

Values are mean±SEM; at each temperature, seven hearts were investigated. Low heart rates are 60 and 100 beats per minute, and high heart rates are 120 and 200 beats per minute at 28°C and 37°C, respectively.

After a 10% reduction in arterial oxygen tension at 37°C, oxygen consumption increased significantly inside the NMR tube by ~7%. This was associated with a very small (~1%) increase in developed pressure after the reduction in arterial oxygen concentration. Although the mechanism of this small increase in oxygen consumption following a decrease in arterial oxygen tension is not known, this phenomenon has been reported before in a study on dog heart in situ. After a 7.3±0.4% reduction of flow at 37°C, there was no statistically significant change in oxygen consumption. These two findings indicate that oxygen supply did not limit oxygen consumption at 37°C. In accordance with several previous studies on the isolated rabbit heart, oxygen supply also did not limit oxygen consumption at 28°C.

Calculations of $t_{\text{mito}}$

In Figs 3C and 4C, recordings of coronary venous oxygen tension obtained during stepwise increases and decreases in heart rate are presented. From such recordings, $t_{\text{mito}}$ was determined (see "Materials and Methods").

![Fig 2](https://example.com/f2.png)

Fig 2. Left ventricular (LV) systolic and diastolic pressures plotted during several interventions at both 28°C and 37°C. Heart rate was 60 beats per minute at 28°C and 100 beats per minute at 37°C, except during doubling of heart rate ($\Delta$ HR). Symbols represent mean±SEM. The error bars for diastolic pressure were omitted when they fell within the symbol. C indicates control before and after the respective intervention; $\Delta$ F, decrease in perfusion flow; and $\Delta$ PO₂, 10% decrease in arterial oxygen tension. *Statistically significant difference compared with C value.

![Fig 3](https://example.com/f3.png)

Fig 3. Time courses of inorganic phosphate (Pi) content (A), phosphocreatine (PCr) content (B), coronary venous oxygen tension (C), and product of heart rate and peak systolic pressure (rate-pressure product [RPP], D) during heart-rate steps at 28°C. 

$dw$ indicates dry weight; BPM, beats per minute; and arrows and dashed lines, the time points at which the heart rate was changed. The heart rate was initially 60 beats per minute. Metabolite contents were determined at 2-second intervals and were corrected for partial saturation. For each experiment, the nuclear magnetic resonance data for every single time point consisted of 64 accumulated free induction decays (see "Materials and Methods"). All data were averaged for seven experiments; bars represent SEM, which mainly reflects interindividual variation. The SEM of oxygen tension (~26 mm Hg) and of RPP (~730 mm Hg·min⁻¹) are omitted for clarity.

Within all perfusion conditions the $t_{\text{mito}}$ obtained from upward and downward steps in heart rate was not significantly different. Thus, the average $t_{\text{mito}}$ of the upward and downward changes in heart rate is presented in Table 2. At both temperatures, $t_{\text{mito}}$ increased significantly from the beginning to the end of the experiment, with 5.1 and 3.8 seconds at 28°C and 37°C, respectively. In Table 2, $t_{\text{mito}}$ averaged over time is given.

$t_{\text{transport}}$ was obtained from venous oxygen tension transients after steps in flow, down by 9.4±0.3% at 28°C and by 7.3±0.4% at 37°C, or after steps in arterial oxygen tension. $t_{\text{transport}}$ values obtained at the high and low heart rates did not show any difference. No significant difference was found between $t_{\text{transport}}$ obtained at the beginning or at the end of the experiment, indicating stability of oxygen transport. Therefore, the overall average $t_{\text{transport}}$ for each temperature is used to calculate $t_{\text{mito}}$ (Table 2).

Changes in force production in isolated heart muscle, which are comparable with changes in developed pressure in our isolated heart preparation, correspond with...
immediate changes in ATP hydrolysis during each contraction.\textsuperscript{27} If rate-pressure product (RPP), ie, heart rate times peak systolic pressure, is taken as a measure for metabolic demand,\textsuperscript{6} we can investigate the time course of metabolic demand (ATP hydrolysis) after a step in heart rate on a beat-to-beat basis. The RPP reached a steady state after an initial overshoot (Figs 3D and 4D), because there is a gradual decrease in systolic pressure after the upward step in heart rate. The mean response time for RPP ($t_{\text{RPP}}$) is negative because of this overshoot (see Reference 9 for the mathematical definition of $t_{\text{RPP}}$). The negative values of $t_{\text{RPP}}$ (Table 2) indicate that $t_{\text{RPP}}$ slightly underestimates the delay between ATP hydrolysis and mitochondrial ATP synthesis.\textsuperscript{9}

