Temperature, skeletal muscle mitochondrial functions, and oxygen debt

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Oxygen debt was defined by Hill et al. (15) in 1924 as the difference between the increased \( O_2 \) consumption after exercise and a resting metabolic base line. Initially, the increased \( O_2 \) consumption after exercise was related to and used to quantify the amount of anaerobic metabolism that occurred during exercise. However, agreement has still not been reached on the portion of the postexercise \( O_2 \) consumption that constitutes the repayment of the \( O_2 \) debt (35). Basal (30), resting (14), and exercise (35) \( O_2 \) consumption levels have all been used as base lines from which to estimate the \( O_2 \) debt. Regardless of the base line used, experimentally determined values of \( O_2 \) debt have invariably been larger than the debt which can be accounted for physiologically (Table 1) (7, 13, 35, 36).

Originally, Hill et al. (15) attributed the increased \( O_2 \) consumption after exertion to the energy requirements for the reconversion of lactate to glycogen in muscle and liver, but subsequent observations indicated that most of the \( O_2 \) debt was repaid by the time the highest concentrations of lactate appeared in the blood (27). To resolve this threat to the concept of the \( O_2 \) debt, Margaria et al. (27) proposed that the \( O_2 \) debt consisted of alactacid and lactacid components.

Kayne and Alpert (17) observed that eviscerated dogs had \( O_2 \) debts of the same magnitude as control animals. Since gluconeogenesis, with lactate as the carbon source, does not occur to any appreciable extent in skeletal muscle (28, 31), the bulk of the \( O_2 \) debts in these experiments must have been of lactacid origin. Alpert and Root (1) also observed that infusion of lactate did not produce an \( O_2 \) debt. These data raised serious doubts about the validity of the classical concept of \( O_2 \) debt.

To bring the alactacid component of the \( O_2 \) debt closer to a theoretical value, Margaria (26) recently presented a correction for the \( O_2 \) cost of cardiorespiratory work and the resaturation of blood with \( O_2 \). Welch et al. (35) also concluded that \( O_2 \) consumption in recovery from exercise was too high to be attributed only to the resynthesis of depleted energy stores and proposed a role for the exponential increase in the \( O_2 \) cost of breathing with increasing ventilation (35).

Oxygen debts averaging 28 liters were reported by Barnard and Foss (4) for dogs whose thermoregulation was limited by tracheostomy. In a dog gastrocnemius-plantaris in situ muscle preparation, Barclay (3) observed a 50–130% increase in \( O_2 \) debt associated with a 3 C rise in muscle temperature. Furthermore, during recovery \( O_2 \) consumption returned to resting levels with the same basic time course as muscle temperature. The extremely high \( O_2 \) debts and the relationship between postsccric \( O_2 \) consumption and muscle temperature suggested to us that a heat load might contribute to the magnitude of the \( O_2 \) debt. It is possible that in the intact animal exertion stimulates muscle respiration, whereupon increased respiration elevates muscle temperature; the elevated muscle temperature results in a further increase in respiration, initiating a positive feedback effect. Because of the large \( O_2 \) debts measured under circumstances when muscle temperatures might be high, it seemed appropriate to investigate the effects of temperature on some functions of skeletal muscle mitochondria in vitro and to relate the findings to the functions of skeletal muscle in vivo within the observed physiological temperature range.
weights were approximately 250-300 g. For determinations described by Chance and Williams (8). Measurements were trode mounted in a temperature-controlled, all glass reac-

At exhaustion, animals were killed by a blow to the head for an hour at 18 m/min up a 10% grade. After an hour the driven treadmill for 1 hr at 18 m/min on a 10% grade. Control animals were killed no sooner than 48 hr after the last bout of physical exercise. Exercised animals were run to exhaustion, at which time temperatures and all other preparatory procedures were performed at 25, 31, 37, 40, 43, and 45 C. The effect of aging at 0 C was observed to be insignificant, but with alternate preparations the sequence of incubation temperatures, either low to high or high to low, was reversed. Respiratory rates in the presence of oligomycin (3.3 μg/ml) and FCCP (1 μM), and with NADII as the substrate were also determined.

