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Chemical energy usage during isometric twitches of frog sartorius muscle intoxicated with an isomer of creatine, β-guanidinopropionate

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Abstract. Frogs were injected for several weeks with β-guanidinopropionate, an isomer of creatine. Their sartorius muscles were isolated, poisoned with iodoacetate and stimulated isometrically with 75 shocks/min in nitrogen until rigor. In comparison with sartorius muscles of untreated frogs, they contained more free creatine and less phosphocreatine, but the same content in total creatine and ATP. They also contained β-guanidinopropionate both free and phosphorylated. However, muscles in rigor contained the same concentration of the phosphorylated form as resting muscles, i.e., phospho-β-guanidinopropionate was not split during contraction. The number of twitches performed before rigor was decreased. There was no change in the chemical energy usage (sum of phosphocreatine breakdown and twice ATP breakdown) per twitch.

Key words: Frog muscle – Isometric twitch – Phosphocreatine – β-guanidinopropionate

Introduction

Resynthesis of ATP during muscular contraction occurs rapidly through phosphorylation of ADP by phosphocreatine (Lohmann 1934). Thus phosphocreatine concentration is proportional to the energy stored in the muscle in a form rapidly available to the contractile machinery. Creatine is the only important guanidine compound in vertebrate, but several others have been observed in invertebrate muscle, the most important being arginine (Ennor and Morrison 1958; di Jeso et al. 1967; Thoaï and Roche 1964).

Fitch et al. (1974, 1975) have reported that the creatine isomer β-guanidino propionate, fed to rats, enters into striated muscle cells, is phosphorylated (probably by creatine kinase) and is apparently used as a substitute for creatine. This interesting observation raises the possibility of interfering with the energy transfer between phosphocreatine and actomyosin during contraction. Accurate measurements of chemical energy usage are however difficult to perform on mammalian muscle. This is the reason why we set out to investigate the possibility of substituting β-guanidinopropionate for creatine in frog sartorius muscle, a material in which energy transfers have been thoroughly investigated (Aubert 1956; Maréchal 1964; Hill 1965; Mommaerts 1969; Curtin and Woledge 1978). This work demonstrates that the presence of β-guanidinopropionate in frog muscle cells does not change the energy usage during twitches.

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Methods

Summer frogs (*Rana temporaria*) were injected three times a week through the mouth into the ventral lymphatic space with 1 ml of 127 mM NaCl containing 6–60 mg of β-guanidinopropionate during 20–120 days. They were kept at room temperature in running tap water at 14° C. Control frogs were kept in separate tanks.

Sartorius muscle pairs were dissected out, their standard length l_0 (Hill 1952) was measured and the paired muscles, still attached to the pelvic bone, were stored overnight at 5° C in Ringer solution (113 mM NaCl; 2.0 mM KCl; 1.8 mM $CaCl_2$; 1.9 mM phosphate buffer; pH 7.3), which was gassed with air. The following morning the muscle pairs were poisoned 30 min in a Ringer solution containing 0.4 mM iodoacetate at 20° C. They were slightly blotted to remove surface water and they were mounted at length l_0 on a rapid freezing apparatus (Maréchal 1964). Each muscle of the pair was attached with a light silver chain to a strain gauge force transducer (compliance 0.3 mm/N) the output of which was displayed on a fast pen recorder (Sanborn, model 320). The muscle chamber was then flushed with moist nitrogen during 10 min at 2° C. One muscle of the pair, chosen at random, was stimulated isometrically through three silver electrodes with supramaximal (120%) condenser discharges (time constant: 5 ms) at 75 shocks per minute until rigor had set in. Muscles were frozen in isopentane cooled to −160° C, weighed and ground to powder in steel cartridges (Mommaerts and Schilling 1955). The powder was extracted twice on ice with 1 ml of 0.5 N perchloric acid. The extracts were combined and neutralized with 1 N KOH. The $KClO_4$ precipitate was removed by centrifugation (10 min at 4,000 rpm; 3° C) and the supernatant was diluted to a final volume of 10 ml.

