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# $^{31}$P NMR studies of control of mitochondrial function in phosphofructokinase-deficient human skeletal muscle

(metabolic control/exercising human skeletal tissues/ADP control)

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**ABSTRACT** **Metabolic control of mitochondrial respiratory activity by $P_i$ and ADP has been evaluated by $^{31}$P NMR measurements of the levels of $P_i$ in normal exercising human skeletal tissues in the resting–active–resting transition and, in this contribution, in the phosphofructokinase (PFK)-deficient leg. The latter studies show near constancy of $P_i$ in the recovery from maximal exercise of the leg, with large changes of sugar phosphate (*SP*) complementary to the changes of phosphocreatine (*PCr*). The PFK deficiency permits observation of *PCr* resynthesis in postexercise recovery under conditions of nearly constant $P_i$ and ATP—a phenomenon not evident in normal exercising muscle. The constancy of free $P_i$ is inconsistent with its role in control of mitochondrial activity, leaving ADP as a key metabolic control element. These results help clarify previous controversies on the nature of control of metabolic activity of mitochondria and extend the idea of ADP control of mitochondrial metabolic states *in vivo* and, in addition, provide an appropriate exercise protocol for the evaluation of a genetic deficiency affecting mitochondrial metabolism.**

The control of cell respiration in response to the needs of energy metabolism is one of the most obvious and strongest responses of body biochemistry to energy-requiring function. Very large changes of respiration rate (>20-fold) are observed in the transition from the resting state (state 4) to the active state (state 3) and their impacts upon the redox state of electron transfer components have been observed in isolated mitochondria (1), in cells (2), and in tissues (3).

The nature of metabolic control in skeletal tissues has been a topic of great interest and, indeed, controversy since the early 1950s, when Lynen (4) published his landmark paper on phosphate control of metabolism in yeast cells and Racker (5) followed with similar ideas on tumor cells (see also ref. 6). The role of ADP was emphasized by Chance and Williams (7) and by Lardy and Wellman (8), who found that both ADP and $P_i$ were pivotal in the control of oxidative metabolism of isolated mitochondria and in ascites tumor cells (9). Their views were extended to include the phosphate potential (10–12) and "energy charge" (13) also has been proposed.

One problem of identifying the roles of ADP and $P_i$ *in vivo* has been the difficulty in analysis for $P_i$, which was underlined by the paper of Mommaerts and co-workers (14). They showed that errors are present in the extraction of phosphate compounds from muscle and that serious overestimates of the tissue $P_i$ can easily occur. For instance, Chance and Maitra (15) concluded that analytical chemistry gives a level of $P_i$ that exceeds by a factor of 80 that which is determined spectroscopically from their measurement of the phosphate potential of ascites tumor cells. Determination of cytosolic phosphate in tissues by phosphorous NMR (16, 17) supported the previous findings (14, 15) and showed that the phosphate levels indeed were incompatible with those usually accepted for $P_i$ determinations in skeletal tissues (14). Monitoring $P_i$ levels quasi-continuously by $^{31}$P NMR in the resting state 4 to the active state 3 transition has shown $P_i$ concentrations that are consistent with its role as an activity-controlling metabolite (17). Furthermore, special attention has been called to the very low phosphate levels and high *PCr*/$P_i$ values observed in hyperemia following transition from activity state 3 to resting state 4 (17) after exercise of muscle. Under these conditions, the phosphate levels were less than the $K_m$ for respiratory activation, as observed *in vitro*, and would be regulatory in state 4, seemingly consistent with the proposals of Lynen and Racker for phosphate control (4–6). Nevertheless, further evidence seems necessary, particularly on phosphate levels in the active state 3.

There is the possibility that the trapping of phosphate as sugar phosphate (*SP*) in glycolysis would afford an intracellular phosphate deficiency and limit state 3 respiratory activity, as indeed has been pointed to by Uyeda and Racker (18). One example is provided by the widespread studies of the accumulation of deoxyglucose 6-phosphate, particularly in the brain (19). The possibility that glycogenolysis and glucose phosphorylation also would provide a similar phosphate trap *in vivo* in human tissues is examined here by observing a human who is deficient in muscle phosphofructokinase (PFK). This demonstration stands in contrast to the case of McArdle disease that was examined recently by $^{31}$P NMR (20), in which glycogen utilization was not possible and the flow of substrate into the glycolytic system was inhibited and no trapping of phosphate occurred. In this case of PFK deficiency, glycogenolysis is fully functional and *SP* compounds are accumulated up to the PFK step. Thus, we have the opportunity of observing in a human subject the effects of a physiological phosphate trap caused by fully activated glycogenolysis and to obtain therefrom a more precise identification of metabolic control components.

