observed by the senior author in Slippery Rock Creek in western Pennsylvania and in the headwaters of the Schuylkill River in eastern Pennsylvania where acid mine wastes enter circumneutral waters which are apparently very low in humates.

Several human activities produce SO_x and NO_x. The question is, which combination of these activities produces the most serious effects? From the data analyzed it appears that it is not the burning of coal per se that is producing acid precipitation, but rather a combination of many activities, including the treatment of stack effluents, the increased use of petroleum, and methods of combustion of fossil fuels.

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Transport of Energy in Muscle: The Phosphorylcreatine Shuttle

Samuel P. Bessman and Paul J. Geiger

The performance of muscle work was early recognized to be dependent on energy derived from chemical reactions within the tissue. In 1907, Fletcher and Hopkins (*1*) measured increased lactic acid in muscles fatigued to the point of rigor. Parnas and Wagner (*2*) showed that lactic acid was derived from muscle glycogen. Meyerhof (*3, 4*) and his coworkers related lactic acid production to work done by the muscle.

Experiments on heat production, largely done by A. V. Hill (*5*) and his colleagues, revealed that the chemistry of the contraction itself was nonoxidative. Much later, in 1940, D. K. Hill (*6*) showed that oxygen was used only when contraction and relaxation were over. This "aerobic recovery heat" was virtually equal to the anaerobic work of contraction and relaxation. The initial heat of contraction was, however, much greater than could be accounted for by lactic acid formation.

The discovery of "phosphagen," that is, phosphorylcreatine, by Eggleton and Eggleton (*7, 8*) and Fiske and Subbarow (*9*) supplied the answer to this question. Phosphorylcreatine was the first compound found to carry the "high energy" phosphate bond (*10, 11*).

Meyerhof and Lohmann (*12, 13*) quantified the energetics of phosphorylcreatine hydrolysis, and Lipmann and Meyerhof (*14*) showed that phosphorylcreatine breakdown during muscle activity was a reality, and not an artifact, by correlating contraction with the liberation of free creatine. Lundsgaard (*15*) observed phosphorylcreatine breakdown in contracting muscle poisoned with iodoacetate, which prevents lactic acid formation, and suggested that phosphorylcreatine was the energy donor for contraction, the role of glycolysis being to supply energy for resynthesis of the phosphorylcreatine (*16, 17*). It was agreed that lactic acid formation was only indirectly related to muscle contraction. Phosphorylcreatine was thus established as the direct energy donor.

At this time, the discovery of adenosine triphosphate (ATP) complicated the picture (*18, 19*). Meyerhof and Lohmann (*20*) observed the liberation of about 12 kilocalories per mole for each of the two terminal phosphates released from ATP by hydrolysis. Work in Englehardt's laboratory (*21*), amplified by Banga and Szent-Györgyi (*22*) and Straub (*23*), demonstrated that actomyosin contracts only upon addition of ATP. Lohmann

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Summary. In order to explain the insulin-like effect of exercise, it was proposed in 1951 that contracting muscle fibers liberate creatine, which acts to produce an acceptor effect—later called respiratory control—on the muscle mitochondria. The development of this notion paralleled the controversy between biochemists and physiologists over the delivery of energy for muscle contraction. With the demonstration of functional compartmentation of creatine kinase on the mitochondrion, it became clear that the actual form of energy transport in the muscle fiber is phosphorylcreatine. The finding of an isoenzyme of creatine phosphokinase attached to the M-line region of the myofibril revealed the peripheral receptor for the mitochondrially generated phosphorylcreatine. This established a molecular basis for a phosphorylcreatine-creatine shuttle for energy transport in heart and skeletal muscle and provided an explanation for the inability to demonstrate experimentally a direct relation between muscle activity and the concentrations of adenosine triphosphate and adenosine diphosphate.

(*24*) proved that phosphorylcreatine splitting in muscle cannot take place without the presence of adenylate compounds, and Parnas *et al.* (*25*) showed the reversibility of the Lohmann reaction (phosphorylcreatine + ADP ⇄ ATP + creatine) (ADP, adenosine diphosphate). The picture now seemed clear enough. Before phosphorylcreatine can yield energy for muscle contraction, ATP breakdown must occur. The role of phosphorylcreatine seemed to be that of a buffer for the resynthesis of ATP in muscle metabolism.

