Mitochondrial uncoupling is often invoked as a mechanism underlying cellular dysfunction; however, it has not been possible to study this phenomenon directly in intact cells and tissues. In this paper, we report direct evaluation of mitochondrial uncoupling in the intact myocardium using \(^{31}\)P NMR magnetization transfer techniques. Langendorff perfused rat hearts were exposed to either a known uncoupler, 2,4-dinitrophenol (DNP), or a potential uncoupler, octanoate. Both DNP and octanoate decreased mechanical function as measured by the rate pressure product and caused an increase in the oxygen consumption rate (MVO\(_2\)) with DNP this increase in MVO\(_2\) was dose-dependent. The ATP synthesis rate measured by \(^{31}\)P NMR, however, was not elevated commensurately with MVO\(_2\); instead, the P/O ratio declined. In contrast, the linear relationship between the ATP synthesis rate and rate pressure product was not altered by the uncoupling agents. These data demonstrate that 1) \(^{31}\)P NMR magnetization transfer can be utilized to measure uncoupling of oxidative phosphorylation in intact organs, 2) octanoate does not induce excess ATP utilization in the intact heart, and 3) high levels of octanoate induce mitochondrial uncoupling in the intact myocardium; and this may, in part, be the cause of the toxic effects associated with fatty acid exposure.

Mitochondrial uncoupling results in decreased ATP synthesis for a given level of oxygen consumption and has been proposed as the underlying mechanism for postischemic or fatty acid-induced myocardial dysfunction. This hypothesis was not based on direct experimental demonstration of mitochondrial uncoupling in the intact tissue; rather, it was suggested by two different types of observations: 1) reversible periods of ischemia (1-4) or exposure to high concentrations of fatty acids (5-18) depress cardiac mechanical function while myocardial oxygen consumption (MVO\(_2\)) remains unchanged or increases, and 2) fatty acids reduce the P/O ratio in suspensions of isolated mitochondria (19-22). In the absence of a direct determination of the P/O ratio in the intact tissue, however, alternative explanations for the divergent response of mechanical function and MVO\(_2\) are possible, and the elevated MVO\(_2\) may reflect excess ATP utilization or "wastage" as opposed to uncoupling of oxidative phosphorylation (2, 17).

The rigorous demonstration of mitochondrial uncoupling as a possible mechanism of cellular dysfunction requires the determination of the ATP synthesis rate and the oxygen consumption rate in the intact cell rather than in isolated mitochondria. It is not possible to perform this measurement, however, using the methods employed to study isolated mitochondria. We have recently demonstrated that \(^{31}\)P NMR magnetization transfer methods can be utilized to determine the net oxidative ATP synthesis rate in the intact myocardium provided that a prominent glycolytic contribution to the NMR-measured P\(_1\) → ATP rate is eliminated (23). Similarly, we illustrated the use of a multiple magnetization transfer technique (24) to determine the net ATP hydrolysis rates in intact perfused rat hearts (23). These magnetization transfer techniques in principle provide the means by which mitochondrial uncoupling can be investigated in the intact cell. Even with this methodology, however, conducting such kinetic measurements in the complex environment of the intact cell is complicated and requires rigorous validation (23). In view of this necessity and the potential mechanistic importance of mitochondrial uncoupling in myocardial dysfunction, we undertook \(^{31}\)P NMR studies of ATPase kinetics in hearts perfused with either a known uncoupler or fatty acids at supraphysiologic concentrations. Specifically, we have 1) established that \(^{31}\)P NMR and magnetization transfer methods can measure mitochondrial uncoupling in the intact tissue, 2) examined directly the relationship between mechanical function and the net ATP synthesis rate under normal and uncoupled conditions, 3) determined that the effect of fatty acids on MVO\(_2\) is due to mitochondrial uncoupling and not to excess ATP utilization, and 4) provided additional evidence for the validity of our conclusions on ATP synthesis kinetics in postischemic hearts where mitochondrial uncoupling was excluded as the mechanism responsible for elevated MVO\(_2\) relative to mechanical function (2).