Venous oxygen tension first increased transiently after the upward step in heart rate and subsequently decreased to a new steady state because of the increase in oxygen consumption (shown in Fig 3C and, barely visible, in Fig 4C). During a decrease in heart rate, the inverse was found (Figs 3C and 4C). This initial deflection is not related to changes in mitochondrial oxygen consumption but is presumably caused by a transitory increase in venous outflow, resulting in a decrease of coronary vascular volume due to the more frequent contractions of the myocardium.\textsuperscript{8-10,28} The initial deflec-

![Fig 4. Time courses of inorganic phosphate (Pi) content (A), phosphocreatine (PCr) content (B), coronary venous oxygen tension (C), and product of heart rate and peak systolic pressure rate-pressure product [RPP], D during heart-rate steps at 37°C. dw indicates dry weight; BPM, beats per minute; and arrows and dashed lines, the time points at which the heart rate was changed. The heart rate was initially 100 beats per minute. Metabolite contents were determined at 1.8-second intervals and were corrected for partial saturation. The SEM values of oxygen tension (≈31 mm Hg) and of RPP (≈950 mm Hg · min$^{-1}$) have been omitted for clarity. For further explanation see legend to Fig 3.](http://circres.ahajournals.org/)

**Table 2.** Measured Venous Response Time, the Transport Time Characterizing the Total Delay Caused by Diffusion and Vascular Transport, and Mean Response Time of Mitochondrial Oxygen Consumption After Steps in Heart Rate

<table>
<thead>
<tr>
<th>Temperature</th>
<th>28°C</th>
<th>37°C</th>
</tr>
</thead>
<tbody>
<tr>
<td>$t_{\text{1}}$, s</td>
<td>22.6±1.5</td>
<td>20.8±1.8</td>
</tr>
<tr>
<td>$t_{\text{transport}}$ (ΔF), s</td>
<td>8.2±0.7</td>
<td>9.9±1.1</td>
</tr>
<tr>
<td>$t_{\text{transport}}$ (ΔPO$_2$), s</td>
<td>ND</td>
<td>8.8±1.4</td>
</tr>
<tr>
<td>$t_{\text{transport}}$ (mean), s</td>
<td>ND</td>
<td>9.4±1.5</td>
</tr>
<tr>
<td>$t_{\text{mito}}$, s</td>
<td>14.4±1.5</td>
<td>11.3±1.1</td>
</tr>
<tr>
<td>$t_{\text{RPP}}$, s</td>
<td>−3.5±0.3</td>
<td>−1.2±0.1</td>
</tr>
<tr>
<td>$t_{\text{initial deflection}}$, s</td>
<td>2.4±0.3</td>
<td>0.09±0.02</td>
</tr>
</tbody>
</table>

$t_{\text{1}}$ indicates measured response time obtained from venous oxygen transient after a heart-rate step; $t_{\text{transport}}$ (ΔF), transport time obtained from step in perfusion flow; $t_{\text{transport}}$ (ΔPO$_2$), transport time calculated from the step in arterial oxygen tension; $t_{\text{transport}}$ (mean), average of $t_{\text{transport}}$ (ΔF) and $t_{\text{transport}}$ (ΔPO$_2$); $t_{\text{mito}}$, mean response time of mitochondrial oxygen consumption, calculated from $t_{\text{1}}$ and $t_{\text{transport}}$ (see "Materials and Methods"); $t_{\text{RPP}}$, mean response time rate-pressure product; $t_{\text{initial deflection}}$, contribution of initial deflection to mean response time (see Reference 10); and ND, not determined. Values are mean±SEM.

**NMR Measurements**

Fig 5 shows two NMR spectra obtained at 28°C at a heart rate of 60 and 120 beats per minute, respectively. From these spectra, differences in PCr and Pi content with different work loads can be clearly observed. Figs 3 and 4 show the time courses of PCr, P, coronary venous oxygen tension, and RPP during heart-rate steps at 28°C and 37°C, respectively. After an increase in heart rate, P$_i$ increased and PCr decreased by almost the same amount, whereas for a decrease in heart rate, the opposite was found. The time constants of the changes was quantified according to a previously published method\textsuperscript{10} and turns out to be small (Table 2), so that $t_{\text{RPP}}$ is only slightly overestimated.

