THE EFFECTS OF FEEDING B-GUANIDINOPROPRIONIC ACID ON CREATINE METABOLISM AND SKELETAL MUSCLE FUNCTION AND STRUCTURE OF RATS

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ABSTRACT

THE EFFECTS OF FEEDING B-GUANIDINOPROPRIONIC ACID ON CREATINE METABOLISM AND SKELETAL MUSCLE FUNCTION AND STRUCTURE OF RATS

By

Robert Pierce Shields

This research was conducted to test the hypothesis that high intracellular concentrations of creatine are essential to the integrity of vertebrate skeletal muscle; and to provide new information on the relationship between abnormal creatine metabolism and muscle disease. To accomplish these objectives, the structure and function of muscles were evaluated after abnormal creatine metabolism was induced by feeding rats a creatine analog, B-guanidinoproprionic acid (B-GPA).

The abnormal metabolism of creatine that occurred when rats were fed diets containing 1% B-GPA was characterized by decreases in muscle and brain levels of creatine, muscle N-phosphorylcreatine, urinary excretion of creatinine, muscle activity of creatine phosphokinase (CPK) and by an increase in urinary excretion of creatine. The white-type fibers from *gastrocnemius* muscles of normal rats contained more creatine than the red-type fibers from the same muscles; however, the creatine levels of both were proportionally lower than normal in fibers from rats fed B-GPA. While pregnant rats consumed less food when their diets contained B-GPA, there was no significant effect on food intake

of young male rats when they were pair-fed rations with or without B-GPA. In an *in vitro* experiment, B-GPA inhibited the reaction of creatine with rat muscle CPK. Beta-guanidinoproprionic acid apparently induces abnormal creatine metabolism in rats by inhibiting the mediated entry of creatine into tissues from plasma--and possibly by competing with creatine for the active site of the CPK enzyme.

When muscle function was evaluated by running, the group of rats fed B-GPA did not perform as well as did those that had not received B-GPA. There were also structural changes in muscle fibers of exercised rats fed B-GPA. The white fibers from the *gastrocnemius* of rats fed B-GPA were significantly smaller than those from rats not fed the test compound; nevertheless, normal histochemical profiles were maintained in these fibers. Gross pathological changes were not observed in experimental rats. Thus, when the normal creatine complement of skeletal muscle is depleted (75%) by feeding B-GPA, the normal structure and function of muscle is not maintained.

This is the first report that tissue levels of creatine can be reduced by altering creatine metabolism directly at a point near its functional site. The ability to deplete skeletal muscle of its normally high intracellular concentrations of creatine and N-phosphorylcreatine will be of value in clarifying the relationship between abnormal creatine metabolism and neuromuscular disease. In addition, the possibility that the CPK enzyme acts as a translocating molecule during its reaction with creatine should enhance our understanding of trans-membrane transport of small molecular weight compounds.

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By

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Dedicated to my wife and family

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INTRODUCTION

The loss of muscle creatine, excessive creatinuria, and a reduced excretion of creatinine in the urine are diagnostic signs of abnormal creatine metabolism in all vertebrates. For more than 50 years these changes have been directly associated with diseases of skeletal muscles; nevertheless, the metabolic defect(s) responsible has not been clearly determined. In addition, knowledge of the role of normal creatine metabolism in the function and structure of normal muscle is incomplete.

These relationships have not been studied critically partly because of an inability to directly alter creatine metabolism at or near its functional site in muscle. Abnormal creatine metabolism associated with primary muscular dystrophy or myopathy experimentally induced by surgery, drugs, or diet may or may not result from a primary effect on creatine metabolism.

Creatine or, more specifically, its functional metabolite, N-phosphorylcreatine, is of a class of organic compounds which function in the animal kingdom as phosphagens. A brief summary of the biological significance of phosphagens is included, therefore, as an introduction to a review of normal and abnormal creatine metabolism.

Ennor and Morrison (1958) defined phosphagens as:

> "naturally occurring phosphorylated guanidines which function as stores of phosphate-bond energy from which phosphoryl groups may be transferred to adenosinediphosphate to form adenosinetriphosphate as a result of enzymatic catalysis."

Although several different phosphagens such as phosphorylarginine, phosphoryltaurocyanamine, phosphorylglycocyanime, and phosphoryllombricine have been isolated from non-vertebrates, the only phosphagen found in vertebrates is phosphorylcreatine. In each case a specific phosphoryl transferase enzyme has been reported. Thus, phosphagens and their phosphoryltransferase enzymes are found throughout the animal kingdom and may be evidence of biochemical evolution. There is no direct support that phosphorylarginine is ontogenetically older than phosphorylcreatine; nevertheless, since arginine is a constituent of all proteins, it may be, and the basic phosphagen system may have survived evolutionary selection in vertebrates as phosphorylcreatine.

REVIEW OF LITERATURE

This review focuses on the normal and the abnormal metabolism of creatine in vertebrates. Special emphasis is placed on the relationship of creatine metabolism to the function and structure of skeletal muscle.

Historical

Creatine or N-methylguanidinoacetate acid was first isolated by Chevreal in 1832 from an extract of muscle (cited by Hunter, 1928; Wang, 1939; Milhorat, 1953). Within the next few years creatine and its anhydride creatinine were isolated from the muscle and urine of many vertebrate species. During the latter half of the 19th century investigators were primarily concerned with the chemical nature of these 2 compounds and fundamental studies such as those by Leibig (1847) were instrumental in determining their structure. Quantitative estimates of creatine or experimental studies of creatine metabolism were not practical, however, until Folin (1905) developed a colorimetric assay. The Folin method was based on the reaction of creatinine with picric acid that had been previously described by Jaffe. The Folin method, while not specific for creatine, permitted reasonably accurate estimates of muscle levels of creatine and creatinine as early as 1914. Another metabolic derivative of creatine, N-phosphorylcreatine, was isolated in 1927 (Eggleton and Eggleton, 1927; Fiske and Subbarow, 1927). Thus, by 1930 all the presently known metabolic derivatives of creatine had

been isolated and an analytical method was available for experimental consideration of creatine metabolism.

Normal Creatine Metabolism

The origin, biological significance and fate of creatine and its metabolic derivatives N-phosphorylcreatine and creatinine will be considered separately.

Creatine. Although creatine is absorbed from the gastrointestinal tract of mammals, it has not been found to be an essential dietary constituent (Block and Schoenheimer, 1941). Significant quantities of creatine are synthesized endogenously from glycine, arginine, and methionine (Borsook and Dubnoff, 1940, 1941). The first step in the synthesis is the formation of glycocyanamine from glycine and arginine which occurs primarily in the kidney. Glycocyamine is subsequently methylated to form creatine. These data were confirmed by Block *et al.* (1941) and DuVigneaud *et al.* (1941) using N^{15} labelled compounds. As a result of their studies, Borsook and Dubnoff and Block *et al.* (1941) proposed a mechanism (below) accommodating all findings whereby the total body requirement of creatine might be endogenously synthesized.

(1) arginine + glycine $\xrightarrow{\text{(aminidinotransferase)}}$ guanidinoacetic acid + ornithine

(2) guanidinoacetic acid + methionine $\xrightarrow{\text{(methyltransferase)}}$ creatine + homocysteine

Van Pilsum *et al.* (1963) detected L-arginine-glycine aminidinotransferase (E.C.2.6.2.1.) activity in most body tissues but the greatest activity per unit weight of tissue was found in the kidney and pancreas. Studies by Cantoni *et al.* (1954, 1957) characterized the last reaction

as one which requires S-adenosylmethionine as the methyl donor. S-adenosylmethyltransferase (E.C.2.1.1.2.) activity has been detected in the liver (Cantoni and Vignos, 1954), pancreas (Walker, 1959) and testicle (Salvatore and Schlenk, 1962). The tissue distribution of the enzymes responsible for creatine synthesis are in accord with *in vitro* studies by Azzone and Carafoli (1956), who failed to detect creatine synthesis in muscle. Earlier Baker and Miller (1940) had failed to detect creatine synthesis in any tissues except liver and kidney. Some synthesis of creatine in rat brain (Defalco and Davies, 1961) and testicle (Alekseeva and Arkhangel'skaya, 1964) have been reported, but there has been no quantitative evaluation with respect to total body creatine.

While most creatine synthesis seems to occur in the liver, kidney, and pancreas, it has been estimated that over 95% of the total creatine in the body is in skeletal muscle (Block and Schoenheimer, 1941). Various types of muscle contain between 100 and 600 mg of creatine per 100 g wet weight with the concentration arranged in descending order of skeletal muscle > diaphragm > cardiac muscle > smooth muscle (Folin and Buckman, 1914; Baker and Miller, 1939; Gross and Sandberg, 1942; Eggleton *et al.*, 1943; Borsook and Dubnoff, 1947). The remaining 2 to 5% of the body's creatine has been reported to be widely distributed with the most significant concentrations in nerve and testicular tissue (Wang, 1939). The brain contains approximately 132 mg per 100 g and the testicles approximately 267 mg per 100 g (Hunter, 1928; Eggleton *et al.*, 1943).

Since creatine synthesis has not been detected in skeletal muscle, it has been proposed that plasma creatine is the source of creatine that accumulates in muscle. Values of 2.0 ml/100 ml of blood have

been reported in rats (Fitch and Payne, 1965) and rabbits (Tanzer and Gilvarg, 1959). Blood levels of creatine are maintained by endogenous synthesis (mentioned above), by ingestion from dietary sources (Block and Schoenheimer, 1941), and by reabsorption in the renal tubules (Pitts, 1943; Gayer and Krentz, 1962). *In vitro* studies have been used to demonstrate a mediated system of creatine entry into muscles from plasma (Fitch and Shields, 1966). This system was highly specific for creatine (Fitch *et al.*, 1968b) and could account for the normal accumulation of creatine in muscles from plasma despite a significant concentration gradient.

The major excretory route for creatine is as urine creatinine (Borsook and Dubnoff, 1947). Small amounts of creatine have been detected in bile, but this is not considered a major excretory route (Jannakopulu and Impicciatore, 1961). Creatine excretion in feces has also been reported (Fitch and Maben, 1964). Molecular degradation of creatine may occur in the gastrointestinal tract, possibly by microorganisms (Twort and Mellenby, 1912), but no molecular decomposition of creatine has been detected within mammalian tissues.