**MATERIALS AND METHODS**

**Perfused Heart Preparation**—Details of the preparation of Langendorff perfused hearts and the experimental arrangement that allowed continuous and simultaneous measurement of left ventricular pressure (LVP), heart rate (HR), and MVO\(_2\) were described previously (2, 23, 26). Hearts from male Sprague-Dawley rats weighing 300-400 g were perfused with a modified phosphate-free Krebs-Henseleit buffer containing 108 mmoles/L Na\(_2\)HPO\(_4\), 27 mmol/L NaCl, 11.5 mmoles/L KCl, 1.15 mmoles/L MgCl\(_2\), 1.15 mmoles/L NaHCO\(_3\), 5 mmoles/L glucose, adjusted to pH 7.40 with NaOH.

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\(1\) The abbreviations used are: MVO\(_2\), myocardial oxygen consumption in micromoles O\(_2\) min\(^{-1}\) (g dry weight)\(^{-1}\); ATP, the \(\gamma\)-phosphate of ATP; CP, creatine phosphate; DNP, 2,4-dinitrophenol; FID, free induction decay, HR, heart rate (mum\(^{-1}\)); LVP, left ventricular pressure; MVO\(_2\), myocardial oxygen consumption in micromoles of oxygen atoms min\(^{-1}\) (g dry weight)\(^{-1}\); P\(_i\), inorganic phosphate; P/O ratio, the ratio of the net oxidative ATP synthesis rate to the rate of oxygen atom utilization; RRP, rate pressure product, the product of heart rate and peak left ventricular systolic pressure.
Mitochondrial Uncoupling in Isolated Hearts

buffer (119 mM NaCl, 5.9 mM KCl, 1.2 mM MgCl, 1.8 mM CaCl, 28 mM NaHCO, 0.1 mM EDTA equilibrated with 85% O2, 5% CO2 gas mixture). The perfuse was supplemented with sodium octanoate and/or sodium pyruvate as a carbon source, and the NaCl content was decreased by the same amount to maintain a constant ionic strength and sodium content. The index of mechanical performance utilized was the product of the peak systolic LVP and HR (RPP, mm Hg x beats/min). LVP was measured via intraarterial pulmonary catheter, the volume of which could be adjusted to set the end diastolic pressure. Measurement of effluent O2 content and coronary flow allowed calculation of MVO2.

2,4-Dinitrophenol (DNP) was selected as the known uncoupling agent because it can be used at relatively high concentrations and consequently all oxygen consumption was assumed of mitochondrial origin. Octanoate was chosen as the fatty acid instead of a long-chain acid such as palmitate or oleate for the following reasons. 1) Octanoate at high perfusate concentrations has been shown to increase MVO2 and decrease mechanical function in the myocardium (8-14). 2) The long-chain fatty acids used have serum albumin as a carrier and the concentration of the former in the perfusate would be limited by the bovine serum albumin concentration. 3) The myocardium contains peroxisomes, where long chain fatty acids can undergo β-oxidation to acetyl-coenzyme A and short-chain acyl-coenzyme A (26, 27). The P/O ratio for this pathway is lower than for the mitochondrial pathway because the substrates peroxisomal β-oxidation need bovine serum albumin as a carrier and the concentration of the former in the perfusate would be limited by the bovine serum albumin concentration.

Typical 3P NMR spectra (40 FIDs) were recorded before and after the acquisition of kinetic data for each heart using 90° pulses and a 15-s interpulse delay. The Pi, ATP, and CP contents were determined from the fully relaxed spectra. The protocol to generate the fully relaxed spectra involved incubating the heart for 100 min in a 100 mM phenylphosphonate solution in the left ventricular balloon as a standard. At the end of each experiment, the balloon volume was increased by 0.100 ml, corresponding to an increase of 10 μmol of phenylphosphonate contained in the balloon. Another fully relaxed 3P NMR spectrum was recorded, and the increase in the integrated intensity of the phenylphosphonate resonance was calculated. This corresponded to 10 μmol of 3P nuclei. This provided a direct calibration for the conversion of NMR intensity to micromoles for each spectrum.

Intracellular pH was calculated from the chemical shift difference between Pi and CP (28). Differences and changes in pH can be measured accurately even though the precise calculated pH may vary slightly among different laboratories because of different calibration curves.

