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^{31}P NMR study of improvement in oxidative phosphorylation by vitamins K_3 and C in a patient with a defect in electron transport at complex III in skeletal muscle

(metabolic diseases/exercise physiology/lactic acidosis/hypoxia/genetic deficiency)

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ABSTRACT **The bioenergetic capacity of skeletal muscle in a 17-year-old patient with a severe defect in complex III of the electron transport chain has been examined by ^{31}P NMR measurements of the molar ratio of phosphocreatine to inorganic phosphate (PCr/P_i). Resting ratios were 1.3–2.5, which can be compared with roughly 8.6 for a young, normal female control at rest. Quantitative evaluation of the activity of oxidative metabolism was afforded by the rate of recovery of PCr/P_i from exercise and was found to be 2.5% of normal. After administration of menadione and ascorbate, we found a 21-fold increase of the recovery rate relative to the pretherapy value, to within 56% of the recovery rate of the young female control. Thus, NMR examinations of skeletal muscle at rest and in recovery from activity document marked improvement to specific drug therapy in the electron transport capabilities and the ATP synthesis rate of a patient with a deficiency in a cytochrome *b*-containing complex III. Improvements in functional ability, although not as dramatic as biochemical changes, are also apparent.**

NMR examination of excised skeletal muscle *in vitro* has enabled us to define limits to the thermodynamically related parameter of the molar ratio of phosphocreatine to inorganic phosphate (PCr/P_i) from its maximum >10 at rest (state 4) to a value of 1 for fully activated (state 3) oxidative metabolism, beyond which the activation of glycolytic energy metabolism causes severe tissue lactic acidosis. Similar observations of the human arm during measured exercise using a Cybex isokinetic ergometer and phosphorus NMR determination of PCr/P_i indicate that the ratio of energy expenditure to work output is approximately constant from the resting (state 4) to the maximally "aerobic" contracting muscle (state 3) (1). Human *in vivo* NMR spectroscopy has offered quantitation of energy performance in naive and trained skeletal muscle of the arm and the leg (2) as well as evidence of deficient oxygen supply to calf muscle in peripheral vascular disease (3, 4). Similar studies have been performed on several inborn errors of metabolism, including McArdle disease (5), phosphofructokinase deficiency (3, 6), and an idiopathic cardiomyopathy (7). In moderate peripheral vascular disease and some metabolic diseases, oxygen delivery and tissue metabolism appear sufficient to allow adequate tissue level of ATP to maintain PCr/P_i > 2 with a correspondingly adequate thermodynamic capability.

To quantitate the limits of PCr/P_i and improve the functional capability of patients with bioenergetic disabilities, we have studied the resting and limited-activity states of a patient with a deficiency of several polypeptides of complex III, including reducible cytochrome *b*, and greatly diminished ubiquinol–cytochrome *c* reductase activity. In attempting to improve the patient's thermodynamic capabilities by increasing the resting levels of PCr/P_i and accelerating recovery times, we have designed an appropriate bypass of the cytochrome *b*-deficient sites by redox mediators, menadione and ascorbate.

Case Report. The patient is a 17-year-old girl with an 8-year history of progressive muscle weakness associated with a ragged-red fiber myopathy and lactic acidosis. A detailed case history is presented elsewhere (8, 9). Blood lactate levels are 3–13 meq/liter and lactate-to-pyruvate molar ratios on two occasions were 26 and 29 (normal < 20). Cerebrospinal fluid lactate, measured once, was 10.1 meq/liter (lactate/pyruvate = 31) when blood lactate was 8.4 meq/liter (lactate/pyruvate = 22). This, together with the presence of substantial amounts of malate and an abnormally high β-hydroxybutyrate-to-acetoacetate ratio in fasting urine suggests an altered redox state with an increased ratio of NADH to NAD in both cytosol and mitochondria (10).