N-phosphorylcreatine. Soon after the characterization of N-phosphorylcreatine (Fiske and Subbarow, 1929), Meyerhof and Lohmann (cited by Kuby and Moltman, 1962) reported the synthesis of this compound in muscle extracts and the simultaneous hydrolysis of adenosinetriphosphate (ATP). The enzyme adeniosinetriphosphate : creatine transphosphotransferase (CPK) [E.C.2.7.3.2.] which was responsible for this synthesis was isolated and crystallized by Kuby *et al.* (1954).

The physiological function of N-phosphorylcreatine as an energy source for skeletal muscle was proposed soon after its characterization

as a "high-energy" compound (Fiske and Subbarow, 1929). Nevertheless, it was not until 1962 that Cain and Davies (1962) were able to detect experimentally the depletion of muscle ATP during a single muscle twitch. This was done by inhibiting the CPK enzyme which prevented the rapid resynthesis of ATP from N-phosphorylcreatine. It is now recognized that the phosphorylated form of creatine serves as an energy store to rapidly regenerate ATP during muscle metabolism and/or the contraction processes (West *et al.*, 1966).

The tissue distribution of N-phosphorylcreatine is identical to its precursor creatine but many of the reported tissue levels are open to criticism. Details of these criticisms are beyond the scope of this review; however, of primary importance is the extremely labile nature of N-phosphorylcreatine (Ennor and Morrison, 1958). Reported tissue values are between 60 and 80% of the total creatine of the analyzed tissue (Ennor and Rosenberg, 1952). Values of 2.4 mg of N-phosphoryl-creatine per g of rat muscle have been reported by Fawz *et al.* (1962). It has also been suggested (Beatty *et al.*, 1970) that the white type fibers of skeletal muscle contain more N-phosphorylcreatine than do the red type fibers. N-phosphorylcreatine has not been detected in urine or plasma (Ennor and Morrison, 1958). The metabolic fate of N-phosphoryl-creatine is assumed to involve its hydrolysis to creatine or to creatinine and subsequent urinary excretion.

Creatinine. *In vitro*, a rapid conversion of creatine to creatinine has been observed (Borsook and Dubnoff, 1947). No enzyme has been detected to mediate this conversion and it has been estimated that the loss of body creatine at the rate of 2% per day could occur by a non-enzymatic conversion of creatine to its anhydride creatinine under body conditions

(Block and Schoenheimer, 1939, 1941; Borsook and Dubnoff, 1947). These studies have also established that urinary creatinine is derived from body creatine and that most urine creatinine comes from muscle creatine.

Tissue levels of creatinine usually range from 1 to 5 mg per 100 g of tissue (Folin, 1914; Borsook and Dubnoff, 1947). Its concentration in human plasma has been reported to be between 1 and 8 mg per 100 ml. Creatinine present in the blood is filtered by the kidney and excreted unchanged in the urine. The rate of creatinine excretion is relatively constant and is proportional to the body muscle mass (Melvin *et al.*, 1958; Kleiner and Orten, 1962; Despopoulous and Kolber, 1964).

Abnormal Creatine Metabolism and Neuromuscular Disease

Abnormalities in the metabolism of creatine were the first biochemical lesion detected in patients with muscular dystrophy (Adams *et al.*, 1967). Between 1870 and 1953 it was noted that patients with a wide variety of neuromuscular diseases exhibited one or more of the following signs of abnormal creatine metabolism: a decreased urinary excretion of creatine, an increase in urinary excretion of creatine, or a decrease in muscle levels of creatine (Nevin, 1934; Milhorat, 1953). These changes were observed in humans as well as in experimental animals with generalized myopathy, regardless of the etiologic agent. Thus, in 1953 Milhorat (1953) concluded that the relationship between abnormal creatine metabolism and neuromuscular disease lacks etiological specificity. The abnormal patterns of creatine excretion and loss of muscle creatine were therefore assumed to be secondary to muscle cell injury and not related to the cause of such maladies. Since 1953 newer knowledge of the dynamics of body creatine metabolism, of muscle function and structure, and improvements in analytical procedures suggest that

etiologic specificity exists and that probably the relationship is more complex.

Using isotopic creatine, Fitch and Sinton (1964) provided evidence that although the clinical signs of abnormal creatine metabolism may be the same regardless of the etiology of the muscle injury, the mechanism by which these signs develop may differ. They were able to measure the turnover (T 1/2-time) of muscle creatine in patients with abnormal creatine metabolism due to various neuromuscular diseases. The T 1/2-time of muscle creatine of normal patients and of patients with polymyositis was approximately 38 days. Patients with neurogenic (amyotrophic lateral sclerosis) or primary muscular dystrophy (Duchenne's type) had significantly increased and decreased T 1/2-times, respectively. These data were interpreted in relation to the ability of skeletal muscle to maintain its normal complement of creatine. In one case (neurogenic) it was proposed that plasma creatine could not enter muscle at the normal rate and was excreted in the urine. In the case of primary muscular dystrophy, it was porposed that muscle could not successfully trap or bind creatine that had entered and it returned to the blood and was excreted. With polymyositis creatine entry or binding within the muscle was normal but muscle cells were being destroyed by the inflammatory process and their creatine was lost more rapidly than normal. In each case there was creatinuria and lower-than-normal levels of muscle creatine, but the mechanism by which these signs occurred seemed to differ. These data (Fitch *et al.*, 1964, 1968a) were the first evidence relating abnormal creatine metabolism to the etiology of neuromuscular disease. Subsequent studies of the entry of creatine from plasma (Fitch *et al.*, 1966, 1968b) indicated that (at least in rats) creatine enters muscle by a specific mediated process.

To more clearly understand the relationship between creatine metabolism and skeletal muscle function and structure one must also consider certain adaptive responses of muscle. In the muscle of the newborn, CPK activity is lower than in adults (Perry, 1970). Within the first 10 days after birth when walking begins, there is a dramatic increase in muscle CPK activity. There are also changes in CPK isoenzyme patterns from the BB type in fetal muscle to the MM type which is predominant in adults (Goto *et al.*, 1969). As the animal matures certain muscle fibers develop a different biochemical profile (white type fibers) that also seems to be dependent upon muscle activity (Dubowitz, 1970). These adaptive changes also suggest that there may be a direct relationship between creatine metabolism and normal skeletal muscle function.

In addition to the above data, indirect evidence, such as the role of N-phosphorylcreatine in maintaining tissue ATP levels, the ability of muscle to store large quantities of creatine, and the preservation of the phosphagen system through evolutionary selection, indicate that additional knowledge of creatine metabolism could provide a better understanding of vertebrate muscle function. It would also clarify the relationship between abnormal creatine metabolism and neuromuscular disease.

OBJECTIVES

The purpose of this research was to provide a better understanding of the role of creatine in muscle integrity and to clarify the relationship between abnormal creatine metabolism and pathological changes in skeletal muscle. Previous research by the author had demonstrated that an analog of creatine, beta-guanidinoproprionic acid (B-GPA), would inhibit creatine entry into muscle *in vitro*. The initial phase of these studies was to determine the *in vivo* effect on creatine metabolism when B-GPA was fed to rats. The second phase included an evaluation of the performance and structure of muscles after they had been deprived of their normal complement of creatine by feeding B-GPA.

The specific objectives were: (1) to determine the effects of feeding B-GPA on creatine metabolism of rats; (2) to determine the gross and microscopic tissue changes that occur when rats are fed B-GPA; (3) to evaluate muscle performance or rats that have been fed B-GPA; and (4) to correlate the biochemical and morphologic data from the above experiments to permit a better understanding of the role of creatine in the function, structure and disease processes of skeletal muscle.

MATERIALS AND METHODS

General Plan

Creatine metabolism of rats fed B-GPA was evaluated by determining urine, muscle, and brain creatine; urine creatinine, muscle B-GPA; and muscle CPK activity. Possible effects on muscle function and structure were studied by gross and microscopic examination of selected muscles and by evaluating individual animal performance when subjected to controlled muscular stress in a running wheel. General or whole body effects were studied by determining body weight gain, food intake, and routine cage performance.

Source and Maintenance of Animals

Rats of the Sprague-Dawley strain purchased from commercial sources* were used for all experiments. Except during nursing periods, they were housed in individual wire-bottomed cages. Stainless steel metabolism cages were used for urine collection and for obtaining food-intake data. Water was given free choice at all times. Diets consisted of either commercial laboratory chow** or a diet prepared from purified ingredients (Fitch *et al.*, 1960). The ingredient analysis as well as subsequent chemical analysis during the course of these studies indicated that the commercial diet contained creatine. The purified diet

*Spartan Research Animals, Inc., Haslett, Michigan, or Hormone Assay Laboratories, Chicago, Illinois.

**Ralston Purina Co., St. Louis, Missouri.

was therefore used in one study to test the possible influence of dietary creatine on the effect of B-GPA. The test compound, B-GPA, was incorporated into both of these diets by batch mixing in a commercial paddle-type table mixer. In one trial pregnant rats were fed 0.5, 1.0 or 2% B-GPA. In additional feeding trials young male rats were pair-fed diets containing 1 or 2% B-GPA.

Source of B-guanidinoproprionic Acid

The B-GPA used in these experiments was obtained by special synthesis from the Cyclo Chemical Company, Los Angeles, California. Purity was determined by gas and thin layer chromatography.

Chemical Analyses

Urinary creatine and creatinine were determined on 24-hour urine samples collected under toluene by the alkaline picrate method described by Folin (1914). Muscle creatine was estimated on water homogenates by the alkaline picrate method and by the enzymatic analysis of Tanzer and Gilvarg (1959) as modified by Bernt *et al.* (1963) and by Marymont *et al.* (1968). Muscle B-GPA was determined on water homogenates by Sakaguchi reaction using 8-hydroxyquinoline as proposed by Gilboe and Williams (1955) and by paper chromatography (Block *et al.*, 1958). The enzymatic assay of Tanzer and Gilvarg (1959) was also used to determine CPK activity but modifications were necessary. Immediately after decapitation, the left *gastrocnemius*, *soleus*, and *plantaris* were removed as a unit and frozen by submersing in isopentane that had been precooled in liquid nitrogen. The frozen muscles were stored in individual sealed containers at -20 C. Muscle for analysis was sliced from the posterior aspect of the *gastrocnemius* (Zone I, see Figure 1) without thawing the sample. Creatine phosphokinase was extracted according to Oliver (1955)

except that the muscle was homogenized in 20-volumes of ice-cold 0.1M KCl instead of 6-volumes. After homogenization in an all-glass homogenizer the sample was centrifuged at 15,000 x g for 20 minutes while at 0 to -4 C. The resultant supernate was diluted 1:200 in 1.0M glycine buffer and assayed for CPK activity. Protein of the diluted extract was determined by the Lowry (1951) method. The final assay mixture was that recommended by Marymont *et al.* (1968) except that 0.01M mercaptoethanol was included (Kar and Pearson, 1965) and the amount of creatine and B-GPA varied as described for each individual experiment.