In the three different octanoate groups were 30 ± 6, 34 ± 4, and 29 ± 4%, significantly greater than the 11 ± 5% decline observed in control pyruvate-perfused hearts without octanoate (p < 0.025). ATP loss in DNP-perfused hearts was not significantly different from controls (12 ± 4, 6 ± 4, and 19 ± 4% in DNP20, DNP20, and DNP40 groups, respectively).

Average intracellular pH was 7.03 ± 0.01 (mean ± S.E.) in control hearts, and 7.04 ± 0.01 and 7.00 ± 0.01 in hearts perfused with DNP and octanoate, respectively. These small differences in mean pH values are not large enough to be biologically significant.

Saturation of the ATP resonance caused an easily detect-
resonance intensity is shown as a function of pulse repetition time. Spectrum 192 FIDs were collected in a time-averaged manner as described under "Materials and Methods" using 90° pulses. The solid and C were recorded prior to the kinetic measurements, and spectra in B and D were acquired immediately following the kinetic measurements. Assignment of peaks: R, phenylphosphonate reference; P, inorganic phosphate; CP, creatine phosphate; ATP, ATPA, and ATPβ, the γ-, α-, and β-phosphate groups of ATP. For each spectrum 40 FIDs were collected with 90° pulses, a 15 s repetition time. Chemical shifts are referenced to 85% H₃PO₄ at 0.0 ppm using CP at -2.5 ppm as an internal reference.

Addition of DNP (20 or 40 μM) to the perfusate in order to induce mitochondrial uncoupling increased the coronary flow by approximately 15%. The increases in coronary flow and MVO₂ with DNP were consistent with previous reports (29, 30). Reversibility of DNP action was demonstrated in a single heart perfused with 1 mm pyruvate and without dobutamine. DNP (40 μM) was alternately added and removed, each time allowing a 5-min equilibration period followed by a 10-min ³¹P NMR spectrum. Peak systolic LVP decreased and end diastolic pressure and MVO₂ increased with DNP in the perfusate; all parameters returned to control levels after DNP was removed. The reversibility of the DNP effect was similar to that described in previous reports (31, 32). The ³¹P NMR spectra indicated that CP and ATP levels decreased and Pᵢ levels increased when DNP was added. All three levels recovered substantially, but not completely to the original levels, when DNP was removed (data not shown).

The effects of DNP and octanoate on RPP and MVO₂ recorded during the kinetic measurements are presented in Table I. The control data included in this table have been reported previously as part of a study conducted over a large range of MVO₂ and workstate conditions (23). This control group had the same pyruvate and dobutamine concentrations as the DNP20 group.

Average values for Pᵢ levels maintained during collection of the magnetization transfer data are shown along with kinetic data in Table II. It should be re-emphasized that the pyruvate concentration was adjusted in order to maintain similar Pᵢ levels in all groups. Average ATP levels in DNP- or octanoate-perfused hearts ranged from 21.1 ± 0.8 to 24.6 ± 1.0 μmol·(g dry weight)⁻¹, similar to values of 21.2-30.4 μmol·(g dry weight)⁻¹ observed in other groups of pyruvate- or glucose-perfused hearts operating at similar or lower workstates (2, 23, 33). The slightly low values in the present study are due largely to the greater loss of ATP during the kinetic measurements in hearts perfused with octanoate than in control hearts (see above).

The relationship between MVO₂ and RPP obtained during the kinetic measurements in pyruvate-perfused normal hearts (with or without iodoacetate) (23) and in hearts exposed to DNP and octanoate is shown in Fig. 3A. The MVO₂ and RPP measured in normal pyruvate-perfused hearts displayed the expected linear dependence similar to that seen in the working heart (34). The MVO₂-RPP data of hearts perfused with DNP or octanoate, however, were displaced upward in this plot, with higher MVO₂ values at a given RPP.

