Proc. Natl. Acad. Sci. USA
Vol. 78, No. 11, pp. 6714–6718, November 1981
Biochemistry

Mitochondrial regulation of phosphocreatine/inorganic phosphate ratios in exercising human muscle: A gated ^{31}P NMR study

(noninvasive biochemistry/muscle exercise performance/mitochondrial function)

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Contributed by Britton Chance, July 22, 1981

ABSTRACT **^{31}P NMR is used to determine the relationship between work output in the exercising human forearm and the steady-state capability of oxidative phosphorylation as measured by the phosphocreatine/inorganic phosphate ratio (*PCr*/P_i). Exercise intensities (one contraction per 5 sec) permitting comfortable continuation of activity for >1 hr produced *PCr*/P_i of about 1 for a subject of moderate training. Linear relationships between work rate per unit volume of muscle and the 5-min mean *PCr*/P_i were found for the subject's left and right arms. The protocol affords sensitive criteria of muscle performance in normal subjects and of biochemical or vascular disease in abnormal subjects. The P_i, *PCr*, and ATP levels found by ^{31}P NMR represent the initial values in the cycle of contraction and relaxation which permit restitution of resting state 4 prior to the next contraction and the continuation of steady-state work performance.**

Regulation of the bioenergetic state of body tissues by mitochondrial respiratory control (1) is exemplified in skeletal muscle by the state 4–3 response of tissue mitochondria in the oxidation of the matrix space NADH (2) and of cytochrome *b* (3, 4) of the frog gastrocnemius muscle in response to electrically induced contractions. Activation of energy metabolism has been attributed to the arrival of ADP at mitochondria or, as more recently suggested by ^{31}P NMR studies, to the fact that increased levels of free P_i elicit the resting → active (state 4–3) transition in the tissue mitochondria (5–9). In either case, one would expect the state 4–3 transition of mitochondria to regulate the rate of ATP synthesis as the work load is increased in a linear fashion up to the point of maximum state 3 electron transport, respiration rate, and ATP production.

The noninvasive ^{31}P NMR determination of tissue P_i concentration is extended in detail to the human arm and leg in these studies and supports the earlier finding of low P_i in resting tissue (6–9, ‡), confirming initial studies (10) of phosphate potential in suspensions of ascites tumor cells in which a value of $10^{4.9}$ was found by a noninvasive optical measurement and differed by a factor of >85 from the chemical data which gave $10^{3.2}$.

Much work has been done with paired samples of amphibian muscle by both analytical biochemistry (11) and by ^{31}P NMR (12). In humans, however, continual biopsy and biochemical analysis are not practical because even needle biopsies cause a loss of functional muscle. The object of the present experiments was to maintain steady states of wrist flexor work output and to determine the corresponding stimulation of mitochondrial activity by measuring the phosphocreatine (*PCr*)/P_i ratio in a noninvasive, nondestructive manner.

The first noninvasive human studies with ^{31}P NMR permitted only mild exercise of an extremity within the magnet (6). These studies evaluated *PCr*/P_i values during continuous exercise of the human wrist flexor musculature, monitored by direct coupling to the Cybex ergometer. In two other recent applications to arm exercise with NMR monitoring, ergometer coupling was not possible and the changes of *PCr* and P_i were artificially magnified by a tourniquet or pressure cuff (13, ‡). In our experiments, an attempt was made to evaluate *PCr*/P_i quantitatively just after muscle contraction with known exercise rates and over a range from rest to the maximal obtainable in the steady state without discomfort. It has been possible to explain the relationships in terms of metabolic control by the mitochondrial resting–to–active transition (state 4–3) in the resting and working human arm (1).

METHODS

In order to measure the work output of the wrist flexors, a Cybex ergometer (14) was mechanically coupled to a specially constructed wrist flexion exercise probe by a nonmagnetic linkage. Wrist flexor work output was recorded while *PCr* and P_i were measured in the forearm musculature which overlay the NMR probe inside the magnet (15). For example, the work rate during the initial phase of activity in Fig. 2 is 1.7 J per repetition (at 1 repetition per 5 sec; average power output, 0.34 W).

