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THE INVESTIGATION OF MECHANICAL AND HEMODYNAMIC PARAMETERS IN NORMOXEMIA AND HYPOXEMIA IN HEARTS OF CREATINE DEPLETED RATS

by

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A thesis submitted to the School of Graduate Studies of the University of Ottawa in partial fulfillment of the requirements for the degree of Doctor of Philosophy in Physiology, Faculty of Health Sciences.

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DEDICATION

To my Mother and Father who have provided encouragement and inexhaustable support throughout my scholastic endeavors.

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LIST OF ABBREVIATIONS

acetyl CoA - acteyl coenzyme A

ADP - adenosine diphosphate

AMP - adenosine monophosphate

ATP - adenosine triphosphate

CO - cardiac output

$C O_2$ - oxygen content

CK - creatine kinase

CP - creatine phosphate

Cr - releasable fraction of intracellular calcium

Cs - slowly recycling pool of intracellular calcium

DF - maximum developed force

dF/dt - rate of force development

dP/dt - rate of pressure development

-dP/dt - rate of relaxation

2,3-DPG - 2,3-diphosphoglycerate

dT/dt - rate of tension development

EDL - extensor digitorum longus

F - statistical ratio used in one-way analysis of ANOVA

$FADH_2$ - 1-fluoro-2,3-dinitrobenzene

GBA - β-guanidinobutyric acid

GPA - β-guanidinopropionic acid

GPAP - phosphorylated GPA

Hb - hemoglobin

HPLC - high pressure (performance) liquid chromotography

IOSB - 2-iodosodium benzoate

Lmax - maximum length of a muscle

LVEDP - left ventricular end-diastolic pressure

NaCN - sodium cyanide

NADH - reduced nicotinamide adenine dinucleotide

NMR - nuclear magnetic resonalce

Pi - inorganic phosphate

pCO_2 - partial pressure of carbon dioxide

pO_2 - partial pressure of oxygen

p50 - the pO_2 at which hemoglobin is ½ saturated with oxygen

RBC - red blood cell

RCC - red cell concentrate

SR - sarcoplasmic reticulum

TCA - tricarboxylic acid cycle

TMT - time to maximal tension

T ½ R - time to half-relaxation

$\Delta T/\Delta t$ - mean rate of tension development

W - total external power

W_i - power input

W_k - kinetic power

W_p - pressure power

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ABSTRACT

The essential role of creatine phosphate (CP) in energy utilization in the myocardium was investigated in creatine depleted rats. Depletion was achieved by feeding rats a diet supplemented with structural analogues; either with 2% β-guanidinobutyric acid (GBA) or 1% β-guanidinopropionic acid (GPA). Both analogues are poor substrates for creatine kinase. After 7 weeks, total creatine (creatine and CP) decreased in the left ventricle by 75% in both GBA and GPA groups as compared to pair fed controls. After 10 weeks, the decline in total creatine was by 90% in the GBA group and by 84% in the GPA group.

The effects of creatine depletion on myocardial performance were tested first at the tissue level, by assessing the contractile properties of isolated ventricular papillary muscles and second, at the whole organ level by investigating the hemodynamic performance of the isolated blood-perfused working rat heart.

Creatine depletion of 75-90% did not alter contractile characteristics of single isometric twitches. Also, the imposition of trains of high frequency stimuli did not significantly alter the performance of the creatine depleted papillary muscles from that of control papillary muscles at 25°C or 31°C. However, the subsequent recovery from the trains of high frequency stimuli was significantly longer in creatine depleted muscles than in the controls. Paired stimulation did

not affect the creatine depleted muscles more than it did the controls at intervals longer than 180 ms. However, fusion of the two twitches occurred at significantly longer intervals in the creatine depleted muscles than in controls. Hypoxia did not alter the contractile characteristics more adversely in creatine depleted muscles than in controls at a low stimulation rate.

Hemodynamic performance was measured in the isolated blood perfused rat heart preparation and no significant differences were observed in the creatine depleted hearts when compared to controls in the maximum and submaximum cardiac output at a constant arterial pressure of 110 cm H_2O. However, the length of time the creatine depleted hearts were able to maintain the submaximal performance was decreased with decreasing levels of total myocardial creatine. Under hypoxemic conditions, the submaximal performance in creatine depleted hearts began to decline at a significantly higher partial pressure of oxygen. The number of hearts that survived in the GPA group was significantly less than in the control or GBA groups at higher partial pressures of oxygen.

