Experimental Depletion of Creatine and Phosphocreatine from Skeletal Muscle*

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SUMMARY

To evaluate the long term effects on skeletal muscle of feeding a competitive inhibitor of creatine transport, $\beta$-guanidinopropionic acid was fed to rats as $1\%$ of their diet for 6 weeks or longer. Although these rats appeared healthy on casual inspection, the P-creatine concentration in their gastrocnemius muscles decreased from a mean value of 22.5 $\mu$moles per g wet weight to 1.6. Instead of P-creatine, the muscles contained a new phosphorylated guanidino compound in concentrations as high as 30 $\mu$moles per g wet weight. By chromatography on Sephadex QAE-A25 a new compound was indistinguishable from phosphorylated $\beta$-guanidinopropionate formed in vitro in a reaction mixture containing $\beta$-guanidinopropionate, rabbit muscle creatine kinase, and an ATP generating system. Hydrolysis of the new compound liberated $\beta$-guanidinopropionic acid and orthophosphate in a 1:1 molar ratio. When muscles from rats fed $\beta$-guanidinopropionic acid were caused to contract under anoxic conditions, the concentration of phosphorylated $\beta$-guanidinopropionate decreased dramatically, raising the possibility that it, like P-creatine, can serve in a system to regenerate ATP. On the other hand $\beta$-guanidinopropionate is a relatively ineffective substrate for creatine kinase in vitro, and animals fed $\beta$-guanidinopropionic acid have sub-normal concentrations of glucose 6-phosphate, ATP, and ADP in resting muscle in vivo. These animals may prove useful in studies of the metabolic adaptations of skeletal muscle.

Creatine and P-creatine are known to have several potentially critical roles in skeletal muscle. With creatine kinase, P-creatine participates in a system that regenerates ATP during contraction (2); P-creatine inhibits P-fructokinase (3) and pyruvate kinase (4) and activates fructose 1,6-diphosphatase (5); and creatine in the bathing medium of skeletal muscle cells in tissue culture stimulates incorporation of $[\text{H}]$leucine into myosin heavy chain (6). Despite knowledge of these roles, however, it is not certain that high concentrations of creatine and P-creatine in skeletal muscle are essential either for function or for viability. It is true that low concentrations of these compounds are associated with pathologic changes in the muscles of subjects with muscular dystrophy and other myopathies (7), but in no case has it been possible to determine which came first, the abnormality of creatine metabolism or the muscle pathology.

To evaluate the essentiality of high concentrations of creatine and P-creatine in skeletal muscle, a specific means of chronically depleting muscle of these two compounds is needed. Therefore, the present feeding trials with $\beta$-guanidinopropionic acid were initiated. $\beta$-Guanidinopropionic acid is related to creatine structurally and is known to compete with creatine for transport into muscle. Shields also observed that this diet had only a minimal effect, if any, on ability to perform in an exercise test and did not reduce the growth rate of male rats (10).

EXPERIMENTAL PROCEDURES

Rats of Wistar origin (NLR strain, National Laboratories, Creve Coeur, Missouri) were given ground laboratory chow (Ralston Purina, St. Louis) and water ad libitum. Normal and experimental rats were treated identically except that the diet for experimental rats contained $1\%$ of $\beta$-guanidinopropionic acid (Cyco Chemical Corp., Los Angeles). The diets were fed for 6 weeks or longer before the present studies were performed.

To obtain resting muscle, the rats were anesthetized with pentobarbital, the muscles were exposed surgically using care not to provoke contraction, and biopsies were taken with rongeurs prechilled in liquid nitrogen. These biopsies were kept solidly frozen as they were weighed, pulverized, and mixed with frozen perchloric acid prior to preparing a neutral extract for measurement of muscle phosphates.

