Metabolic Cost of the Stimulated Beating of Isolated Adult Rat Heart Cells in Suspension

Robert A. Haworth, Douglas R. Hunter, Herbert A. Berkoff, and Richard L. Moss
From the Department of Surgery, and of Physiology, University of Wisconsin, Madison, Wisconsin

SUMMARY. Heart cells from adult rats were induced to beat in suspension by electric field stimulation. We have gained evidence that all the rod-shaped cells in suspension were indeed beating, and that the beat had dynamic characteristics similar to those of intact heart muscle contracting under zero load. The cells were undamaged in the process, as judged by maintenance of ATP levels, morphology, and ability to beat. In gaining such evidence, we also measured the metabolic cost to the cells of beating under zero load. In cells with oxidative phosphorylation inhibited by rotenone plus oligomycin (termed anaerobic), the rate of beat-dependent lactate production suggested an equivalent rate of ATP utilization of 0.126 ± 0.013 nmol ATP/beat per mg protein (plus isoproterenol), and 0.058 ± 0.005 nmol ATP/beat per mg protein (minus isoproterenol). In respiring cells, the rate of beat-dependent oligomycin-sensitive oxygen consumption gave an equivalent rate of ATP utilization of 0.198 ± 0.009 nmol ATP/beat per mg protein (plus isoproterenol), and 0.126 ± 0.013 nmol ATP/beat per mg protein (minus isoproterenol). The cells beat with the same approximate maximum velocity whether isoproterenol was present or not. We calculate that—in the case of anaerobic cells without isoproterenol—this rate of ATP utilization can account for only about a 15% degree of activation of the contractile proteins. In addition, we have found an oligomycin-insensitive beat-dependent mitochondrial respiration of 0.023 ± 0.006 nanoatom O/beat per mg. The cause of this respiration is not known. The total rate of oxygen consumption of cells and also the rate per beat was comparable to that measured in nonworking whole hearts. (Circ Res 52:342–351, 1983)

SUSPENSIONS of cells isolated from whole organs offer an experimental system which has unique advantages: vascular factors are eliminated, the cells see an identical environment, and experimentation is facilitated by the ease of control and manipulation. Procedures for isolating adult rat heart cells have been available for many years, but the usefulness of such preparations was limited by their inability to remain viable in media containing Ca++. Recently, several methods have been published which claim to yield suspensions of cells that resist Ca++ (Powell and Twist, 1976; Haworth et al., 1980; Kao et al., 1980; Frangakis et al., 1980; Montini et al., 1981). Such Ca++-resistant cells are quiescent in the presence of Ca++, but beat when depolarized by electric field stimulation (Krueger et al., 1980; Haworth et al., 1980). If cells could be stimulated to beat in bulk suspension, many beat-related processes would be opened to scrutiny in a new way, using the powerful methodology of solution biochemistry. However, the application of electric pulses to cells in suspension with a view of studying beat-related processes raises a number of questions. How can we know whether or not the cells indeed are beating, how many are beating, and how strongly they are beating—and will the cells sustain damage in the process? The experiments reported here were designed to speak to these questions. We conclude that we have succeeded in so stimulating suspensions of heart cells that all the rod-shaped cells beat, that the beat has dynamic characteristics similar to those of intact heart muscle contracting under zero load, and that the cells are undamaged in the process. In so doing, we have also measured the metabolic cost to the cell of beating under zero load.

Methods

Isolation of Ca++-Resistant Myocytes

Cell suspensions were prepared as previously described (Haworth et al., 1980). Two female retired breeder Sprague-Dawley rats were anesthetized by intraperitoneal injection of 0.4 ml 5% thiamylal sodium (Surital), and the hearts excised. After retrograde (Langendorff) perfusion at 37°C for 5–10 minutes with a Krebs-Henseleit bicarbonate medium, first with Ca++ and then without Ca++, the hearts were digested for 40 minutes by recirculating perfusion with a medium containing collagenase (0.55 mg/ml) and hyaluronidase (0.33 mg/ml), and no added Ca++. Such Ca++-resistant cells are quiescent in the presence of Ca++, but beat when depolarized by electric field stimulation (Krueger et al., 1980; Haworth et al., 1980). If cells could be stimulated to beat in bulk suspension, many beat-related processes would be opened to scrutiny in a new way, using the powerful methodology of solution biochemistry. However, the application of electric pulses to cells in suspension with a view of studying beat-related processes raises a number of questions. How can we know whether or not the cells indeed are beating, how many are beating, and how strongly they are beating—and will the cells sustain damage in the process? The experiments reported here were designed to speak to these questions. We conclude that we have succeeded in so stimulating suspensions of heart cells that all the rod-shaped cells beat, that the beat has dynamic charac-
5 mM Na acetate. When the rate of oxygen consumption was to be measured, 5 mM Na acetate was replaced by 11 mM glucose. The pH had been adjusted to 7.0 with NaOH. The suspension was incubated at 37°C in a Dubnoff metabolic shaking incubator set at 80–90 cycles/min, and allowed to equilibrate with air. The protein concentration was measured at this time by the biuret procedure and adjusted to 5.0 mg/ml. Insulin (2 μM) was added, and experiments were initiated typically after 30 minutes of incubation.