Reproducibility of muscle performance was assisted by initiating each contraction in response to a 0.2-Hz periodic sound from the computer. The subject also observed the intensity of his own contraction, displayed on an oscilloscope, and attempted to duplicate the previous contraction exactly. We also used a premeasurement "warm-up" interval of 4 min to allow for circulatory adjustments. The free induction decay (FID), which contains information concerning the amounts and types of P-containing compounds present in the muscle, was gated for an interval of 100 msec at the end of each muscular contraction and prior to relaxation.

In contrast to our earlier study in which a demonstration of feasibility was paramount (6), systematic variation of *PCr*/P_i in the human forearm was required. To improve the accuracy of the ratio, repetition rate of the rf pulse was decreased from 25 to 0.2 Hz and accumulation time was increased from 2 to 5 min. A single rf pulse at 24 MHz gave a 10:1 signal-to-noise ratio for determination of *PCr* in the human forearm. By averaging 60 FIDs, a *PCr*/P_i at very nearly the calculated signal-to-noise level of 70:1 was obtained. The lower repetition frequency favored a complete relaxation of the ^{31}P nuclei and a more accurate measurement of relative concentrations of *PCr*/P_i.

The Johnson Foundation ^{31}P NMR instrument described previously (6, 16) was used in these studies. Improvements in surface coil design over that described (6) have been detailed elsewhere (16) and afforded an increased signal-to-noise ratio. The electronic circuits used in these studies used an improved

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Abbreviations: *PCr*, phosphocreatine; FID, free induction decay.

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‡ Dawson, M. & Wilkie, D. (1981) *International Biophysics Congress*, Mexico City, Abstract TH-P3, p. 260.

preamplifier (courtesy of James Engle). The 7-inch (17.8 cm) magnet (Oxford) was operated at 1.5 tesla without room temperature shims or profiling coils because the full clear bore was needed to permit wrist exercise. Maximum field homogeneity was obtained by adjusting the helium temperature shims to optimize the spectrum of the water protons in the muscle (60 MHz). Under these conditions, the half-width of the *P*Cr peak was ≤26 Hz at 24 MHz and the characteristic 20-Hz doublet of the γ resonance of ATP could be resolved. Baselines are derived for P_i in resting state 4 when P_i is minimal and for *P*Cr in active state 3 at maximum exercise when *P*Cr is minimal. At the levels of steady-state exercise described here, the sugar phosphate levels are small compared with P_i; no correction for their contribution to the NMR spectra was made.

The exercise protocol involved an initial steady state (rest) followed by incremental levels of steady-state exercise (as measured by work rate) to a level at which discomfort was discernable. The time of transition between steady states (as determined by *P*Cr/P_i) was determined by observing sequential 2-min (24 FID) spectra. Upon initiation of activity, a full 4 min was required; transitions from one exercise state to another involving work increments of 20% to 50% required less than the allotted 4 min. The inverse protocol—namely, violent exercise followed by more moderate exercise during recovery—was explored but complete data are not yet available.

The volunteer selected for these tests was one who has maintained a steady and high level of exercise training (17).

EXPERIMENTAL RESULTS

Establishment of a Steady State. Fig. 1 shows a gradation of mitochondrial metabolic states due to contractile activity from near rest (*A*), which we term state 4, according to evaluations from isolated mitochondria, toward an active steady state 3 (*B*) at a low level of work rate. This low-power output was continued for 5 additional minutes (*C*) in an effort to determine how steady the work might be maintained. Thereafter was a more energetic steady state (*D*).

The characteristic spectrum of the near-resting muscle (Fig. 1*A*) shows the *P*Cr peak (displaced 3 ppm from a phosphoric acid standard) with the three peaks of ATP (γ, α, β). α and β are clearly resolved, and the γ is fused with *P*Cr, in agreement with other results (18). However, the quality of the resolution is indicated by the splitting of the 20-Hz γ phosphate peak (19). P_i appearing at 5 ppm from *P*Cr in the nearly resting muscle suggests that the pH of the subject's tissue is greater than 7. The sugar phosphates are at a low level and appear as a broad resonance shifted further from *P*Cr than P_i. The progressive changes of the various components after exercise in the ergometer set at 16 rpm (14) are indicated in Fig. 2. The first level of exercise is one that just exceeds the frictional losses in the ergometer coupling (≈1.0 J per repetition). The second exercise level (sections *B* and *C*) is significantly higher; section *D* is near maximal for this subject. *P*Cr/P_i fell initially from 10 to 3.4 in the first 5-min interval and then from 3.4 to 3.1 in the second interval, indicating the possibility of maintaining the bioenergetic activity to within about 10%. In the third interval, the work rate increased by 30% (mean power output = 0.5 W) and *P*Cr/P_i fell 36%, suggesting a proportionality between these two quantities in the range where the mitochondrial state 4–3 transition is governing the metabolism.