Running Performance

Muscle performance was tested by running rats in the Controlled-Running Wheel of Wells and Heusner (1971). Rats to be tested were run according to the 4-week, short duration, high intensity endurance program developed by these investigators (Appendix A). This was a progressively increasing program in which rats eventually ran at speeds of 4 feet-per-second for 4 bouts of 10 seconds each. Individual performance was monitored by calculating the percent-of-expected revolutions (PER) and the percent-shock-free-time (PSF). This program was designed for at least 75% of the rats to perform at better than a 75% rate in both parameters. Body weights were recorded both before and after running and each animal was necropsied after the program was completed. Multiple tissues were taken for histopathologic examination and muscles from the rear limbs (*gastrocnemius*, *soleus* and *plantaris*) were frozen for chemical and histochemical analyses.

Histologic Techniques

All experimental animals were killed by decapitation and exsanguination and examined grossly for lesions. Multiple tissues were taken at time of necropsy for histologic examination and placed either in Zenker's

fixative or in 10% neutral buffered formalin. After 24 to 48 hours tissues were retrimmed, embedded in paraffin, sectioned, and stained with hematoxylin and eosin, Giemsa, Gomori's trichrome and the periodic acid-Schiff reaction (PAS). All procedures were those recommended in the *Manual of Histologic Staining Methods* of the Armed Forces Institute of Pathology (1968).

Histochemical procedures were performed on muscles removed and frozen immediately after decapitation. The rear legs were skinned and the superficial posterior crural muscles were exposed by reflecting overlying tissue. The right *gastrocnemius, soleus* and *plantaris* were removed as one unit, rolled in talcum powder and lowered with forceps into isopentane for approximately 60 seconds. The isopentane was precooled to -140 C. to -185 C. by liquid nitrogen. The frozen muscle was stored in individual sealed containers at -20 C. At a later, more convenient time small sandwich blocks (10 mm thick) were cut from the midsection of each muscle unit with a precooled microtome knife. These smaller blocks were immediately mounted on chucks with 5% gum tragacanth. Serial cross sections 10 microns thick were then prepared using a rotary microtome-cryostat.* The sections were mounted on glass coverslips and air dried.

The following histochemical procedures were applied to sections from each muscle unit: phosphorylase (PPL), by the method of Takeuchi (1958); succinate dehydrogenase (SDH), by the method of Barka and Anderson (1963); intermyofibrillar adenosine triphosphatase (ATPase), by the method of Wachstein and Meisel (1957) as described by Thompson and Hunt (1966); cytochrome oxidase (CYO), by the method of Burstone

*International-Harris Microtome-Cryostate, Model CTI International (IEC) Equipment Co., Needham Heights, Massachusetts.

(1959) as modified by Pearse (1960); and lactic dehydrogenase (LDH), by the method of Hess *et al.* (1958) as described by Pearse (1960). Sections were also stained with Sudan black B (Lillie, 1954), Gomori's trichrome (Engle and Cunningham, 1963), and with Giemsa, PAS and hematoxylin (Harris alum) and eosin as described in the *Manual of Histologic Staining Methods* of the Armed Forces Institute of Pathology (1968).

Tissue morphology and staining reactions were evaluated by light microscopy. In addition, specific areas of these muscle units were selected for more critical study. The areas selected corresponded to foci where the fibers were predominantly red (Zone-I), white (Zone-II) or were of intermediate (Zone-III) fiber type (Figure 1). In these zones the intensity of staining was determined by the percent light transmitted through each of 30 fibers in the zone. To be sure of identifying the same fibers in each stain, the hematoxylin and eosin stained sections were projected with a microprojector* on a white sheet of paper (x200) and at least 30 cross sections of adjacent individual fibers were sketched. These drawings were then used to identify individual and groups of fibers. The percent of transmitted light was then determined by projecting each muscle fiber in an area over a photocube coupled to a digital readout (Wells *et al.*, 1972). The fiber sketches were also used to determine mean fiber area after tracing with a compensating polar planimeter.** Fiber area was read in square centimeters but, because of the initial magnification, divided by 40,000 to indicate actual fiber diameter in square centimeters.

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*Prado Universal, Ernst Leitz GMBH, Wetzlar, Germany.

**Keuffel and Esser Co., New York.

Statistical Analyses

Data were analyzed statistically by methods described by Lewis (1966).

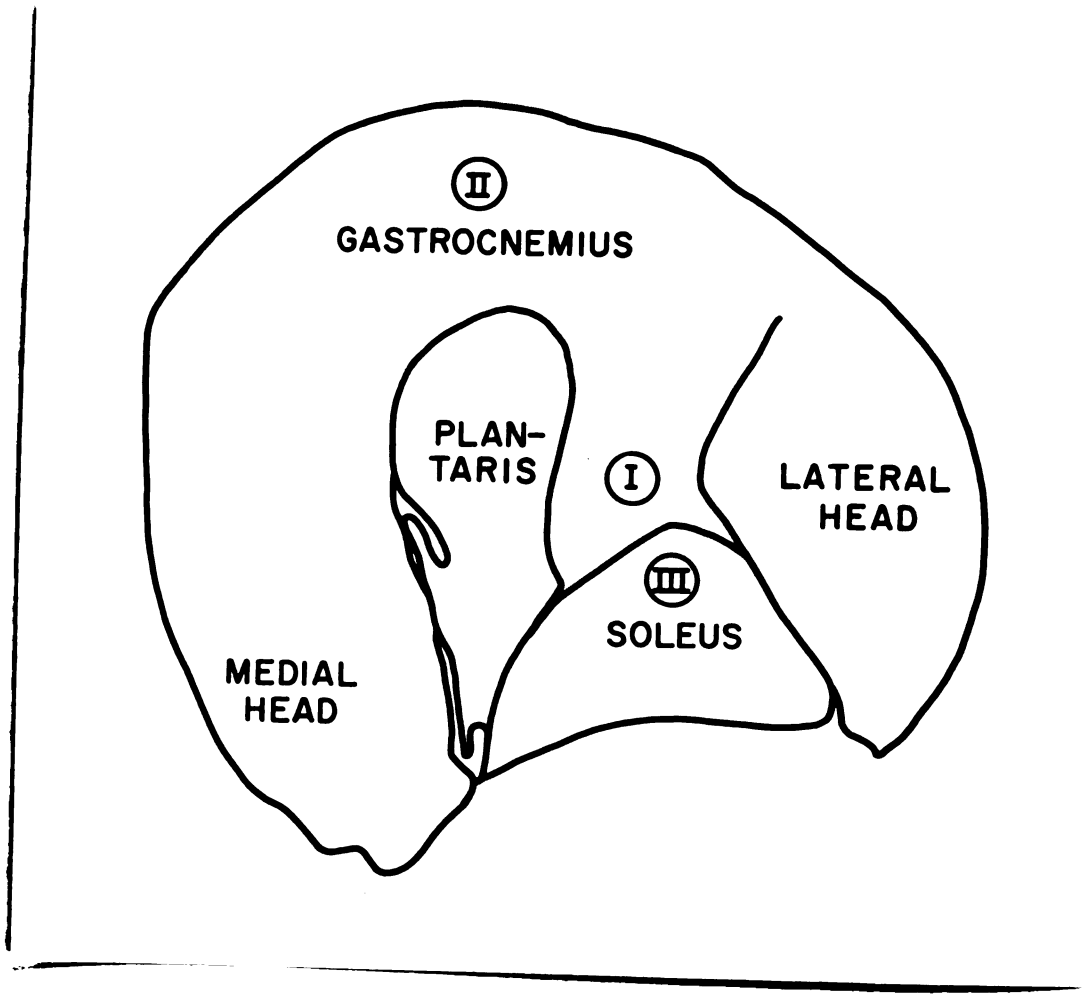

Figure 1. Diagrammatic cross section of muscle unit from the rear leg of a rat. Specific regions studied are designated by Roman numerals. Zone I contained predominantly red (Type I) fibers, Zone II contained predominantly white (Type II) fibers, and Zone III contained fibers classified as intermediate (from Edgerton *et al.*, 1969).

RESULTS

Feeding Trials

Pregnant Females. Eight pregnant rats (first litter) were fed various levels of B-GPA during the last 6 to 8 days of pregnancy and during lactation, as outlined in Table 1. Three others that received no B-GPA served as controls. The females fed 1 or 2% B-GPA in a commercial laboratory chow failed to consume adequate amounts of the diet to maintain normal weight gains. Therefore, 4 days after parturition, 2 of the rats that had received 1% B-GPA were changed to a 0.5% level for the remainder of the test period. Only 1 female that had received 1 or 2% B-GPA weaned any pups. The other pups were either eaten by the mother or died--apparently from starvation. The effects of feeding B-GPA to pregnant female rats is summarized in Table 1. Pregnant rats receiving B-GPA gained less weight during the last 7 days of pregnancy, gave birth to fewer pups and weaned fewer pups than did the control animals. In addition, there was a marked effect on creatine metabolism, as indicated by the drop in levels of muscle creatine and the increase in the urinary creatine : creatinine ratio.