Stimulation of Beating in Suspension

"Anaerobic" Conditions

At t = 0, a 0.5-ml aliquot of cell suspension was transferred to the stimulation chamber (Fig. 1). At t = 30 sec, 11 mM glucose was added, followed at 15-second intervals by 4.5 mM Na ascorbate, 10 μM 1-isoproterenol (if required), and 10 μM verapamil (if required). Measurements in the presence and absence of verapamil were done simultaneously in two identical chambers. At t = 2 minutes, oxidative phosphorylation and respiration were inhibited by adding 10 μM oligomycin and 10 μM rotenone. At t = 3 minutes, a 0.1-ml aliquot of cell suspension was removed and added to 0.1 ml 16% perchloric acid on ice, and the stimulating pulses were switched on. The shape of the pulse is shown in the inset to Figure 2. This biphasic pulse was derived from a monophasic square pulse using a 0.033 μF capacitance, power amplified, and applied across the carbon electrodes. An oscilloscope also was connected across the electrodes to monitor the magnitude and frequency of the applied pulses. Further 0.1-ml aliquots of cell suspension were removed at t = 5 and t = 8 minutes and added to 0.1 ml 16% perchloric acid on ice. The chamber was washed with water, with ethanol, and then with water again before the next 0.5-ml aliquot of cell suspension was added. Lactate content of the perchloric acid extracts was measured as described below. The linearity of rates of lactate production under these conditions was evident from the similarity of the rates calculated using the three combinations of pairs of the three time points. The mean of these three values was taken as the rate. When ATP and creatine phosphate were to be measured, 1 ml of cell suspension was added to the chamber. At the required time, 0.76 ml of this was removed and added to 0.76 ml 16% perchloric acid on ice.

Aerobic Conditions

To measure the rate of oxygen consumption of beating cells, a chamber was used similar to that in Figure 1, except that an additional port allowed access of a Clark oxygen electrode to the suspension. One and one-half milliliter of cell suspension was put in the chamber at t = 0. This was sufficient to exclude all air, and the rate of oxygen consumption could be measured in the conventional manner. Since all measurements of oxygen consumption were made using a strong pulse (40 V/cm), we think that most of the observed increase in the rate of oxygen consumption catalyzed direct oxidation of buffer components. Res-
ported values for the rate of oxygen consumption have been corrected for this effect by subtracting the rate of oxygen consumption of stimulated buffer plus ascorbate and isoproterenol at the oxygen tension at which the cellular respiration was measured.

Lactate Measurement

A procedure based on the method of Marback and Weil (1967) was used. To the cell suspension plus perchloric acid was added 0.76 ml medium (at room temperature) containing 0.5 mM glycine, 0.4 M hydrazine, and sufficient KOH to bring the final pH (plus sample) to 9.9. The suspension was mixed by inversion. To this was added 20 μl 0.1 mM nicotinamide adenine dinucleotide (oxidized form), and 20 μl 2 mg/ml lactic dehydrogenase. The suspension was mixed again, centrifuged for 1 minute in a Beckman microfuge B centrifuge, and the absorbance of the supernatant at 340 nm was read after 30 minutes of incubation at room temperature.

ATP and Creatine Phosphate Measurement

ATP and creatine phosphate were measured on neutralized perchloric acid extracts by the enzymatic assay of Lowry and Passonneau (1972). Particular care was taken to minimize and allow for contaminating myokinase activity in the measurement of creatine phosphate.

Glycogen Measurement

Aliquots (0.5 ml) of cell suspension were layered on top of 0.5 ml bromodecane, itself on top of 0.1 ml 0.5 M HCl and NaCl, and were centrifuged for 1 minute in a Beckman microfuge B centrifuge. This separated the cells from the glucose-containing medium. Each HCl-NaCl layer plus cell pellet was made up to 1 ml with water, and the glycogen content was determined as previously described (Haworth et al., 1981).