This explanation, satisfying as it might have been biochemically, never really fit the physiological facts. In 1950, A. V. Hill challenged biochemists to prove that ATP was the energy donor in the contraction of living muscle (*26*), since there was no experimental evidence that ATP broke down during contraction. With either single twitches or tetany, the only changes in high energy compounds seemed to be the breakdown of phosphorylcreatine. This question seemed to be answered when Davies (*27*) and his coworkers observed some twitches in muscle poisoned with fluorodinitrobenzene, a specific inhibitor of creatine phosphokinase, and showed that even though ATP broke down to ADP, phosphorylcreatine remained unchanged (*27*). A direct transfer of energy from phosphorylcreatine to the contractile system was thus ruled out.

The demonstrable fact that muscle twitches and tetany are not accompanied by measurable changes in ATP but are accompanied by hydrolysis of phosphorylcreatine should have stimulated more thought on the role of this compound. Even if one accepted phosphorylcreatine as a buffer for rephosphorylation of ATP, there was still no clear view of the way that the creatine liberated by this rephosphorylation could again be converted to phosphorylcreatine.

A recent review (*28*) on energy metabolism in the heart states that the primary function of heart mitochondria is the production of ATP for contraction and ion pumping in the myocardium. Also, "Several enzymes of importance in energy metabolism, notably adenylate kinase, are associated with the outer mitochondrial membrane or the intermembrane space, but these, like the mitochondrial creatine phosphokinase, react functionally with substrates in the extramitochondrial space." Thus, the review repeats the conventional view of the creatine adenylate system as an energy buffer. Another recent essay (*29*) also concluded that phosphorylcreatine acts as a store of high energy phosphorus to be delivered to ADP "for use *in an emergency*" (our italics). Figure 1 illustrates the conventional view of the energy transfers leading to muscle contraction.

Woledge (*30*) and Curtin and Woledge (*31*) presented another view, saying, "It will now be apparent to the reader that the last few years have seen the reopening of several questions that previously seemed settled. The largest of these is the nature of the primary reaction which drives contraction."

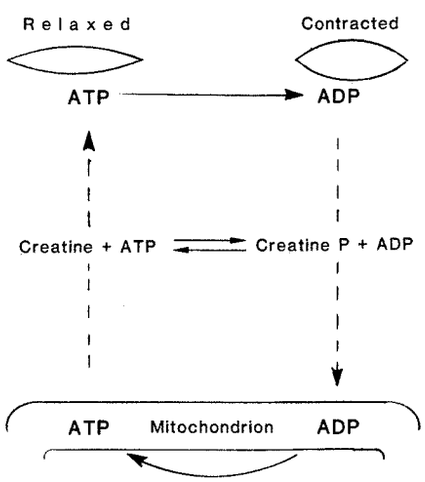

Fig. 1. The energy transport-respiratory control system of muscle based on an ATP-ADP shuttle and a buffer system of creatine and phosphorylcreatine (*Creatine P*).

Evidence for a Central Role of Phosphorylcreatine

The first evidence for a central role of creatine in muscle metabolism was the demonstration by Thunberg (*32*) in 1911 of a significant increase in respiration of minced muscle after the addition of creatine. Eggleton and Eggleton (*7*, *8*) observed that depleted frog muscle regained phosphagen only aerobically.

Katz (*33*) found that exogenous creatine increased the resting respiration rate of frog muscle. Belitzer and Tsybakova (*34*) reported that creatine stimulated oxidation of substrate in muscle, with the simultaneous formation of phosphorylcreatine from inorganic phosphate. This was probably the first evidence of what is now called the "acceptor effect": creatine phosphokinase removes ATP (reverse Lohmann reaction) and provides ADP to stimulate aerobic phosphorylation by mitochondria, the end product being phosphorylcreatine.

Lack of recognition of the key role of phosphorylcreatine in energy metabolism of muscle was probably reinforced by the fact that the "phosphagen era" of the late 1920's and early 1930's had come and gone with the discovery of ATP and the working out of the reversible Lohmann reaction. To quote A. V. Hill (*26*) recalling earlier comments (*35*), ". . . after phosphagen had deposed lactic acid from pride of place as the chief chemical agent in contraction . . . , one could write: 'on stimulation, phosphagen breaks down. . . . This is the primary change by which energy is set free,' " but, "now, in the adenosine triphosphate era, lactic acid and phosphagen have been relegated to recovery and ATP takes their place."