Creatine and β-guanidinopropionate react with a mixture of diacetyl and α-naphthol (Ennor and Stocken 1948). The reaction with creatine is somewhat enhanced (+30%) in the presence of propanol, whilst that of β-guanidinopropionate is strongly enhanced (+335%) (Rosenberg et al. 1956). We have carefully measured the absorption spectra and the time courses of the color

developed in the presence or in the absence of propanol, using various mixtures of diacetyl and α-naphthol. The following procedure was found to give optimal results. A first reaction was performed in duplicate in the absence of propanol. The reaction tube contained: 0.5 ml of extract; 1 ml of diacetyl diluted 1 : 2,500; 2 ml of an alcaline solution of α-naphthol (10 g of α-naphthol dissolved in 1 l of an alcaline solution containing 60 g of NaOH and 160 g of Na_2CO_3). The reacting solution was diluted with water to a final volume of 10 ml. The reaction was allowed to proceed in the dark during 40 min. The absorbance (E_1) of the solution was read at 520 nm. A second reaction was performed, also in duplicate, in the presence of propanol. The reaction tube contained: 0.5 ml of extract; 1 ml of 3 N NaOH; 2 ml of a solution containing 5 g of α-naphthol, 2.5 ml of diacetyl diluted 1: 100 v/v with water and 100 ml of n-propanol. The reacting solution was diluted with water to a final volume of 10 ml and stored in the dark during 20 min. Its absorbance (E_2) was read at 535 nm. Standards of creatine and of β-guanidinopropionate were similarly treated. If A_1 and A_2 are the absorbances of creatine standards respectively with and without propanol, and if B_1 and B_2 are the corresponding absorbances for β-guanidinopropionate standards, then the creatine concentration of an extract is equal to $(E_1 \cdot B_2 - E_2 \cdot B_1)/(B_2 \cdot A_1 - B_1 \cdot A_2)$ and its β-guanidinopropionate concentration is equal to $(E_2 \cdot A_1 - E_1 \cdot A_2)/(B_2 \cdot A_1 - B_1 \cdot A_2)$. The accuracy of this procedure is better than 5%.

Phosphocreatine and phospho-β-guanidinopropionate were measured by the following indirect method. The extract was heated 10 min at 65° C in 0.4 N HCl and neutralized with 2 N NaOH in order to hydrolyze the phosphorylated guanidines. Total creatine (sum of creatine and phosphocreatine) and total β-guanidinopropionate (sum of β-guanidinopropionate and phospho-β-guanidino propionate) were measured in the extract with the spectrophotometric method described above. Phosphocreatine (or phospho-β-guanidinopropionate) was estimated by the difference between total creatine (or total β-guanidinopropionate) and creatine (or β-guanidinopropionate). Phosphocreatine but not β-guanidinopropionate was multiplied by 1.11, to allow for creatinine formation during acid hydrolysis of phosphocreatine. We checked with internal standards the reliability of these procedures.

ATP was measured with the method described by Jaworek et al. (1970). Amino acids were measured with liquid chromatography in an automatic analyzer (Liquimat III, Kontron). Some extracts were analyzed by one dimensional thin layer chromatography on cellulose sheets (Merck) at 5° C. The solvent was propanol, water, acetic acid in proportions 8 : 2 : 1 (di Jeso 1968). This procedure separates creatine and β-guanidinopropionate (Fig. 2). Guanidine derivatives were stained on the cellulose sheet with a spray containing 1 ml of diacetyl diluted 1 : 2,500 (v/v) and 2 ml of an α-naphthol solution (1 g of α-naphthol in 100 ml of an alcaline solution containing 60 g NaOH and 160 g of Na_2CO_3 per liter).

β-guanidinopropionate used as standard was synthesized with the method of Rowley et al. (1971). The phosphorylated form was obtained using creatinekinase and ATP. (Fitch et al. 1974). Phosphocreatine was obtained from Sigma Chemicals, diacetyl from Light and the other reagents were of analytical grade.

Results

Figure 1 shows one typical experiment performed on frog sartorius muscles. One muscle of the pair remains at rest (Fig. 1, A2, B2), whilst the other one contracts isometrically (Fig. 1, A1, B1). The twitch force at first increases slowly (positive staircase). It remains fairly constant for most of the experiment. Then it decreases slowly, whilst rigor sets in. This experiment is similar to that reported by Lundsgaard, a long time ago (Lundsgaard 1930). In order to evaluate precisely the effect of treating frogs with β-guanidinopropionate, we have measured the following parameters: the maximum tension of the twitches, P_{to}; the time elapsed between the first twitch and the twitch giving a tension equal to $P_{to}/2$ above rigor, t_a; the maximal tension developed in rigor, P_r; the time elapsed between the first twitch and the moment when the rising rigor is equal to half P_r, t_r. Table 1 demonstrates that administration of β-guanidinopropionate to frogs significantly shortens t_a. Examination of the records shows that the number of full-sized twitches is reduced. We have also computed the number N of "the equivalent number of maximal twitches", using for its evaluation the method proposed by Carlson and Siger (1960): N is equal to the sum of the tension of each twitch divided by P_{to}. N is reduced by 27% (Table 1) in frogs intoxicated with β-guanidinopro-