Stimulation of Beating on a Hemocytometer

Two platinum wire electrodes were fixed to a hemocytometer so that cells on the platform could be uniformly subjected to electric pulses of the same strength, form, and frequency as those applied to cells in suspension. Aliquots (0.5 ml) of cell suspension were placed in the stimulation chamber (Fig. 1) and treated as for the anaerobic lactate measurement (see above). After the addition of rotenone plus oligomycin, drops of cell suspension were transferred to the hemocytometer platform at room temperature (24°C) and a coverslip was placed on top. Cells on the platform were observed through a Zeiss binocular microscope. At t = 3 minutes, the cells were stimulated at a frequency of 2.173 Hz. The rod-shaped cells that beat in response to pulses of a given strength were counted, as were the rod-shaped cells that did not beat. No cells were counted after t = 8 minutes. The term “rod-shaped” designates those cells that showed a relaxed sarcomere band pattern, which, in our preparation under these conditions, has a period of 1.8 to 1.9 μm (Haworth et al., 1981).

Measurement of Shortening Velocity

Cells were applied to the hemocytometer as described above. Individual cells were then photographed by a Polaroid camera plus a Xenon flash unit programmed to flash (duration 1 msec) at a given interval after a maximally effective pulse was applied. The magnification on the prints was 453X. Sarcomere lengths were measured on unstimulated cells, as were the cell lengths. We then measured cell lengths in stimulated cells, and converted these values to sarcomere lengths.

Stimulation of Glycolysis by Uncoupler

A 0.5-ml cell suspension was placed in a glass vial under aerobic incubation conditions at t = 0. Glucose (11 mM) was added at t = 30 seconds, followed at 15-second intervals by 4.5 mM Na ascorbate and 10 μM isoproterenol. At t = 2 minutes, 10 μM rotenone was added, followed at t = 2.5 minutes by the amount of uncoupler (carbonylcyanide p-trifluoromethoxyphenylhydrazide, FCCP) shown. Then 0.1-ml aliquots were removed at t = 3, 5.5, and 8 minutes and added to 0.1 ml perchloric acid on ice, and lactate was measured as described.

Results

When cell suspensions under anaerobic conditions plus 10 μM isoproterenol at 37°C were subjected to biphasic pulses of increasing magnitude at a frequency of 2.173 Hz, they showed an increased rate of lactate production (Fig. 2B). When verapamil (10 μM) was included in the medium, no increase in the rate of lactate production was observed. If aliquots of a similar anaerobic cell suspension were placed on a hemocytometer (at 24°C) fitted with platinum wire electrodes and exposed to similar pulses at the same frequency, the response of any cell to pulses of a given size was evident from inspection through a microscope. The fraction of rod-shaped cells that beat in response to pulses of increasing magnitude is shown in Figure 2A. The fraction of beating rod-shaped cells increased from zero to near 100%, with a dependence on pulse magnitude very similar to that of the increased rate of lactate production measured on cells stimulated in suspension at 37°C (Fig. 2B). For any pulse strength, no diminution of the ability of cells to beat was observed for the time period over which the rate of lactate production was measured. Also, the beating of the cells was completely inhibited by 10 μM verapamil (Fig. 2A). When cells on the hemocytometer were photographed at time intervals after a single pulse of excitation, the time course of contraction shown in Figure 3A was found. The average maximal degree of shortening for these cells was 0.334 μm/sarcomere. By photographing a number of cells at both 20 and 60 msec after excitation, we gained a measure of the cell population heterogeneity with respect to the approximate maximum rate of shortening (Fig. 3B). After combining data from three preparations, we found that the approximate maximum rate of shortening, by this method, was 2.12 ± 0.62 μm/sec per half sarcomere (mean ± SD, n = 67 cells).

To check for cell damage during the anaerobic incubation and stimulation, we measured cellular levels of ATP and creatine phosphate before and after the addition of rotenone plus oligomycin, and before and after the 5-minute stimulation period. It is seen, in Table 1, that although there was a decline in ATP on anaerobic incubation, as previously reported (Haworth et al., 1981), there was no ATP decline associated with stimulation or with beating. The cells that
were stimulated and allowed to beat did appear to contain less creatine phosphate than the cells stimulated in the presence of verapamil (p < 0.05, paired t-test); however, the significance of this is uncertain, since the level of creatine phosphate in the unstimulated cells was closer to that of the cells stimulated without verapamil. It is clear that the creatine phosphate loss was determined principally by the addition of rotenone plus oligomycin. The percentage of cells which showed rod-shaped morphology also was determined before and after stimulation, and was found not to have changed (Table 1).