At about the same time that Hill delivered his "challenge" it was necessary, from a completely different theoretical standpoint, to postulate a creatine-phosphorylcreatine shuttle. Bessman (*36*, *37*) suggested (in the original statement of the hexokinase-acceptor theory of the mechanism of insulin action in 1952) that, during exercise, muscle contraction provides mitochondria with creatine, which they do not have at rest. "The powerful acceptor system provided by the creatine-ATP reaction in muscle makes muscle independent of insulin for all reactions except glycolysis and can explain the well-known observation that the oxidation of ketone acids by

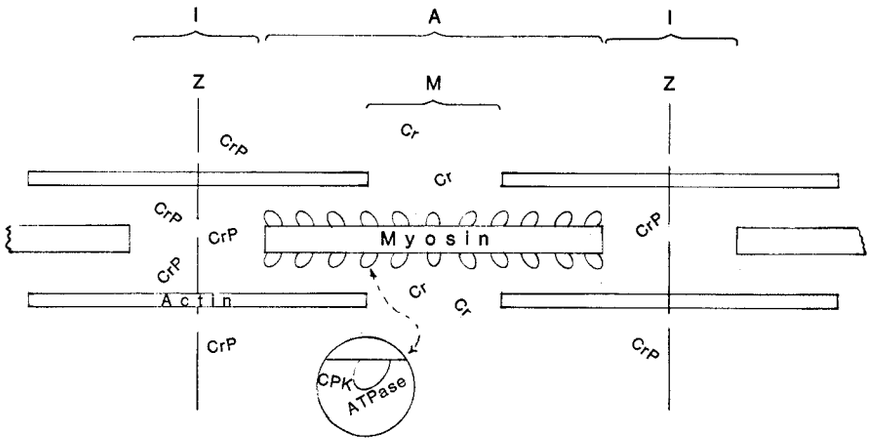

Fig. 2. Schematic relation between heavy myosin and thin-filament actin in striated muscle. The location of adenosinetriphosphatase (*ATPase*) and creatine phosphokinase (*CPK*) is shown in the inset, which represents a meromyosin head. Creatine (*Cr*) diffuses out primarily along the M band (*M*), and phosphorylcreatine (*CrP*) moves along the I bands (*I*). The A band (*A*) and Z lines (*Z*) of one sarcomere are shown.

diabetic muscle is normal." However, the hexokinase reaction is necessary at rest, as the creatine phosphokinase system is then inactive. In this way the insulin-like effect of exercise was compatible with the proposed insulin-hexokinase acceptor mechanism.

In Klingenberg's laboratory, Jacobs *et al.* (*38*) some years later demonstrated the presence of a separate and distinct isoenzyme of creatine phosphokinase bound to mitochondria of heart, brain, and skeletal muscle. Bessman and Fonyo (*39*) then presented quantitative data for the respiratory control of pigeon breast muscle mitochondria by creatine. This provided a microanatomical basis for the mitochondrial end of the creatine-phosphorylcreatine shuttle, the replenishment of the phosphorylcreatine. They suggested that the feedback regulation of respiration in response to muscular activity was mediated through the shuttle of creatine and phosphorylcreatine (*39, 40*). Jacobus and Lehninger (*41*), using rat heart mitochondria, verified and extended these observations.

Saks *et al.* (*42*) showed that, in the presence of a high initial concentration of phosphorylcreatine, the creatine phosphokinase from rat heart mitochondria permitted the phosphorylation of creatine at nearly the maximal rate, even though the substrate-binding and product-binding properties of the mitochondrial enzyme were not compatible with this finding. They reported that ATP synthesized by oxidative phosphorylation seemed to be more effective than exogenous ATP in stimulating phosphorylcreatine production (*42, 43*).

In the same year that the insulin-hexokinase acceptor mechanism was enunciated, Lardy and Wellman (*44*) demonstrated that the concentration of ADP controlled the rate of mitochondrial respiration. Chance (*45*) then showed that the amount of ADP formed after a muscle twitch is only a few percent of the amount expected. He said, ". . . we are faced with the fact that the ADP concentration *arriving at the mitochondria* is, on a per twitch basis, only a few percent of that to be expected if ATP were expended directly during a twitch." He did not refer to the possibility that creatine might indeed be the actual respiratory control intermediate.