Six animals, one male and five females, were used for determinations of mitochondrial ATPase activities. Measurements were made in a medium of 25 mM TES, pH 7.5, 5 mM ATP, 150 mM sucrose, and 5 mM MgCl2. Oligomycin was used at a final concentration of 10 μg/ml of incubation medium. Inorganic orthophosphate, as a product of the ATPase reaction, was extracted according to Lindberg and Ernster (24) and measured spectrophotometrically according to the method of Berenblum and Chain (5). Determinations were made in duplicate upon reactions carried out at 25, 37, and 45 C.

For determinations of the rate of oxidation of NADH, NADII was added to a substrate concentration of 1.67 mM. ADP was added to the incubation medium to a final con-

Sprague Dawley rats, fed ad libitum on standard labora-
tory diet, were used for this study. At the time of kill the weights were approximately 250-300 g. For determinations of mitochondrial respiratory rates and ADP:O ratios, two groups of 10 animals each were used. The 20 animals were arbitrarily selected on the basis of willingness to participate in forced exercise. Each group was comprised of five males and five females. One group was a control group; the other was used immediately after a period of severe exercise. All animals were trained daily to be able to run on a motor-driven treadmill for 1 hr at 18 m/min on a 10% grade. Control animals were killed no sooner than 48 hr after the last bout of physical exercise. Exercised animals were run for an hour at 18 m/min up a 10% grade. After an hour the workload was increased to 27 m/min at 10% grade; most rats continued for at least another 30 min at this workload. At exhaustion, animals were killed by a blow to the head and then decapitated. Exhaustion was determined to be that point at which animals were no longer able to keep pace with the treadmill even in response to electrical, mechanical, and acoustical stimuli. The hindlegs were rapidly removed, skinned, and immersed in ice-cold isotonic KCl. Dissection and all other preparatory procedures were performed at 0-2 C.

Skeletal muscle mitochondria were isolated according to the method of Makinen and Lee (25). Oxygen uptake was measured polarographically with a Clark type oxygen elec-
trode mounted in a temperature-controlled, all glass reac-
tion vessel. * Assumptions: ADP, ATP: adenosine di- and triphosphate, respectively; ATPase: ATP phosphohydrolase, EC 3.6.1.3.; EDTA: ethylenediaminetetraacetic acid; FCCP: &-trifluoromethoxy carbonyl cyanide phenylhydrazone; NADH: nicotinamide adenine dinucleotide, reduced; SMM: skeletal muscle mitochondria; TES: N-tris (hydroxymethyl)methyl-2-aminoethane sulfonic acid; Tris: tri(hy-
droxyaminomethyl)aminomethane.

**TABLE 1. Maximum accountable postexercise O2 consumption for a 70-kg human**

<table>
<thead>
<tr>
<th>Source</th>
<th>Ox Equival L.</th>
<th>Cumulative Total L. O2</th>
</tr>
</thead>
<tbody>
<tr>
<td>Adenosine triphosphate*</td>
<td>0.45</td>
<td>0.45</td>
</tr>
<tr>
<td>Creatine phosphate*</td>
<td>1.05</td>
<td>1.50</td>
</tr>
<tr>
<td>Resaturation of tissue H2O</td>
<td>0.05</td>
<td>1.55</td>
</tr>
<tr>
<td>Resaturation of venous blood</td>
<td>0.44</td>
<td>1.99</td>
</tr>
<tr>
<td>Resaturation of blood in muscle</td>
<td>0.40</td>
<td>2.39</td>
</tr>
<tr>
<td>Resauration of myoglobin</td>
<td>0.20</td>
<td>2.59</td>
</tr>
<tr>
<td>Extra cardiorespiratory work during recovery†</td>
<td>0.40</td>
<td>2.99</td>
</tr>
</tbody>
</table>

* ATP and C-P values for human skeletal muscle, J. Bergstrom (6). † Discussed by R. Margaria (26) and Welch et al. (35).
exercise-exhausted animals were not demonstrable, the mitochondrial data in the remainder of this paper will deal with results obtained from control animals only.

Figure 1 shows the effect of temperature on states 3 and 4 respiration, the ADP:O ratio, and the respiratory control ratio (RCR). Between 25 and 37 °C, both states 3 and 4 respiratory rates increased fairly linearly. Above 37 °C, both rates of respiration continued to increase, but at a far greater rate than below 37 °C. Because the increase in state 4 respiration with increasing temperature was particularly pronounced, respiratory control decreased rapidly at temperatures above 37 °C.