To determine whether the lactate produced under anaerobic conditions came principally from glycogen or glucose, we measured the glycogen loss during the 5-minute stimulation period. We found that the gly-

<table>
<thead>
<tr>
<th>Table 1</th>
<th>Effect of Stimulation (S) and Verapamil (V) on ATP, Creatine Phosphate, and Percent Rod-Shaped Cells, in Cells Inhibited with Rotenone (R) Plus Oligomycin (O)</th>
</tr>
</thead>
<tbody>
<tr>
<td>ATP (nmol/mg)</td>
<td>+S -V</td>
</tr>
<tr>
<td>Before R + O</td>
<td>16.8 ± 1.1</td>
</tr>
<tr>
<td>1 min after R + O (start of S)</td>
<td>18.2 ± 0.9</td>
</tr>
<tr>
<td>5 min after S</td>
<td>20.3 ± 1.1</td>
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</table>

| Creatine phosphate (nmol/mg) | +S -V | +S +V | -S -V | -S +V |
| Before R + O | 32.7 ± 5.9 | 32.7 ± 5.9 | 32.7 ± 5.9 | 32.7 ± 5.9 |
| 1 min after R + O (start of S) | 20.2 ± 2.3 | 20.2 ± 2.3 | 20.2 ± 2.3 | 20.2 ± 2.3 |
| 5 min after S | 13.9 ± 1.7 | 22.5 ± 2.1 | 17.2 ± 0.8 | 15.4 ± 2.1 |

| Rod-shaped cells (% of total) | +S -V | +S +V | -S -V | -S +V |
| Before R + O | 56.8 ± 0.9 | 56.8 ± 0.9 | 56.8 ± 0.9 | 56.8 ± 0.9 |
| 1 min after R + O (start of S) | 57.2 ± 2.4 | 57.2 ± 2.4 | 57.2 ± 2.4 | 57.2 ± 2.4 |
| 5 min after S | 54.2 ± 3.4 | 54.2 ± 3.4 | 54.2 ± 3.4 | 54.2 ± 3.4 |

Values shown are mean ± so, n = 3 preparations. Cells were stimulated at 2.173 Hz with pulses of 40 V/cm under anaerobic conditions in the presence of isoproterenol.
cogen loss (approximately 30% of total) was sufficient to account for all the lactate produced, even though glucose and insulin were present (Table 2).

When the frequency of stimulation of cells under anaerobic conditions plus isoproterenol was varied, a linear relationship between stimulation frequency and the rate of lactate production was found (Fig. 4A). The stimulation-dependent rate was almost completely verapamil sensitive (Fig. 4A). By contrast, if glycolysis was stimulated artificially by the addition of rotenone plus FCCP (an uncoupler which induces a mitochondrial ATPase activity), the rate of lactate production was only slightly (if at all) inhibited by verapamil (Fig. 4C). When isoproterenol was omitted from the medium, the stimulation-dependent rate of lactate production was reduced (Fig. 4B), and the dependence on stimulation frequency could deviate significantly from linearity (P = 0.652, from analysis of variance [Bliss, 1967]). The solid lines are drawn on this presumption; other biphasic combinations are possible. Cells without isoproterenol but with FCCP showed a rate of verapamil-insensitive lactate production similar to that of cells with isoproterenol (data not shown). When the approximate maximum velocity of contraction of the cells was measured as in Figure 3, but without isoproterenol, no significant change was found (V_max = 2.556 ± 0.386 μm/sec per half sarcomere without isoproterenol, n = 46 cells, compared with V_max = 2.45 ± 0.687 μm/sec per half sarcomere with isoproterenol, n = 41 cells; data from two preparations, in each of which V_max was measured with and without isoproterenol).

When both rotenone and oligomycin were omitted from the cell suspension, the metabolic cost of beating could be measured from changes in the rate of oxygen consumption. Figure 5A shows the dependence of the rate of oxygen consumption on stimulation frequency for cells in the presence of isoproterenol. Similar to the rate of lactate production, there is no significant deviation from linearity up to 5 Hz. Acetate was not included in the experiments shown in this figure. The effect of acetate was to elevate rates of oxygen consumption by a small amount, with or without stimulation (data not shown). If oligomycin was included (Fig. 5A), the rate of beat-dependent oxygen consumption was reduced. The amount of oligomycin required to inhibit the oligomycin-sensitive respiration by 50% was approximately 0.2 nmol/mg protein. The level (2 nmol/mg) used in Figure 5 was sufficient for maximum inhibition: higher levels of oligomycin inhibited no further (data not shown). There does, therefore, appear to be a significant amount of beat-dependent oligomycin-insensitive respiration remaining. If verapamil was included, stimulation caused very little increase in oxygen consumption. Oligomycin and verapamil together had no greater effect than verapamil alone (Fig. 5A). When isoproterenol was not included, the beat-dependent oligomycin-sensitive respiration was approximately half that seen in the presence of isoproterenol (Fig. 5B), consistent with the effect of isoproterenol on the rate of lactate production observed under anaerobic conditions (Fig. 4A and B). There was also a trend toward a decrease in oligomycin-sensitive respiration per beat at high frequencies of stimulation (5 Hz) in the absence of isoproterenol (Fig. 5B), but the trend was not statistically significant.