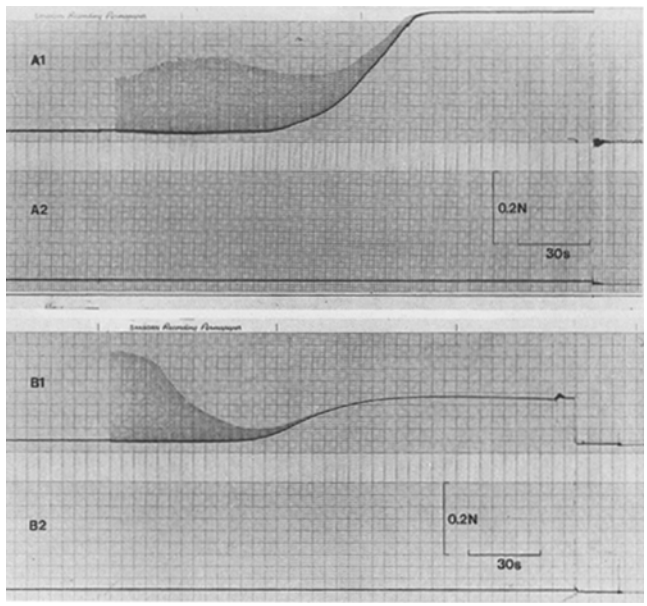

Fig. 1A, B. Mechanograms of sartorius muscle pairs in nitrogen and poisoned with 0.4 mM of iodoacetate. **A** Control muscle pair: A1 muscle stimulated at 75 shock/min; A2: muscle at rest; 1_0: 28 mm; weight of stimulated muscle: 75 mg; equivalent number of maximal twitches: 93; difference of concentration between control and stimulated: phosphocreatine: 19,8 μmol/g; ATP: 3.7 μmol/g. **B** Frog intoxicated with injections of 60 mg of β-guanidinopropionate every two days for 42 days; B1: muscle stimulated at 75 shock/min; B2: muscle at rest; 10: 26 mm; weight: 40 mg; equivalent number of maximal twitches: 43. Difference between control and stimulated muscles: phosphocreatine: 12.0 μmol/g; ATP: 2,3 μmol/g; phosphorylated β-guanidinopropionate: 0 μmol/g. Each muscle contained 7.8 μmol/g of β-guanidinopropionate and 7.6 μmol/g of phosphorylated β-guanidinopropionate. The large force drop at right-hand side of figure signals the moment of rapid freezing

pionate. In a few experiments, the time course of the twitches was displayed on an oscilloscope and photographed. The shape of the first twitches did not change, but the later ones shortened somewhat. This is an already known effect of iodoacetate (Aubert et al. 1957) which appears not to be modified by intoxication with β-guanidinopropionate.

Figure 2 shows analyses of muscle extracts by thin layer chromatography. It demonstrates that muscle of fresh frogs contains creatine as the only guanidine in major concentration. Muscle of frogs treated with β-guanidinopropionate contains the analog besides creatine. This method is quite dependable to demonstrate the presence of the analogue in muscle, but it is not suited for quantitative measurements, which are best performed with a spectrophotometric method.

Table 2 reports the results of the spectrophotometric analyses of the extracts. Control muscle has ATP, creatine and phosphocreatine content that is comparable to that observed by other workers (cf. Maréchal 1964, p 69). It appears to contain some β-guanidinopropionate, free or phosphorylated, although in low concentrations statistically not different from zero. Since we know that these fresh muscles do not contain the analogue, these results show the extent of the error made when creatine and β-guanidinopropionate are simultaneously measured by our spectroscopic method (see Methods). At rest, muscle of frogs treated with β-guanidinopropionate has lower content in ATP and phosphocreatine, but higher content in creatine, with the consequence that its total creatine content is unchanged. It also contains sizeable amount of β-guanidinopropionate both free and phosphorylated in concentrations more or less proportional to the total quantity of analogue injected to the frog (not shown). It is interesting