In skeletal muscle mitochondria, succinate–cytochrome *c* reductase was <5% of normal and rotenone-sensitive NADH–cytochrome *c* reductase was undetectable, localizing the defect to the ubiquinol–cytochrome *c* reductase segment (complex III) of the electron transport chain (8, 9). Spectral analysis and antibody-binding experiments revealed a lowered level of reducible cytochrome *b* (16% of normal), approximately normal levels of cytochrome $c + c_1$ and $a + a_3$, and deficiency of at least four additional polypeptides of complex III (8).

Rationale for Drug Therapy. In the usual circumstances, electrons are transferred from succinate or NADH to ubiquinone, then via cytochromes *b*, c_1, *c*, and aa_3 to molecular oxygen. The defect in our patient lies between coenzyme Q and cytochrome *c* (complex III) and includes a deficiency of reducible cytochrome *b* that should render this whole mitochondrial pathway nonfunctional, effectively preventing aerobic metabolism and oxidative phosphorylation. From published *in vitro* data, we felt that we could bypass the deficient complex III by making menadione (vitamin K_3) and ascorbate (vitamin C) available to mitochondria. These vitamins, in their novel capacity as electron transfer mediators, should bypass cytochrome *b* and carry the electrons to cytochrome *c* (Fig. 1), theoretically increasing the ATP production rate to a maximum of 67% of that possible in a normal tissue. The 33% loss exists because one of the three phosphorylation sites is bypassed by these redox agents.

We elected to treat our patient with the maximal dose of

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Abbreviation: PCr, phosphocreatine.

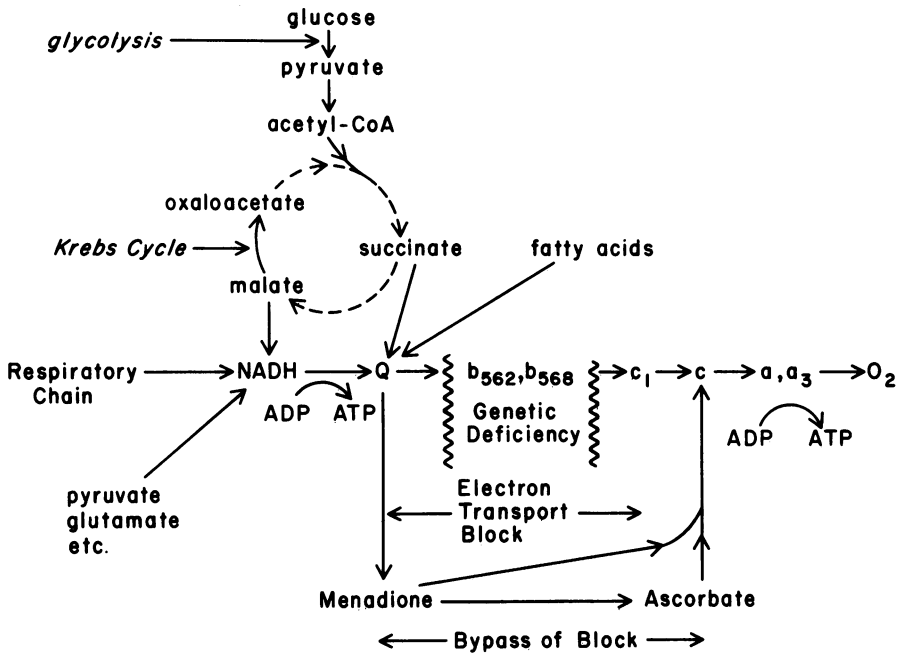

FIG. 1. Diagram of the patient's respiratory chain, indicating deficiency of complex III. The coenzyme Q pool and Q protein are indicated by the symbol Q, and a number of iron–sulfur proteins participating in succinate dehydrogenase and NADH dehydrogenase are omitted. The diagram indicates equilibration of menadione with the Q pool and the possibility of direct interaction of menadione with cytochrome oxidase or through ascorbate as an intermediary. The system would then have two intact phosphorylation sites.

menadione recommended by the manufacturer (10 mg every 6 hr). The dose of ascorbate was 1 g every 6 hr. We also elected to give the first dose of menadione intramuscularly to allow more rapid uptake by body stores. She has been maintained on this regimen since the studies described in this paper.