The interplay between mitochondrial use of $P_i$ and phosphorylation of sugars by the glycolytic pathway can be evaluated. The competition allows the phosphorylation of sugars to the extent that the $P_i$ pool is maintained at a relatively constant level throughout metabolic transitions, presumably rest (state 4) to active (state 3) and a return to the resting state 4. In the PFK-deficient leg, substrate deficiency (state 2) may occur, prolonging the recovery. This constancy of $P_i$ sheds light not only on the *in vivo* reaction rates and equilibria but also on the role of phosphate controlling the mitochondrial function in living tissues (21, 22). Furthermore, simultaneous NMR determinations of ATP levels confirm that ATP is not an important

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Abbreviations: *PCr*, phosphocreatine; PFK, phosphofructokinase; *SP*, sugar phosphate.

variable in the active state 3–resting state 4 transitions evoked by skeletal muscle exercise in the arm and leg and calls especial attention to ADP control (1, 23, ¶).

## EXPERIMENTAL METHODS

The determination of *SP*, ATP, *PCr*, and $P_i$ by $^{31}P$ NMR has been demonstrated for excised amphibian muscle (24), for hearts (25), for living mouse brain (26), and for human skeletal tissues (16, 17). Because 3 sec intervened between NMR interrogations, the ratios of metabolites are approximately correct. The quantitative values of the parameters in the recovery intervals of the PFK-deficient arm and leg are based upon the analytical value for the ATP content of tissue, 5 mM/kg of wet weight, and are assumed to apply to normal and PFK-deficient tissues.‖ Integration of areas and peak heights gives similar results (16, 17). On this basis, the ordinates of Fig. 3 *A* and *B* are scaled to give mM/kg of fresh weight.

Two NMR spectrometers have been used in these tests: one developed at The Johnson Foundation, which uses a 7-inch (17.8 cm) clear bore system (16) for arm exercise protocols and small animal brains, and an instrument built by Oxford with an 8-inch (20.3 cm) clear bore system appropriate to human arms with their focus coil and, without the focus coil, a 10-inch (25.4 cm) clear bore is obtained suitable for human legs. The calculation of the results has been described (17).

An exercise protocol, developed specifically for arm exercise (17), employs direct coupling to a quantitative exercise modality such as the Cybex ergometer and is used for normal subjects, whereas a hand clenching routine is used for patients who tire easily. The hand clenching exercise of the flexor muscle group occurs at a rate of one per second and allows a suitable steady-state level of activity to be established. The total averaging time is usually 36 sec (12 scans, 3 sec per scan) (Fig. 1), and data are collected just prior to contraction.

Because "in magnet" leg exercise of others than those having relatively small feet (male shoe size, <8; female shoe size, <9) is somewhat uncomfortable within a 10-inch bore magnet, an "outside magnet" exercise routine of plantar flexions (on toes, 3 sec; rest, 2 sec) is employed and the exercised leg then is rapidly inserted (10–20 sec) in the magnet. Recovery from the active metabolizing state 3 to the resting state 4 is followed continuously by NMR. This is the leg exercise routine developed by us for the study of peripheral vascular disease (H. Berkowitz, personal communication). Both arm and leg protocols were used here.