Table 1. Effect of β-guanidinopropiate on iodoacetate rigor

Parameter	Control	Intoxicated
P_{to} (kN/m²)	112 ± 16	116 ± 10
t_a (s)	59 ± 5	43 ± 2
P_r (kN/m²)	73 ± 10	65 ± 7
t_r (s)	107 ± 8	91 ± 6
N	71 ± 6	51 ± 4
n	7	13

Sartorius muscles were intoxicated with iodoacetate and stimulated in nitrogen. P_{to}: maximal tension of twitches; t_a: time between first twitch and the twitch at which tension equals $P_{to}/2$; P_r: maximal tension of rigor; t_r: time between forst twitch and moment when rigor equals $P_r/2$; N: equivalent number of maximal twitch, equal to the sum of tension of all twitches divided by P_{to}; n: number of experiments; Intoxicated: frogs poisoned with β-guanidinopropionate. The numbers are means with their standard errors

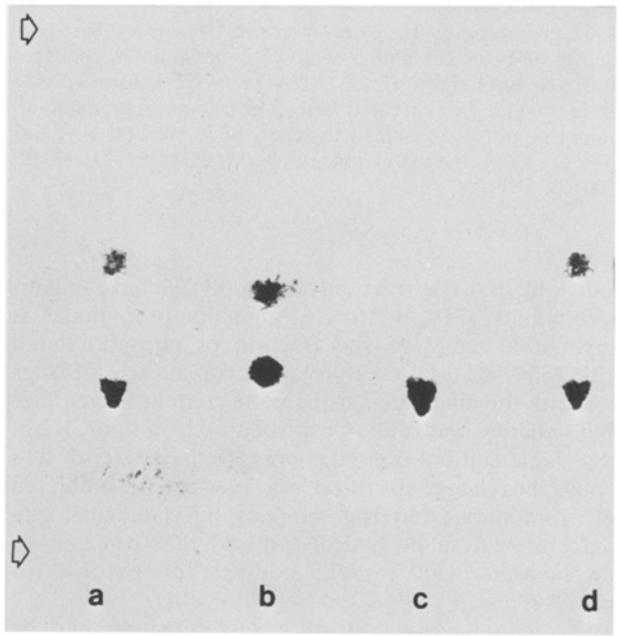

Fig. 2a-d. Thin layer chromatograms on cellulose of standard solutions and of muscle extracts. Developing solution: propanol, water, acetic acid, 8 : 2 : 1. Lane **a**: muscle from frogs intoxicated with β-guanidinopropionate. Lane **b**: standard solutions of creatine (lower spot) and β-guanidinopropionate (upper spot). Lane **c**: muscle from untreated frog. Lane **d**: standard solution of β-guanidinopropionate mixed with an extract of muscle from untreated frog: the upper spot is similar to that observed in **a**. Note the slightly different Rf of standards in comparison to muscle extracts. The arrows indicate the starting line and the solvent front

Table 2. Effects of β-guanidinopropionate on the ATP and guanidine content (μmol/g) of resting or stimulated frog sartorius muscle

	Control			Intoxicated		
	Resting muscle	Stimulated muscle	Difference (stim.-rest.)	Resting muscle	Stimulated muscle	Difference (stim.-rest.)
Creatine	8.7 ± 1.3	25.5 ± 0.5	16.8 ± 1.1	15.6 ± 1.3	24.3 ± 1.5	8.7 ± 1.3
Total creatine	24.8 ± 0.6	25.5 ± 0.5	0.7 ± 0.4	23.9 ± 1.3	24.4 ± 1.4	0.5 ± 0.4
Phosphocreatine	16.1 ± 1.3	0.0 ± 0.1	−16.1 ± 1.4	8.3 ± 1.2	0.1 ± 0.1	−8.2 ± 1.2
β-guanidinopropionate	0.7 ± 0.4	− 0.4 ± 0.7	− 1.1 ± 0.5	5.3 ± 1.1	5.1 ± 1.2	−0.2 ± 1.1
Total β-guanidinopropionate	− 0.2 ± 0.7	0.7 ± 0.5	0.9 ± 0.6	7.7 ± 1.7	7.0 ± 1.5	−0.7 ± 0.8
Phospho β-guanidinopropionate	− 0.9 ± 0.8	1.1 ± 0.6	2.0 ± 0.7	2.4 ± 1.3	1.9 ± 0.6	−0.5 ± 0.7
ATP	3.0 ± 0.4	0.3 ± 0.1	− 2.7 ± 0.3	2.6 ± 0.2	0.3 ± 0.1	−2.3 ± 0.2
n	7	7	7	13	13	13