Young Male Rats. Since pregnant rats ate the diet containing 1 or 2% B-GPA reluctantly, 2 paired-feeding trials were conducted to determine the effect of B-GPA of food intake in younger rats. In the first, 10 28-day-old rats were divided into 3 groups. Group I, containing 4 rats, received 1% B-GPA in a commercial ration for 8 days followed by 2%

Table 1. Effects of feeding B-GPA upon weight gain, number and weight of offspring and creatine metabolism of pregnant rats.†

Wt. gain last 7 days of pregnancy	Number pups in litter and (average wt.)	Number pups weaned and (average wt.)	Muscle creatine (mg/g) and no. days on test	Urine $\frac{\text{Creatine}}{\text{Creatinine}}$ Ratio*
			Control Diet	
+122	16 (6.4g)	7 (41.3g)**	4.38 (34 days)	0.515
+112	14 (6.8g)	4 (41.5g)**	4.64 (34 days)	0.576
+119	13 (9.3g)	12 (41.2g)	5.42 (128 days)	
			0.5% B-GPA	
+ 66	10 (7.8g)	6 (27.9g)	1.88 (128 days)	
+ 71	10 (7.0g)	9 (35.4g)	2.23 (128 days)	
			1.0% B-GPA	
+ 58	7 (5.7g)	none	1.10 (34 days)	3.98
+ 63	10 (5.0g)	none	1.02 (34 days)	3.89
+ 58	9 (7.2g)	none***	1.77 (10 days 1%-122 days at 0.5%)	
+ 44	9 (6.9g)	9 (29.9g)***	1.86 (10 days 1%-122 days at 0.5%)	
			2.0% B-GPA	
+ 10	14 (3.8g)	none	1.04 (39 days)	3.30
+ 18	8 (5.2g)	none	1.42 (35 days)	3.25

†Sixteen days after mating rats were fed a commercial ration either with or without B-GPA.

*Mean of at least 2 determinations on different 24-hr. urine samples during test period.

**Males only - females sacrificed before weaning.

***Changed to 0.5% B-GPA 4 days after parturition.

B-GPA for 6 days. Group II, which also contained 4 animals, were pair-fed the same diet but without B-GPA. The remaining 2 rats (Group III) were fed the same diet without B-GPA but in *ad libitum* quantities. There was no significant difference in the average daily body weight gain or in the average daily food intake of the groups of rats in this study (Table 2, Part A).

The second paired-feeding trial included 17 21-day-old male rats that were divided into 3 groups. Beta-guanidinoproprionic acid was mixed into a purified ration (Fitch *et al.*, 1960) at the 1% level and was fed for 21 days. As in the first study, Group I (6 rats) received diet containing B-GPA, Group II (6 rats) was pair-fed with Group I but received no B-GPA, and Group III (5 rats) served as *ad libitum* controls. Data from this study are given in Table 2, Part B. The rats fed B-GPA did not have a significantly different mean daily food intake or weight gain than rats in the control groups.

Chemical Analyses

Urine Excretion of Creatine and Creatinine. Urine excretion of creatine and creatinine was determined at selected intervals during all feeding trials as well as on samples from animals used in the running experiments. The pregnant females were removed from their cages and placed in metabolism cages for 24 hours for urine collection. Feeding trials in younger rats were conducted in metabolism cages and 24-hour urine collections were made without moving them. In every case, feeding B-GPA significantly increased urinary levels of creatine, decreased the excretion of creatinine, and therefore increased the urinary creatine : creatinine ratio. These data are summarized in Table 3.

Table 2. Effects of feeding B-GPA upon body weight and food intake of young male rats

Group	Average daily weight gain*	Average daily food intake*
Part A: Commercial Laboratory Diet		
Data for 14-day feeding trial:		
I (4)**	6.52 ± .50	18.0 ± 1.20
II (4)	7.04 ± .29	17.8 ± .87
III (2)	7.57	19.1
Data for 8 days - fed 1% B-GPA:		
I	7.84 ± .81	17.6 ± 2.10
II	8.12 ± .82	16.9 ± 1.20
III	9.18	18.6
Data for 6 days - fed 2% B-GPA:		
I	4.75 ± .58	18.7 ± .39
II	5.58 ± 1.10	18.5 ± .51
III	5.45	19.8
Part B: Purified Ingredient Diet		
Data for 20 days - fed 1% B-GPA:		
I (6)	5.76 ± .69	12.6 ± 1.1
II (6)	4.87 ± .67***	10.7 ± 1.3
III (5)	5.53 ± .35	12.0 ± .8

*Mean values ± S.D. of mean in g per day.

**Number in parentheses indicates no. of rats in group.

***Probability = .3>P>.2; between this value and mean from Group I of this trial.

Table 3. Effect of feeding B-GPA on indices of creatine metabolism of young male rats[†]

Analysis	Diet	Postweaning Days on Test			
		7th day	14th day	50th day***	75th day**
Urine Creatine (mg/ml)	B-GPA	1.47±.10(4)	1.26±.14(10)*	0.84±.03(7)	0.73±.05(8)
	Control	0.40±.05(6)	0.37±.04(19)	0.10±.01(9)	0.29±.05(4)
Urine Creatinine (mg/ml)	B-GPA	0.43±.01(4)	0.34±.07(10)*	0.21±.02(7)	0.29±.03(8)
	Control	0.73±.06(6)	0.80±.04(19)	0.62±.07(9)	0.82±.06(4)
$\frac{\text{Creatine}}{\text{Creatinine}}$ ratio	B-GPA	3.38±.18(4)	4.63±.57(10)*	4.25±.48(8)	2.55±.12(8)
	Control	0.56±.07(6)	0.47±.04(19)	0.19±.03(9)	0.35±.06(4)
Muscle Creatine (mg/g)	B-GPA		1.43±.01(6)	0.82±.01(6)	1.08±.02(8)
	Control		3.35±.16(6)	4.94±.24(5)	4.87±.01(4)
Muscle B-GPA (mg/g)	B-GPA		5.07±.69(4)*	4.84±.20(6)	4.34±.18(8)

[†]Urine values were obtained from 24 hr. urine samples. Results are mean values ± S.E. The number of rats tested is given in parentheses. All animals were fed 1% B-GPA except as noted.

*Four rats fed 1% B-GPA for 8 days and 2% for 6 days.

**Dams fed 0.5% B-GPA during nursing period.

***These received purified ingredient diet.

Muscle Creatine. Muscle creatine was determined on the *triceps brachii* of male rats that had received B-GPA for 14, 50 and 75 days postweaning. In each case, the muscles from rats that had been fed B-GPA contained less creatine than did the muscles from rats that were fed diets containing no B-GPA (Table 3). Muscle creatine levels decreased to approximately 1.0 mg/g of muscle in rats fed B-GPA for 50 days and, in spite of continued feeding, the values did not decrease further.

The values for muscle creatine given in Table 3 were estimated by the alkaline-picrate method. Muscles from rats fed B-GPA for 75 days were also analyzed for creatine by the enzymatic assay of Tanzer and Gilvarg (1959). The mean values for the alkaline-picrate method were 4.87 mg/g muscle for control rats and 1.08 mg/g muscle for the rats fed B-GPA. Mean values using the enzymatic assay on normal and test rats were 4.54 mg/g muscle and 0.73 mg/g muscle, respectively. Thus, there was close agreement between the 2 methods.

Muscle creatine was also determined on small samples of muscles taken from areas of the *gastrocnemius* that contained either predominantly red (Zone I) or predominantly white (Zone II) type fibers. The samples were taken from rats that had received 1% B-GPA in a purified ration for 50 days. The results, summarized in Table 4, indicate that the white fibers normally contain more creatine than the red type and that feeding B-GPA resulted in proportionally lower levels of creatine in both fiber types.

Exercise did not seem to affect the amount of creatine in the muscles of rats. Muscle creatine levels of rats that were used in the running experiments were compared to those of rats of the same age that had not been forcibly run. Four exercised control rats had a mean muscle creatine level of 4.87 $\pm$ .02 S.D. mg/g muscle compared to that

Table 4. Effects of feeding B-GPA on muscle creatine concentration in predominantly red and white fiber areas of the rat *gastrocnemius*

Rat No.	Diet	Muscle creatine (mg/g wet wt.) Red fiber area	White fiber area
1	1% B-GPA*	0.920	1.051
2	1% B-GPA	0.980	.935
3	1% B-GPA	0.540	.669
4	1% B-GPA	0.478	.717
5	1% B-GPA	0.544	.560
6	1% B-GPA	0.462	.707
	Mean =	0.654 ± .23**	0.773 ± .18
13	Control	5.647	7.159
14	Control	4.389	5.526
15	Control	5.066	6.095
16	Control	4.374	5.711
17	Control	4.553	5.838
18	Control	5.235	5.329
	Mean =	4.877 ± .52	5.943 ± .65

Summary:

(a) Control: white fiber areas = 5.943 ± .65
red fiber areas = 4.877 ± .52 [P < .05]

(b) Effect of B-GPA:
white fiber areas - 87% decrease in creatine
red fiber areas - 87% decrease in creatine

*Animals fed 1% B-GPA in a purified ingredient diet for 50 days.

**Mean ± S.D. of mean.

of 4.93 for 2 sedimentary controls. Of the groups that had received B-GPA, the 8 exercised rats had a mean muscle creatine of 1.08 ± .05 S.D. and 5 non-exercised rats had a mean of 0.99 ± .63 S.D. mg/g muscle.

Brain Creatine. Although most of the body's creatine is in skeletal muscle, significant quantities are present in nerve tissue (Wang, 1939). It was of interest, therefore, to determine if feeding B-GPA to rats would also lessen the concentration of creatine within brain tissue. Analyses were done by the enzymatic assay on frozen brains from control rats as well as rats that had been fed B-GPA for 50 and for 75 days. These data (Table 5) indicate that feeding B-GPA will also effectively lower brain creatine concentration.

Muscle B-GPA. Muscle levels of B-GPA were determined on the *triceps brachii* of rats fed the experimental rations for 14, 50 and 75 days. The results, which are included in Table 3, indicate that significant quantities of B-GPA would accumulate in muscles when it was included in the diet at the 1% level.