Creatine depletion of 75-90% did not alter contractile characteristics of papillary muscles, nor the initial performance of the isolated blood perfused rat heart. Therefore, it does not appear that CP is essential in the heart on a beat-to-beat basis, but it is important in buffering ATP levels.

INTRODUCTION

Living cells use adenosine triphosphate (ATP) as a source of energy in numerous functions. In the myocardium, for example, fatty acids and glucose are oxidized aerobically to produce ATP and the energy generated by ATP production is used in the steps of excitation, contraction and relaxation (as reviewed by Bing 1965; Ross and Sobel 1972; Neely and Morgan 1974). Creatine phosphate (CP) also plays a role in energy provision but has always been considered a buffer, or a secondary store of energy maintaining ATP levels. This classical view of CP has been accepted since CP is present in the myocyte at a concentration five times that of ATP. Also, during contraction, the terminal phosphate group (~Pi) is cleaved off from ATP and is replenished by CP, preventing a decline in ATP levels.

However, with the discovery of creatine kinase (CK) isozymes and their specific location within the cell, the concept of the relative importance of CP in cellular energetics has changed. Moreover, several investigators (Seraydarian et al. 1961; Gudbajarnason et al. 1970; Dhalla et al. 1972) who attempted to correlate some index of myocardial contractility with tissue levels of ATP, illustrated in experiments using ischemic and anoxic models, that ATP did not correlate well with contractility, but CP did. These findings led to "functional compartmentation" of ATP and then to the hypothesis about CP, acting as a shuttle, transferring high energy phosphate between discrete ATP pools in cellular sources and sinks (Bessman and Geiger 1981; Saks et al. 1978). The hypothesis was well received, particularly because within cells, creatine kinase (CK)

isozymes had been discovered to be in the vicinity of organelles involved in energy production and consumption.

On the other hand, it has also been proposed by Meyer et al. (1984) that CP functions as an energy buffer which can provide a mechanism for facilitated diffusion of high energy phosphates. Mainwood and Rakusan (1982) proposed that CP maintains a high ATP/ADP ratio at the sites of utilization by its ability to diffuse faster throughout the cell. In addition, Garkinkel and Kohn (1980) suggested that CP buffers against sudden changes in oxygen supply and demand during transition from low to high workloads by maintaining high ATP levels in a myocyte.

In attempts to determine what the function of CP is in energy transduction, researchers have used creatine analogues to study the effects of creatine depletion on skeletal muscle function (Fitch et al. 1974, 1975, 1978; Fitch and Chevli 1980; Petrofsky and Fitch 1980). These studies have illustrated that CP is not essential for skeletal muscle function. In fact, endurance of some muscle types was improved in CP depleted animals.

This study was undertaken to determine if the reduction of total creatine (creatine and CP) will affect the normal functioning of the heart, thus determine if CP is an essential transporter of energy or an energy buffer in the myocardium. We will describe experiments in which contractile characteristics and hemodynamic parameters have been investigated in rats that were depleted of total creatine by the substitution of structural analogues, β-guanidinobutyric and β-guanidinopropionic acids.

REVIEW OF LITERATURE

A) Myocardial Energy Production and Consumption

The heart works continuously even when an organism is at rest, and unlike skeletal muscle it can work for only several seconds in the absence of oxygen. When the heart is deprived of oxygen, aerobic energy production falters and the pump's performance deteriorates within seconds (Braunwald, 1979). The beating heart, per gram, consumes 0.08 to 0.15 ml of oxygen per min and the energy derived from this oxygen consumption is used to maintain normal pump function (Gibbs 1978).

Energy is generated with or without oxygen in one of two metabolic pathways, glycolysis or oxidative phosphorylation. As has been reviewed by Gibbs (1978), in the heart under normoxic conditions, the major energy producing reaction is oxidative phosphorylation which must be sustained if the heart is to maintain normal function. Under hypoxic or ischemic conditions, ATP production takes place predominantly by the glycolytic pathway and compensates for the cessation of aerobic ATP production for a short period of time. Ultimately, under ischemic conditions accumulation of glycolytic intermediates and protons occurs, leading to the inhibition of glycolysis and to the development of intracellular acidosis which results in inhibition of all energy production (Ach et al., 1979).