METHODS

^{31}P NMR Evaluation of Bioenergetic State (*P*Cr/P$_i$). A ^{31}P NMR spectrometer was connected to a 5-cm-diameter single-turn circular surface coil, which was placed inside a 1.9-T magnet with a 27-cm-diameter clear bore and a 27-cm^3 region with a homogeneity better than 0.7 ppm with an arm in place. The coil was attached to a rigid framework that was in turn connected to an isokinetic ergometer (1, 3). The phosphorus spectrum of the forearm flexor muscles was accumulated with 1-min resolution (1) while the subject was at rest, while she was performing isokinetic work at a known rate and force, and during recovery from work. The *P*Cr and P$_i$ concentrations were measured from peak amplitudes with respect to a baseline drawn through the traces from 15 ppm on the left to 25 ppm on the right. The tissue pH was calculated by the chemical shift of P$_i$ with respect to *P*Cr (11).

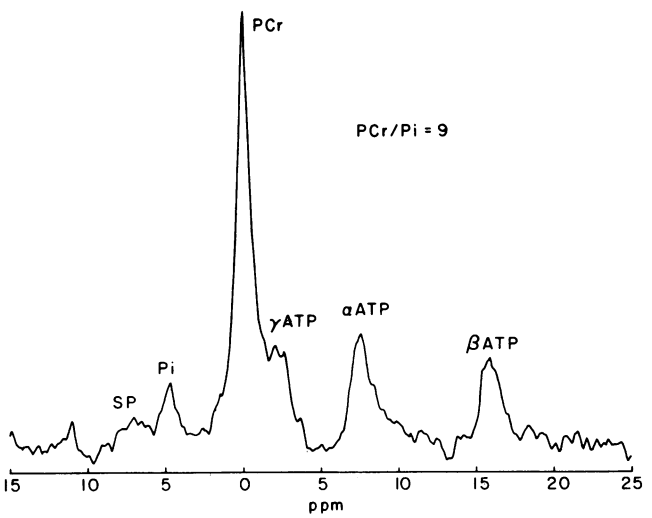

FIG. 2. A phosphorus NMR spectrum of the arm of a normal young female control at rest. *P*Cr/P$_i$ as measured by the peak values is 8.6. A low concentration of glycolytic intermediates (SP) is present.

Exercise Evaluation. We have already established a routine for measuring the efficiency of muscle exercise on a biochemical basis (1, 3) and have employed it to evaluate exercise performance in normal subjects and oxygen delivery to claudicated legs in patients with peripheral vascular disease (figure 2 of ref. 3). The exercise protocol is intended to diminish *P*Cr/P$_i$ to 1 and to measure the rate of recovery to the initial resting state. The ergometer is used to ensure that the total exercise is roughly constant throughout the exercise studies. A 1.5-sec near-maximal isokinetic contraction repeated every 4.5 sec diminished *P*Cr/P$_i$ consistently to 1 within a period of 4 min.

RESULTS

Normal Control. Figs. 2 and 3 demonstrate the NMR spectra and responses of a 20-year-old female with normal mitochondrial metabolism. The resting arm spectrum shows on

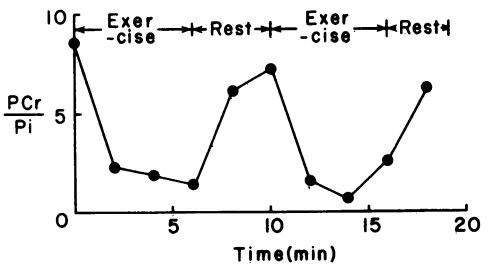

FIG. 3. Isokinetic ergometer exercise of control female, indicating intervals of rest and exercise; a spectrum taken every 2 min. The return from the initial interval of exercise is accomplished in the first 2 min. The second exercise bout reduced the *P*Cr/P$_i$ to much less than 1, and recovery occurred in the second 2-min interval.