The details of preparation of the muscle extract and of the automated, chromatographic method used to separate and quantitate muscle phosphates have been published (11). Briefly, the extract is applied to a column of Sephadex QAE-A25, and the phosphate compounds are eluted with a mixture of solutions which form a continuous gradient of decreasing sodium sulfate.
Fig. 1. Automated, chromatographic measurement of muscle phosphates and β-guanidinopropionate. A brief description of methods is given in the text. The elution pattern obtained by monitoring for phosphate is shown in the upper tracing for resting muscle of a normal rat and in the middle tracing for resting muscle of a rat fed β-guanidinopropionic acid; the peaks representing glucose 6-phosphate, P-creatine, P_i, ADP, and ATP are identified. The elution pattern obtained by monitoring for Sakaguchi positive compounds is shown in the bottom tracing for resting gastrocnemius muscle of a rat fed β-guanidinopropionic acid; the first peak corresponds to β-guanidinopropionate and the other peak represents a new guanidino phosphate. Neither of these guanidino peaks was present in the elution patterns of extracts from normal muscles.

Figures have not been included in the text as it would be disorganized and difficult to follow. The figures should be referenced in the text with figure numbers and descriptions.

Methods of obtaining samples and chemical analysis were given in the text. The elution pattern obtained by monitoring for Sakaguchi positive compounds is shown in the bottom tracing for resting gastrocnemius muscle of a rat fed β-guanidinopropionic acid; the first peak corresponds to β-guanidinopropionate and the other peak represents a new guanidino phosphate. Neither of these guanidino peaks was present in the elution patterns of extracts from normal muscles.

Enzymatic measurements of Kcreatine (14) and P_i (15) were made in certain instances, using enzymes and other reagents obtained from Sigma Chemical Company; and the total creatine contents of some of the muscle samples were measured by the method of Rose et al. (16) after ascertaining that β-guanidinopropionic acid causes no interference in this method. The outputs from monitoring colorimeters were recorded to obtain elution patterns (Fig. 1), from which the concentrations of the various compounds were calculated.

Enzymatic measurements of P-creatine (14) and P_i (15) were made in certain instances, using enzymes and other reagents obtained from Sigma Chemical Company; and the total creatine contents of some of the muscle samples were measured by the method of Rose et al. (16) after ascertaining that β-guanidinopropionic acid causes no interference in this method. Total water content was determined by drying muscle samples to constant weight at 85°.

The ability of β-guanidinopropionate to serve as a substrate for creatine kinase was studied in the coupled spectrophotometric system of Tanzer and Gilvarg (17) as modified by McLaughlin et al. (18) for kinetic studies. The enzymes and other reagents for this system were purchased from Sigma Chemical Company.

### Results and Discussion

Although the rats fed β-guanidinopropionic acid appeared healthy on casual inspection throughout the feeding trials, the P-creatine concentrations in their gastrocnemius muscles were reduced to less than 10% of the normal value (Fig. 1 and Table 1). This reduction in concentration was apparent from an examination of the elution patterns from the Sephadex QAE-A25 column (Fig. 1), and it was confirmed by enzymatic measurements of P-creatine (Table 1). In agreement with the work of Shields (10), the total content of creatine also was reduced from 38.7 ± 1.7 (mean ± S.E.) μmoles per g wet weight of muscle for nine normal rats to 9.62 ± 0.92 for seven rats fed β-guanidinopropionic acid. These changes in content of creatine and P-creatine are not due to replacement of muscle cells by fatty or connective tissue (10), and they are not due to changes in water content. We found the water content to be 76.7 ± 0.4% of wet weight of muscle for nine normal rats and 77.6 ± 0.4% for eight rats fed β-guanidinopropionic acid.

Instead of a large P-creatine peak, the elution pattern obtained from muscle extracts of rats fed β-guanidinopropionic acid displayed an exceptionally large peak in the position normally occupied by the P_i peak (Fig. 1). Although the new peak at first was considered to represent P_i (19) subsequent enzymatic measurements revealed normal concentrations of P_i in skeletal muscle of rats fed β-guanidinopropionic acid (Table 1). Thus, the existence of a new organic phosphate was apparent.