### Discussion

**Evidence That Cells in Suspension Beat when Stimulated**

To gain evidence that cells stimulated in suspension actually do beat, we have looked for beat-associated metabolic changes. It proved advantageous in two ways initially to measure such changes in cells with oxidative phosphorylation blocked by the addition of rotenone plus oligomycin: first, the extra metabolism associated with beating turned out to be rather small and was easier to measure under conditions where glycolysis supplied the entire demand for energy, and, second, by blocking oxidative phosphorylation, we made the cells in suspension more directly comparable to cells on the hemocytometer, where otherwise differences in oxygen supply would have been a complicating factor. Isoproterenol was included initially because it enhanced the beat-related rate of lactate production and, hence, made it easier to measure. Thus, Figure 2 provides two pieces of evidence that the cells in suspension actually do beat when subjected to an electric pulse: first, cells on a hemocytometer exposed to a pulse of similar strength can be seen to beat; and second, the percentage of cells seen to beat on the hemocytometer and the verapamil-

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**Table 2**

Comparison of Glycogen Lost and Lactate Produced in Cells Inhibited with Rotenone Plus Oligomycin, ± Stimulation (S) ± Verapamil (V)

<table>
<thead>
<tr>
<th></th>
<th>+S - V</th>
<th>+S + V</th>
<th>-S - V</th>
<th>-S + V</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glycogen loss</td>
<td>22.97 ± 2.11</td>
<td>11.05 ± 2.03</td>
<td>11.17 ± 2.15</td>
<td>9.21 ± 1.82</td>
</tr>
<tr>
<td>Lactate produced</td>
<td>17.99 ± 1.05</td>
<td>10.32 ± 0.76</td>
<td>9.32 ± 0.85</td>
<td>8.92 ± 0.61</td>
</tr>
</tbody>
</table>

Values shown are mean ± SD, n = 3 preparations, calculated from measurements made on samples taken before and after stimulation for 5 minutes. Cells were stimulated at 2.173 Hz with pulses of 40 V/cm under anaerobic conditions in the presence of isoproterenol.
sensitive lactate production of cells in suspension show a similar dependence on the strength of the stimulating pulse.

Given that almost all the rod-shaped cells beat, when stimulated in suspension, it is important to know how the dynamic characteristics of the beat compare with the beating of intact unloaded heart muscle. Figure 3 shows that the cells behave fairly homogeneously with respect to their approximate maximal velocity of shortening, $V_{\text{max}}$. The mean value for $V_{\text{max}}$ is similar to that found in intact papillary muscle at a similar temperature (Pollack and Krueger, 1976). It is, however, somewhat less than that found on selected isolated cells by laser light diffraction (Krueger et al., 1980).

**Cell Damage**

The data in Table 1 show that the cells are not damaged in the process of stimulation, as measured by the maintenance of rod-shaped morphology and ATP levels. The observation that the rate of ATP utilization in cells stimulated in the presence of verapamil differs little from the rate in unstimulated cells (Figs. 2, 4, and 5) is additional evidence for this conclusion. Ascorbate was included in the medium to protect the cells and isoproterenol from oxidative damage.
FIGURE 5. Dependence of rate of oxygen consumption on stimulation frequency. Cells were stimulated with pulses of 40 V/cm in a stimulation chamber modified to allow monitoring by an oxygen electrode under aerobic conditions at 37°C (see Methods). Values shown are corrected for the rate of oxygen consumption of the buffer alone (plus ascorbate), at the oxygen tension at which cell respiration was measured. This was: 34.3 ± 6.1 nanoatom O/min per ml (5 Hz), 16.4 ± 1.3 nanoatom O/min per ml (2.173 Hz), 5.4 ± 1.6 nanoatom O/min per ml (0 Hz). Panel A: effect of oligomycin and verapamil in the presence of isoproterenol. The solid lines are calculated from the data by linear regression, and correspond to the relations: y = (3.38 ± 0.09)x + (9.19 ± 0.25), no addition; y = (0.35 ± 0.01)x + (8.34 ± 0.03), +verapamil; y = (1.37 ± 0.21)x + (8.85 ± 0.61), +oligomycin; and y = (0.44 ± 0.11)x + (8.20 ± 0.31), +oligomycin and verapamil. Panel B: The effect of isoproterenol. The solid lines are: y = (1.94 ± 0.04)x + (20.94 ± 0.12), no addition; y = (0.81 ± 0.12)x + (10.23 ± 0.33), + oligomycin; y = (4.07 ± 0.29)x + (10.70 ± 0.78), + isoproterenol; y = (1.12 ± 0.08)x + (10.19 ± 0.23), + isoproterenol + oligomycin.