the chemical shift or frequency axis the three peaks characteristic of ATP (the α, β, and γ phosphorus atoms), a very large *P*Cr peak, and a small P_i peak with a shoulder partly due to glycolytic intermediates (SP). It has been customary to evaluate the bioenergetic capability of the tissue or organ through the ratio *P*Cr/P_i. The sum of the concentrations of these compounds is constant under normal conditions of exercise, *P*Cr being broken down to P_i in the phosphorylation of ADP produced from muscle contraction. The sum *P*Cr + P_i is nearly constant in our experiments. The resting *P*Cr/P_i of the control was 8.6 (Fig. 2). The chemical shift of P_i with respect to *P*Cr was 5.2 ppm, corresponding to a tissue pH of 7.2. Fig. 3 shows that during exercise in the Cybex ergometer *P*Cr/P_i fell abruptly to approximately 1, a value below which painful lactic acidosis usually soon occurs. The steady state was maintained for 4 min. The chemical shift of P_i to *P*Cr was 4.8, indicating a tissue pH of 6.9. Cessation of exercise caused an abrupt increase of *P*Cr/P_i to 7 within 2 min. The slope is at least 5 units of *P*Cr/P_i per 2 min; i.e., 2.5 units/min. A second exercise interval diminished *P*Cr/P_i to less than 1; the recovery of the exhausted, painful arm was slower and occurred over a 4-min interval. The slope is 1.4 *P*Cr/P_i units per min.

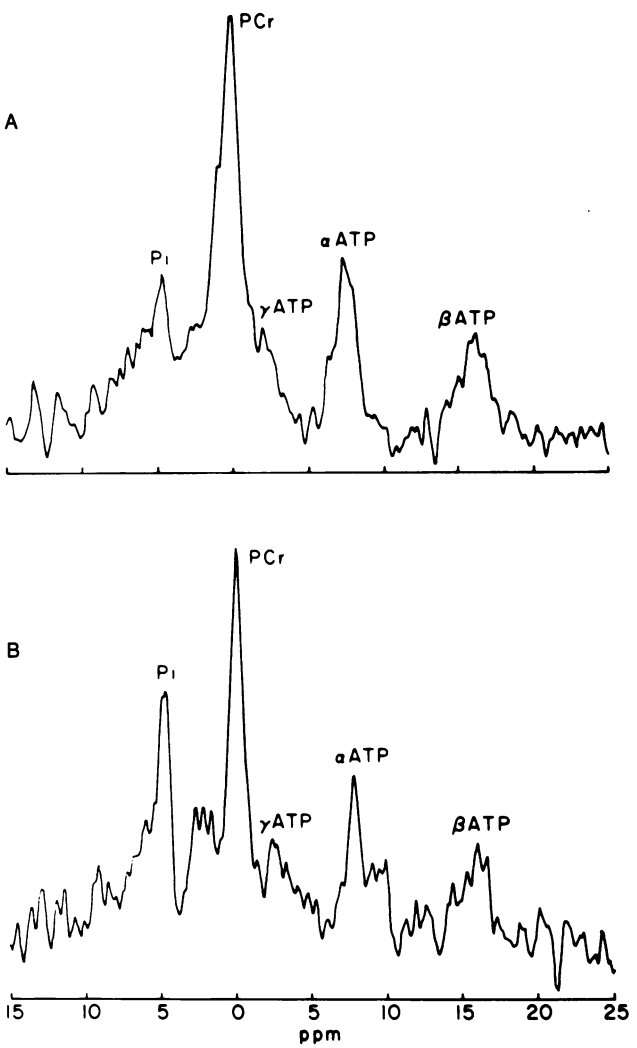

FIG. 4. Two spectra of the patient's arm. (*A*) At resting state prior to any exercise evaluation that day, *P*Cr/P_i is 2.5. The chemical shift of P_i is 4.7 ppm (pH 6.8). (*B*) At the end of the day, after two exercise evaluations and a 2-hr recovery period. This resting scan shows *P*Cr/P_i = 1.3 and the chemical shift of the P_i peak is 4.7 ppm (pH 6.8). The postexercise spectrum (*B*) is characteristically more "noisy" than a rest spectrum (*A*).