Oxidative phosphorylation takes place within the mitochondria that are lined up in parallel with the myofibrils. Fats, carbohydrates and lactate are the main substrates for aerobic metabolism and are oxidized to carbon dioxide and water by the tricarboxylic acid (TCA) cycle (summarized by Lehninger

1971). Carbohydrates or fatty acids are degraded to acetyl-CoA (acetyl coenzyme A) and enter the TCA cycle which generates ATP through a series of oxidative reactions, catalyzed by enzymes of the respiratory chain. These enzymes are tightly bound to the inner surface of the inner mitochondrial membrane, coupling ATP formation to oxidation of nicotinamide-adenine dinucleotide (NADH) and flavin-adenine dinucleotide ($FADH_2$). These reduced coenzymes have been produced when carbodydrates and free fatty acids were oxidized in the TCA. During the oxidation of NADH and $FADH_2$ in the respiratory chain, a large amount of energy is liberated and can be trapped in the form of high-energy phosphate bonds of the ATP. From one single mole of glucose, 36 moles of ATP can be produced through this oxidative process while through anaerobic glycolysis only two moles of ATP can be produced.

The rate of ATP production in the mitochondria is determined primarily by the availability of substrates, oxygen and the relative concentrations of ATP, ADP and inorganic phosphate (Pi) (Kohn et al., 1979). As long as the myocardium remains well oxygenated, ATP is formed at the rate at which it is utilized and a fine balance is usually maintained between oxygen supply and demand. However, pathological conditions creating chronic overload situations, increase the requirement for oxygen that may surpass the aerobic capacity and compromise the function of the myocardium (Braunwald, 1971).

Researchers as early as 1915 (Evans and Matsuoka 1915) noted that a direct correlation between cardiac oxygen consumption and the work of the heart existed. Sarnoff et al. (1958) recognized the importance of oxygen demand and discovered

that greater oxygen cost was incurred when cardiac work increased during "pressure work" as opposed to "volume work". These authors realized that tension development against augmented pressure (increased afterload) required greater expenditure of energy than when increased work was the result of greater volume. Thus, wall tension was observed to be a fundamental determinant of oxygen consumption in the working heart. A later study showed that other parameters such as the velocity of contraction was also a determinant of oxygen consumption (Sonnenblick et al. 1966). By the early 1970's, the requirement for oxygen was accepted as a function of several components of ventricular contraction (Braunwald 1971):

- tension development
- velocity of contraction
- contractile state
- heart rate
- basal metabolism
- depolarization
- repolarization
- direct metabolic effects of catecholamines
- activation
- maintenance of active state
- shortening against a load -- the Fenn effect
- relaxation

The supply of oxygen was recognized as being determined by (Weber 1979):

- arterial oxygen content
- hemoglobin concentration

- myoglobin concentration
- coronary flow and its distribution through the microcirculatory network, such as redistribution of flow from non-exchanging to exchanging vessels
- density of open vessels
- anatomic characteristics of the coronary microcirculation, including the relation of capillaries to myocardial fibers and the diffusion distances for oxygen
- the proportion of the delivered oxygen that is driven from the capillaries into the intracellular compartment.

Balance between oxygen demand and supply is essential to maintain optimal myocardial function. An increment in ventricular chamber pressure, volume, inotropic or chronotropic state of the heart, increases the metabolic demand and an increase in oxygen supply is essential. Since it takes time (30 to 60 seconds) for adjustments to take place to increase oxygen and substrate delivery, the heart must have some readily mobilizable reserve of chemical energy to meet sudden increases in ATP utilization. This role has been given to CP in the early 1900's after the discovery of ATP (Lohmann, 1929).

B) High Energy Phosphogens

Creatine was first described by Chevreul in 1835 who isolated it in a crystalline form from meat extract. Thunberg (1911) was the first to show that adding creatine to respiring muscle homogenate increased the rate of oxygen uptake. Riesser (1922) who was impressed by the distribution of creatine within tissues found that it was present in greater amounts in rapidly contracting white muscle than in the slower red muscle.