The Sakaguchi reaction used for these analyses is not specific for B-GPA but only for a monosubstituted guanidine (Gilboe and Williams, 1955). Thus arginine and other monosubstituted guanidines present in the water extracts of muscle might be expected to react positively. Because the concentration of free arginine in muscle is relatively low (approximately 0.25 μM/g, Rogers and Harper [1968]), it was possible to dilute the muscle extracts and obtain virtually a zero optical density reading on control samples while still maintaining an adequate reading from the samples of muscle taken from animals fed B-GPA. To confirm that the reacting compound was B-GPA, aliquots of extracts were compared

Table 5. Effects of feeding B-GPA to rats on the concentration of creatine in the brain

Animal No.	Diet	Days fed Diet	Brain Creatine (mg/g wet weight) ± S.D. of mean
2/2	1% B-GPA	75	0.975
3/2	1% B-GPA	75	1.040
3/4	1% B-GPA	75	0.970
8	1% B-GPA	75	1.003
11	1% B-GPA	75	0.830
13	1% B-GPA	75	0.910
15	1% B-GPA	75	1.050
			(Mean = 0.968 ± .08)
2	1% B-GPA	50	1.310
4	1% B-GPA	50	0.810
6	1% B-GPA	50	0.974
			(Mean = 1.031 ± .26)
	Mean for all rats fed B-GPA = 0.987 ± .14*		
2	Control	75	1.250
5	Control	75	1.485
			(Mean = 1.368)
14	Control	50	1.340
16	Control	50	1.400
18	Control	50	1.418
			(Mean = 1.386)
	Mean for all rats fed control diets = 1.379 ± .09*		

*P < .001.

to standard solutions of B-GPA, creatine, creatinine and arginine by paper chromatography using 2 different solvent systems.

Running Performance

Sixteen 68-day-old male rats were used in these experiments. Ten were raised by females that had received 0.5% B-GPA during pregnancy and lactation. These 10 were then continued from weaning on 1% B-GPA. Six rats of the same age but which had received no B-GPA served as controls. All rats were exercised in the controlled running wheel of Wells and Heusner (1971). After a 4-day training period, 2 rats from each group were removed on the basis of their performance and the remaining 12 rats (8-test and 4-controls) were run according to the 4-week running program given in Appendix A. Data pertaining to body weight gain during these experiments are given in Appendix B. The final mean body weight and the average daily weight gain for the rats that were fed B-GPA were slightly less than for control animals; nevertheless there was overlap in these data.

The running performance of the rats in this study was evaluated in terms of the percent of expected revolutions (PER) and by percent of shock free time (PSF). These results are expressed graphically in Figures 2 and 3, respectively. The expected daily PER of 75% was accomplished by 3 of the 4 rats in the control group and therefore the overall group performance was within the expected range of 75%. Four of the animals fed B-GPA (50%) performed below 75% PER on more than 5 (75%) days, indicating that the group performance was less than the expected 75% PER. A critical comparison of these groups is not possible because of the small numbers of rats tested. Individual rats in the test group, however, performed as well or better than individuals in

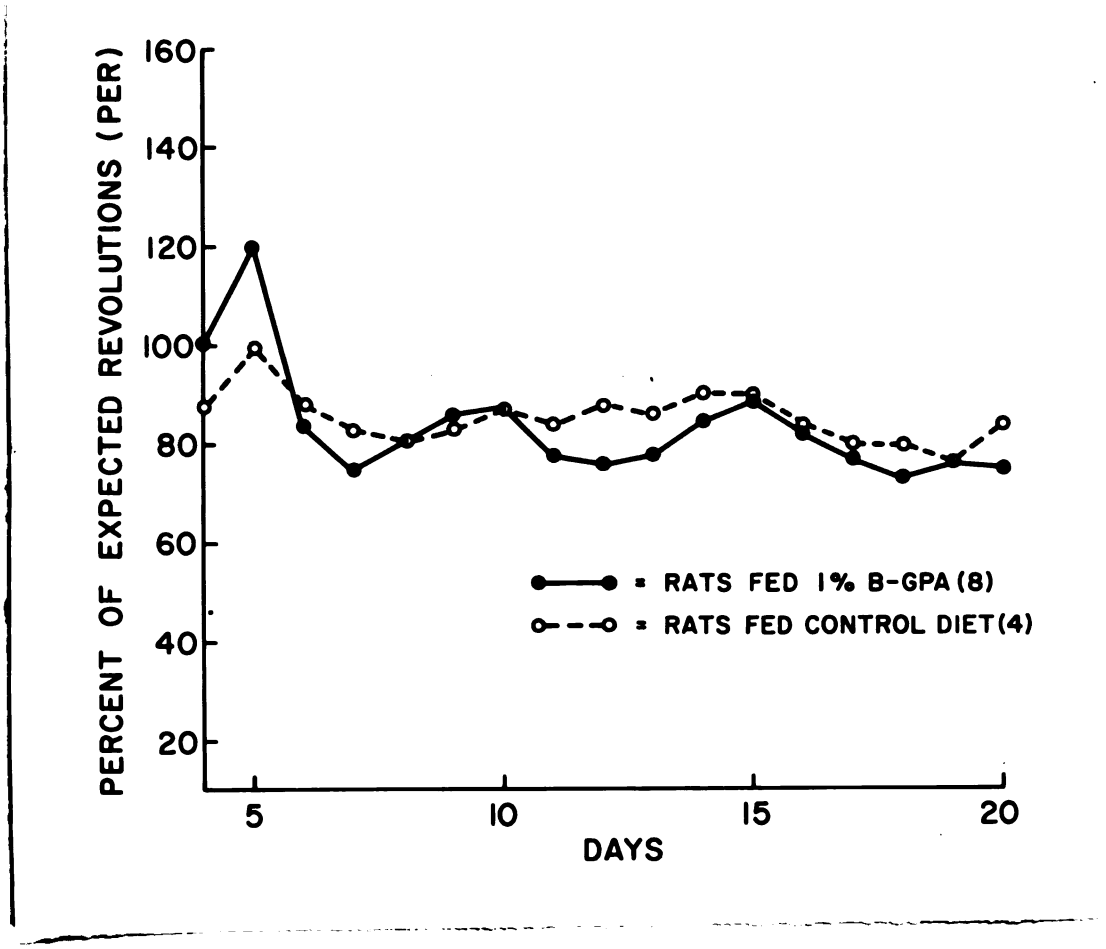

Figure 2. The effect of feeding B-GPA on the running performance of rats expressed as the percent of expected revolutions. The number of rats is given in parentheses.

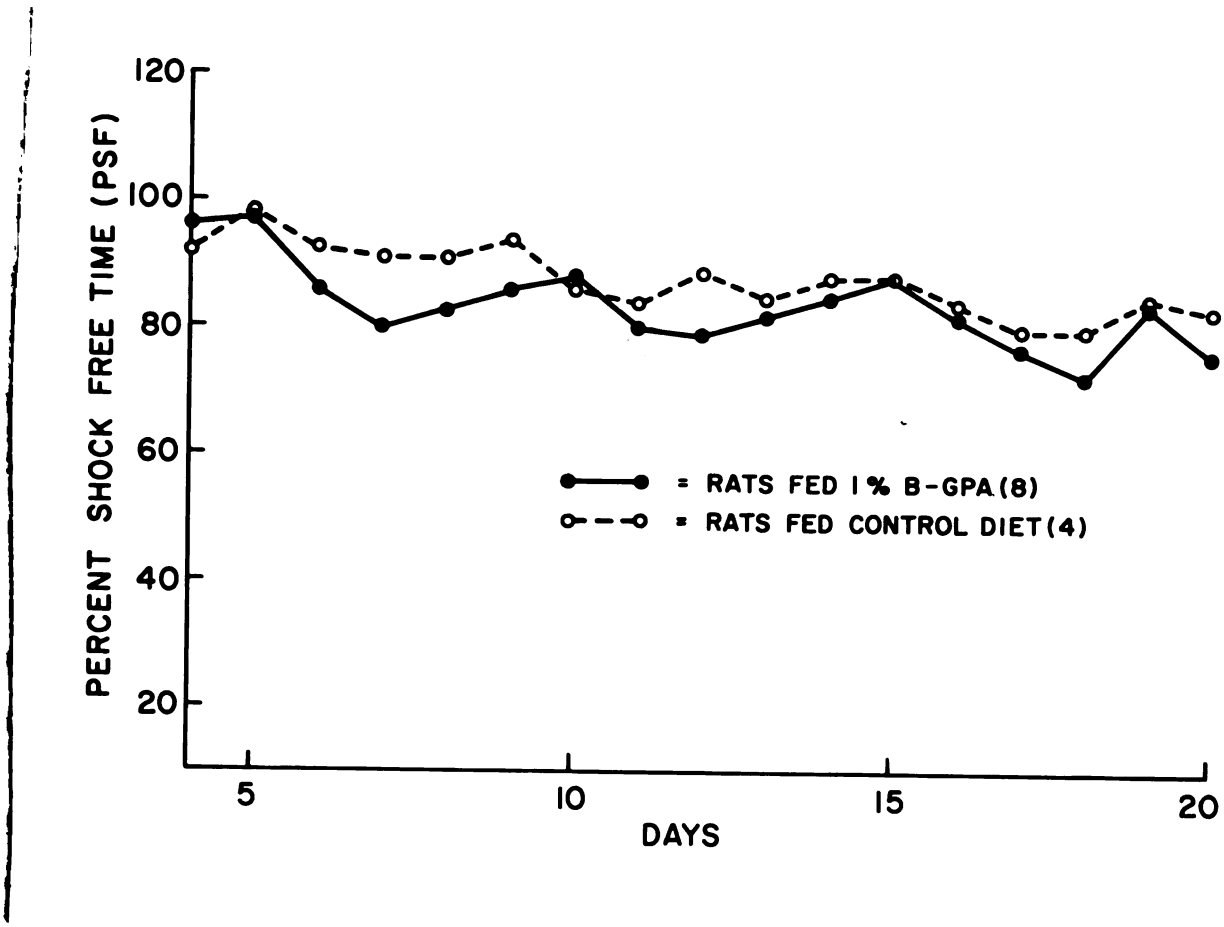

Figure 3. The effect of feeding B-GPA on the running performance as expressed as the percent of shock free time. Number of animals is given in parentheses.

the control group in spite of the fact that their muscles contained less than the normal amount of creatine.

Histologic Results

Histologic studies consisted of (1) the examination of multiple paraffin-embedded tissues taken at time of euthanasia; (2) the histochemical analysis of frozen muscle sections; and (3) the measurement of individual muscle fiber cross-sectional area.

Paraffin-embedded Tissue. Multiple tissues were taken from all animals and embedded in paraffin. Sections were prepared from the following organs: liver, kidney, heart, lungs, testicle, thyroid, intestine, thymus, spleen, and the *triceps brachii*, *psoas major*, and *tibialis anterialis* muscles. Paraffin sections were also prepared from the *gastrocnemius* from all animals except those in the performance studies. At the time of necropsy the brains of experimental animals were removed whole and either frozen or fixed in formalin.