From the following observations we conclude that the new organic phosphate is phosphorylated β-guanidinopropionate. (a) Hydrolysis of that fraction of the eluate which contained the new compound liberated P_i and a Sakaguchi positive compound² in a 1:1 molar ratio (Fig. 1). (b) Acid hydrolysis of the muscle extract prior to Sephadex QAE-A25 chromatography liberated a Sakaguchi positive compound that chromatographed as β-guanidinopropionate. The position of the β-guanidinopropionate peak in the chromatographic tracing is shown in Fig. 1. In this system β-guanidinopropionate is easily distinguished from the new compound and from arginine because it emerges from the column much sooner than the new compound and slightly later than arginine. β-Guanidinopropionate and guanidinoacetate are not separated by the Sephadex QAE-A25 column. (c) By paper chromatography using two different solvent systems, the major Sakaguchi positive compound in muscle extracts from rats fed β-guanidinopropionic acid behaved identically to authentic

² The quantity of Sakaguchi positive compound was estimated from a standard curve prepared from authentic β-guanidinopropionic acid.
P-Guanidinopropionic acid.

3 mM ATP, 6 mM MgSO₄, 0.33 mg of rabbit muscle lactate dehydrogenase containing P-guanidinopropionate, rabbit muscle creatine kinase, P-guanidinopropionate formed in muscle extracts of rats fed P-guanidinopropionic acid was indistinguishable from phosphorylated arginine or guanidinoacetic acid (Table II). And (d) by chromatography on Sephadex QAE-A25, the new phosphorylated arginine and guanidinoacetic acid (Table II). And (d) by chromatography on Sephadex QAE-A25, the new phosphorylated arginine and guanidinoacetic acid (Table II).

Unknown.

Arginine.

Guanidinoacetic acid.

The reaction mixture consisted of 120 ml of n-butyl alcohol, 30 ml of glacial acetic acid, and 80 ml of water. Solvent B consisted of 65 ml of n-butyl alcohol, 65 ml of pyridine, and 65 ml of water. Extracts of muscles for chromatography were prepared as follows. Samples of skeletal muscle were excised from anesthetized rats and allowed to stand at room temperature for 30 to 60 min prior to freezing for storage. Subsequently the samples were thawed slowly, homogenized at room temperature, and treated with BaOH and ZnSO₄ to precipitate protein (13). Such extracts from muscles of rats fed P-guanidinopropionic acid contained large amounts of an unknown Sakaguchi positive compound, approximately 37 μmoles per g wet weight of muscle as estimated by the method of Van Pilsum and associates (13).

**Table II**

| Compound                  | Solvent A | Solvent B |
|---------------------------|-----------|-----------|
| P-guanidinopropionic acid | 53        | 32        |
| Unknown                   | 53        | 32        |
| Arginine                  | 17        | 19        |
| Guanidinoacetic acid      | 34        | 30        |

Fig. 2. β-Guanidinopropionate as a substrate for creatine kinase. The reaction mixture consisted of ATP, 3 mM; MgSO₄, 6 mM; P-enolpyruvate, 1 mg per ml; DPNH, 0.1 mg per ml; glucose 6-phosphate, 100 mM, pH 9; rabbit muscle lactate dehydrogenase, 0.33 mg per ml; rabbit muscle pyruvate kinase, 0.5 mg per ml; and creatine or P-guanidinopropionate to achieve the concentrations shown in the figure; 1 mg of rabbit muscle creatine kinase per ml was added at 4 min. This mixture was incubated at 25° under room air, and the change in optical density at 340 nm was recorded as a function of time.

β-guanidinopropionic acid (20) and was easily distinguished from arginine and guanidinoacetic acid (Table II). And (d) by chromatography on Sephadex QAE-A25, the new phosphorylated guanidino compound was indistinguishable from phosphorylated β-guanidinopropionate formed in vitro in a reaction mixture containing β-guanidinopropionate, rabbit muscle creatine kinase, and an ATP generating system.²

² The reaction mixture had the following initial composition: 4 mM β-guanidinopropionate, 4 mM DPNH, 14 mM P-enolpyruvate, 3 mM ATP, 6 mM MgSO₄, 0.33 mg of rabbit muscle lactate dehydrogenase per ml, 0.5 mg of rabbit muscle pyruvate kinase per ml, 3 mg of rabbit muscle creatine kinase per ml, and 100 mM glycine, pH 9. The reaction was allowed to proceed for 21 hours at 25° after which a perchloric acid extract was made for analysis by the automated, chromatographic procedure (11). Monitoring for β-guanidinopropionate yielded an elution pattern identical with those obtained from extracts of muscle from rats fed β-guanidinopropionic acid (bottom tracing of Fig. 1). Approximately 90% of the β-guanidinopropionate in the reaction mixture was converted to phosphorylated β-guanidinopropionate.