Data points show mean ± SD for n = 6 preparations in both panel A and panel B. The 2.173 Hz data points are from three preparations; the 5 Hz data points are from three more preparations, and the 0 Hz points are from all six preparations.

species generated by the electrodes. Stimulation of buffer alone causes an increased rate of oxygen consumption if the buffer contains oxidizable components such as glucose (see legend to Fig. 5); inclusion of ascorbate greatly increases this oxygen consumption rate, suggesting that it is an avid substrate for the reactive species generated by the electrical pulses. However, we have not been able to detect any effect of ascorbate on the rate of lactate production in experiments such as that in Figure 4A, so it could be that the ascorbate is superfluous. Although the cells appear undamaged, as judged by the criteria of morphology, ATP level, rate of ATP utilization, and sustained ability to beat when stimulated, it is quite possible that exposure to the stimulating pulses does cause some damage which would be manifest in the longer term viability of the cells. When cells were stimulated at 5 Hz and 40 V/cm for 5 minutes in the stimulation chamber under aerobic conditions, and were then returned to the shaking water bath, they still showed no significant decline in percentage of rod-shaped cells after an hour; however, longer times were not investigated.

Control of Glycolytic Rate

The observation, in Figure 4, that stimulation-induced lactate production is verapamil-sensitive, whereas uncoupler-induced lactate production is not, is evidence that the inhibition of stimulation-induced lactate production is not caused by a direct inhibitory action of verapamil on glycolysis. Also, the linear relationship between the rate of lactate production and stimulation frequency found with isoproterenol suggests that the measured rate of lactate production in the absence of verapamil is not limited by the potential ability of glycolysis to keep up with the demand for ATP. Such a limit certainly exists, and can be found using higher levels of uncoupler in the presence of rotenone. The limit is manifest by a significantly lower rate of lactate production in the second 2.5 minutes of measurement than in the first 2.5 minutes (see Methods). Its value varies from one preparation to another, and generally occurs between 30 and 40 nmol lactate/min per mg. This limit is similar to the maximum work-stimulated glycolytic rate observed in intact working rat hearts (Kobayashi and Neely, 1979).

Correction of Measured Values

To make our measured values of the rates of oxygen consumption and lactate production more comparable to those measured previously on the whole heart, correction must be made for the fraction of cells which are not beating. To do this we divide measured values by 0.665, to give values (termed “corrected”) which are representative of the rates per milligram of
The calculation of this factor was based on the following assumptions: that 57% of the cells are rod-shaped (Table 1), that the remaining 43% of the cells do not contribute to respiration or lactate production significantly, and that they contain two-thirds as much protein as the rod-shaped cells. In support of the last two assumptions, we have found previously that most of the round cells in our preparation will take up trypan blue and are therefore freely permeable (Haworth et al., 1980). Cells made permeable with digitonin lose approximately one-third of their protein (unpublished observation). Such permeable cells appear to be metabolically inert (Haworth et al., 1981). It is not clear what contribution will be made by the small fraction of cells that are round but that still exclude trypan blue. However, even though our correction factor is not rigorous, it certainly provides a figure which is more realistic for comparison with whole tissue data than the uncorrected data in Figure 5.