## SUBJECT HISTORY

The subject is a 29-year-old man who has been diagnosed as lacking muscle PFK activity by quantitative analysis of leg muscle biopsy (unpublished data). The subjective signs of the disease are muscle discomfort whenever certain limits of exercise are exceeded. The involved muscles are firm and painful for hours after exercise and may occasionally be damaged, resulting in myoglobinuria. The exercise regimens employed here were designed to avoid such effects. A similar case has been examined by NMR by Wilkie, who indicated a simple diagnosis by $^{31}P$ NMR.**

For comparison, slow recovery kinetics of a PFK-sufficient subject similar to those of the PFK-deficient subject are presented in Fig. 3*B*, which shows data of a 73-year-old male patient (H. Berkowitz, personal communication) whose left calf pressure index is consistent with superficial and infrapopliteal occlusive disease. The plantar flexion exercise of 15 times over an interval of 75 sec gave changes of *PCr* and recovery times similar to those of the PFK-deficient subject. Normal subjects are unsuitable for this comparison; the plantar flexion exercise does not decrease *PCr* to the levels observed here and the recovery time is within the time required for leg insertion into the magnet. Appropriate informed consent was obtained in all cases.

## EXPERIMENTAL RESULTS

A kinetic analysis of changes in the phosphate compounds in the arm muscle (flexor) of the PFK-deficient subject is illustrated in Fig. 1. Starting from rest (Fig. 1*A*), it is seen that a normal $PCr/P_i$ ratio was observed, 10:1 (24 scans, 3 sec per scan). In spectra of Fig. 1 *B–E*, the interval of observation was decreased to 36 sec, sufficiently short to give a kinetic picture of the metabolic changes and long enough to record the *PCr* peak to an accuracy of 10%. Hand clasping for an initial interval of 36 sec (12 scans, 3 sec per scan) decreased the $PCr/P_i$ value to 2.5 (Fig. 1*B*). Continued activity for another 36 sec (Fig. 1*C*) caused a further increase of the $P_i$ peak and an unprecedented rise in the level of *SP*. This corresponds to a drop of the $PCr/(P_i + SP)$ ratio to 0.7, a relatively low value. Because a contracture was subjectively imminent in the arm, the exercise was terminated and two 36-sec scans (Fig. 1 *D* and *E*) and three 72-sec scans (Fig. 1 *F–H*) represent the recovery interval. In the spectrum of Fig. 1*D*, the ratio of $PCr/(SP + P_i)$ was still below 1; yet, the distribution of the $SP/P_i$ had drastically altered from 1:1 in the spectrum of Fig. 1*C* to 2:1 in the spectrum of Fig. 1*D*. In the spectrum of Fig. 1*E*, the $P_i$ had dropped to insignificance, as is characteristic of a hyperemic interval (17) and yet, the value of $PCr/(SP + P_i)$ was still low (1.3). In the spectrum of Fig. 1*F*, recovery had proceeded and the $PCr/(P_i + SP)$ had risen to 2. In spectra of Fig. 1 *G* and *H*, *SP* decreased further and the $P_i$ peak reappeared from the noise level. The metabolic response to activity consisted initially of a rise in $P_i$, a redistribution of $P_i$ into the *SP* pool and, on recovery, a depletion of cytosolic $P_i$, a rise of *SP* to a maximum, and a final recovery of the $P_i$ level to the resting level as the *SP* disappeared.

The kinetic plot of these data in Fig. 2 confirms and extends what can be seen from Fig. 1, particularly because better time continuity is achieved by interpolation between the data points and the times at which exercise was activated and stopped. The *PCr* fall was precipitous and was reflected in the immediate increase of $P_i$. A further fall of *PCr* was reflected in this case by increases of both *SP* and $P_i$, with $P_i$ increasing more rapidly in the initial phase than *SP*. At the cessation of exercise, the rise of *PCr* and the fall of $P_i$ were rapid due to the utilization of $P_i$ in oxidative phosphorylation and the formation of ATP, which, in turn, increased the *PCr* level. $P_i$ decreased and *SP* continued to increase; $P_i$ fell below the initial resting level in a characteristic hyperemic recovery interval. *SP* then fell to a plateau and the $P_i$ rose approximately to its initial or resting level prior to stimulation. The ATP concentration remained at the resting level to within the experimental error.

## LEG METABOLISM FOLLOWING EXERCISE

To ensure that the deficiency also could be observed in the leg skeletal tissues and to follow the balance of *PCr* and $(P_i + SP)$, a series of studies was made, employing the plantar flexion (tip-

¶ Chance, B. (1955) Proceedings of the Third International Biochemistry Congress, 1955, Brussels, Belgium, pp. 301–304 (abstr.).