After activity ended, scans taken every 2 min showed rapid recovery in the course of 5 min. Final resting values indicate a lower P_i and a higher *P*Cr/P_i level than was present in the initial state. From *A* to *B*, the change in ATP was scarcely detectable. The ATP level then remained constant throughout the remaining exercises and did not increase after termination of activity. Thus the ATP change is not significant in these studies.

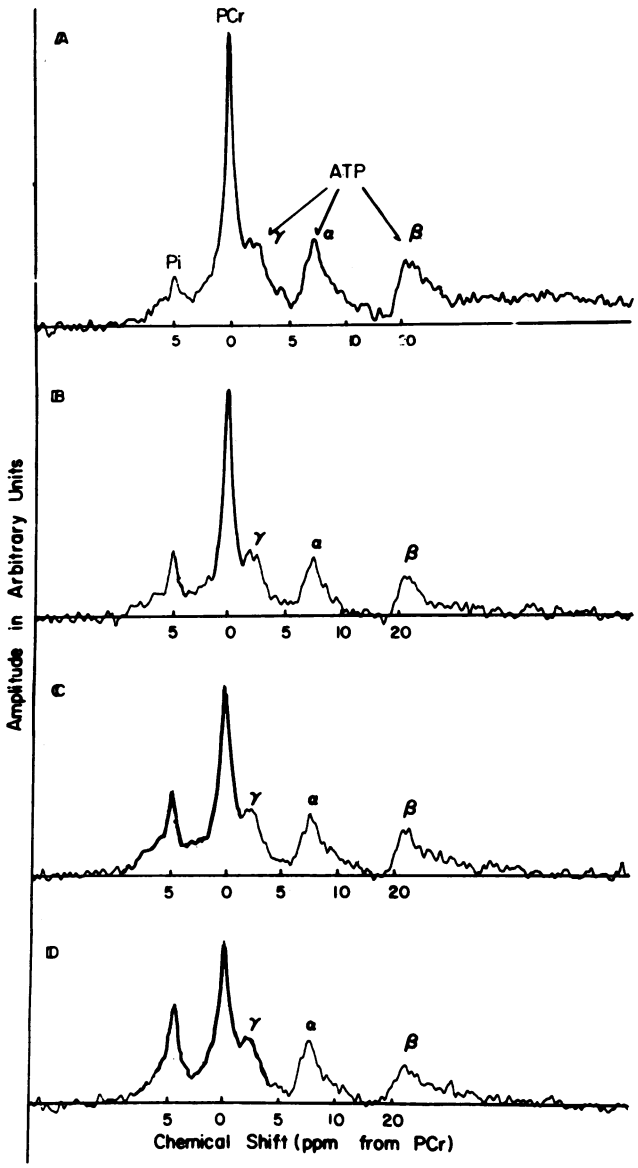

FIG. 1. ^{31}P NMR spectra of resting and exercising arm.

A corresponding progression of pH shift can be calculated from the chemical shift of the P_i toward the *P*Cr peak (20) which increased considerably in *D* so that 0.4 unit acidification with respect to the resting pH of 7.0 (13) was observed. Characteristically, this shift was not restored by the time *P*Cr/P_i had returned to rest values. The level of acidosis caused no discomfort at these low work rates. In summary, the state 4–3 transition in the human forearm musculature can be effected over a wide range of *P*Cr/P_i in a highly consistent manner.

Maintenance of Steady State at High Workload. In Fig. 3 *Left*, the upper trace represented a characteristic resting state 4 (*P*Cr/$P_i \approx 20$). The arm then was exercised at a work output per repetition similar to that produced in the more active portion of the previous protocol, but here it was repeated every 2½ sec (after an initial 5-min warm-up interval). Under these conditions, P_i and *P*Cr were approximately equal, and ATP was maintained at initial resting value. As frequently observed, noise level of the exercised arm was more than that of the resting arm (13).