Prior to 1927, creatine was considered to be the only essential energy component of muscle function. But in 1927, a "phosphogen" (now referred to as CP) was discovered by the Eggletons (Eggleton and Eggleton 1927) and independently by Fiske and Subbarow (1927). It was the first compound to be characterized as an energy metabolite carrying a "high energy phosphate bond". Subsequently the energetics of CP hydrolysis were quantified by Meyerhof and Lohmann in 1928, and in 1930, Lipman and Meyerhof had shown that CP broke down during muscular activity liberating free creatine. It was at this time that CP was established as a direct energy donor.

The role of CP in muscle contraction became complicated by the discovery of ATP by Fiske and Subbarow in 1929 and by Lohmann (1929). In 1934, Banga and Szent-Györgyi demonstrated that actomyosin contracts only upon the addition of ATP. In 1934 Lohman proved that CP splitting in muscle could not occur unless adenylate compounds were present and showed that the "Lohman reaction" (as it became known) was reversible:

$$ATP + C \underset{r}{\overset{f}{\rightleftharpoons}} ADP + CP$$

In the early 1940's, CP was considered to be an energy buffer made available for resynthesis of ATP, which was used in muscle contraction. However, in physiological terms no direct experimental evidence showed that ATP was the true energy donor since no one had demonstrated that ATP broke down in living muscle. So far, with either single twitches or tetany, the only changes in high-energy compounds seemed to be the breakdown of CP (Meyerhof and Suranyi 1927; Meyerhof and Schultz 1935). Hill (1950) challenged biochemists to show that ATP was truly the source of energy for muscle contraction. In fact, in 1954 Mommaerts showed that ATP levels did not change during contraction, supporting Hill's challenge. Direct proof that ATP was the immediate source of energy for contraction was finally provided by Cain and Davis (1962), who demonstrated a decrease in ATP in single twitches of muscle poisoned with 1-fluoro-2,3-dinitrobenzene (FDNB, a non-specific inhibitor of creatine kinase). Adenosine diphosphate increased while no changes in levels of CP were observed.

This presumably settled the role of ATP and CP in muscle contraction; when ATP was consumed during the contraction, it was replenished by CP. However, the details of energy supply and demand within a cell were still nebulous.

Studies on energy utilization in cardiac tissue by Gudbjarnason et al. (1970), Seraydarian et al. (1961), and Dhalla et al. (1972) emerged with the conclusion that under conditions of hypoxia, ischemia or energy restriction by inhibitors of glycolysis and oxidative phosphorylation, a rapid decrease in CP content (by 75%) accompanied the decline in contractile force.

The modest changes in ATP (decline by 20%) were difficult to explain when cardiac contractions stopped. Moreover, Gercken and Schlette (1968) showed that CK inhibition in intact cardiac cells allowed only a small pool of ATP to be used directly for contraction.

Similar results were obtained in experiments that correlated the declining CP levels with the decrease in the ability to develop tension both in the frog sartorius (Seraydarian et al. 1961), and in the mouse soleus (Spande and Schottelius 1970). Furthermore, increased rates of relaxation in frog gastrocnemius correlated with diminishing CP levels in experiments where a stimulus was given until the muscles were fatigued (Dawson et al. 1980).

A postulate arose from these experiments that a large part of the cellular ATP is not directly accessible for use in contraction, that only a small amount of ATP around the myofibrils is available and has a high rate of turnover. When CP declines and is not regenerated, this portion of the ATP pool is not replenished and contraction ceases (Gudbjarnason et al. 1970). It was further postulated, that this small pool of ATP is normally replenished at the expense of CP, which had been suggested to be a carrier of high energy phosphate bonds connecting mitochondrial ATP pools and possibly other pools within a cell.

Studies conducted between 1964 and 1973 lead to the recognition of two important aspects of intracellular energy transport. One, that functional compartmentation of ATP in the cell may prevent rapid transport of energy within muscle cells and two, that a Ca^{2} pathway for energy transport from the

compartmentalized sites of ATP to the sites of utilization involving several creatine kinase isozymes may exist.