‖ Dawson, M. J. & Wilkie, D. R. (1981) Seventh International Biophysics Congress and Third Pan-Am Biochemistry Congress, Aug. 23–28, 1981, Mexico City, p. 260 (abstr. TH-P-2).

** Wilkie, D. R. (1982) Tenth International Conference on Magnetic Resonance in Biological Systems, Aug. 29–Sept. 3, 1982, Stanford, CA, p. S3 (abstr.).

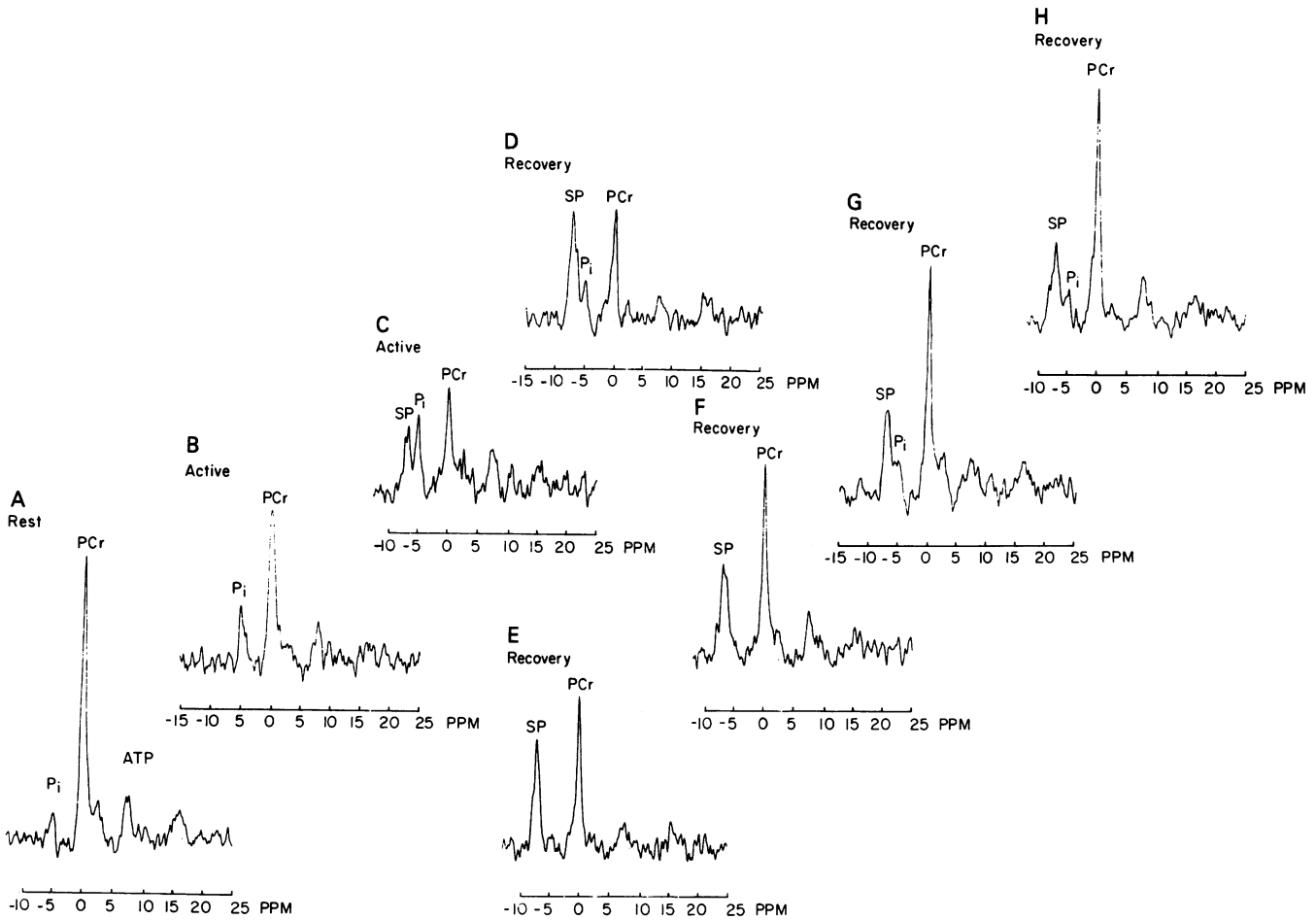


FIG. 1. Sequence of $^{31}P$ NMR spectra from a PFK-deficient individual's arm from rest (state 4) to activity (state 3) and during recovery (state 3 to state 4). Spectra: (*A*) 72 sec; (*B–E*) 36 sec; (*F–H*) 72 sec. Zero ppm was set at the *P*Cr peak; one scan per 3.0 sec.