At this level of exercise, *P*Cr/P_i could not be maintained constant. It decreased 20% over the 10-min interval, indicating that, for this subject, $[P_i] = [PCr]$ was the upper limit of metabolic activity that could be maintained relatively constant for the 10-min interval.

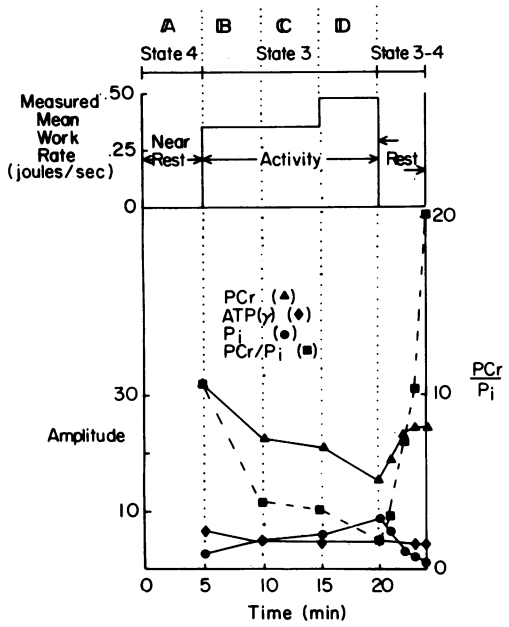

FIG. 2. Correlation of Cybex work rate and NMR data.

Lactic acidosis, as measured by the chemical shift of the P_i peak toward the *P*Cr peak, was not detectably increased during the 10-min interval. This may reflect a greater precision of *P*Cr/P_i determination than of the pH determination. This was borne out in a further series of steady-state work in which the rate was maintained at one contraction every 5 sec for 10 min (*P*Cr/P_i = 0.76) and then decreased to about half by doubling the interval between contractions (10 sec) for 20 min (*P*Cr/P_i = 1.06). The pH increased <0.1 unit while *P*Cr/P_i increased 40%. Thus, *P*Cr/P_i seems to be a much more sensitive indicator of work rate for *P*Cr/P_i values as low as 0.76.

The Relationship Between Muscular Activity and Mitochondrial Metabolic State. Because Fig. 2 suggested a proportionality between muscular power output (21) and the metabolic level as indicated by *P*Cr/P_i, we next attempted to quantify the relationship between the activity of the musculature and the activity of the mitochondria for this subject.

In this specific protocol, the subject performed a series of steady-state exercise trials, each at a different intensity. The protocol was based on previous experiments; a 4-min warm-up interval preceded the 6-min recording period. The subject proceeded from low to progressively higher power outputs over 1 hr, maximal output being set below the level of momentary discomfort during the contraction. In the initial test run, some difficulty in maintaining uniform contractions over the hour was experienced but in the second trial, shown here, consistent work rates were achieved as a result of the subject's prior training.

The average power output over each interval was computed from the Cybex ergometer traces. Because power output is related to muscle volume, we have normalized the data for the right and left forearms on the basis that the lengths of the muscle in both forearms are the same and that their cross-sectional areas differ. We approximated the ratio of the muscular areas by squaring the ratio of the circumferences of the two forearms, assuming that the life of the muscles is identical, which yields a normalized factor of $(27\ cm/25\ cm)^2 = 1.2$ for the right arm area compared to the left. *P*Cr/P_i values were obtained by measuring the peak amplitudes for each averaged 6-min spectrum.

Instead of plotting *P*Cr/P_i against work rate per unit volume of muscle, we plot the linearly increasing quantity of the reciprocal shown (Fig. 4). The normalized data (work rate/unit volume muscle) for each forearm is fitted by two straight lines which could be extrapolated to intercept the abscissa at 0.05, which is consistent with the independently determined resting level (*P*Cr/P_i = 20). This suggests that the standardized performance curves can be obtained for the bioenergetic system with normal blood flow and mitochondrial function. Deviations from these curves would result in a larger value of P_i/*P*Cr for a given power output tissue volume, as may be the case in peripheral vascular disease.