Patient's Resting Values. Two ^{31}P NMR spectra are presented. Fig. 4*A*, obtained before any exercise that day, shows a high P_i peak, equal to the ATP peak; *P*Cr/P_i is low (2.7). The chemical shift of P_i with respect to *P*Cr is 4.7 ppm, corresponding to an acidic pH of 6.8. Fig. 4*B* is a resting scan taken 2 hr after completion of two exercise bouts. The P_i peak is twice the ATP peak. *P*Cr/P_i is 1.6. The chemical shift of P_i was again 4.7 ppm (pH 6.8).

Recovery from Activity. Since the patient agreed to exercise to the point of discomfort every day, we were able to measure the *P*Cr/P_i at this point and to use it as a uniform baseline from which recovery of the *P*Cr/P_i could be followed throughout the week.

In the example shown in Fig. 5*A*, the first exercise test of the day gave a resting *P*Cr/P_i value of 1.7, similar to that of Fig. 4*B*. The traces are typical of the patient's ability to exercise to the point at which *P*Cr/P_i fell to 1. Thereafter, the distinctive feature of her recovery is the slow progression of *P*Cr/P_i back to the normal level over 20 min. This slope is 1.0 unit of *P*Cr/P_i per 15 min (0.07 unit/min) as compared with 2.5 units/min in Fig. 3, a decrease of about 40-fold (to 2.5%) with respect to the normal control.

Effect of Ascorbate and Menadione: The Resting State. The effect of the redox therapy upon the resting state of the patient's arm is shown in Fig. 6. Twelve hours after initiation of treatment, *P*Cr/P_i is increased to 4.1 and the chemical shift is changed to 4.9 ppm (pH 7.0, a nearly normal value).

Exercise Evaluation of Redox Therapy: Recovery from Activity. Two exercise trials are presented here (Fig. 5*B*, right arm; Fig. 5*C*, left arm). These show the following characteristics: (*i*) resting *P*Cr/P_i values of 4.0 and 3.6, higher than before treatment; and (*ii*) a fast phase of the recovery kinetics. As in previous exercise, the patient was able to achieve *P*Cr/P_i of 1, under a similar work load. However, in the recovery phase there was a rapid increase of *P*Cr/P_i over 40% of the recovery kinetics for the right arm. The left arm, which prior to redox therapy had a similar slow recovery,

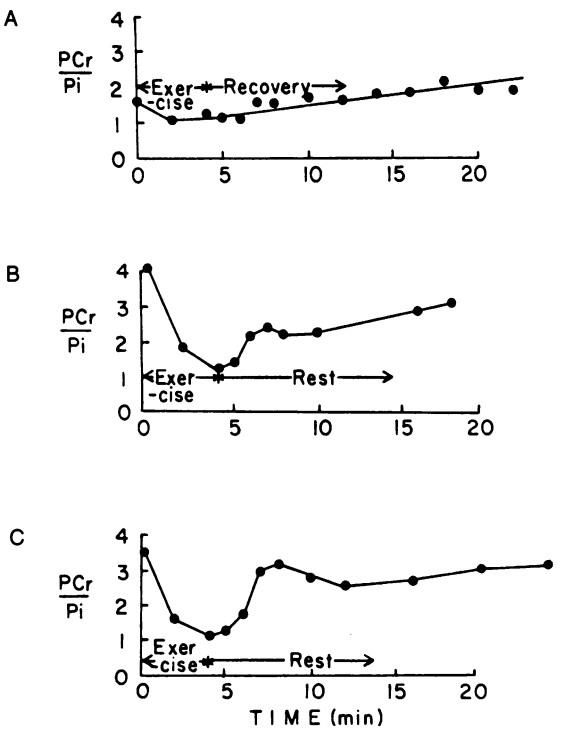

FIG. 5. (*A*) Pretreatment exercise evaluation of the patient, illustrating an initial *P*Cr/P_i value of 1.7 (similar to that of Fig. 4*B*), an exercise level of 1.0, and a recovery extending over 20 min. (*B* and *C*) Exercise evaluation after therapy, showing a higher resting *P*Cr/P_i and faster recovery kinetics. (*B*) Right arm, (*C*) left arm.