![Fig 5. 31P nuclear magnetic resonance (NMR) spectra of an isolated perfused rabbit heart paced at 60 (A) or 120 (B) beats per minute at 28°C obtained during the cyclic heart-rate-step NMR protocol at time points of 118 and 24 seconds (see Fig 3), respectively. See text for signal acquisition parameters. Similar results were found at 37°C. MDP indicates methylenediphosphonate (external reference); Pi, inorganic phosphate; PCr, phosphocreatine; and PPM, parts per million.](http://circres.ahajournals.org/)
in phosphate metabolites did in no case differ significantly between the upward and downward steps. The time constant for the changes in \( P_i \) content was 5.3 ± 1.3 seconds at 28°C. The same value, 5.3 ± 1.7 seconds, was found for the changes in PCr content. Toward the end of the period of high heart rate at 28°C, \( P_i \) content slightly decreased toward a new level. At 37°C, the time constants were 2.3 ± 1.2 and 2.7 ± 2.2 seconds for changes in \( P_i \) and PCr content, respectively. The time constants at 37°C are not much longer than the time resolution and could therefore not be estimated very accurately.

Table 3 summarizes data on PCr, \( P_i \), and ATP during the cyclic heart-rate–step NMR protocol. PCr declines ≈11% after an increase in heart rate from 60 to 120 beats per minute at 28°C, whereas \( P_i \) content increases by about the same absolute amount as PCr decreases. The calculated [ADP] at 28°C was 32 and 37 μmol · L⁻¹ at a heart rate of 60 and 120 beats per minute, respectively. The calculated [P] increased from 0.77 to 1.44 mmol · L⁻¹ after doubling the heart rate at this temperature. Compared with 28°C, at 37°C the changes of PCr and \( P_i \) are somewhat smaller (Table 3), and the ATP content does not change. At this latter temperature [ADP] changes from 56 to 63 μmol · L⁻¹ at 100 and 200 beats per minute, respectively. The calculated \([P] \) values at 100 and 200 beats per minute are 0.55 and 0.83 mmol · L⁻¹, respectively.

pH, was 7.10 ± 0.05 at 28°C and 7.05 ± 0.04 at 37°C. There were no significant changes in steady-state pH with heart rate. However, at 2 to 16 seconds after doubling the heart rate at 28°C, pH was increased significantly by 0.02 to 0.04 pH unit and then decreased again toward the control value (Fig 6). At 37°C, a similar increase in pH at 2 to 9 seconds after the heart-rate step was also significant. The opposite was seen after the decrease in heart rate.

### Comparison of \( t_{\text{mito}} \) and the Time Constants of PCr and \( P_i \)

Values for \( t_{\text{mito}} \) are presented in Table 2. At both 28°C and 37°C, \( t_{\text{mito}} \) is significantly longer, by a factor of 2.7 and 4.5, respectively, than the time constants of the changes of both \( P_i \) and PCr at the same temperature.

### Discussion

**Critique of Methods**

Since the expected changes in phosphate metabolites were quite small, we tried to impose changes in heart rate that were as large as possible without causing problems with oxygen supply. It has been suggested that at 37°C the oxygen supply might be somewhat impaired in the isolated perfused rabbit heart. Therefore, we did half of our studies at 28°C, and we used adenosine to obtain maximal vasodilation. Earlier studies from this laboratory showed that the heart rate could be increased to 120 beats per minute at 28°C without causing problems in oxygen supply or mechanical function. In pilot experiments, we found that we could increase the heart rate to ≈200 beats per minute at 37°C before oxygen supply would become limiting. This is in agreement with a report by Murashita et al on the isolated working rabbit heart. To obtain a basal heart rate as low as possible, we crushed the atrioventricular node of the heart, after which we could pace the heart at 100 beats per minute. Compared with normal in vivo heart rates of a rabbit, which vary between 200 and 300 beats per minute, the heart rates we used in the present study are thus relatively low, necessitated by the requirement of a good oxygen supply. The temperature of rabbits in vivo is relatively high (on average, 39.5°C). We followed the practice of the majority of laboratories and obtained measurements at 37°C, despite the small difference this implies with in vivo conditions.

The calculation of \( t_{\text{transport}} \) depends on our model for oxygen transport. Since several assumptions, which have been discussed by Van Beek and Westerhof, however, the two methods to calculate \( t_{\text{transport}} \), which use two independent data sets, give identical results. Further, comparison of \( t_{\text{mito}} \) with the time course of oxidative phosphorylation obtained from recovery heart-rate measurements in isolated heart muscle showed excellent correspondence.