The ADP:O ratio remained fairly constant in the temperature range from 25 to 40 °C. Above 40 °C the ratio declined linearly until at 45 °C a significant decrease (P < 0.01) of 18% was observed compared to the ADP:O ratio at 37 °C.

Figure 2 depicts two typical records of the O2 electrode from which the data in Fig. 1 were calculated. For comparison, the same time scale has been used for both 25 and 45 °C. In practice, for purposes of accuracy, the time base on the O2 electrode recorder was expanded 5 times for readings at temperatures above 37 °C. Figure 2 also shows the large difference in the amount of O2 initially in solution at the two temperatures.

In addition to the large differences in rates of O2 consumption, skeletal muscle mitochondria incubated at 25 and 45 °C also showed a striking difference in response to oligomycin. The rate of respiration after administration of oligomycin at 25 °C was not very different from that seen in state 4. However, at 45 °C oligomycin inhibited respiration to about 50% of the state 4 rate. The effect of oligomycin in inhibiting respiration below the level of state 4 (i.e., the percent inhibition of state 4 respiration) was not linear with respect to temperature but increased (Fig. 3) very rapidly between 40 and 45 °C.

Since oligomycin inhibits mitochondrial ATPase activity (16, 21), the results shown in Figs. 2 and 3 suggest that the mitochondrial ATPase activity might vary with incubation temperature and be responsible for the elevated state 4 respiratory rates and decreased ADP:O ratios observed. To test this hypothesis, oligomycin-sensitive ATPase activity of isolated skeletal muscle mitochondria was assayed at 25, 37, and 45 °C. A greater increase in oligomycin-sensitive ATPase activity was observed between 37 and 45 °C than between (Fig. 4) 25 and 37 °C. These data are in agreement with the effect of temperature on state 4 respiration (Fig. 1), and with the effect of oligomycin on respiration (Fig. 3).

The effect of temperature on the RCR is also depicted in Fig. 4. A differential temperature effect, although the inverse of that on ATPase activity, is apparent in the RCR. In this case there was a slow decrease in RCR between 25 and 37 °C and a rapid decrease between 37 and 45 °C. As
exercise. The normal temperature gradient existing from core to periphery during rest was reversed in the immediate postexercise period.

shown in Fig. 1, these changes in the RCR were due primarily to increased state 4 respiration relative to state 3 respiration.

All the above results are based on studies of isolated skeletal muscle mitochondria in vitro. If the hypothesis is valid that increased tissue temperature contributes to the increased rate of O\textsubscript{2} consumption during and subsequent to exertion, increased tissue temperatures should be observed at these times. Although we were unable to measure tissue temperatures while rats were running on the treadmill, we have measured temperatures immediately after exercise, during the initial period of repayment of O\textsubscript{2} debt.

The resting skeletal muscle temperature in rats was observed to be 36.0 ± 0.2 °C (mean ±SE). After severe exercise, this temperature increased to 44.1 ± 0.1 °C, with a maximum recorded value of 44.3 °C. Liver temperature increased from 38.6 ± 0.2 at rest to 43.4 ± 0.1 °C after exercise. The normal temperature gradient existing from core to periphery during rest was reversed in the immediate postexercise period.

**DISCUSSION**

The increased tissue temperatures following severe exercise and the effects of elevated temperatures on mitochondrial respiration in vitro suggest that current concepts of O\textsubscript{2} debt require modification (7). Our results indicate that in addition to lactacid and alactacid components (27), there is a third component contributing to the total O\textsubscript{2} consumption observed after exercise. The third component is not related to the repayment of an energy debt. It is, in fact, largely nonconservative in nature and is a consequence of the well-known effect of temperature on the rates at which biochemical reactions proceed. Therefore, it follows that increased O\textsubscript{2} consumption resulting from such a temperature effect on metabolic processes is not restricted to the musculature and the liver but will occur at any site at which temperature is elevated above normal.