toe exercise) protocol outside the magnet. Thus, the data are interrupted during the 125 sec of exercise and the 10-sec delay due to insertion of the leg into the magnet.

The time course of phosphorous metabolites in recovery from leg exercise shows (Fig. 3*A*) distinctive features that are worthy of comment. ATP and $P_i$ were nearly constant in the recovery interval. The variable features of the recovery metabolism were the increase of the *P*Cr towards the resting level and a decrease of *SP*.

pH changes seemed to be very small in the PFK-deficient tissues; no time-dependent chemical shift correlated with exercise or recovery either of $P_i$ or *SP* was observed either in the arm or leg, consistent with the patient's inability to produce lactic acid. The uptake of $H^+$ in *P*Cr utilization appeared to be balanced by the release of $H^+$ in glycogen phosphorylation. The lack of a pH shift made it difficult to identify the components of *SP* by their characteristic pH-dependent chemical shift.

By way of contrast, we indicate in Fig. 3*B* the time course of energy-related metabolites in a peripheral vascular-diseased leg having a recovery time roughly equal to that of the PFK-deficient leg. In Fig. 3*B*, beginning on the left are the preexercise levels, intervals of exercise and insertion of the leg into the NMR, and the kinetics of the phosphate compounds during the rest and recovery intervals. In Fig. 3*B*, the recovery of *P*Cr roughly paralleled the kinetics of Fig. 3*A*. $P_i$ had complementary kinetics to *P*Cr, and the sum of the two (*P*Cr + $P_i$) was nearly constant, phosphate being conserved in the *P*Cr (Fig. 3*B*). *SP* rose slightly during exercise but was maintained at a constant low level in the recovery interval, as followed out to 10 min after exercise. The ATP level was maintained constant at the preexercise level during the recovery interval.

A comparison of Fig. 3 *A* and *B* clearly shows very similar responses to *P*Cr and ATP and opposite responses of *SP* and $P_i$. In the peripheral vascular-diseased leg and normal leg (17), the changes of the *SP* pool during recovery were not detectable (Fig. 3*B*), and in the PFK-deficient leg (Fig. 3*A*), the changes in $P_i$ were not detectable.

The metabolic state of the leg muscle during the final stages of recovery from exercise is shown in Fig. 4. The spectrum represents the average of 4.5 min of scans taken between 7 and 11.5 min after the start of the recovery interval and the signal-to-noise ratio was sufficient to delineate clearly the phosphorous metabolites of the leg. *SP* and $P_i$ were approximately equal at this point in the recovery while *P*Cr had recovered towards a value of 8:1 with respect to $P_i$. The scan also shows a phosphodiester peak at 1/2 the ATP level, in agreement with our previous finding of phosphodiester in the human gastrocnemius muscle, presumably glycerophosphorylcholine (27).

## DISCUSSION

In a previous paper (15), a constancy of ATP concentration was observed in a human arm exercise regime from rest to the maximal load that could be maintained in a steady state without undue discomfort (*P*Cr/$P_i$ varied from >10 at rest in state 4 and decreased very nearly to 1:1 in state 3 under exercise protocol). The data showed an increase of $P_i$ commensurate with the changes of *P*Cr so that the sum of the two was nearly constant;