No significant histological changes were observed in sections from paraffin-embedded tissues when stained with hematoxylin and eosin, Giemsa, Gomori's trichrome, or PAS.

Frozen Tissue. Muscles (*gastrocnemius*, *soleus* and *plantaris*) were removed as a unit and rapidly frozen. Subsequently, serial sections prepared from these tissues were stained with a series of histochemical and conventional stains. Some of the differential staining reactions of the 3 fiber types studied are presented in Figures 4, 5 and 6.

The staining reactions of red (Zone I) and white (Zone II) fibers were compared by determining the percent of light transmitted through 30 adjacent fibers in each zone. These data are summarized in Table 6.

Figure 4. Transverse section of muscle unit from the rear leg of a normal rat stained for LDH activity. Section includes parts of Zone I (a), Zone III (b) and an area containing many pale Zone II type fibers (c). x 75.

Figure 5. Transverse section of muscle unit from the rear leg of a normal rat stained for myofibrillar ATPase activity. Areas identified by letters are given in the legend of Figure 4. x 75.

Figure 6. Transverse section of muscle unit from the rear leg of a normal rat stained for PPL activity. Areas marked by letters are given in the legend of Figure 4. x 75.

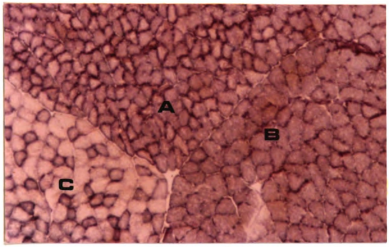

Figure 4

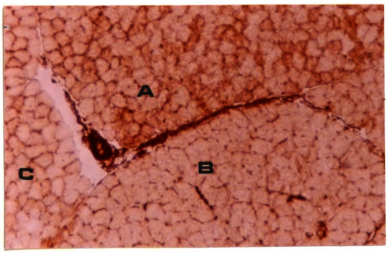

Figure 5

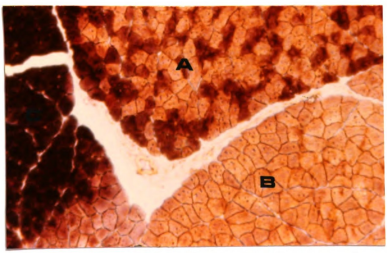

Figure 6

Table 6. Effect of exercise and B-GPA on the histochemical reaction in predominantly red and white fiber areas of the *gastrocnemius*

Stain	Muscle Zone*	% light transmitted**	
		1% B-GPA(5)	Control(4)
PAS	I	43±10.6	55±12.9
	II	41± 5.8	60± 6.2
PPL	I	26± 8.1	20± 6.8
	II	6± 2.6	11± 4.8
LDH	I	40± 0.9	42± 3.2
	II	68± 5.1	70± 6.6
ATPase	I	50± 2.7	53± 6.5
	II	66± 4.4	68± 6.2
SDH	I	35± 7.8	34± 3.0
	II	54± 3.3	52± 2.4
Cy-O	I	75± 2.4	80± 4.7
	II	76± 4.0	81± 0.7
Sudan	I	80± 2.4	79± 5.0
	II	82± 4.1	85± 2.8

*Zone I=predominantly red fibers, Zone II=predominantly white fibers. See Materials and Methods.

**% light transmitted through 30 individual fibers in each area and from each animal. Values given are means ± S.D. Numbers in parentheses indicate number of animals tested.

The red fibers of control rats stained more intensely (lower % transmission) with stains for SDH, LDH, and ATPase activity and possibly with the Sudan stain. White type fibers stained more intensely with the reaction for PPL. The staining of red and white fibers from rats fed B-GPA was, with one exception, the same as was observed in the control animals. The only exception was the PAS reaction which, in Zone II, was slightly darker in the rats fed B-GPA. Thus, in these studies both the red and white type fibers stained normally (Engle, 1970) and no significant changes were detected in animals fed B-GPA. Typical staining reactions of fibers from Zone II of test and control rats are illustrated by Figures 7 through 10.

Fiber Measurements. The cross-sectional area of 30 adjacent muscles was determined in each of the 3 different zones of the *gastrocnemius* that are shown in Figure 1. The mean cross-sectional area of fibers from each zone is given in Table 7. Fibers in Zone I (predominantly red) were approximately the same size in test and control rats and were the smallest of the fibers measured. The fibers in Zone II (predominantly white) were the largest and the white fibers from the control rats were larger than those from rats receiving B-GPA. Zone III fibers were intermediate in mean fiber area and also somewhat larger in the control animals.

Since the mean body weight of the rats that received B-GPA was slightly less than the mean body weight of control rats, a ratio was calculated which consisted of the mean fiber area divided by the individual rat body weight. These data are given in Table 7 and support the conclusion that fibers in Zone II of test rats were proportionally smaller than those in Zone II of the animals that did not receive B-GPA.

Figure 7.* Cross section of muscle fibers in Zone II of the *gastrocnemius* of exercised rats stained for LDH activity. Figure 7a was made from muscle of a control rat and Figure 7b was prepared from a rat fed 1% B-GPA for 75 days. White fibers stain lighter than other fiber types. x 189.

Figure 8.* Cross section of muscle fibers in Zone II of the *gastrocnemius* of exercised rats stained for PPL activity. Figure 8a, control; Figure 8b, test. White fibers stain darker than other fiber types. x 189.

Figure 9.* Cross section of muscle fibers in Zone II of the *gastrocnemius* of exercised rats stained for myofibrillar ATPase activity. Figure 9a, control; Figure 9b, test. White fibers stain lighter than other fiber types. x 189.

Figure 10. Cross section of muscle fibers in Zone II of the *gastrocnemius* of exercised rats stained with PAS. White fibers stain darker than other fiber types and the intensity of staining in the test rat (10b) is greater than in the fibers from the control rat (10a). x 189.

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*These sections are serial sections taken from control rat #2 and test rat #8 (see Appendix).

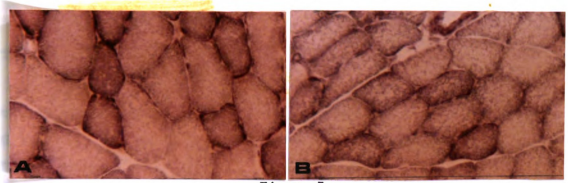

Figure 7

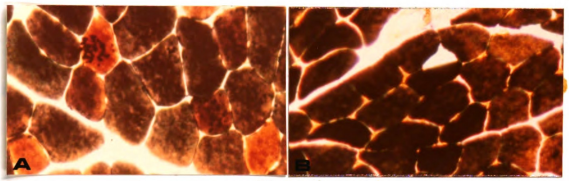

Figure 8

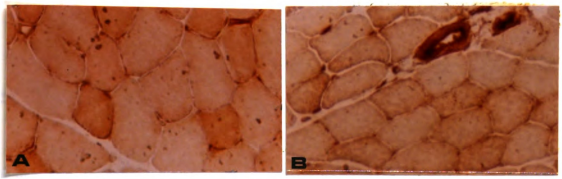

Figure 9

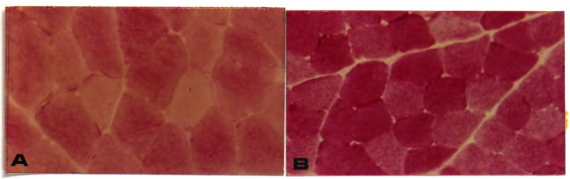

Figure 10

Table 7. The effects of feeding B-GPA on muscle fiber size of exercised rats††

Group Treatment	Zone I	Zone II	Zone III
A. Mean Fiber Area*			
Control (4)†	1.18±0.3[a]	0.86±.009[b]	0.56±.004
Fed 1% B-GPA (5)	0.61±0.1[a]	0.64±.004[b]	0.49±.004
B. Mean Fiber Area/Total Body Weight†††			
Control (4)	3.96±.27[c]	2.87±.14	1.90
Fed 1% B-GPA (5)	2.27±.11[c]	2.46±.90	1.85

[a] $P < .001$.

[b] $P < .005$.

[c] $P < .001$.

*Area expressed in sq.cm. x 2.5 x 10^{-5} and are mean values ± S.D.

†Number in parentheses = number of animals tested.

††From dams that received 0.5% B-GPA during nursing and fed 1% B-GPA for 75 days. Exercised for 20 days in running wheel.

†††Figures given are mean values ± S.D. in sq.cm. x 2.5 x 10^{-8}.

The small difference in mean area of fibers in Zone III was not evident when divided by body weight. Thus, the only real difference in fiber size between test and control animals would appear to be in Zone II. This difference in fiber size is visible in Figure 12.

Creatine Phosphokinase Results

It had been previously reported (Fitch *et al.*, 1968) that B-GPA would not serve as substrate for rabbit muscle CPK in the *in vitro* assay of Tanzer and Gilvarg (1959). This fact was confirmed during the course of these studies. In addition, it was found that B-GPA would not interfere with the reaction of creatine under the assay conditions. These data, however, may not apply to rat muscle CPK. Under the assay conditions the enzyme is not saturated with substrate which would limit one's ability to detect interference between B-GPA and creatine for the active site of the enzyme. Because the mediated entry of creatine into rat muscle from plasma could involve the CPK enzyme, studies were initiated to determine the effect of feeding B-GPA on the activity of rat muscle creatine phosphokinase and to study the *in vitro* reaction of creatine and B-GPA with this enzyme. The results given in Table 8 indicate that rats fed B-GPA have lower muscle CPK activity and that B-GPA effectively inhibits the reaction of creatine with the enzyme.

Figure 11. Cross section of muscle fibers in Zone I of the *gastrocnemius* of exercised rats. Cross-sectional areas are similar in control animal #2 (11a) and animal fed 1% B-GPA, #8 (11b). H & E stain. x 189.

Figure 12. Cross section of muscle fibers in Zone II of the *gastrocnemius* of exercised rats. The cross-sectional area of the fibers from control rat #2 (12a) is greater than that of test rat #8 (12b). x 198.