All muscle pairs were treated with iodoacetate and nitrogen. One muscle of the pair was not stimulated; the other was stimulated with 75 shocks/min until rigor had set in. Pairs labelled "intoxicated" were obtained from frogs injected with β-guanidinopropionate. The means are given with their standard errors; the differences are calculated pairwise

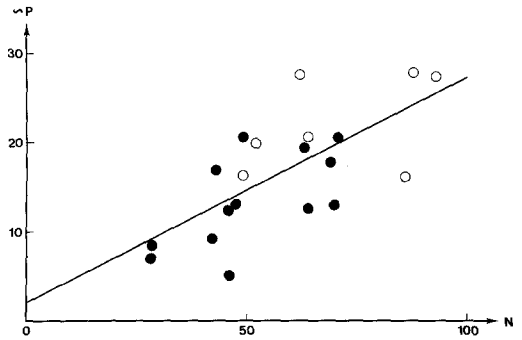

Fig. 3. Chemical energy usage during a series of twitches. Sartorius muscles have been stimulated with 75 shocks/min in iodoacetate-nitrogen until rigor. *Open circles* (n = 7): control; *closed circles* (n = 13): frogs treated with β-guanidinopropionate. N: equivalent number of maximal twitches. ~ P: sum of ΔPC and 2ΔATP. The line is the regression line, ~ $P = 2.34\ (\pm 3.24) + 0.25\ (\pm 0.06)\ N$

to point out that the fraction of phosphorylated β-guanidinopropionate (31% of total β-guanidinopropionate) is approximately equal to the fraction of phosphorylated creatine (35% of total creatine). In rigor, muscle of frogs treated with the analogue has the same content in creatine, phosphocreatine and ATP as control muscle in rigor. It has also the same content in β-guanidinopropionate, both free and phosphorylated, as those observed in unstimulated muscle from intoxicated frog: the phosphorylated analogue does not appear to be hydrolyzed and thus can neither rephosphorylate ADP to ATP nor prevent the onset of rigor.

The mean energy cost of a single twitch may be computed from the results reported above. As muscles are stimulated in nitrogen and poisoned with iodoacetate, oxidation and glycolysis are inhibited. The only source of energy is then phosphocreatine and ATP, which contributes for two high energy phosphate since it is dephosphorylated and deaminated to inosine monophosphate (Canfield and Maréchal 1973). Phosphorylated β-guanidinopropionate can be neglected since it is not split. The mean energy cost of an equivalent twitch is then computed by the equation

$$(2\Delta\text{ATP} + \Delta\text{PC})/N$$

in which ΔATP is the difference in ATP between resting and stimulated muscle, ΔPC is the breakdown of phosphocreatine estimated by the difference in free creatine between resting and stimulated muscle, and N is the equivalent number of maximal twitches. The energy cost is equal to 0.32 ± 0.07 μmol ~ P/g (n = 7) in fresh muscle, a result in good agreement with that reported by Carlson and Siger (1960) and Carlson et al. (1967). It is unchanged in muscle of frog intoxicated with β-guanidinopropionate: 0.27 ± 0.08 μmol ~ P/g (n = 13). Figure 3 shows the individual experiments. The energy cost of the series of twitches is plotted as a function of the equivalent number of maximal twitches: the regression line is equal to $0.25\ (\pm 0.06)\ N + 2.3\ (\pm 3.7)$. The graph demonstrates that muscle of frogs treated with β-guanidinopropionate is not able to contract as many times as fresh muscle does. This reduction in contractile capacity is proportional to the decrease in the initial stores of high energy phosphate and it cannot be explained by a higher energy cost of twitch.