The resting leg spectrum (gastrocnemius region-BC) shown in Fig. 5 has the normal features of the arm spectra—i.e., a small

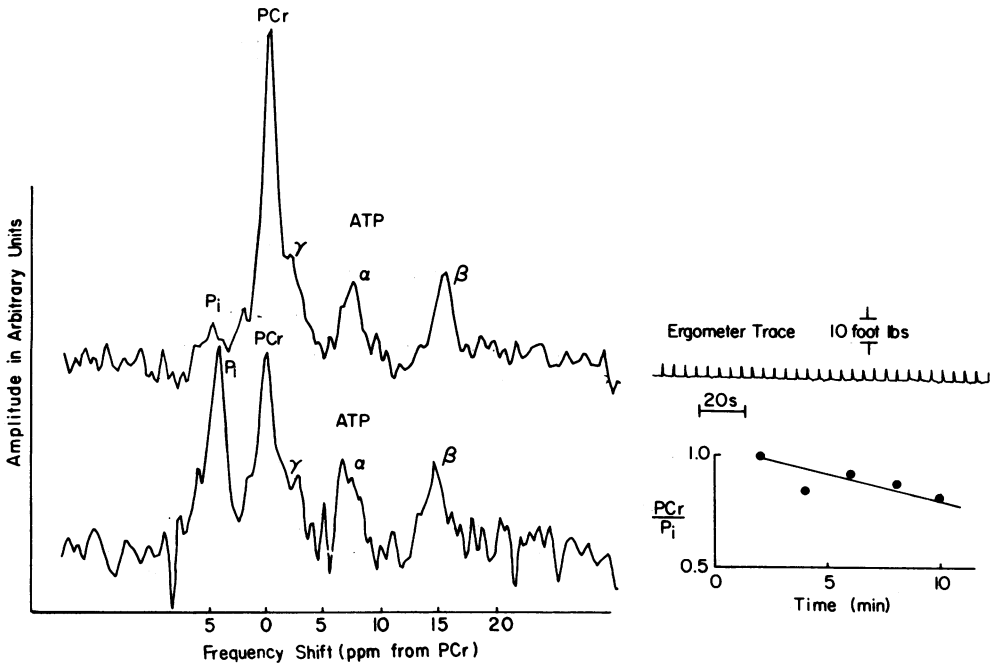

FIG. 3. Steady state of near-maximal work rate. (*Left*) ^{31}P NMR spectra: upper trace, resting; lower trace, exercise. (*Upper right*) Ergometer record. (*Lower Right*) *P*Cr/P_i vs. time.

P_i peak, less than 0.1 that of the *PCr* peak together with the triplet of ATP peaks. The P_i peak is located at 5 ppm from the *PCr* peak. A peak not observed in the arm but characteristic of the slow muscles of the leg is located at 3.2 ppm from *PCr* with an intensity 1/3rd that of the ATP peak, in agreement with animal models (22), and is attributed to phosphodiesters such as glycerol 3-phosphorylcholine or possibly to serine ethanolamine phosphodiester (22). During mild exercise (6), the P_i peak increased but the phosphodiester peak stayed nearly constant.

DISCUSSION

The arm exercises described here were intended to explore, by a gated noninvasive recording, the relationship between metabolism and work rate under conditions such that discomfort due to lactic acidosis was minimal and the aerobic energy metabolism of mitochondria regulated the metabolic response to muscle energy demand. The PCr/P_i ratios at which this was feasible were found to range from rest $PCr/P_i \approx 20$ to approximately 1.0 in accord with previous tests (6), and here these tests were extended to 0.67. At that point, lactic acidosis was felt momentarily during the ≈1-sec contraction but passed rapidly during the ensuing ≈3-sec rest period. However, measurements of average pH by the NMR chemical shift method (19) showed only <0.1 pH unit shift between an uncomfortable exercise level ($PCr/P_i = 0.76$) and a comfortable level ($PCr/P_i = 1.06$). The muscle pH seemed to be a relatively insensitive indicator of muscle energy metabolism in the range $PCr/P_i = 20$–1.0.

Thereafter, the pH change between the low and high work rates of Fig. 4 is <0.1 pH. We conclude that steady-state aerobic metabolism is predominant over the interval of exercise and that momentary glycolytic activity during maximal contractions is not of significance in the following discussion. Thus, mitochondrial control characteristics regulate the muscle energy metabolism over the remarkable range of P_i and *PCr* values shown in Fig. 4.