The elevated temperatures in both core and muscle, following extreme exertion in rats, corroborate reports of similar results on humans (32, 33) and guinea pigs (David R. Lamb, personal communication). Saltin (32) has reported muscle temperatures in humans between 35 °C at rest and 40.6 °C in submaximal exercise, indicating the same 6-7 °C operating range we have found in rats. In humans, muscle temperatures were found to be about 2 °C above rectal during submaximal exercise (32, 34). Rectal temperatures as high as 41 °C have been observed in humans after exercise (9, 29). It is particularly interesting that at rest core temperature always exceeds that of skeletal muscle (34) whereas the gradient is reversed following extreme exertion. Apparently, the heat load generated by severe exercise is so great that the core may in effect function as a heat sink for the skeletal musculature maintaining muscle temperature below the point where a significant reduction in the ADP:O\textsubscript{2} ratio occurs (7).

If elevated muscle temperatures produce the same effects in vivo that we were able to demonstrate in vitro, the question arises why we were unable to isolate mitochondria from exercise-exhausted rats which displayed, at lower temperatures in vitro, the aberrant effects we were able to induce at high temperatures. Published reports indicate that exercise causes both cardiac (2, 18) and skeletal muscle (11) mitochondria to swell to several times their normal size. It is possible that disruption of the tissue during the mitochondrial isolation procedure results in fragmentation of some swollen mitochondria and their loss during differential centrifugation. However, since there was no obvious difference in the mitochondrial yield from control and exhausted animals, a more likely explanation is that the exposure of mitochondria from exercised animals to isotonie solutions containing substantial amounts of bovine serum albumin, ATP, and EDTA during isolation may reverse the disruptive effects of exercise, greatly increasing the probability that mitochondria isolated will be normal.

Our studies on isolated rat skeletal muscle mitochondria in vitro provide some insight into possible metabolic mechanisms whereby postexercise O\textsubscript{2} consumption is increased. The most marked effect of temperature on isolated skeletal muscle mitochondria was the effect on the state 4 respiratory rate. Within the physiological range of 37–43 °C the rate of state 4 respiration was increased over 200%. Within this same temperature interval state 3 respiration increased only about 60%. Since the rate of state 4 respiration of mitochondria can be taken as the lowest possible level of resting metabolism in skeletal muscle, and since this very high state 4 respiration would not be expected to be associated with resynthesis of ATP or with extramitochondrial energy requiring functions, exaggerated rates of nonconservative, state 4, respiration after exercise may explain, in part, the discrepancy between measured oxygen debts and estimates of debts based on the sum of lactacid and alactacid components.

State 4 mitochondrial respiration may be increased by 1) loosening of the coupling between electron transport and
phosphorylation, 2) stimulation of the mitochondrial ATPase, 3) partial uncoupling, or 4) a combination of these phenomena. It would appear that in skeletal muscle mitochondria incubated at elevated temperatures, the rate of state 4 respiration is increased primarily on the basis of stimulation of mitochondrial ATPase activity. This contention is supported by three observations: 1) oligomycin-sensitive ATPase activity increased markedly as a function of temperature; 2) reasonable ADP respiratory control existed at all temperatures; and 3) both oligomycin (16, 21) and FCCP (14) markedly affected respiration and phosphorylation at all temperatures.

A direct relationship between incubation temperature and the inhibition of state 4 respiration by oligomycin was observed (Fig. 3). Since the rate of state 4 respiration is supposedly independent of the availability of ADP, there would seem to be no reason to expect such a differential effect of oligomycin on the state 4 respiratory rate unless there were, at higher temperatures, a higher rate of ATP turnover to generate ADP and thus increase the rate of respiration.

Since the mitochondrial ATPase is stimulated at elevated physiological temperatures (Fig. 4), the phosphorylative mechanism is probably exposed to an elevated, but still limiting, concentration of ADP. Our observations indicate that this concentration of ADP is not enough to increase respiration to the full state 3 rate. Under such conditions a pseudo-state 3 rate, rather than a true state 4 rate, is observed. This has been referred to as state 3½ respiration (19).

A more mechanistic explanation of the effects of oligomycin on inhibiting respiration below the state 4 rate is given in Fig. 5. If, as proposed by Lee and Ernster (23) for submitochondrial particles, oligomycin repaired an energy leak at the level of X-I in the energy coupling mechanism, the results obtained would be expected if the primary effect of temperature was manifested at the leak site. A scheme for the chemical coupling hypothesis is presented in Fig. 5A. The effect of temperature may be to induce a conformational change in the enzyme which catalyzes the reaction at X-I by either altering the active site so that X-I is no longer a substrate at the site or by destroying the hydrophobicity of the active site, allowing the hydrolysis of X-I by H⁺ or OH⁻ ions (Fig. 5B). The actions of oligomycin could then be as depicted in Fig. 5C, to prevent a conformational change in the enzyme at X-I and to inhibit the ATPase. By inducing an energy leak at X-I, temperature would stimulate ATPase activity. The energy required to push the reaction catalyzed by the ATPase (EC 3.6.1.3) in the direction of ATP would not be available and the enzyme would, because of its equilibrium constant, hydrolyze ATP to ADP and P_i (Fig. 5B).