Creatine kinase (ATP:creatine-phosphotransferase, EC2.7.3.2) was first described by Burger et al. (1964) who identified three isozymes of CK separable on agar gel electrophoresis and named them MM, MB and BB. That same year, Jacobs et al. (1964) isolated a fourth CK present in mitochondria from isolated brain, heart and skeletal muscle. This CK was further identified by and specified by Sobel et al. (1972) as CK_m. By 1973, Scholte had proven that CK_m is bound to the outer side of the inner mitochondrial membrane and that it accounts for approximately 40% of the total enzyme activity within a cell.

Of the other isozymes, MB, BB and half of MM are located in the cytoplasm and account for another 40% of the total CK activity. The remaining MM isozymes are bound to myofibrils, sarcoplasmic reticulum and plasma membrane, accounting for 20% of the total CK activity. The fraction of MM bound to the myofibrils was shown to be located at the M-lines of the sarcomeres (Turner et al. 1973; McClellan et al. 1983; Savabi et al. 1984) and appears to be an integral element of the M-line structure and the A bands (Sharov et al. 1977; Mani and Kay 1976). In contrast the BB isozymes were demonstrated to exist on the Z line region, but only in the chicken skeletal muscle and heart (Willimann et al. 1977).

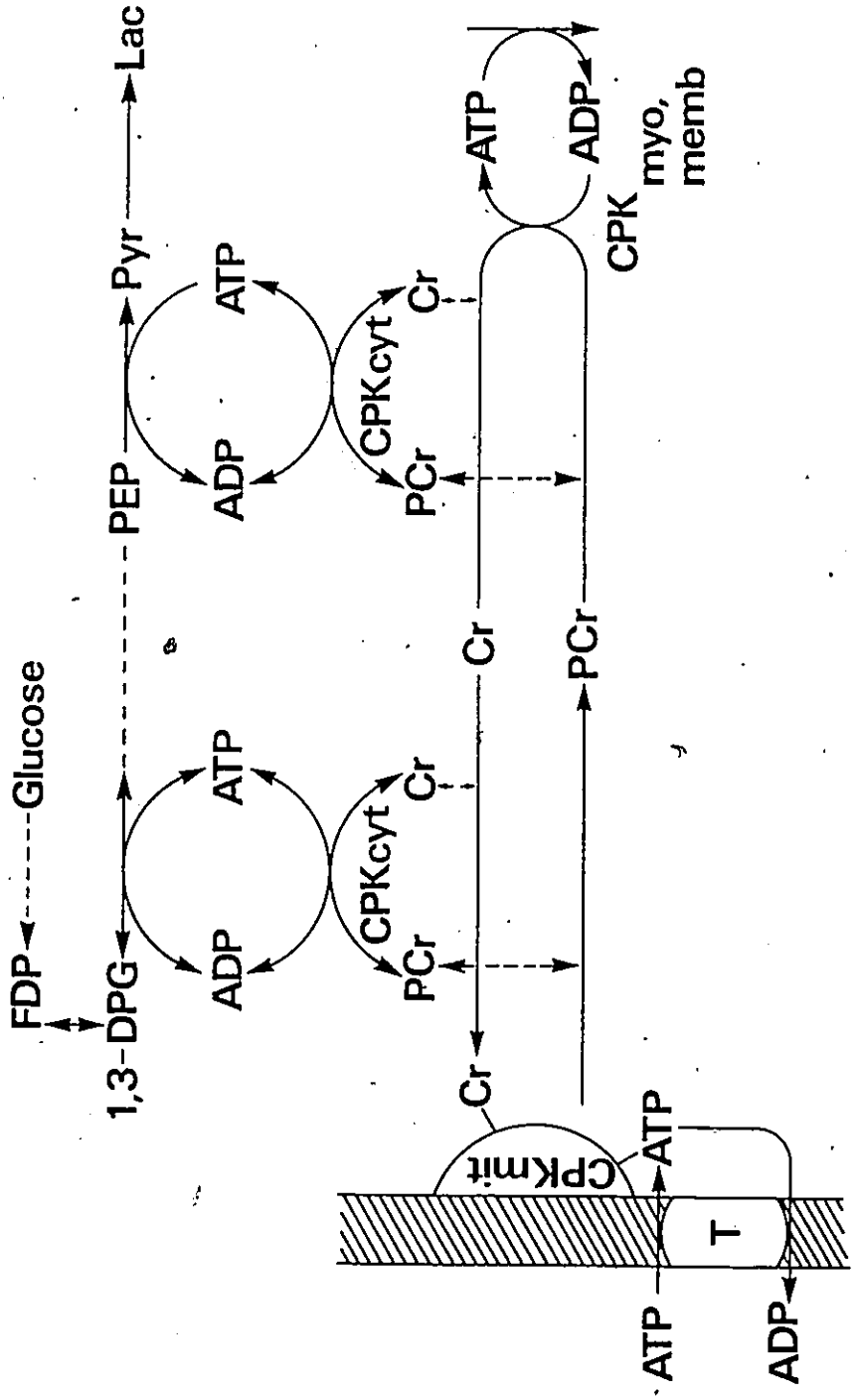
Yagi and Mase (1962) and Mani and Kay (1976) proved that the MM isozyme binds to isolated, purifed myosin. When CP and ADP were supplied to the myosin, the myosin ATPase activity was much higher than when ATP was added alone in the presence of calcium