In 1966, it was shown by Szent-Györgyi and Prior (*46*) that one molecule of ADP remains firmly bound to each unit of G-actin after its polymerization to F-actin, more evidence that ADP could not move to the mitochondrion to produce the respiratory control postulated by the conventional view (Fig. 1).

D. K. Hill (*47, 48*) studied the endogenous distribution of ATP and phosphorylcreatine with the use of ^{3}H-labeled compounds. Almost all of the phosphorylcreatine was shown by radioautography to be localized near the edges of the I bands of the myofibrils (Fig. 2).

In the study of the compartmentation of phosphorylcreatine within muscle, Lee and Visscher (*49*) found a pattern of labeling in [1-^{14}C]creatine-perfused rabbit hearts that showed a specific activity of creatine well above that of phosphorylcreatine. They interpreted their data to mean either that transphosphorylation was very slow or that there is more than one compartment of phosphorylcreatine and creatine in the heart. During washout (pulse labeling experiments), the ^{14}C disappeared from creatine much faster than from phosphorylcreatine. In addition, there was rapid labeling of a small pool of both creatine and phosphorylcreatine. Their observations were consistent with the presence of two or more phosphorylcreatine pools within the heart. These data, we now suggest, also support a creatine shuttle mechanism.

If we disregard mitochondrial creatine phosphokinase for a moment and consider nonmitochondrial creatine phosphokinase, we ask where is it located? Much of it is not within the sarcoplasmic reticulum (adenylate kinase, sarcoplasmic reticulum-adenosinetriphosphatase, adenylate cyclase, adenosine 3′,5′-monophosphate-dependent kinase), but is elsewhere (*43*). The M-band protein, which can be extracted with dilute salt solution, has been shown beyond reasonable doubt (*50*) to contain as a major component the MM isoenzyme of creatine phosphokinase. This isoenzyme is also the major component of "soluble" creatine phosphokinase. Discovery of the M-band creatine phosphokinase again supports the idea of a shuttle, the M-band enzyme using phosphorylcreatine diffusing along the I band from the mitochondria.

DeHaan *et al.* (*51*) recounted anatomical details of the mitochondrial (granule) arrangement in muscle. Muscles of the intermittent type (skeletal muscle of many vertebrates) have granules primarily at the isotropic (I) bands (Fig. 2). The aerobic energy supply is positioned exactly where needed. The two major locations of creatine phosphokinase provide appropriate receptors for the creatine-phosphorylcreatine shuttle at the mitochondrial and myofibrillar ends.

Primary Importance of the Creatine-Phosphorylcreatine Shuttle

On the basis of work by Bessman and Fonyo (*39*), the existence of a phosphorylcreatine shuttle in muscle was formally proposed in 1972 (*52*) (Fig. 3). Jacobus and Lehninger (*41*) and Saks [summarized in (*43*)] verified and amplified the evidence for the shuttle, and Turner *et al.* (*50*) showed the presence of the isoenzyme that supplied the distal end of the shuttle. The loop or shuttle provides for utilization of chemical energy and its smooth replenishment on demand, particularly during exercise of skeletal muscle, or in a continuous manner, as with normal contraction of the heart. It now appears to be established that the transport of energy in heart and skeletal muscle cells is carried out as a "conversation" between two isoenzymes of creatine phosphokinase, one integral

with mitochondria and one with the myofibril (Fig. 3).

Because the attachment of hexokinase to mitochondria had been shown to increase the efficiency of transfer of phosphate to ATP (*53*), an experimental approach to the functional nature of the connection of creatine phosphokinase to sarcosomes appeared feasible. This would provide an experimental basis for the suggestion of Saks that the mitochondrial kinetics of phosphorylcreatine formation requires a special relation between translocase and creatine phosphokinase.

To improve understanding of this relation, we investigated how radioactive phosphate is incorporated into phosphorylcreatine by respiring mitochondria (*54, 55*). In initial rate experiments, new techniques with the radioactive tracer ^{32}P (or ^{33}P) permitted us to measure specific activities and pool sizes of all of the components of the system (*56*).