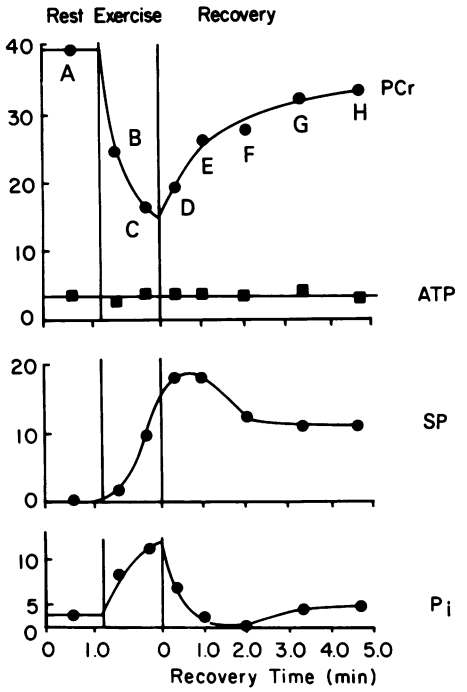


FIG. 2. Kinetics of *PCr*, ATP, *SP*, and $P_i$ in exercised PFK-deficient human arm. Data are from the experiment shown in Fig. 1. Concentrations of metabolites are expressed in mmol/kg of wet weight.

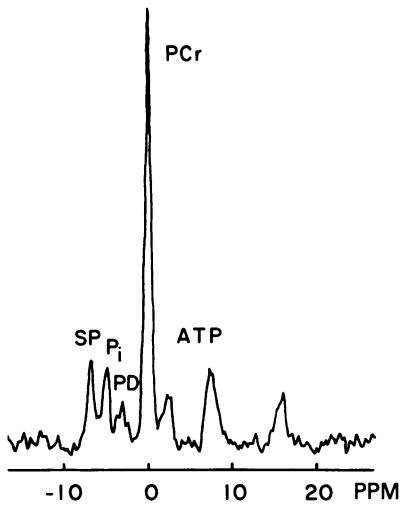


FIG. 4. Intermediates of phosphorous metabolism in leg muscle of PFK-deficient human averaged over 7-11.5 min after the termination of exercise. PD, phosphodiesters (17). Zero ppm was set at the *PCr* peak.

$P_i$ obtained from *PCr* appeared as $P_i$. This paper contributes further to the study of metabolic control by $P_i$ by taking advantage of a genetic deficiency in human skeletal muscle in which the partition of phosphate between the glycolytic system and the mitochondrial system is altered.

The arm and leg exercise data on the PFK-deficient subject illustrate different and highly significant aspects of metabolic regulation, particularly when contrasted with data on the subject with the peripheral vascular-diseased leg. In the human leg, the recovery kinetics show striking features of the PFK-deficient metabolism in which both ATP and $P_i$ are maintained constant and the interplay of phosphates is between *PCr* and *SP*. This may be contrasted to the peripheral vascular-diseased leg shown in Fig. 3*B*, in which case, ATP and *SP* are maintained constant and the interplay of phosphates is between *PCr* and $P_i$.

The phosphate level during the recovery interval is approximately equal to ATP and is high enough to fully activate mitochondrial metabolism because the $K_m$ for phosphate is 1/5th as much [1 mM (28)]. In fact, the phosphate level is maintained higher than the $K_m$ and is unaltered during the entire course of metabolic recovery, which corresponds to an active state 3 (or state 2; see below) to a resting state 4 transition (1). For this reason, the metabolic control in the recovery interval appears to follow the model of ADP control identified in isolated mitochondria and tumor cells (7, 15) and illustrated by the Crabtree reaction (29, 30) in ascites tumor cells and by the transient stimulation of respiration in yeast cells following deoxyglucose addition, causing a rise of ADP and a precipitous fall of $P_i$ (31).