In order to examine further the MVO₂-RPP relationship in octanoate-perfused hearts without the prolonged data acquisition time required for kinetic measurements, two groups of hearts were subjected to four different workstates as described under "Materials and Methods." One group was perfused with 5 mm pyruvate, the other with 4 mm octanoate. Dobutamine was absent in the two low workstates and present in the two high workstates. Kinetic measurements were not performed, and the RPP and MVO₂ data were recorded rapidly as described under "Materials and Methods." In both groups, the RPP-MVO₂ relationship was linear (Fig. 3B). The MVO₂ was higher in octanoate-perfused hearts than in pyruvate-perfused hearts over all four workstates examined, though the differ-

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**Fig. 1.** ³¹P NMR spectra of hearts perfused with either 40 μM DNP (A and B) or 6 mM octanoate (C and D). Spectra in A and C were recorded prior to the kinetic measurements, and spectra in B and D were acquired immediately following the kinetic measurements. Assignment of peaks: R, phenylphosphonate reference; P, inorganic phosphate; CP, creatine phosphate; ATP, ATPA, and ATPβ, the γ-, α-, and β-phosphate groups of ATP. For each spectrum 40 FIDs were collected with 90° pulses, a 15 s repetition time. Chemical shifts are referenced to 85% H₃PO₄ at 0.0 ppm using CP at -2.5 ppm as an internal reference.

**Fig. 2.** Measurement of $T₁^*$ and $ΔM/M₀$ for $Pᵢ$ during saturation transfer in an octanoate-perfused heart. Steady-state $Pᵢ$ resonance intensity is shown as a function of pulse repetition time with (●) and without (○) saturation of the ATP resonance. For each spectrum 192 FIDs were collected in a time-averaged manner as described under "Materials and Methods" using 90° pulses. The solid line was obtained by fitting the solid points to a single exponential relaxation equation as described under "Materials and Methods."
Substrate conditions and functional data for DNP-perfused hearts and octanoate-perfused hearts

All values are the mean ± S.E. Hearts were perfused as described under “Materials and Methods.” n is the number of hearts on which these measurements were performed.

<table>
<thead>
<tr>
<th>Group</th>
<th>n</th>
<th>Substrate</th>
<th>[DNP]</th>
<th>Heart rate</th>
<th>RPP</th>
<th>MVO₂</th>
<th>μmol·min⁻¹</th>
<th>(g dry wt)</th>
<th>μmol·min⁻¹</th>
<th>(g dry wt)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>9</td>
<td>1 mM pyruvate</td>
<td>0</td>
<td>432 ± 12</td>
<td>55.5 ± 2.8</td>
<td>56.1 ± 3.1</td>
<td>10³ mm Hg·min⁻¹</td>
<td>56.1 ± 3.1</td>
<td>48.4 ± 1.1</td>
<td>76.0 ± 2.6</td>
</tr>
<tr>
<td>DNP20</td>
<td>12</td>
<td>1 mM pyruvate</td>
<td>20</td>
<td>368 ± 7</td>
<td>45.6 ± 2.8</td>
<td>84.4 ± 2.6</td>
<td>10³ mm Hg·min⁻¹</td>
<td>84.4 ± 2.6</td>
<td>76.0 ± 2.6</td>
<td>118.6 ± 2.6</td>
</tr>
<tr>
<td>DNP20'</td>
<td>8</td>
<td>0.75 mM pyruvate</td>
<td>0</td>
<td>450</td>
<td>38.9 ± 2.2</td>
<td>72.6 ± 2.6</td>
<td>10³ mm Hg·min⁻¹</td>
<td>72.6 ± 2.6</td>
<td>84.4 ± 2.6</td>
<td>118.6 ± 2.6</td>
</tr>
<tr>
<td>DNP40</td>
<td>7</td>
<td>2 mM pyruvate</td>
<td>40</td>
<td>322 ± 7</td>
<td>28.7 ± 1.4</td>
<td>69.3 ± 3.7</td>
<td>10³ mm Hg·min⁻¹</td>
<td>69.3 ± 3.7</td>
<td>84.4 ± 2.6</td>
<td>118.6 ± 2.6</td>
</tr>
<tr>
<td>OCT4</td>
<td>10</td>
<td>4 mM octanoate + 0.5 mM pyruvate</td>
<td>0</td>
<td>376 ± 6</td>
<td>45.3 ± 2.5</td>
<td>72.6 ± 2.6</td>
<td>10³ mm Hg·min⁻¹</td>
<td>72.6 ± 2.6</td>
<td>84.4 ± 2.6</td>
<td>118.6 ± 2.6</td>
</tr>
<tr>
<td>OCT6</td>
<td>9</td>
<td>6 mM octanoate + 0.5 mM pyruvate</td>
<td>0</td>
<td>369 ± 3</td>
<td>35.2 ± 3.9</td>
<td>68.6 ± 1.5</td>
<td>10³ mm Hg·min⁻¹</td>
<td>68.6 ± 1.5</td>
<td>84.4 ± 2.6</td>
<td>118.6 ± 2.6</td>
</tr>
<tr>
<td>OCT6'</td>
<td>8</td>
<td>6 mM octanoate (no dobutamine)</td>
<td>0</td>
<td>450</td>
<td>42.4 ± 2.1</td>
<td>68.6 ± 1.5</td>
<td>10³ mm Hg·min⁻¹</td>
<td>68.6 ± 1.5</td>
<td>84.4 ± 2.6</td>
<td>118.6 ± 2.6</td>
</tr>
</tbody>
</table>