The Metabolic Cost of Beating

The data in Figures 4 and 5 can be used to compute the extra metabolism associated with beating, under anaerobic and aerobic conditions. These values can be translated into ATP per beat and compared directly if several assumptions are made: that the ATP yield per lactate molecule was 1.5 (i.e., glycogen rather than glucose is the substrate (Table 2)); that the ATP yield per oxygen atom was 3.17; that the rate of lactate formation under aerobic conditions was negligible (values of 1 to 2 nmol/min per mg were found); that respiration unrelated to ATP synthesis is unaffected by oligomycin; and that the only source of ATP was either oxidative phosphorylation or glycolysis. Corrected values so calculated are shown in Table 3. Two points are noteworthy: the values for ATP/beat are somewhat greater under aerobic conditions than under anaerobic conditions, and each beat uses twice as much ATP when isoproterenol is present. We can use these values to calculate the maximum possible rate of ATP hydrolysis by the cross-bridges per pass of an actin site under these conditions. Heart muscle contains approximately 0.464 nmol myosin/mg protein [Katz, 1970, and using a myosin molecular weight of 466,000 (Harrington, 1979)]. Thus, if all the beat-dependent ATP hydrolysis was caused by actomyosin ATPase, this would only amount to the hydrolysis of at most one molecule of ATP per eight myosin molecules per beat under anaerobic conditions in the absence of isoproterenol. Each beat results in a relative translation of the actin and myosin filaments of approximately 167 nm (Fig. 3A; isoproterenol made very little difference to the extent of contraction). Therefore, since the actin filament helix repeat distance is approximately 36 nm, and assuming that cross-bridges can interact with the actin filament at only one position per helix repeat (Eisenberg et al., 1980), we conclude that cross-bridges hydrolyze a maximum of one molecule of ATP per 40 passes of an actin site under these conditions. This low rate of ATP hydrolysis can be explained by two mechanisms. The first is that, at high contraction velocities, the rate of translation of cross-bridges past actin sites becomes greater than the rate of attachment, resulting in a significant number of "misses" (Huxley, 1957; Eisenberg et al., 1980). Second, the actomyosin ATPase may not be fully activated. One test of this possibility is to compare the measured ATP utilization per beat with that expected just for activation processes in muscle which is fully activated. Solaro and Briggs (1974) have calculated that full activation of heart muscle requires approximately 0.43 nmol Ca ++/mg protein (converted using factors in Table 4). Since the sarcoplasmic reticulum requires one molecule of ATP to take up two atoms of Ca ++ (Weber et al., 1966), each fully activated beat would require 0.216 nmol ATP just to sequester Ca ++, on the (controversial) assumption that most of the Ca ++ was taken up by the sarcoplasmic reticulum. Thus, from Table 3, if all the beat-dependent ATP hydrolysis was used for Ca ++ metabolism during recovery, this would only allow for at most a 27% activation of the troponin for each beat under anaerobic conditions in the absence of isoproterenol. This is an upper limit, since some ATP must be hydrolyzed by the actomyosin ATPase. These calculations therefore suggest that, under these conditions, the magnitude of Ca ++ release per beat is sharply reduced to perhaps 10% to 20% of that needed

### Table 3

<table>
<thead>
<tr>
<th>Summary of Corrected Values Obtained for the Metabolic Cost of Beating</th>
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<tbody>
<tr>
<td><strong>I. Beat-dependent ATP utilization (nmol ATP/beat per mg)</strong></td>
</tr>
<tr>
<td><strong>A. Anaerobic conditions</strong></td>
</tr>
<tr>
<td>0.033 ± 0.009 (0-5 Hz)</td>
</tr>
<tr>
<td>0.058 ± 0.005 (0-3 Hz)</td>
</tr>
<tr>
<td><strong>B. Aerobic conditions</strong></td>
</tr>
<tr>
<td>0.089 ± 0.010</td>
</tr>
<tr>
<td><strong>II. Beat-dependent non-ATP-linked respiration (nanoatom O/beat per mg)</strong></td>
</tr>
<tr>
<td>+Isoproterenol</td>
</tr>
<tr>
<td>0.126 ± 0.013</td>
</tr>
<tr>
<td>0.196 ± 0.009</td>
</tr>
<tr>
<td>0.023 ± 0.006</td>
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</tbody>
</table>

IA was calculated from the data in Figure 4, from the difference in slopes with and without verapamil. IB was calculated from the data in Figure 5, from the difference in slopes with and without oligomycin; the value with isoproterenol was calculated from the combined data of Figure 5, A and B. II was calculated from the data in Figure 5A, from the difference in slopes with and without verapamil, in the presence of oligomycin.
Table 4
Comparison of the Corrected Oxygen Consumption of Isolated Cells and Intact Tissue

<table>
<thead>
<tr>
<th></th>
<th>KCl-arrested rat heart</th>
<th>Unstimulated cells</th>
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<tbody>
<tr>
<td>Basal oxygen consumption (nanoatom O/min per mg)</td>
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</tr>
<tr>
<td>[Challoner, 1968]</td>
<td>19.03 ± 1.43</td>
<td>16.44 ± 0.19</td>
</tr>
</tbody>
</table>