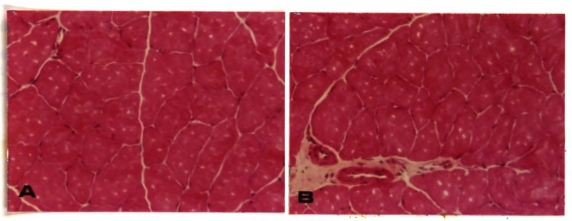

Figure 11

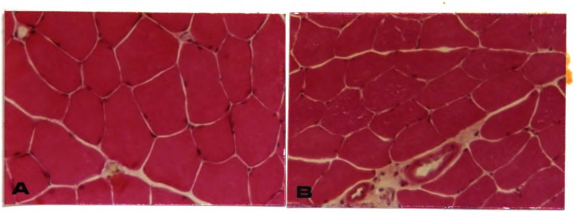

Figure 12

Table 8. CPK activity of rats fed B-GPA and effects of B-GPA on rat CPK activity *in vitro*

PART A: Muscle CPK activity of normal rats and rats fed 1% B-GPA*

mM Creatine in Assay		Activity** + B-GPA	Activity** Control
42		6.96	8.18
		6.31	7.77
			9.91
	Mean	6.64	8.62
25		3.08	5.31
		3.00	3.60
	Mean	3.04	4.45

PART B: Effect of B-GPA (52.5 mM) on rat CPK activity *in vitro*

mM Creatine in Assay	Activity Without B-GPA	Activity With B-GPA
25.20	3.60	3.39
18.90	3.17	---
12.60	2.94	2.14
7.65	2.13	1.48
6.30	2.00	1.44
2.52	1.09	.75

*Fed 1% B-GPA for 75 postweaning days.

**Activity expressed as μmoles creatine phosphorylated/minute/mg protein in extract.

DISCUSSION

The ultimate objective of these studies was to provide a better understanding of the relationship between creatine metabolism and skeletal muscle function and structure. To this end, muscle function and structure were evaluated after abnormal creatine metabolism was induced by feeding B-GPA. There were, therefore, 2 separate but interrelated phases of this research. One served to monitor creatine metabolism and in the other muscle function and structure were evaluated. To facilitate an analysis of the data obtained, this discussion has therefore been divided into 2 parts. In the first part, the data pertaining to the effects of feeding B-GPA on creatine metabolism will be considered. The second part of this discussion will deal more specifically with the effects of abnormal creatine metabolism on skeletal muscle function and structure.

The Effects of Feeding B-GPA on Creatine Metabolism of the Rat

To accurately evaluate the effects of any dietary supplement it must be ascertained if the addition of the test compound to the diet will alter or otherwise modify the intake of other essential dietary ingredients. During the studies using pregnant rats, a marked difference in food intake was observed when 1 or 2% B-GPA was included in the diet. With the possible exception of a significant effect on creatine metabolism, it is believed that most of the effects observed when pregnant rats were fed B-GPA were manifestations of a reduced food intake.

In contrast to the studies in mature female rats, the inclusion of 1 or 2% B-GPA did not alter the food intake in young male rats. There was no significant difference in mean daily body weight gain or in mean daily food intake when young rats were pair-fed diets containing B-GPA for periods of up to 21 days. Food intake was not measured over longer periods; nevertheless, the body weights of rats fed 1% B-GPA for 75 days were slightly less than those of rats that did not receive B-GPA (Appendix B). The difference in these 2 groups might represent a moderate, long-term effect on food intake; or, in light of the smaller size of the Type II fibers in the rats fed B-GPA, the difference in body weight might be a reflection of the failure of these white fibers to hypertrophy. Since there are no estimates of the size of the total-body white fiber population, this latter explanation would be difficult to evaluate further. With this one possible exception, it would appear that feeding B-GPA to young male rats had no significant effects on food intake. It would, therefore, be reasonable to conclude that any effects of B-GPA such as those on creatine metabolism occur directly because of the presence of B-GPA in the diet and are probably not due to a secondary effect on food intake.

All tests employed in this research to monitor the effects of B-GPA indicated that there were marked changes in creatine metabolism. The alterations were manifested by a decrease in muscle and brain levels of creatine, an excessive creatinuria and a diminished excretion of creatinine in the urine.

The mechanism(s) by which B-GPA alters creatine metabolism is probably complex; however, based on these studies and previous data the most plausible explanation would be that it inhibits creatine entry from plasma into muscle. The *in vitro* studies of Fitch and Shields

(1966) and by Fitch *et al.* (1968b) indicate that creatine enters rat skeletal muscle by a mediated system and that B-GPA will competitively inhibit this entry. When rats that have been fed B-GPA are injected with a small dose of creatine-1-14C, less radioactive creatine accumulates in their muscles and a greater portion is excreted in the urine. These results were attributed to the ability of B-GPA to inhibit the entry of the radioactive creatine into muscle from plasma *in vitro* (Shields and Whitehair, 1972). In these present studies, brain levels of creatine as well as creatine levels in muscle were significantly lowered when rats were fed B-GPA. This suggests that the same entry system may also account for a portion of the normal brain complement of creatine. Moreover, it is tempting to regard a mediated system of creatine entry from plasma as of practical significance for many body tissues.

It has been estimated that between 60 and 80% of the creatine in muscle is in the form of N-phosphorylcreatine. When muscle creatine is lowered to only 25% of its normal concentration as in these present studies, the muscle concentration of N-phosphorylcreatine must also be significantly lower than normal. This conclusion has been recently substantiated by Jellinek and Fitch (1972). In addition, they found that after feeding rats 1% B-GPA for 26 days, muscle ATP, ADP and phosphoenolpyruvate (PEP) levels were also significantly lower than normal.

The inability to lower tissue levels of creatine appreciably below 1.0 mg/g tends to support the theory that more than one entry system is involved in the entry of creatine into muscle (Fitch and Shields, 1966). Based on *in vitro* data a small increment of creatine enters muscle by simple diffusion. At physiologic plasma levels of creatine (ca. 0.1 mM),

however, this mechanism alone might not be capable of maintaining an intracellular concentration of approximately 7.6 mM. Entry by diffusion plus intracellular binding, as has been proposed (Fitch and Sinton, 1964), might be a better explanation.

The excessive excretion of creatine in the urine that developed in all animals fed B-GPA could have resulted from (a) a blockage of renal reabsorption, (b) an excessive loss of creatine from tissues, (c) an increase in the synthesis of creatine and/or (d) a blockage of creatine entry into tissue. There is no evidence to implicate any of these mechanisms except that already presented regarding the blockage of creatine entry into muscle. The reabsorption of creatine by the kidney has been reported (Gayer and Kienitz, 1962), but the specificity of this mechanism has not been studied in detail. Since most mechanisms at renal reabsorption, however, are subject to some analog competition (West *et al.*, 1966), it would seem possible that B-GPA could inhibit creatine reabsorption and result in creatinuria. Of the mechanisms proposed, only an increase in the synthesis of creatine might have been capable of maintaining normal tissue creatine levels. In this research, however, normal muscle creatine values were not maintained regardless of the status of creatine synthesis.

The excretion of creatinine in the urine of all rats fed B-GPA was decreased. Since urine creatinine is derived primarily from muscle creatine (Block and Schoenheimer, 1939, 1941; Borsook and Dubnoff, 1947), a depletion of muscle creatine concentration should result in a decreased creatinine excretion in the urine. Patients with myopathy and concomitant low levels of muscle creatine also exhibit this same change (Milhorat, 1953).

Although creatine entry into skeletal muscle by a mediated system has been proposed (Fitch and Shields, 1966), there has been no indication of the specific mechanism involved. The CPK studies of this research suggest that this enzyme may be at least a part of the creatine entry mechanism. The phosphorylation of creatine during transport across a cellular membrane would possibly remove the then intracellular phosphorylated creatine from a gradient against entry. The conformational changes that reportedly occur during the reaction of this enzyme with its substrates (James and Morrison, 1966), as well as the fact that most CPK is bound to cellular membranes (Baskin and Deamer, 1970; Jacobus, 1972) would tend to add support to the participation of CPK in the transport of creatine. In addition, it has been recently proposed that B-GPA is phosphorylated when it accumulates in muscle after feeding (Jellinek and Fitch, 1972). Future studies of the kinetics of the reaction of creatine and B-GPA with rat muscle CPK may clarify this hypothesis.

The Effects of Abnormal Creatine Metabolism on Skeletal Muscle Structure and Function

No marked differences were observed in the histochemical staining reactions of white (Type II) or red (Type I) fibers when abnormal creatine metabolism was produced by feeding B-GPA to rats. The small difference in the intensity of PAS staining in Type II fibers of rats fed B-GPA may have been a result of the smaller size of these white fibers. Thus muscle fibers seemed to maintain most of their characteristic histochemical patterns in spite of the low levels of creatine.

Of particular significance is the fact that the white fibers of rats fed B-GPA were not as large as those of control rats after both groups had been exercised. This difference in fiber size was not due

to a difference in total body weight as was indicated by the body weight : fiber size ratio (Table 7). This is even apparent when fibers from animals of similar body weights are visually compared (Figure 12).

The specific cause of the difference in fiber size is not known. Also, it is not known if this change represents atrophy or a failure of the normal processes of hypertrophy. It has been demonstrated that the Type II white fibers differentiate soon after birth and hypertrophy is concomitant with increasing muscular fiber activity (Ashmore *et al.*, 1972). During this same period the metabolic profile of these fibers also changes. There is an increase in CPK activity and N-phosphoryl-creatine levels (Perry, 1970) and, since muscle creatine levels and CPK activity were below normal in rats fed B-GPA, the normal processes of differentiation may have been at least partially limited. Not all normal differential processes were impaired, however, because the Type II fibers retained most of their normal histochemical characteristics.

In a review, Engle (1970) has summarized experimental and clinical findings of Type II fiber atrophy. Atrophy of Type II fibers is one of the most common myopathies. It has been observed experimentally in cats and guinea pigs after nerve resection and clinically in cases of cachexia, disuse, chronic corticosteroid intoxication, myasthenia gravis and untreated collagen vascular diseases such as panencephalitis. It has been also proposed by Engle that preferential atrophy of Type II fibers occurs because they are more susceptible to a lack of trophic influence than are the Type I fibers. In the case of the rats in this present study, their running performance would make it difficult to argue that there was a marked difference in the nerve impulses received by the white fibers of control and test animals. A more plausible

explanation might be that the fibers were unable to respond to the impulses in a normal manner to produce fiber enlargement.