*Significantly different from the control values, p < 0.05. Statistical tests were not performed for heart rate.

**Table II**

Kinetic data for DNP-perfused hearts and octanoate-perfused hearts

All values are mean ± S.E. Hearts were perfused as described under “Materials and Methods.” Metabolite contents are in units of micromoles/g dry weight.

<table>
<thead>
<tr>
<th>Group</th>
<th>[P]</th>
<th>T₁(P)</th>
<th>∆M/M₀</th>
<th>P_1 → ATP rate</th>
<th>μmol s⁻¹</th>
<th>(g dry wt)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>14.3 ± 1.3</td>
<td>1.15 ± 0.11</td>
<td>0.328 ± 0.034</td>
<td>4.26 ± 0.46</td>
<td>4.26 ± 0.46</td>
<td></td>
</tr>
<tr>
<td>DNP20</td>
<td>16.7 ± 1.1</td>
<td>1.22 ± 0.07</td>
<td>0.396 ± 0.013</td>
<td>5.40 ± 0.33</td>
<td>5.40 ± 0.33</td>
<td></td>
</tr>
<tr>
<td>DNP20'</td>
<td>13.5 ± 1.4</td>
<td>1.27 ± 0.09</td>
<td>0.398 ± 0.012</td>
<td>3.95 ± 0.40</td>
<td>3.95 ± 0.40</td>
<td></td>
</tr>
<tr>
<td>DNP40</td>
<td>19.2 ± 1.8</td>
<td>1.75 ± 0.08</td>
<td>0.319 ± 0.019</td>
<td>3.50 ± 0.33</td>
<td>3.50 ± 0.33</td>
<td></td>
</tr>
<tr>
<td>OCT4</td>
<td>14.9 ± 0.9</td>
<td>1.38 ± 0.09</td>
<td>0.398 ± 0.022</td>
<td>4.40 ± 0.33</td>
<td>4.40 ± 0.33</td>
<td></td>
</tr>
<tr>
<td>OCT6</td>
<td>15.7 ± 0.7</td>
<td>1.53 ± 0.10</td>
<td>0.324 ± 0.032</td>
<td>3.50 ± 0.50</td>
<td>3.50 ± 0.50</td>
<td></td>
</tr>
<tr>
<td>OCT6'</td>
<td>15.2 ± 1.2</td>
<td>1.54 ± 0.09</td>
<td>0.322 ± 0.019</td>
<td>3.86 ± 0.54</td>
<td>3.86 ± 0.54</td>
<td></td>
</tr>
</tbody>
</table>

* T₁(P) is the P₁ spin-lattice relaxation time when ATP₃ spins are null.

**Table I**

Mitochondrial Uncoupling in Isolated Hearts

ences was not significant (analysis of covariance with repeated measures grouped by workstate). Comparison of Fig. 3, A and B, illustrates that the data obtained with this protocol are in excellent agreement with the data obtained during the kinetic measurements on octanoate- or pyruvate-perfused hearts.