Linearity of the control characteristics or transfer function between respiration and the ADP and phosphate concentrations has been demonstrated for isolated mitochondria using P_i or ADP concentrations that cause the state 4–3 transition (1, 23). Similarly, the Crabtree phenomenon in ascites tumor cell (24) and changes in muscle contractile activity (2–5) give the mitochondria state 4–3 transition. P_i and ADP control of mitochondrial electron transfer and phosphorylation are linear at or below their respective K_m values of 10^{-3} and 10^{-5} (1, 23) and could give the linear relationship between work rate and P_i/*PCr* as indicated in Fig. 4 providing that one or the other was not far above its K_m. Because the sum of P_i and *PCr* is constant, the P_i concentrations in resting muscle are calculated to be less

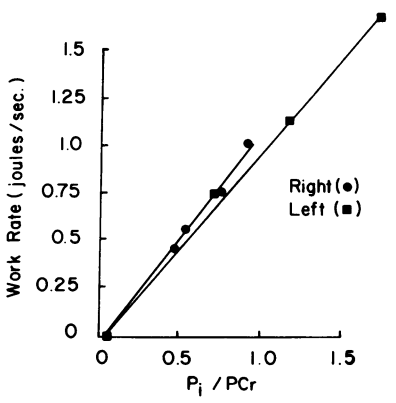

FIG. 4. Work rate vs. P_i/PCr for 6 min steady-state work intervals. Day 1, right arm (●); day 2, left (■) and right (●) arms.

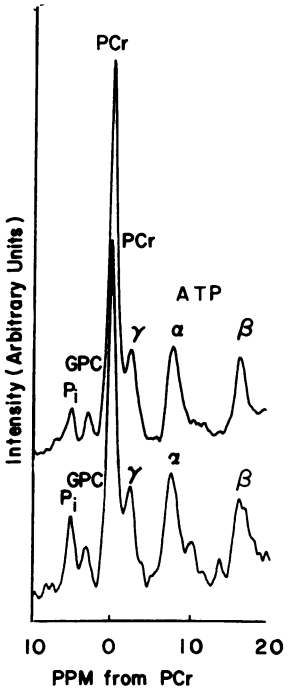

FIG. 5. ^{31}P NMR of resting and mildly exercised leg. Oxford TMR-32/200 without field profiling coils, Department of Surgery, Hospital of the University of Pennsylvania. GPC, Phosphodiester.

than the K_m for P_i based upon the *PCr* concentration as determined by metabolic assay (≥20 mM) (25). Similarly, the ADP concentration at rest is less than K_m for ADP (25).

In the muscle exercise recorded here, the NMR was timed to measure the metabolites just at the end of the contraction—i.e., when PCr/P_i is lowest and the mitochondrial activation is maximal. We can estimate the P_i and ADP levels from the NMR data at this time. P_i levels exceed K_m at $PCr/P_i \approx 10$, or ≈10% of the full range of the abscissa of Fig. 4. Because ADP is released from the breakdown of ATP in amounts equal to P_i, it is apparent that ADP similarly increases above its K_m for the major portion of the graph of Fig. 4. Thus, their values would maximally activate the mitochondria immediately after the contraction. However, these values are restored nearly to their resting state 4 values prior to the next contraction. Consequently, the subject's sensations were clearly periodic, ranging from lactic acidosis during the actual exertion to rest prior to the next contraction. This periodicity is also identified in experimental studies on exercised or intact muscles of animal models in which optical monitoring of mitochondrial response to single twitches or tetanic concentrations clearly shows the corresponding intense response of mitochondrial electron transport activity beginning a few tenths of a second after the stimulus and lasting an appropriate interval for restitution of the ATP level and restoration of the resting state 4 (2–4).