Because of the noiselessness of our data on the phosphate metabolites, the results of the curve-fitting procedure exhibited a relatively large standard error. Despite this scatter in the data, the differences between the response times of the phosphate metabolites and oxygen consumption were highly significant. Extrapolation of the present results in the isolated saline-perfused rabbit heart to in vivo conditions should be done with caution. Especially the exogenous substrate available to the heart (in the present study, glucose) might have a large effect on the behavior of energy metabolism.

### Physiological Implications

The present study demonstrates that in the isolated Tyrode-perfused rabbit heart, high- and low-energy phosphates change with work load in contrast to many of the results obtained in vivo. The changes in high- and low-energy phosphates suggest that phosphate metabolites could participate in the control of oxidative phosphorylation in this experimental model. In the isolated rabbit heart, oxygen consumption following a step in heart rate adapts with a response time of many seconds and is substantially longer than the corresponding time constants of the phosphate metabolites.
were not the view that the observed low-energy phosphate
content is most likely caused by the fall in PCr content, which leads to an absorption of protons via the creatine kinase reaction.25-27 The reverse is seen after a decrease in heart rate. According to Connell,25 a decrease in [PCr] of ∼1.5 mmol · L⁻¹ in skeletal muscle would cause an increase in pH of ∼0.03 to 0.05 pH units. Since in the present study the changes in both [PCr] and pH are in these ranges, this would mean that the alkaline shift observed could be totally attributed to the change in [PCr]. The return of pH to baseline after the initial increase during the heart rate step (Fig 6) may then be caused by the extra production of protons due to the formation of intracellular acid such as lactic acid (see end of “Discussion”).

Several mechanisms for the control of oxidative phosphorylation involving ADP and Pi have been put forward; these include control via the phosphorylation potential (ATP/ADP · P),38 the ATP-to-ADP ratio,39 and [ADP]2 and [Pi].40 The small but significant changes in ADP that occur after changes in heart rate in the present study suggest that the change in [ADP] could contribute to the regulation of oxidative phosphorylation. Furthermore, [ADP] at 28°C seems to be in the range of its mitochondrial Km value, which is believed to be 20 to 30 mmol · L⁻¹ at similar temperatures.2,40 Recently, it has also been suggested that the Km of mitochondria for ADP increases significantly when the suspending medium has a colloid osmotic pressure comparable to that of the cytosol (References 41 and 42; F.N. Gellerich, personal communication). Also, [Pi] in the present study is in the range of its Km value, 330 to 800 mmol · L⁻¹,43 indicating that the observed changes in [Pi] (from 0.77 to 1.44 mmol · L⁻¹ at 28°C and from 0.55 to 0.83 mmol · L⁻¹ at 37°C) may very effectively stimulate mitochondrial ATP synthesis.

In isolated mitochondria, the response of oxidative phosphorylation to changes in phosphate metabolites is very fast (half time, ∼70 milliseconds).44 In contrast, in intact myocardium the time constants of the changes in PCr and Pi (and by inference, also ADP) are smaller by several seconds than t_mito for a step in heart rate (see “Results”). This contrast indicates that factors other than phosphate metabolites very likely also contribute to the regulation of mitochondrial ATP synthesis. Intramitochondrial calcium and the delivery of reducing equivalents to the mitochondrial respiratory chain have been proposed to be potential regulators in the control of oxidative phosphorylation.31,45-48 We have previously shown that the response time of cardiac oxygen consumption to demand was significantly diminished when the NADH supply to the respiratory chain was increased by replacing glucose by supraphysiological concentrations of pyruvate in the perfusate of the isolated rabbit heart, revealing the control exerted by the NADH-producing processes.14 Further, it has been suggested that mitochondrial proteins regulating ATP synthase play a role in the adaptation of ATP synthesis to work load.49

Oxygen consumption at a heart rate of 60 beats per minute at 28°C is larger than the oxygen consumption at a heart rate of 100 beats per minute at 37°C (Table 1), although the RPP, which correlates well with myocardial oxygen consumption in other cases, is smaller at 28°C. Furthermore, several reports in the literature have appeared that showed increases in systolic pres-

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**Table 3. Metabolite Contents of Isolated Perfused Rabbit Hearts at Two Heart Rates**