Table 3. Amino acid contents of control and intoxicated muscle

	Control	Intoxicated
Taurine	0.3	0.4
Aspartic acid	0.2	0.0
OH-Proline	0.0	0.0
Threonine	0.5	0.3
Serine	0.5	0.3
Glutamic acid	1.4	0.8
Proline	0.2	0.3
Glycine	1.0	0.5
Alanine	1.1	0.6
Valine	0.2	0.2
Cystine	0.0	0.0
Methionine	0.1	0.1
Isoleucine	0.1	0.0
Leucine	0.3	0.2
Tyrosine	0.2	0.2
Phenylalanine	0.0	0.2
Ornithine	0.0	0.0
Lysine	0.4	0.4
Histidine	0.4	0.2
Arginine	0.2	0.2
Carnosine	7.8	11.1

Amino acids are expressed in μmol per gram fresh muscle. They were measured in a 127 mM NaCl extract of gastrocnemius muscles of one control and one intoxicated frog. The unstimulated sartorius muscle of the same control frog contained: creatine: 7.6 μmol/g; phosphocreatine: 15.5 μmol/g. The unstimulated sartorius muscle of the same intoxicated frog contained: creatine: 11.6 μmol/g; phosphocreatine: 16.9 μmol/g; β-guanidinopropionate: 3.2 μmol/g; phosphorylated β-guanidinopropionate: 9.5 μmol/g

Finally we have measured the concentrations of twenty amino acids (Table 3). Their concentrations are similar to those reported for human muscle (Bergström et al. 1974), except for taurine and glutamic acids, which are noticeably lower in frog muscles. The concentration of carnosine observed in our experiments is the same than those reported for human muscle by Perry et al. (1967). Intoxication of a frog by β-guanidinopropionate does not change the concentrations of the amino acids. It is especially noticeable that arginine concentration is not affected, since changes in this amino acid might interfere with the spectrophotometric assays of creatine of β-guanidinopropionate.

Discussion

β-guanidopropionate is a molecule similar to creatine. Fed to rats, it enters muscle cells where it is partly phosphorylated; it seems to be dephosphorylated during a long series of twitches (Fitch et al. 1975). It is not known whether this analogue interferes with the energy transfer between phosphocreatine and the actomyosin-ATP system. Accurate studies of energy balance are difficult to obtain in mammalian muscle. This is the reason why we set out to study the effects of β-guanidinopropionate on frogs, because in frog muscles so much is known of the chemical energy mobilization and its transfer in heat and mechanical power (Aubert 1956; Maréchal 1964; Hill 1965; Mommaerts 1969; Curtin and Woledge 1978).

We have observed that β-guanidinopropionate enters into frog muscle cells. Its accumulation in muscle is roughly proportional to the total amount of β-guanidinopropionate injected to the frogs over several weeks. This indicates that the drug is not easily excreted. The frogs do not look healthy. Their mortality is high: we lost 50% of them during the process of intoxication. The treated frogs have lost their muscular tone and move very slowly. Flexion reflexes elicited by cutaneous stimulation are still brisk. It was something of a surprise for us to observe that sartorius muscle isolated from such ill frogs could twitch normally. Chemical analyses show however that β-guanidinopropionate enters into muscle cells, where it accumulates, albeit in low concentrations. The concentration of total creatine is not decreased, but the proportion of creatine which is phosphorylated decreases to 35% of the total amount of creatine, i.e., less than a half of the proportion usually observed in fresh muscle. The analogue is partially phosphorylated. The proportion of phosphorylated analogue is similar to the proportion of phosphorylated creatine. This observation suggests that there could be an equilibrium between the phosphorylated and the unphosphorylated forms of creatine and β-guanidinopropionate, perhaps mediated by creatine phosphokinase. Such an equilibrium however is modified very slowly since phosphorylated β-guanidinopropionate is not split during a series of twitches.

The most likely explanation of these observations is the following: β-guanidinopropionate presumably enters muscle cells using the same channel as creatine. It is phosphorylated by creatine phosphokinase, to the same extent as creatine; thereby a new equilibrium is obtained in which the proportion of phosphorylated creatine is decreased by half. However, the rates of phosphate exchange between phosphorylated β-guanidinopropionate and ATP are so slow that no apparent breakdown of phosphorylated β-guanidinopropionate can occur during the few minutes required to stimulate the muscle to rigor. The muscle only uses its stores of ATP and phosphocreatine albeit at normal rates. Since the initial phosphocreatine store is lowered in the presence of β-guanidinopropionate, the equivalent number of maximal twitches is decreased.

As the chemical cost per twitch is not changed (approximately 0.27 μmol ~ P/g), there is no indication that β-guanidinopropionate interferes with the energy transfer between phosphocreatine and actomyosin. In contractured muscle, ATP and phosphocreatine stores are depleted, but phosphorylated β-guanidinopropionate is left unchanged. It is thus obvious that the phosphorylated analogue cannot maintain a normal level of ATP in the muscle cell.

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