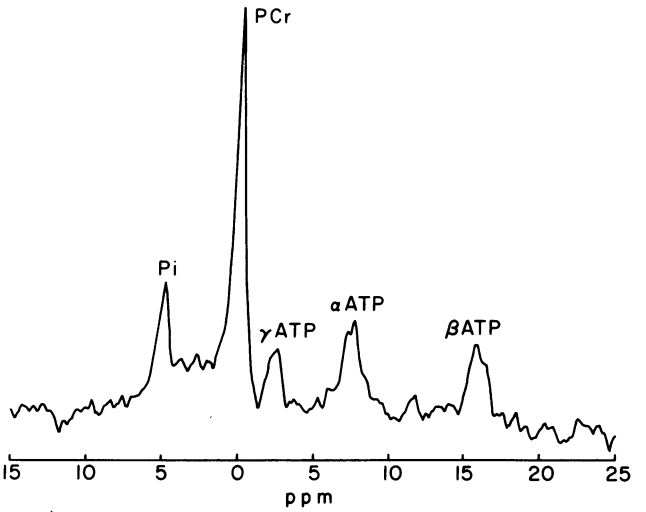

FIG. 6. Resting state of the patient's arm 24 hr after administration of menadione and ascorbate. *P*Cr/P_i value is 3.1 and the chemical shift of P_i is 4.9 ppm (pH 7.0).

exhibited fast recovery over 80% of its extent. In Fig. 5 *B* and *C*, *P*Cr/P_i rises with a maximum slope of 0.5 and 1.4 *P*Cr/P_i units per min, respectively, 8- and 21-fold faster than the pretherapy study of Fig. 5*A*, 21% and 56% of the value for the young female control. The terminal phase of the recovery remains slow.

Subjective Response to Therapy. In the year prior to NMR testing, our patient's tolerance to exercise was severely compromised. She could walk only half a block or climb 5–10 steps without resting, and she frequently used a wheelchair. Exercising on the ergometer was fatiguing and she felt "tired." Within 24 hr of starting redox therapy, there was a marked contrast, the patient claiming to have "more energy." Within 2 days she ceased using her wheelchair, walking several blocks without stopping and no longer complaining of fatigue. Over the next few months, this improvement has been maintained; she can walk two blocks without tiring, can climb 30–40 steps, and has not used her wheelchair at all. However, blood lactate remains elevated (10.2 meq/liter) and the lactate-to-pyruvate ratio of 28 is also still elevated.

DISCUSSION

The severity of the metabolic problem in this patient has previously been documented by studies in isolated muscle mitochondria, which showed succinate–cytochrome *c* reductase activity to be <5% of normal. Other activity measurements, spectral analysis, and antibody-binding studies localized the defect to the ubiquinol–cytochrome *c* reductase portion of the electron transport chain and indicated a deficiency of at least 5 of the 10 polypeptides of complex III, including reducible cytochrome *b*. The predictable consequences of such a defect include (*i*) deficient production of ATP and therefore *P*Cr in muscle, supported by the patient's severe muscle weakness; (*ii*) abnormal redox state with an increased ratio of NADH to NAD in mitochondria and, secondarily, in cytoplasm; this is supported by the increased lactate-to-pyruvate ratio observed in both blood and cerebrospinal fluid and by the pattern of urine organic acids after fasting or medium-chain triglyceride load which showed, in particular, an unusually high ratio of β-hydroxybutyrate to acetoacetate; and (*iii*) chronic lactate/pyruvic acidemia, documented on many occasions in our patient.