ions. McClellan et al. (1983) also provided evidence for the presence of adenine nucleotides at the myofibrils, proving that the two were tightly bound. They concluded that the bond necessitates the phosphorylation of ADP to ATP at the site and that the conversion was done by the CP-dependent CK at the myofibrils. These findings have led investigators to conclude that both the energy production and its consumption depends on highly active CK and that the very specific localization and activity of the isozymes is unique to cardiac cells, playing a key role in intracellular energy regulation.

The first investigators to formulate the CP-shuttle hypothesis were Gercken and Schlette (1968). They postulated that once the ATP produced by oxidative phosphorylation is transferred from the inner mitochondria to the intermembranous space, CP is formed and diffuses to cytoplasm. It then can be used for ATP synthesis by the cytoplasmic CK isozymes located near the myofibrils. The creatine formed in this reaction shuttles back to the mitochondria to stimulate respiration and produce more ATP and ultimately CP that shuttles to the sites of utilization (Figure 1). This hypothesis in the last two decades has been supported and expanded by a number of investigators (Bessman and Fonyo 1966; Saks et al. 1974; 1975; 1976; 1978; 1980; Gudbjarnason et al. 1970; Seraydarian et al. 1974; Jacobus and Lehninger 1973; McClellan et al. 1983; Savabi et al. 1984; Jacobus 1985; Bessman and Carpenter 1985).

The most convincing evidence to support this hypothesis came from studies on isolated mitochondria, which under appropriate experimental conditions (high creatine to total

Figure 1: Schematic representation of the creatine phosphate shuttle. CK_{mit}, CK_{cyt}, $CK_{myo,membr}$, are creatine kinase isozymes located in mitochondria (mit), cytoplasm (cyt) and bound to myofibrils (myo) or sarcoplasmic reticulum and plasma membrane (membr), respectively. T is adenine nucleotide translocase; FDP, 1,3-DPG, PEP, Lac, are: frucose 1,6-diphosphate, 1,3-diphosphoglycerate, phosphophenolpyruvate, and lactate respectively. (redrawn with permission from Jacobus & Ingwall, 1980)


Lac
Pyr
PEP
Glucose
FDP
1,3-DPG
ATP
ADP
CPKcyt
Cr
PCr
CPK
myo,
memb
CPKmit
T
ATP
ADP

adenylate ratio) demonstrated that creatine is a powerful stimulator of respiration and showed that CP is the primary high energy phosphate leaving the mitochondria (Bessman and Fonyo 1966; Jacobus and Lehninger 1973). Also, a study by Yang et al. (1977) revealed that labelled inorganic phosphate had a faster incorporation into CP than ATP in a bath containing isolated muscle mitochondria. Such experimental evidence led several investigators (Saks et al. 1976; Bessman and Geiger 1982) to conclude that mitochondrially produced ATP had preferential access to the reactive sites on the CK_m. This finding was further supported by Vignais (1976) who showed that an adenine nucleotide translocator was incorporated into the inner mitochondrial matrix and functioned to import ADP from the cytosol and simultaneously export ATP into the intermitochondrial space.