Despite the increase in MVO₂ for a given RPP observed with DNP and octanoate, the relationship of the Pi + ATP rate to RPP was not significantly altered (Fig. 4). (Statistical significance was calculated using the predicted mean value of the Pi + ATP rate for a given RPP from the regression line of Pi + ATP rate versus RPP.) However, both DNP and octanoate caused a decrease in the (Pi + ATP rate)/MVO₂ ratio (the measured P/O ratio) (Fig. 5). The P/O ratios were 2.23 ± 0.19, respectively, in the DNP 20, 20', and 40 groups, and 1.82 ± 0.12, 1.48 ± 0.16, and 1.67 ± 0.22, respectively, in the OCT 4, 6, and 6' groups. The solid bar in Fig. 5 illustrates the P/O ratio (2.23 ± 0.19) in the control group given in Table II and is typical of the P/O ratio measured over a large MVO₂ range (23), which is indicated by the dashed line (24.1 ± 0.14) in Fig. 5.

Because octanoate and DNP altered MVO₂ and RPP at a given workstate, there does not exist for the purpose of comparing P/O ratios a single “control” group where all conditions (i.e., workstate, MVO₂, and RPP) are identical to those of octanoate- or DNP-perfused hearts. Therefore, the statistical significance of the differences in P/O ratios was evaluated in two ways. First, the octanoate and DNP groups were compared to a control group operating with identical workstate and similar carbon substrate conditions; in this comparison, the MVO₂ levels achieved by the controls differed, as expected, from those of the octanoate- and DNP-perfused hearts. Second, the octanoate and DNP groups were compared to control hearts operating at different workstates over a wide MVO₂ range. The latter comparison is justified by the fact that the P/O ratio is a fundamental parameter that has been shown experimentally to be independent of MVO₂, as expected (23).

In the first comparison, the P/O ratios measured with the uncoupling agents were compared to a single group of control hearts that were perfused with 1 mM pyruvate and 50 ng/ml dobutamine and operating with end diastolic pressure of 8 mm Hg and HR ~ 430 min⁻¹. The data on this group are given in Tables I and II and in Fig. 5 as Control. When compared to this control group, the decrease in the (Pi + ATP rate)/MVO₂ ratio was significant in the DNP20', (p < 0.02), DNP40 (p < 0.001), OCT6 (p < 0.005), and OCT6' (p < 0.05) groups.

The second comparison used the P/O ratio obtained from the Pi → ATP rate measurements on 72 individual hearts operating over a large MVO₂ range. When these hearts were grouped into eight separate groups based on their respective workstates, the P/O ratio calculated for each group was not significantly different from the others despite the large variation in the MVO₂ attained at the different workstates (23); with equal weighting of the eight groups, the data on the 72 hearts gave a P/O ratio of 2.41 ± 0.14 (S.E., n = 8) (23). Since the P/O ratio was independent of MVO₂ (23), as expected, the 72 hearts could also be weighted equally regardless of their workstate and MVO₂. When this was done, the calculated P/O ratio was 2.40 ± 0.10 (S.E., n = 72). This value as shown by the dashed line in Fig. 5. Using this latter value as
Mitochondrial Uncoupling in Isolated Hearts

In previous studies we have demonstrated the ability to measure net oxidative ATP synthesis rates in the intact myocardium using $^{31}$P NMR magnetization transfer techniques and found the P/O ratio to be constant at 2.41 ± 0.14 (S.E., n = 8) over a wide range of oxygen consumption rates (23). After reversible periods of ischemia, mechanical performance decreased while oxygen consumption remained stable or increased. Mitochondrial uncoupling could not be detected, however, since the P/O ratio was not altered (2). Instead, increased oxygen consumption at a given level of performance appeared to be due to excess ATP utilization, perhaps by the contractile process.

Infusion of the classical uncoupler DNP resulted in decreased cardiac performance and increased oxygen consumption (Table I and Fig. 3A). In contrast to the postischemic groups, however, the measured P/O ratio decreased with exposure to DNP (Fig. 5), indicating uncoupling of oxidative phosphorylation. Because the relationship between the Pi + ATP rate and the RPP in the DNP-treated hearts was unchanged from the relationship in hearts not exposed to DNP (Fig. 4), the elevation of MVO$_2$ for a given RPP can be attributed entirely to effects of DNP on the mitochondria.