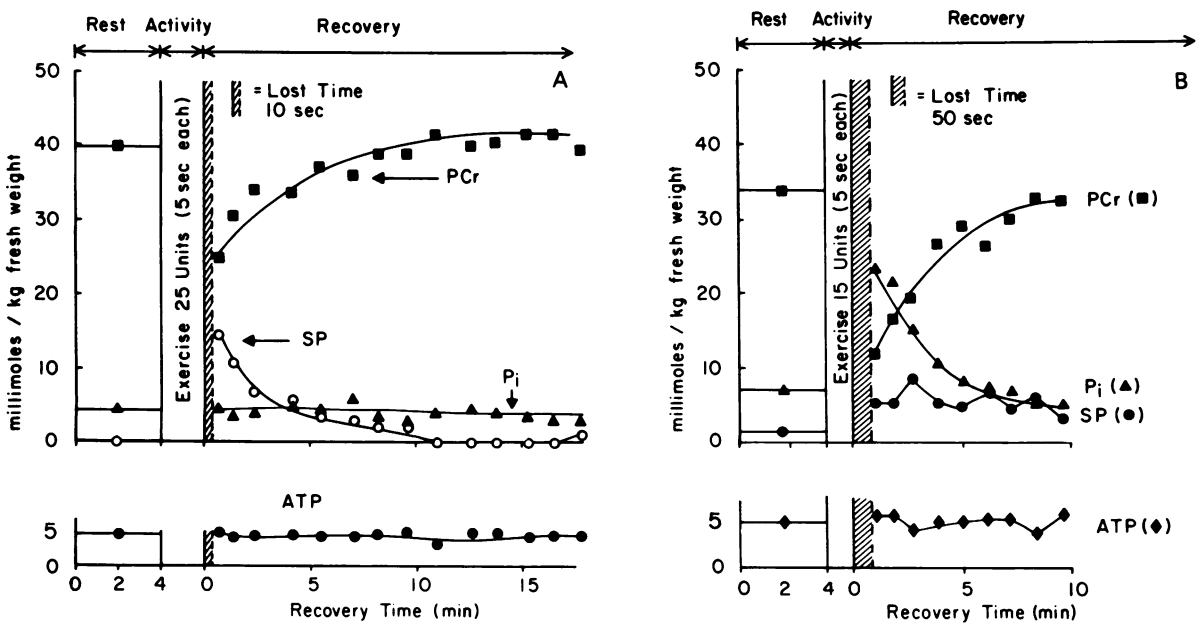


FIG. 3. Comparison of the kinetics of recovery from plantar flexion exercise in PFK-deficient human leg (*A*) and in type II peripheral vascular-diseased leg (*B*) (H. Berkowitz, personal communication). The rest values, together with the interval of "out of magnet" exercise, are indicated.

The latter portion of the recovery interval for the arm ($t_{1/2}$ = 1.4 min) (Fig. 1 *E–H*) is similar to that of the leg ($t_{1/2}$ = 3.7 min) with respect to their showing the resynthesis of *P*Cr. However, the ability to record continuously during the exercise interval affords an insight into the kinetics of the preceding metabolic events in the arm. In the initial phase of the exercise interval, the PFK-deficient arm behaves similarly to that of the normal metabolic control (17) and to the peripheral vascular-diseased leg—i.e., phosphate rises with no perceptible delay on initiation of exercise and adequate substrate; presumably fatty acids are present (32).

However, the abnormally rapid rise of *SP* signals glycogen phosphorylation and has a $t_{1/2}$ of 1 min and reaches a peak in 2 min. The rise of *SP* is accompanied by a fall of $P_i$ to an undetectable level. Thereafter *SP* remains constant and *P*Cr recovers to the resting level. Thus, we see in the arm and leg exercise diagrams, different types of the metabolic regulation. Both show low $P_i$ and high *SP* in the recovery interval while *P*Cr is rapidly increasing. However, the leg shows a monotonic decrease of *SP* consistent with the rise of *P*Cr while the *SP* is relatively constant in the arm. These differences in the two studies are attributed, in part at least, to the vast differences in the total substrate requirement for arm exercise and leg exercise—the arm exercise, which indeed lowers *P*Cr to be less than the sum of *P*Cr + $P_i$, has a rapid recovery time (1.4 min). In the leg exercise, *P*Cr remains greater than *SP* + $P_i$ in the exercise interval but recovers somewhat more slowly ($t_{1/2}$ = 3.7 min). We attribute this to the relatively greater expenditure of substrate in the leg exercise (presumably fatty acid) due to the larger requirement of whole leg exercise (plantar flexor extensions) as compared with the exercise of flexor muscle of the hand. Thus, the NMR of leg exercise is more appropriate for the diagnosis of the PFK deficiency. Indeed, any genetic deficiency affecting metabolic pathways in the body is best evaluated by an exercise level that taxes the whole body metabolism.

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