In 5-second experiments, we found that the specific activity of the phosphorylcreatine formed by mitochondrially bound creatine phosphokinase was consistently smaller than the specific activity of the labeled gamma phosphate of the total pool of ATP. Mitochondrially generated ATP, which was not labeled, must have been used to form the unlabeled portion (approximately one-third) of the phosphorylcreatine measured. That oxidative phosphorylation can supply ATP to creatine phosphokinase without first mixing with the extramitochondrial pool of ATP became even clearer from experiments with the inhibitors carbonyl cyanide chlorophenylhydrazone (CCCH) and atractyloside, where only exogenous ATP, supplied in the incubation medium, could donate ^{32}P-labeled inorganic phosphate (P_i) to the formation of phosphorylcreatine. Although less phosphorylcreatine was formed in the presence of the inhibitors, all of it had the same specific activity as the gamma phosphate of ATP. This showed that, when mitochondrial formation of ATP stopped, the bound creatine phosphokinase could use only the labeled ATP of the medium.

In further work with rabbit heart mitochondria, we used unlabeled ATP and $^{33}P_i$ in the incubation medium and reached the same conclusions that we had reached earlier with gamma-labeled ATP (*57*). Other experiments with mitochondria from the retinas of chick embryos have yielded similar results (*58*). A further conclusion from our data is worthy of mention. Creatine phosphokinase must lie outside the atractyloside barrier, because it appears from the above experiments that creatine phosphokinase can

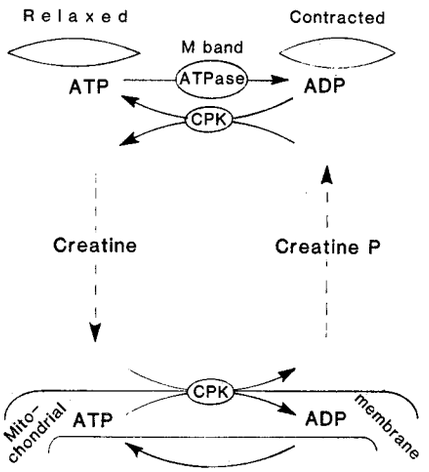

Fig. 3. The phosphorylcreatine mechanism for energy transport, showing the traffic of energy to be a shuttle between two isoenzymes of creatine phosphokinase, one bound to the mitochondrion and one to the M band of the myofibril.

accept high-energy phosphorus from ATP only after this compound has been synthesized and then presented to the enzyme.

These results taken together with the other evidence we have cited lead us to infer that mitochondrial compartmentation of creatine phosphokinase allows the formation of more phosphorylcreatine in the presence of oxidative phosphorylation than the bound creatine phosphokinase can synthesize in the presence of exogenous ATP alone. Accordingly there is now direct support for the postulate (*59*) that a flux of phosphorylcreatine outward from the mitochondrion to the myofibril supplies energy for muscular contraction and that such a scheme is central to aerobic muscle mechanics.

The mitochondrial experiments with [γ-^{32}P]ATP showed that the phosphorylcreatine formed during incubation for 5 to 15 seconds could not all have come from the gamma phosphate of ATP; the phosphorylcreatine was always approximately one-third to one-half as active as the gamma phosphate of ATP. The question arises as to where all of the phosphate for phosphorylcreatine formation actually comes from. It could not come solely from the inorganic phosphate of the medium, in the way that glucose 6-phosphate is synthesized (*53*), nor could it come solely from the gamma phosphate of ATP.

In the mitochondrial experiments, the initial phosphorylcreatine formed had one-third to one-half the specific activity of ATP (*54*). Incubation wtih either an inhibitor of oxidative phosphorylation (CCCH) or with atractyloside, an inhibitor of nucleotide transport, permits the formation of approximately one-third of the control amount of phosphorylcreatine, but this phosphorylcreatine now had exactly the same specific activity as the gamma phosphate of ATP. We conclude that the phosphorylcreatine now formed by the sarcosome is made early, with about one-third formed from exogenous or cytoplasmic ATP and the rest from some other source.

Experiments in which $^{33}P_i$ was incorporated into phosphorylcreatine showed a deficit of approximately 35 to 40 percent in the identifiable sources of phosphate for phosphorylcreatine formation. Other experiments involving incubation with beta- and gamma-labeled ATP resulted in formation of phosphorylcreatine with high specific activity, almost equal to that of the phosphate-labeled ATP. We have tentatively concluded that adenylate kinase (myokinase) could take part in a special connection of creatine kinase to the mitochondrion. To account for our observations, there must be a tetrahedral arrangement of four units, the oxidative phosphorylation site, the two myokinase molecules, perhaps as a dimer, and the creatine phosphokinase molecule around a common pool of ADP. Such an arrangement would provide two portals of entry of ATP and one of AMP (adenosine monophosphate) and an exit portal of phosphorylcreatine. We have named this hypothetical unit a comparticle (*55*).