With the validity of our methodology for detecting mitochondrial uncoupling in the intact heart established, we then investigated the bioenergetic consequences of fatty acid toxicity in the myocardium. Although fatty acids are a preferred carbon substrate for the adult mammalian heart, it is well known that high levels of free fatty acids cause mechanical dysfunction (5-9) and elevated MVO$_2$ (7, 10-18). The dysfunction could result wholly or in part from the inhibitory effects of fatty acids and their amphipathic metabolites on certain key enzyme systems (5, 35, 36). The elevated MVO$_2$ could be due to either mitochondrial uncoupling or excess ATP utilization by a futile cycle (17).

When octanoate replaced pyruvate as the carbon substrate, the control value for the P/O ratio, the same four groups of DNP- and octanoate-perfused hearts had significantly reduced P/O ratios: DNP$^{20'}$ (p < 0.02), DNP$^{40'}$ (p < 0.001), OCT$^{6'}$ (p < 0.005), and OCT$^{6'}$ (p < 0.015).

When octanoate replaced pyruvate as the carbon substrate,

**FIG. 3.** Relationship between MVO$_2$ and RPP in hearts perfused with DNP or octanoate. A, data recorded during saturation transfer measurement of ATP synthesis rates. C, DNP-perfused hearts; $\bullet$, octanoate-perfused hearts; $\triangle$, control hearts perfused with pyruvate with or without iodoacetate. The dashed line is the least squares fit to the $\Delta$ points. A lack of error bars (S.E.) for DNP- or octanoate-perfused hearts indicates that the error was within the size of the symbol. B, data from hearts subjected to multiple workstates as described in the text. $\square$, control hearts perfused with 5 mM pyruvate; $\circ$, hearts perfused with 4 mM octanoate. A lack of error bars (S.E.) indicates that the error was within the size of the symbol.

**FIG. 4.** Relationship between Pi + ATP rate and RPP in hearts perfused with DNP (C) or octanoate (O). $\Delta$, control hearts perfused with pyruvate with or without iodoacetate. The dashed line is the least squares fit to the $\Delta$ points. Error bars (S.E.) are shown for DNP- and octanoate-perfused hearts only.

**FIG. 5.** Bar graph comparing the (Pi + ATP rate)/MVO$_2$ ratios in control hearts and hearts perfused with either DNP or octanoate. Error bars indicate S.E. The dashed line represents the average (Pi + ATP rate)/MVO$_2$ ratio obtained over a large MVO$_2$ range at eight different workstates as reported previously (23). Statistical significance of differences is discussed in the text. The groups labeled 20$^\circ$ DNP and 6$^\circ$ mM OCT lacked dobutamine.

A 4.75% decrease in the average P/O ratio was expected due to the large FADH$_2$/NADH ratio in $\beta$-oxidation. When statistical significance of the decreased P/O ratio in octanoate-perfused hearts was recalculated with the control P/O ratios reduced by 4.75% (to 2.12 ± 0.19), the OCT$^{6'}$ group was still significantly different from the control group (p < 0.02). Furthermore, the presence or absence of dobutamine made no significant difference in the measured P/O ratio.

**DISCUSSION**

In previous studies we have demonstrated the ability to measure net oxidative ATP synthesis rates in the intact myocardium using $^{31}$P NMR magnetization transfer techniques and found the P/O ratio to be constant at 2.41 ± 0.14 (S.E., n = 8) over a wide range of oxygen consumption rates (23). After reversible periods of ischemia, mechanical performance decreased while oxygen consumption remained stable or increased. Mitochondrial uncoupling could not be detected, however, since the P/O ratio was not altered (2). Instead, increased oxygen consumption at a given level of performance appeared to be due to excess ATP utilization, perhaps by the contractile process.

Infusion of the classical uncoupler DNP resulted in decreased cardiac performance and increased oxygen consumption (Table I and Fig. 3A). In contrast to the postischemic groups, however, the measured P/O ratio decreased with exposure to DNP (Fig. 5), indicating uncoupling of oxidative phosphorylation. Because the relationship between the Pi + ATP rate and the RPP in the DNP-treated hearts was unchanged from the relationship in hearts not exposed to DNP (Fig. 4), the elevation of MVO$_2$ for a given RPP can be attributed entirely to effects of DNP on the mitochondria.