The relative effectiveness of β-guanidinopropionate as a substrate for creatine kinase may be appreciated from an examination of Fig. 2. Although the kinetics of the reaction deserve additional study, it is apparent that β-guanidinopropionate is a poor substrate in comparison to creatine. Nevertheless, β-guanidinopropionate is phosphorylated at an appreciable rate; in vivo, the phosphorylation might proceed much more rapidly. We have not studied the ability of phosphorylated β-guanidinopropionate to serve as a substrate for creatine kinase in vitro, but it may be noted that phosphorylated β-guanidinopropionate in muscle extracts of rats fed β-guanidinopropionic acid was not detected in our enzymatic measurements of P'-creatine using commercial rabbit muscle creatine kinase. Thus, phosphorylated β-guanidinopropionate at best is a poor substrate for rabbit muscle creatine kinase.

![Fig. 3. Changes induced by anoxic contraction of skeletal muscle.](image-url)

*Fig. 3. Changes induced by anoxic contraction of skeletal muscle. Resting gastrocnemius muscle was obtained by the method described in the text, after which the rat was killed by creating a pneumothorax. Then the other gastrocnemius muscle was stimulated to twitch twice per s using a Harvard Apparatus Company model 340 stimulator to deliver 1.5 volts through platinum electrodes placed directly on the muscle. After repetitively twitching for 10 min, this muscle was biopsied (anoxic, contracting muscle). Pi was measured enzymatically and the other compounds were measured by the automated, chromatographic procedure. The results from six normal male rats weighing in excess of 300 g each are shown in the lower panel, and the results from four male rats that had received β-guanidinopropionic acid as 1% of their diet for 6 weeks or longer and that weighed in excess of 300 g each are shown in the upper panel; means ± S.E. are shown. The abbreviations used are β-GPA, β-guanidinopropionate; β-GPAP, phosphorylated β-guanidinopropionate; CP, P-creatine; and G-6-P, glucose 6-phosphate.*
muscle creatine kinase under the conditions of the assay. This result is in agreement with the knowledge that phosphorylated guanidinoacetate is a poor substrate for this enzyme (21).

To determine whether phosphorylated β-guanidinopropionate is labile under conditions known to deplete P-creatine in vivo, gastrocnemius muscles were caused to twitch under anoxic conditions (Fig. 3). As expected for normal muscle (Fig. 3), the P-creatine concentration fell to very low levels and there were reciprocal increases in glucose 6-phosphate and Pi. The mean total loss of P-creatine and ATP from normal muscle was 15.9 μmoles per g. Under the same conditions the total loss of phosphorylated β-guanidinopropionate and ATP from muscles of rats fed β-guanidinopropionic acid was 12.0 μmoles per g (Fig. 3). Both the initial and final concentrations of phosphorylated β-guanidinopropionate were higher than the corresponding values for P-creatine. Again there were reciprocal increases in glucose 6-phosphate and Pi. These findings clearly demonstrate that phosphorylated β-guanidinopropionate is labile under the conditions produced by anoxic contraction of skeletal muscle, and they raise the possibility that this compound serves in a system to regenerate ATP in a manner similar to P-creatine. The ability of phosphorylated β-guanidinopropionate to function in such a system could explain apparent good health despite severe depletion of P-creatine from skeletal muscle of rats fed β-guanidinopropionic acid. Additional work is needed to prove that phosphorylated β-guanidinopropionate in fact does substitute for P-creatine.

Studies are in progress to evaluate the metabolic adaptations consequent to substituting β-guanidinopropionate for creatine in skeletal muscle. That metabolic adaptations occur is evident from the reduced concentrations of glucose 6-phosphate, ADP, and ATP in resting skeletal muscle of rats fed β-guanidinopropionic acid (Table 1). Elucidation of these adaptations should add to our understanding of the roles of creatine and P-creatine in the regulation of metabolism in skeletal muscle in vivo.

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