II. Total oxygen consumption (nanoatom O/min per mg)

| Rat heart beating at 4 Hz, extrapolated to zero pressure development (Neely et al., 1967) | Cells beating at 4 Hz (from Fig. 5B) |
| 34.06 equation | linear regression |

| Unstimulated | 34.06 | 28.09 ± 0.23 |

III. Oxygen consumption per beat (nanoatom O/beat per mg)

<table>
<thead>
<tr>
<th>Nonworking dog heart</th>
<th>Beating cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>(Gibbs et al., 1980)</td>
<td>(from Fig. 5B)</td>
</tr>
<tr>
<td>0.0716 ± 0.0386</td>
<td>0.0485 ± 0.0011</td>
</tr>
</tbody>
</table>

Factors used to convert published values to comparable units were: 685 mg protein/g dry wt, and 162 mg protein/g wet weight. These factors are as measured in our laboratory on the rat heart.

The same factors were used for converting dog heart data.

to fully activate the actomyosin ATPase, and yet the cells are able to contract with high velocity.

The greater ATP utilization found in the presence of isoproterenol could reflect a greater extent of Ca++ release and actomyosin ATPase activation. This interpretation is consistent with the observation of Allen and Blinks (1978) that isoproterenol strongly enhanced the activation-related luminescence of the Ca++-sensitive protein aequorin microinjected into cells of frog atrial trabeculae.

An estimate of the maximum possible rate of oxidative phosphorylation of the heart cells can be gained from the magnitude of the maximum uncoupler-stimulated rate of respiration. Cells under aerobic conditions and in the presence of 0.3 μM FCCP respire at a rate of 115.9 ± 16.2 nanoatom O/min per mg above the basal rate (mean ± SD, n = 3 preparations). This is equivalent to a rate of oxidative phosphorylation of 347.7 ± 48.6 nmol ATP/min per mg. Thus, cells beating freely at 5 Hz and using approximately 30 nmol ATP/min per mg (Figs. 4 and 5) are using less than 10% of the cells potential rate of energy liberation.

The Effect of Oligomycin

In addition to the oligomycin-sensitive component of the beat-dependent respiration, there is a substantial oligomycin-insensitive component (Fig. 5). Since sufficient oligomycin was added to give a maximal effect, and oligomycin is a total inhibitor of oxidative phosphorylation (Lardy et al., 1958), this oligomycin-insensitive component must be caused by some demand other than oxidative phosphorylation. Since mitochondria are able to accumulate ions (notably Ca++) by active transport independent of ATP synthesis, such ion transport is a possible candidate for explaining this result.

A striking feature of Figure 5 is that no significant oligomycin-sensitive respiration is evident in the unstimulated cells. This is surprising, because oligomycin stimulates glycolytic lactate production by about 8 nmol/min per mg, and we would therefore expect 4 nanoatom 0/min per mg of oligomycin-sensitive respiration. This would have been detectable. The discrepancy therefore suggests that our assumption that oligomycin-sensitive respiration is a good measure of cellular ATP utilization is not valid at the lowest levels of oxidative phosphorylation. The effect of oligomycin on the basal metabolism of isolated rat hearts has been measured by Challoner (1968). He also found no effect of oligomycin on mVo2, if the hearts were treated with epinephrine. However, he did report an 18% reduction in mVo2 in hearts without epinephrine which does convert to 3.43 nanoatom O/min per mg heart protein. We found no significant effect of oligomycin on quiescent cells, whether or not isoproterenol was present (Fig. 5).

Comparison of the Metabolism of Cells in Suspension with That of Whole Tissue

Table 4 shows that the basal rate of oxygen consumption in the KCl-arrested rat heart, as measured by Challoner (1968), is similar to the rate of oxygen consumption of unstimulated cells. An estimate of the metabolic cost of beating with zero pressure development has previously been made on isolated rat hearts by Neely et al. (1967). This estimate was made by extrapolation to zero pressure development from systolic pressures varied by changing the left atrial filling pressure, and is subject to considerable uncertainty. The value found, converted to comparable units, is shown in Table 4, and is seen to be close to that estimated for cells beating at 4 Hz. We are not aware of any previous work on the rat heart which would allow us to make a comparison with cells on a per beat basis. Such data do exist for the dog heart, however, where the mVo2 was measured in the non-working heart as a function of stimulation frequency (Gibbs et al., 1980). Table 4 shows that the value found per beat agrees well with that measured here on rat heart cell suspensions.

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INDEX TERMS: Adult heart cells • Energy metabolism • Oxygen consumption • Isoproterenol • Maximum velocity of contraction
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R A Haworth, D R Hunter, H A Berkoff and R L Moss

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