This hypothesis fits certain requirements. Creatine kinase differs from hexokinase in its sensitivity to "inhibition" by product ADP (from the reversed Lohmann reaction); the proposed mechanism would provide for prompt and efficient removal of ADP in case the ATP-ADP translocase becomes limited owing to local increase in ATP. It would also explain the efficiency of totally labeled ATP in labeling phosphorylcreatine and would provide an explanation for the inability of mitochondrially generated phosphate to label phosphorylcreatine directly. It would provide an explanation of the inability of gamma-labeled ATP to label phosphorylcreatine totally. What is of further interest is that it might give us an opportunity, for the first time, to understand the function of myokinase, which is now relegated to a scavenger role.

This hypothesis grew out of a theory for the mechanism of insulin action (*36, 37, 59*), and has already proved to be a fruitful mechanism for explaining a number of physiological phenomena.

We have shown that creatine phosphokinase and myosin adenosinetri-

phosphatase are firmly juxtaposed in such a way that even in the presence of 5 m*M* ATP supplied exogenously, the phosphate of phosphorylcreatine (10 m*M*) competes successfully at the myofibrillar creatine phosphokinase–adenosinetriphosphatase site and therefore dilutes the P_i scissioned from the supplied [γ-^{32}P]ATP (*60*).

The myosin heads (see Fig. 2, inset), where the adenosinetriphosphatase is located, probably also contain the bound creatine phosphokinase (*61*), as shown functionally by the above experiments. When muscle contracts, the interdigitation of myosin and actin brings the heads into the region of free phosphorylcreatine around the I band, thereby providing substrate for the transfer of phosphate to the bound ADP on the heads and permitting relaxation to occur. Diffusion requirements for phosphorylcreatine are minimized, and the high-concentration (millimolar) ADP becomes a sink for reception of phosphorylcreatine, which is mediated by the adjacent creatine phosphokinase. The functional data available for myofibrils suggest that there is a nucleotide site which is continually occupied by a mole of nucleotide alternating between triphosphate and diphosphate forms by the sequential action of adenosinetriphosphatase and creatine phosphokinase associated with the heads.

Although it would seem at first glance that an unnecessarily elaborate energy transfer system has evolved for muscle, heart, and brain, certain advantages might accrue to a cell that has such a system. Phosphorylcreatine is about as labile as ATP at intracellular *p*H and could serve equivalently as an energy reserve. Furthermore, with phosphorylcreatine as the transfer agent, a large amount of energy can be available for sudden, immediate use, without the maintenance of large concentrations of ATP. The availability of phosphorylcreatine makes the cell relatively resistant to transitory changes in oxygen availability, whereas an active cell with limited nucleotide in transit might be more easily impaired by anoxia. This is the physiological buffer provided by the system. The minimum values and availability of this energy pool may be elucidated through the analog techniques developed by Fitch (*62*). Finally, because phosphorylcreatine is not a substrate for any enzymes other than creatine phosphokinase isoenzymes, the shuttle system is almost inviolate to cellular activities that might otherwise compete for nucleotide energy. Hall and DeLuca (*63*) and Roberts and Bessman (*64*) showed that in early stages, the fetal heart has little creatine kinase or phosphorylcreatine and is dependent essentially on the ATP shuttle, which dwindles as the creatine phosphokinase shuttle develops to maximum activity shortly after birth.

While evidence for the phosphorylcreatine–creatine phosphokinase shuttle as the source of energy for muscle contraction was being accumulated (primarily by biochemical experimentalists), the clinical evidence was available; in developing a mechanism for the action of insulin, we had postulated the phosphorylcreatine shuttle as a necessary biochemical explanation of the insulin-like effect of exercise (*36*). The physiological approach has also provided experiments [see (*31*, *43*), for instance]. Thus three major lines of biological thought have occupied more or less parallel tracks. Perhaps a new era of understanding of the role of phosphagen is at hand.

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