Direct measurements of oligomycin-sensitive ATPase activity (Fig. 4) show that there is a stimulation of 100% over the physiological temperature range of 37–45°C. When the O_2 required to phosphorylate the extra ADP made available from this source is added to the extra O_2 consumed due to the direct physical effect of increased temperature on the oxidative mechanism, it would seem sufficient to account for the observed 200% increase in the rate of state 4 respiration between 37 and 45°C.

The increased oligomycin-sensitive ATPase activity seen at elevated temperatures (Fig. 4) may account for the decreased ADP:O ratios seen above 40°C in isolated mitochondria (Fig. 1). It is possible that these ADP:O ratios may be spurious because of the method by which they were measured, and the efficiency of energy conservation may be unaltered at high physiological temperatures. Increased oligomycin-sensitive ATPase activity could thus be responsible for the apparent decrease in phosphorylative efficiency of isolated mitochondria in vitro.

On the other hand, the ADP:O ratios measured in this study may, in fact, be representative of the in vivo situation. Either the ATPase or some other temperature-related effect on the energy conserving mechanism, perhaps partial uncoupling due to an energy leak at X-I, may be at work opposing the continuous generation of great amounts of ATP required by the contractile mechanism during exercise. If ATP demands become significantly in excess of the supply, due to a decreased ADP:O ratio at elevated muscle temperatures, work output of the muscle would eventually decrease. This, indeed, may explain fatigue in prolonged, submaximal, aerobic exertions which induce hyperthermia. The greater endurance of swimmers compared to runners (10) can possibly be attributed to the efficacy of water in conducting heat away from the skeletal musculature during exertion. Moreover, since effects of exhaustive exercise are not limited to skeletal muscle but can also be observed in heart (2, 18, 22), liver (vide supra), and most probably central nervous system as well, a discussion of hyperthermic fatigue should include consideration of the effects of prolonged exercise on tissues other than skeletal muscle.

When dealing with mitochondria isolated from skeletal muscle it is important to consider the possibility of error introduced by actomyosin contamination of the preparation. All the results discussed here concerning skeletal muscle mitochondria are confined to the oligomycin-sensitive ATPase, and therefore, represent only mitochondrial ATPase functions. Furthermore, electron micrographs of mitochondria prepared by the method of Makinen and Lee (25) in this laboratory show no evidence of either
actomyosin or sarcoplasmic reticulum (20). Other results on rat liver mitochondria (unpublished data) corroborate the results on rat muscle mitochondria. Since in the preparation of liver mitochondria there is no opportunity for significant actomyosin contamination, and since the effects of temperature on liver mitochondria are similar to those on muscle mitochondria, it must be considered unlikely that the muscle preparations were contaminated by actomyosin. Any contribution of actomyosin ATPase activity to the respiratory rates which were observed would, of course, serve to alter the in vitro results quantitatively. However, this would not in any way affect the conclusion that there is a temperature-stimulated component of O2 consumption present after exercise which is totally independent of the so-called "lactacid" and "alactacid" debs.

At present we are not able to ascertain the portion of the total postexercise O2 consumption which is attributable to respiration stimulated by increased muscle and core temperatures, but the contribution may be considerable. Since at higher physiological temperatures there is both an increase in the rate of nonconservative (state 4) respiration and a decrease in phosphorylative efficiency (ADP:O ratio), and since the elevated temperatures responsible for these effects last for a significant period after exercise and are accompanied by increased levels of O2 consumption of the intact animal (unpublished data), it would appear that it is invalid to equate the extra O2 consumed after exercise with the anaerobic metabolism which occurs during exertion. It becomes important then to reexamine the phenomenon of O2 debt with a view toward partitioning the total extra O2 consumed among lactacid and alactacid debts and the component due to the stimulation of respiration by temperature.

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