<table>
<thead>
<tr>
<th>Heart Rate, bpm</th>
<th>PCr, μmol · g dry wt⁻¹</th>
<th>Pi, μmol · g dry wt⁻¹</th>
<th>ATP, μmol · g dry wt⁻¹</th>
</tr>
</thead>
<tbody>
<tr>
<td>28°C 60</td>
<td>25.3±0.1</td>
<td>2.5±0.1</td>
<td>153.0±2.0</td>
</tr>
<tr>
<td>120</td>
<td>22.6±0.1*</td>
<td>4.7±0.1*</td>
<td>14.9±0.2*</td>
</tr>
<tr>
<td>37°C 100</td>
<td>22.3±0.2</td>
<td>1.8±0.1</td>
<td>20.3±0.3</td>
</tr>
<tr>
<td>200</td>
<td>20.6±0.3*</td>
<td>2.0±0.3*</td>
<td>20.4±0.3</td>
</tr>
</tbody>
</table>

*Significant difference compared with low heart rate at same temperature.

bpm indicates beats per minute; PCr, phosphocreatine; and Pi, inorganic phosphate. Values are mean±SEM (n=7) and are averages of the last 20 or 10 points of each heart-rate period of the cyclic heart-rate-step nuclear magnetic resonance protocol at 28°C and 37°C, respectively (see Figs 3 and 4).

For many of the in vivo NMR studies, it was reported that there were no changes in phosphate metabolites due to changes in work load.4,7 In other studies, changes in these metabolites were reported under specific experimental circumstances.3,32-34 For example, Heineman and Balaban3 showed that in mature dog hearts the PCr-to-ATP ratio only changed with heart rate when there was a concomitant decrease in coronary flow and arterial blood pressure. Portman et al34 showed that changes in the PCr-to-ATP ratio due to increases in work load occurred in newborn lambs but not in mature sheep. Massie et al33 reported a significant decrease in the PCr-to-ATP ratio at high work loads in porcine left ventricle. A possible explanation for the discrepancy between the change in PCr content found in the present study on isolated hearts and results obtained from many in vivo experiments, in which there were often no changes in phosphate metabolites detected,4,7 may be a difference in measurement precision. The standard errors for PCr obtained from in vivo measurements vary between 4% and 10%,6,7 whereas the standard error seen in our study is maximally 1.5%, in most cases even less (Table 3). The standard errors of the steady-state data in the present study are small because the last 10 or 20 time points (see Figs 3 and 4, with each time point being the average of seven experiments) of each heart rate level were averaged. The changes in PCr in the present study are of the same order as the standard error of in vivo NMR measurements in the studies of others, and these changes are significant because of the substantially smaller standard error in the present study.

Changes in phosphate metabolites with work load have been attributed to deficient oxygen supply.3 Because oxygen consumption and developed pressure were not depressed by decreases in perfusion flow and arterial oxygen tension (see “Results” and our previous studies8,10,12,13), it must be concluded that there is no limitation by oxygen supply in our preparation. Furthermore, we have previously shown that the lactate efflux from our preparation is very low.12 Steady-state pH, was constant when the heart rate was doubled, which supports the view that the observed changes in high- and low-energy phosphate levels at different heart rates are not caused by hypoxia. The transient increase of pH, immediately after an increase in heart rate is most likely caused by the fall in PCr content, which leads to an absorption of protons via the creatine kinase reaction.3,32 The reverse is seen after a decrease in heart rate. According to Connell,25 a decrease in [PCr] of ∼1.5 mmol · L⁻¹ in skeletal muscle would cause an increase in pH of ∼0.03 to 0.05 pH units. Since in the present study the changes in both [PCr] and pH are in these ranges, this would mean that the alkaline shift observed could be totally attributed to the change in [PCr]. The return of pH to baseline after the initial increase during the heart rate step (Fig 6) may then be caused by the extra production of protons due to the formation of intracellular acid such as lactic acid (see end of “Discussion”).