During the course of these studies the rats fed B-GPA were able to perform routine cage movements without noticeable impairment, in spite of lower levels of muscle creatine. A more critical test of muscle performance was conducted in the running wheel. Rats fed B-GPA did not run quite as well as did normal rats in this test; however, some of the animals that had low muscle creatine levels performed as well as the controls. It is believed, therefore, that the difference in running ability, as measured in these tests, is minimal (Heusner, 1972). It is tempting to propose that high levels of muscle creatine are not necessary for muscle function but any such hypothesis must be qualified by a consideration of the type of performance test used. The stress required by the running studies in this work may not have been sufficient to stress the performance of white fibers (Faulkner, 1970). Alternative tests such as jumping might be required before a significant impairment of performance is observed.

In these studies high intracellular levels of creatine did not appear essential for survival of muscle cells. Thus, it can be reasoned that either (a) compensatory mecahnisms were adequate for metabolic needs under the experimental conditions or (b) the high levels of intracellular creatine are in excess of metabolic needs. While it is possible that the compensatory reactions may have permitted relatively normal performance, they did not permit the Type II fibers to attain their normal size, nor were they dramatic enough to be detected by histochemistry. Furthermore, the fact that levels of N-phosphorylcreatine, ATP, ADP, and PEP are much lower than normal when rats are fed B-GPA would argue against the presence of effective compensating mechanisms,

especially those functioning as phosphagens. Since the CPK enzyme and N-phosphorylcreatine are detectable in most body tissues, it is possible that part of the normal creatine concentration present in skeletal muscle is acting only as a reservoir to maintain constant blood levels. In this hypothetical case, body tissues would be served with a constant blood supply of creatine and could utilize the N-phosphorylcreatine system to regenerate ATP without the necessity of storing large quantities of creatine.

SUMMARY

This research was conducted to test the hypothesis that high intracellular concentrations of creatine are essential to the integrity of vertebrate skeletal muscle; and to provide new information on the relationship between abnormal creatine metabolism and muscle disease. To accomplish these objectives, the structure and function of muscles were evaluated after abnormal creatine metabolism was induced by feeding rats a creatine analog, B-guanidinoproprionic acid (B-GPA).

The abnormal metabolism of creatine that occurred when rats were fed diets containing 1% B-GPA was characterized by decreases in muscle and brain levels of creatine, muscle N-phosphorylcreatine, urinary excretion of creatinine, muscle activity of creatine phosphokinase (CPK) and by an increase in urinary excretion of creatine. The white-type fibers from *gastrocnemius* muscles of normal rats contained more creatine than the red-type fibers from the same muscles; however, the creatine levels of both were proportionally lower than normal in fibers from rats fed B-GPA. While pregnant rats consumed less food when their diets contained B-GPA, there was no significant effect on food intake of young male rats when they were pair-fed rations with or without B-GPA. In an *in vitro* experiment, B-GPA inhibited the reaction of creatine with rat muscle CPK. Beta-guanidinoproprionic acid apparently induces abnormal creatine metabolism in rats by inhibiting the mediated entry of creatine into tissues from plasma--and possibly by competing with creatine for the active site of the CPK enzyme.

When muscle function was evaluated by running, the group of rats fed B-GPA did not perform as well as did those that had not received B-GPA. There were also structural changes in muscle fibers of exercised rats fed B-GPA. The white fibers from the *gastrocnemius* of rats fed B-GPA were significantly smaller than those from rats not fed the test compound; nevertheless, normal histochemical profiles were maintained in these fibers. Gross pathological changes were not observed in experimental rats. Thus, when the normal creatine complement of skeletal muscle is depleted (75%) by feeding B-GPA, the normal structure and function of muscle is not maintained.

This is the first report that tissue levels of creatine can be reduced by altering creating metabolism directly at a point near its functional site. The ability to deplete skeletal muscle of its normally high intracellular concentrations of creatine and N-phosphorylcreatine will be of value in clarifying the relationship between abnormal creatine metabolism and neuromuscular disease. In addition, the possibility that the CPK enzyme acts as a translocating molecule during its reaction with creatine should enhance our understanding of trans-membrane transport of small molecular weight compounds.

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APPENDICES

APPENDIX A
(Table 9A)

Table 9A. Standard 8-week, short-duration, high-intensity endurance training program for postpubertal and adult male rats in controlled-running wheels*

Wk.	Day of Wk.	Day of Tr.	Accel-eration Time (sec)	Work Time (min:sec)	Rest Time (sec)	Repe-titions per Bout	No. of Bouts	Time Between Bouts (min)	Shock (ma)	Run Speed (ft/sec)	Total Time of Prog. (min: sec)	Total Exp. Revolu-tions TER	Total Work Time (sec) TWT
0	4=T	-2	3.0	40:00	10	1	1	5.0	0.0	1.5	40:00	---	---
	5=F	-1	3.0	40:00	10	1	1	5.0	0.0	1.5	40:00	---	---
1	1=M	1	3.0	00:10	10	40	3	5.0	1.2	1.5	49:30	450	1200
	2=T	2	3.0	00:10	10	40	3	5.0	1.2	1.5	49:30	450	1200
	3=W	3	3.0	00:10	10	40	3	5.0	1.2	1.5	49:30	450	1200
	4=T	4	2.5	00:10	10	40	3	5.0	1.2	2.0	49:30	600	1200
	5=F	5	2.0	00:10	10	40	3	5.0	1.2	2.0	49:30	600	1200
2	1=M	6	1.5	00:10	10	28	4	5.0	1.2	2.5	51:40	700	1120
	2=T	7	1.5	00:10	15	27	4	5.0	1.2	3.0	59:00	810	1080
	3=W	8	1.5	00:10	15	27	4	5.0	1.2	3.0	59:00	810	1080
	4=T	9	1.5	00:10	15	27	4	5.0	1.2	3.0	59:00	810	1080
	5=F	10	1.5	00:10	15	27	4	5.0	1.2	3.0	59:00	810	1080
3	1=M	11	1.5	00:10	15	27	4	5.0	1.2	3.0	59:00	810	1080
	2=T	12	1.5	00:10	20	23	4	5.0	1.2	3.5	59:40	805	920
	3=W	13	1.5	00:10	20	23	4	5.0	1.2	3.5	59:40	805	920
	4=T	14	1.5	00:10	20	23	4	5.0	1.2	3.5	59:40	805	920
	5=F	15	1.5	00:10	20	23	4	5.0	1.2	3.5	59:40	805	920

Table 9A (cont'd.)

Wk.	Day of Wk.	Day of Tr.	Acceleration Time (sec)	Work Time (min:sec)	Rest Time (sec)	Repetitions per Bout	No. of Bouts	Time Between Bouts (min)	Shock (ma)	Run Speed (ft/sec)	Total Time of Prog. (min: sec)	Total Exp. Revolutions TER	Total Work Time (sec) TWT
4	1=M	16	1.5	00:10	20	23	4	5.0	1.2	3.5	59:40	805	920
	2=T	17	1.5	00:10	25	20	4	5.0	1.0	4.0	60:00	800	800
	3=W	18	1.5	00:10	25	20	4	5.0	1.0	4.0	60:00	800	800
	4=T	19	1.5	00:10	25	20	4	5.0	1.0	4.0	60:00	800	800
	5=F	20	1.5	00:10	25	20	4	5.0	1.0	4.0	60:00	800	800

*Wells and Heusner, 1971.

This standard program was designed using male rats of the Sprague-Dawley strain. All animals were between 70 and 170 days of age at the beginning of the program. The duration and intensity of the program were established so that 75% of all such animals should have PSF and PER scores of 75 or higher during the final 2 weeks. Alterations in the rest time, repetitions per bout, number of bouts, or time between bouts can be used to affect changes in these values. Other strains or ages of animals could be expected to respond differently to the program.

APPENDIX B
(Table 10A)

Table 10A. Summary of body weight data from rats used in running experiments

Diet	Dam No.	Rat No.	Average Weaning Wt.	19 days		43 days		72 days	75 days	Mean body wt. of group and average daily gain during 75-day experiment
Control	1	1	41g*	128	4.60g/day	247	4.78g/day (last 23 days)	291	297	297g-3.40g/day
Control		2		122		221		276	285	
Control		4		131		239		320	300	
Control		5		133		247		295	302	
1% B-GPA	2	7	28g	117		212		250	256	
1% B-GPA		8		123		223		274	280	
1% B-GPA	3	9	35g	127	4.68g/day	235	4.34g/day	289	286	268g-3.13g/day
1% B-GPA		11		127		214		264	262	
1% B-GPA		12		122		222		271	271	
1% B-GPA		13		125		219		262	257	
1% B-GPA		14		114		223		280	280	
1% B-GPA		15	30g	117		222		262	252	

*Males only - females sacrificed before weighing.

**Run for 20 days between 46 and 72 days.

VITA

VITA

Robert Pierce Shields was born in Nashville, Tennessee, on June 11, 1932. He was educated in the public school system and was graduated from Hillsboro High School, Nashville, Tennessee, in June 1950.

In September 1950, he enrolled in the pre-veterinary curriculum at the University of Tennessee in Knoxville, Tennessee. In September 1952, he was admitted to the School of Veterinary Medicine of Auburn University at Auburn, Alabama. The degree of Doctor of Veterinary Medicine was awarded to the author in June 1956.

From September 1956 to September 1959 he served as an instructor in the Department of Pathology and Parasitology at the School of Veterinary Medicine of Auburn University. He was awarded a Master of Science degree in August of 1959. In September 1959 he accepted a position as Veterinary Pathologist for the Ralston Purina Company of St. Louis, Missouri.

In 1962 he was awarded a National Institutes of Health Fellowship at the University of Arkansas Medical Center in Little Rock, Arkansas. During the next four years he served as a resident in the Department of Pathology and in 1966 he was awarded a Master of Science degree in Biochemistry from the University of Arkansas.

From 1962 to 1970, the author served as an Associate Professor at Auburn University in the Department of Pathology and Parasitology in the School of Veterinary Medicine and the Department of Animal Science in the School of Agriculture.

In July 1970, he was awarded a Special Postdoctoral Fellowship by the National Institutes of Health for a study of the relationship of phosphogens to neuromuscular disease and the completion of the Doctor of Philosophy degree in Pathology.

The author is married to the former Shirley Clayton Salter of Thomaston, Georgia, and has two children, Carolyn Carey, age eight, and Robert Bradley, age six.