Thus, the time course of the decrease of ADP and P_i will be that of the substrate utilization for a saturated Michaelis–Menton enzyme system (26) whereas the respiratory rate will be maximal (state 3) for an interval required to bring the ADP and P_i down nearly to the state 4 levels. Consequently, the linear response in Fig. 4 is due to the activation of mitochondrial phosphorylative activity for increasing fractions of the interval between contractions. With further work rate increases beyond the range in Fig. 4, continuous state 3 mitochondrial activity throughout the contraction relaxation cycle will occur, linearity between work rate and P_i/PCr will no longer be obtained, and larger pH shifts, lactic acidosis, and discomfort will result. This analysis, therefore, identifies the feasible steady-state work level with the capacity of mitochondrial oxidative phosphorylation to achieve a partial restoration of the *PCr* and P_i levels—i.e., a brief state 4 prior to the next contraction.

Obviously, many other factors must be maintained appropriate to this condition such as the substrate and calcium supplied to the mitochondria and particularly the oxygen supplied by regulation of the microcirculation which proceed hand-in-hand with work rate and mitochondrial respiratory activity (21, 27).

The choice between ADP and P_i as the regulatory elements in metabolic control in the state 4–3 transition has in the past been found to favor ADP (1–5, 23, 24). Because the mitochondria return to state 4 prior to the next contraction, the initial conditions for metabolic regulation do not differ from those previously described for the state 4–3 transition (28).

The response of the muscle to increasing work rate is best considered in terms of the increasing interval of mitochondrial state 3 activity required to pay off the ATP debt in relation to the interval between contractions; as long as the mitochondrial oxidative phosphorylation rate is capable of restoring the state 4 prior to the next contraction, the muscular activity can be continued at a relatively constant work rate output. A limiting case is represented by Fig. 3. Furthermore, the slope and extent of the linear portion of Fig. 4 are pivotal in the identification of normal and pathological functions of the limb. One can evaluate deficits in the function of mitochondrial energy metabolism on the one hand and in the efficiency of myofibrils on the other. A metabolic deficiency would lead to an unusually large change in PCr/P_i response to work (14) and would cause a decrease in the slope of plots similar to those of Fig. 4. Peripheral vascular flow decreases (6) causing diminished oxygen delivery and oxidative phosphorylation or an enzymatic defect in the energy-conserving system would limit the extent of Fig. 4 as would muscular dystrophies (22) affecting the myofibrils and not the metabolic system.

Heterogeneities of muscle fibers (29) and of muscular performance due to dystrophies (22) may not be adequately resolved by the ≈2-cm-diameter sensitive volume which averages normal and diseased tissue performance. In this case, some salient signposts are available—for example, the appearance of the P_i peak in the resting state larger than 1/10th that of the PCr peak would indicate inadequate oxygen delivery to a portion of the tissue under examination. Similarly, any accumulation of sugar phosphate in the resting muscle would signal portions of pericapillary tissue volume to be anoxic with an abrupt transition from anoxia to normoxia as found in studies of heart muscle (5). Deviations from the slope of the normal in diagrams such as that of Fig. 4 may be diagnostic of various genetic and metabolic errors or deficiencies of the bioenergetic system and its associated musculature.

The initial indication of overestimates of intracellular P_i levels of living cells by techniques of analytical biochemistry was based upon studies by Mommaerts and his colleagues (30) who found ≤1 mM P_i by extraction at −45°C and extrapolation to zero time. A further approach involved ATP titration of reversed electron transport in ascites tumor cells where we found an 85-fold discrepancy between the chemical and the noninvasive spectroscopic method which was responsive to the matrix space ADP, P_i, and ATP (10). NMR determinations of the free P_i level in the intact cell (6–10, 13, ‡) identify that which is tumbling sufficiently rapidly to be detected by NMR and, from the mitochondrial standpoint, is P_i that is free to participate in enzyme reactions in the matrix space and not that bound electrostatically or otherwise to other molecules exceeding ≈10 kilodaltons. Many diverse data confirm the low value of P_i in resting tissues of small fish (31) or the muscle of the barnacle where P_i levels are 1/20th or even 1/1000th, respectively, of the phosphogen characteristic of those tissues. Furthermore, our NMR studies of freeze-trapped muscle measured at −10°C to −15°C give values of 13:1 (unpublished data). Further data are provided here for verification of our previous values and extension of them to the human forearm. These values are consistent with others' data (7, 11, 13).

The authors wish to acknowledge research support from National Institutes of Health Grants GM-07612-04, GM-27308, NS-10939, and HL-18708 and from the Johnson Research Foundation, University of Pennsylvania.

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