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Oxygen consumption at a heart rate of 60 beats per minute at 28°C is larger than the oxygen consumption at a heart rate of 100 beats per minute at 37°C (Table 1), although the RPP, which correlates well with myocardial oxygen consumption in other cases, is smaller at 28°C. Furthermore, several reports in the literature have appeared that showed increases in systolic pres-
sure at lower temperature accompanied by an increase in oxygen consumption,15,50 as we found in the present study. A possible explanation for such findings may be an increase in contractility at lower temperature.51 These results demonstrate that RPP is not a good correlate of mitochondrial oxygen consumption during temperature changes.

\[ \Delta P = \Delta m \cdot t_{\text{mito}} \]

where \( \Delta m \) (mol \cdot s\(^{-1}\) \cdot g dry wt\(^{-1}\)) is the change in ATP hydrolysis caused by the sudden change in work load. Here \( \Delta P \) is virtually equal to the change in PCr, since ATP was found to be almost constant. The glycolytic ATP synthesis, which is usually of minor importance in normoxic myocardium during steady states, is not taken into account in this calculation. The ATP to oxygen atom (P/O) ratio of 2.4 that was found in intact myocardium52 was used to calculate \( \Delta m \) from the measured changes in oxygen consumption. If we use the oxygen consumption and \( t_{\text{mito}} \) values measured in the present study, the calculated changes in PCr content are 4.7 and 8.5 \( \mu \)mol \cdot g dry wt\(^{-1}\) for the heart rate steps at 28°C and 37°C, respectively. Using \(^{31}\)P NMR spectroscopy, we actually determined that PCr declined significantly by 11% and 8% after the change in heart rate at 28°C and 37°C, respectively, corresponding with 2.7 and 1.7 \( \mu \)mol \cdot g dry wt\(^{-1}\). Thus, the changes in PCr calculated from \( t_{\text{mito}} \) are much larger than measured by NMR spectroscopy at the same temperature. The difference might be explained by a transiently increased glycolytic ATP production at the higher heart rate,12 which is not taken into account in Equation 3. This temporary glycolytic burst has been predicted by using a computer model of cardiac energy metabolism53 and has also been observed54 in isolated perfused rat heart with pyruvate as exogenous substrate. Furthermore, Connett11 has shown that a large transient glycolytic burst takes place in dog gracilis muscle immediately after a work jump but that the lactate produced remains in the cell. Hak et al12 have shown that the coronary venous lactate efflux doubles when the heart rate was doubled in isolated perfused rabbit heart. However, the calculated glycolytic ATP synthesis related to the lactate efflux measured in our preparation12 was far less than the 2 to 7 \( \mu \)mol \cdot g dry wt\(^{-1}\) difference between the decrease in PCr content measured with \(^{31}\)P NMR spectroscopy and the decrease calculated according to Equation 3. On the basis of our findings, we hypothesize that during fast transitions to higher work load, a brief burst of additional glycolytic ATP production buffers the high-energy phosphates. This would be accompanied by extra NADH production that might stimulate oxidative phosphorylation and presumably by lactate production. Because no burst is detected in the venous lactate efflux in our preparation,12 the lactate produced during the presumed glycolytic burst must have stayed intracellularly. The excess intracellular lactate may be used in the subsequent period as fuel for the mitochondria. The return of pH, to baseline after the initial increase during the heart-rate step (Fig 6) may correspond with the extra production of intracellular lactic acid.

Known biochemical mechanisms could cause the hypothesized fast increase in glycolytic ATP production. Hak et al12 found that lactate production in the isolated rabbit heart during a step in heart rate was increased with a response time of at most 6 seconds at 28°C. The change in phosphate metabolites found in the present study occurred with a similar response time. Because changes in AMP, Pi, and PCr regulate the activity of phosphofructokinase (PFK), an important control point in the glycolytic chain,55 a fast change in glycolytic flux is quite feasible. The small change in pH, also contributes to the regulation of PFK. Citrate, which inhibits the activity of PFK, decreases immediately after an upward step in cardiac work load.55,56 Further, glucose-6-phosphate production from glycogen is stimulated quickly by increases of cytosolic Ca\(^{2+}\) concentration that accompany the increase in heart rate. The increased glucose6-phosphate levels might then accelerate the rate of glycolysis.55

In summary, we found a small but significant decrease in PCr at increased ATP turnover in isolated Tyrodepulsed rabbit heart, in contrast with many in vivo studies. However, these changes in phosphate metabolites were not caused by limited oxygen supply. The time constants of the PCr and Pi changes, which were small compared with \( t_{\text{mito}} \) during a step change in heart rate, suggest involvement of transient additional ATP synthesis early after the heart-rate step via the glycolytic pathway. Although the decrease of PCr and increase in Pi suggest that partial stimulation of oxidative phosphorylation by phosphate metabolites is possible, the discordant time courses of PCr and oxygen consumption indicate that other pathways play a role in the dynamic regulation of cardiac oxidative phosphorylation.

Acknowledgments

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Cardiac high-energy phosphates adapt faster than oxygen consumption to changes in heart rate.

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