With the validity of our methodology for detecting mitochondrial uncoupling in the intact heart established, we then investigated the bioenergetic consequences of fatty acid toxicity in the myocardium. Although fatty acids are a preferred carbon substrate for the adult mammalian heart, it is well known that high levels of free fatty acids cause mechanical dysfunction (5-9) and elevated MVO$_2$ (7, 10-18). The dysfunction could result wholly or in part from the inhibitory effects of fatty acids and their amphipathic metabolites on certain key enzyme systems (5, 35, 36). The elevated MVO$_2$ could be due to either mitochondrial uncoupling or excess ATP utilization by a futile cycle (17).

Octanoate-perfused hearts maintained RPP values similar to or less than those achieved in pyruvate- or glucose-perfused hearts; octanoate-perfused hearts indicated that the error was within the size of the symbol. B, data from hearts subjected to multiple workstates as described in the text. $\square$, control hearts perfused with 5 mM octanoate. A lack of error bars (S.E.) are shown
hearts at a given workstate, but with significantly increased MVO₂ values (Table I and Fig. 3). As with the DNP-treated hearts, uncoupling of oxidative phosphorylation, as reflected in a lower P/O ratio, occurred in a dose-dependent fashion with the noted decrease becoming significant at the higher levels of octanoate (Fig. 5). A few hearts in which perfusion with 8 mM octanoate was attempted developed irreversible ventricular fibrillation; therefore, kinetic measurements at concentrations of octanoate greater than 6 mM were not possible. Analysis of ATP production in relation to RPP yielded a result comparable to that obtained with DNP (Fig. 4). Because the rate of ATP synthesis required to support a rate due to oxidative phosphorylation requires elimination of the glycolytic contribution to this rate (23). In order to do this without using the glycolytic inhibitor iodoacetate, it was necessary to examine the hearts at cardiac performance levels greater than base line (level i in our previous publications (2, 23)). Following the protocols of our previous studies, this was achieved with the use of dobutamine. In order to control for the possibility that a catecholamine such as dobutamine might influence the uncoupling action of DNP or octanoate, two groups of hearts without dobutamine were examined, groups DNP20' and OCT6'. The RPP and MVO₂ levels in these two groups were similar to values in dobutamine-perfused hearts, elevation of MVO₂ by octanoate was not due to significant ATP wastage.

As previously mentioned, measurement of the P_i → ATP rate due to oxidative phosphorylation requires elimination of the DNP reaction contribution to this rate (23). In order to do this without using the glycolytic inhibitor iodoacetate, it was necessary to examine the hearts at cardiac performance levels greater than base line (level i in our previous publications (2, 23)). Following the protocols of our previous studies, this was achieved with the use of dobutamine. In order to control for the possibility that a catecholamine such as dobutamine might influence the uncoupling action of DNP or octanoate, two groups of hearts without dobutamine were examined, groups DNP20' and OCT6'. The RPP and MVO₂ levels in these two groups were similar to values in dobutamine-perfused hearts, elevation of MVO₂ by octanoate was not due to significant ATP wastage.

In conclusion, we have demonstrated that mitochondrial uncoupling in hearts perfused with fatty acids, we can make no conclusion as to the mechanism of uncoupling. The traditional concept of an uncoupler is a weak organic acid that is able to cross the inner mitochondrial membrane in both the protonated and unprotonated forms, thus breaking down the pH gradient and dissociating electron transport and oxygen consumption from ATP synthesis. Recent evidence indicates, however, that some uncoupling agents, including fatty acids, may also decrease the number of protonated protein within the inner mitochondrial membrane during electron transfer in the respiratory chain, which would also result in a lower P/O ratio (37, 38).

In conclusion, we have demonstrated that mitochondrial uncoupling can be studied in intact tissues using 31P NMR and magnetization transfer techniques. The elevated MVO₂ relative to mechanical function observed for the intact myocardium in the presence of high concentrations of fatty acids or known uncouplers of isolated mitochondria, such as DNP, arises by a mechanism different from that seen following a reversible ischemic insult. Fatty acids and DNP cause a relative increase in MVO₂ by mitochondrial uncoupling, while the elevated postischemic MVO₂ is accompanied by a similar rise in ATP synthesis and utilization rates without uncoupling. Cardiotoxic effects of high levels of fatty acids appear to be due, at least in part, to mitochondrial uncoupling.

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