^{31}P NMR provides an ideal tool with which to quantitate the bioenergetic capacity of forearm muscle in patients with genetic or acquired muscle disease. It also provides a means to evaluate the efficacy of experimental therapeutic agents aimed at circumventing a block in energy production, such as the one in our patient. The severity of the problem in this patient is indicated by the prolonged recovery time from mild exercise that uniformly diminished *P*Cr/P_i to a limiting level. One of the few adult human models for such an extended recovery time is the extremely ischemic leg of an adult with occlusive peripheral vascular disease. Recovery time in our patient matches that of calf muscle with greater than 90% occlusion of the superficial femoral artery. Furthermore, and most interesting, the metabolic resting level of the patient's skeletal tissue, reflected by *P*Cr/P_i of 1.8, corresponds to the worst case of peripheral vascular disease that we have seen in 150 patients studied.

The possibility of improving oxidative phosphorylation by bypass of the cytochrome-deficient site has *in vitro* precedence. Menadione, with a redox midpotential of −44 mV is readily reduced to K_3H_2 by ascorbate (midpotential around 0 mV) at physiologic pH. The reaction of both ascorbate and menadione with cytochrome *c* occurs readily since cytochrome *c* is over 200 mV more positive than either menadione or ascorbate. The actual reductant of cytochrome *c* in these reactions may well be superoxide anion as generated from the reaction of reduced menadione with molecular oxygen, as suggested by McCord and Fridovich (12). Thus, it is expected that menadione and ascorbate can serve as cytochrome *c* reductants either directly or via O_2^- generation.

Two types of experiments have been done to demonstrate the effectiveness of menadione and menadione plus ascorbate in reactivating antimycin-a-inhibited respiration and increasing the rate of ATP synthesis, a model for the defect of complex III in our patient (see Fig. 1). In the case of NADH oxidation by yeast, mitochondria stimulated by the addition of menadione were only 25% inhibited by antimycin a. Thus, "menadione seems to bridge the portion of the electron transport pathway of yeast mitochondria involving an antimycin a sensitive site" (13). Similar activities of menadione and ascorbate were observed in the reverse electron transfer reaction (14–16). In this case, the ATP-activated reduction of NAD, ascorbate, and menadione was observed to afford 39% of the rate obtainable with succinate as the electron donor. The rate with ascorbate and menadione was 60% inhibited by antimycin a. Thus, menadione and menadione plus ascorbate seem to be able to bypass the antimycin-a-sensitive site in both forward and reversed electron transport. Maximal therapeutic dosages of menadione and ascorbate seem appropriate to such a reactivation *in vivo* and many naphthoquinones and related compounds may also be so (17, 18).

After therapy with menadione and ascorbate the resting *P*Cr/P_i value rose to 4.0, a level that should be sufficiently high to diminish the glycolytic rate and consequent lactic acidosis. The chemical shift increased to 4.9 ppm, indicative of a normalizing intracellular pH at 7.0. This suggests that the increased rate of *P*Cr resynthesis from P_i does indeed reflect a shift from glycolytic activity to increased mitochondrial oxidative phosphorylation. The results of redox therapy are also quantitated in terms of the rate of recovery of *P*Cr/P_i from an exercise intensity that diminished this ratio to 1 or less. The maximal rate of recovery observed in the patient is 56% of the control value, which can be compared to 2.5% of control before therapy. This can be interpreted as a measure of the improvement in the oxidative phosphorylation activity of the patient's arm after redox therapy.

These indications of improved phosphorylation are consistent with improvements in the patient's functional activity, although this is still far from normal and is less than might be expected from the biochemical evaluation of improved performance. However, we have shown that the size of the pool of menadione and ascorbate that is accumulated in the

vicinity of the mitochondria is sufficient to give accelerated recovery for only 40–80% of the energy expenditure occasioned by this muscle exercise protocol. This phenomenon of only partial recovery needs further study. The value of these mediators in the long term may well be less than that in the short 4-min test described here. Obviously further studies are required to evaluate factors affecting the degree of normal function over long time intervals.

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