Klingenberg (1970), and Jacobus and Lehninger (1973) postulated that some type of "functional coupling" may occur between the adenine nucleotide translocator and CK_m since the latter is localized on the outer side of the inner mitochondrial membrane. Saks et al. (1978) proposed that large amounts of CP are produced not only because the adenine nucleotide translocator increases the concentration of ATP on the outer aspect of the membrane but also because it transports ATP directly to the active sites of CK, due to the spacial proximity of the adenine nucleotide translocator to the CK_m. According to Saks et al. (1978) the rate of CP production depends on the rate of ATP and ADP translocation.

Several investigators tried to provide further experimental

evidence using physiological preparations to support the shuttle. One such group was that of Saks et al. 1976) who placed strips of frog heart in a bath for 8 hours and observed changes in contractile force as well as in intracellular ATP, CP and creatine levels. During the 8 hours, the muscle strips developed a "hypodynamic state" which resulted in a small decline in ATP, and a significant fall in CP and creatine as force development decreased. Adding creatine to the perfusate in concentrations up to 40 mM rapidly increased the intracellular levels of creatine, augmented contractile force three fold but did virtually nothing to the values of ATP. Although these authors claim that the addition of creatine increased contractile force in frog preparations, similar additions of creatine to mammalian skeletal muscles or myocardial preparations have not yielded the same results. Addition of CP to mammalian species has not shown to increase developed force, mainly because phosphorylated compounds do not cross mammalian membranes (Mainwood and Alwart, unpublished data).

Another group of researchers (Rosenshtraukh et al. 1979), who added NaCN to frog ventricle strips, found a rapid decrease in contractile force to 10% of the original value. At the same time, CP levels declined to 10-15% of initial levels, but ATP concentration remained unchanged. They showed that the addition of CP to the system not only increased the force of contraction but also affected the ionic processes of cell membranes. The duration and magnitude of the ATP plateau phase was increased concomitantly with force.

Saks (1980) and other researchers (Bessman and Carpenter 1985; Rosenshtraukh et al. 1979) began to believe that the

hypodynamic state was related to the decreased efficiency of energy transport by CP, the level of which decreased as a result of creatine washout. Since creatine was not present in the perfusing medium, a gradient was set up, allowing diffusion along the gradient to take place. Even though these experiments have never been duplicated in any mammalian species, the conclusion was that the contractile force does not relate to total ATP levels but rather to CP levels.

In summary then, it had been proposed that the movement of ATP through the cytoplasm is restricted because the molecule is compartmentalized and that CP would serve as a mobile high energy carrier between the sites of production to the sites of utilization.

Mainwood and Rakusan (1982) in an attempt to determine if CP is essential for cell function, either as a buffer or a shuttle, analyzed the diffusion profiles of ATP and CP from the cell's periphery to the core. This was done in the presence of adequate CK, insuring that an equilibrium between the high energy phosphates existed. When the diffusion profiles were calculated with ATP alone, ATP concentration decreased and ADP concentration increased substantially from the periphery to the core of the cell. Also, the maximum work per mole of ATP hydrolyzed $(-dG/d\xi)$, which is dependent on the ATP/ADP ratio, fell from about 60 kJ/mole to about 50 kJ/mole when calculated at the periphery and the core of the cell. When ATP and CP were considered together, with enough CK present to ensure that ATP, ADP, CP and creatine were maintained in equilibrium at all rates of ATPase activity, then ATP levels at any point in the cell were

maintained within 1% of their initial resting value. This was true until about half of the CP in that total area was utilized. Similarly, ADP gradients were virtually abolished and $-dG/d\xi$ was maintained at a high level. These observations were made on the assumption that ATP diffusion is not restricted but diffusion of CP in the cytosol is 30% greater than that of ATP.

Based on these calculations, Mainwood and Rakusan (1982) proposed that CP can be an effective agent for the transfer of energy over large distances in the cytosol, which is able to maintain an adequate supply of high energy phosphates from the mitochondria to sites of utilization, with little change in $-dG/d\xi$. These authors stated that diffusion of ATP is probably not restricted nor compartmentalized, but CP can diffuse faster than ATP from the periphery to the core of the cell, increasing the efficiency of high energy phosphate transport through the cell.

This concept of efficient energy transfer by CP had been further expanded by Meyer et al. (1984). These investigators proposed that it is more appropriate to consider CP as an energy buffer which provides a mechanism for facilitated diffusion of high energy phosphates. This idea was based on facilitated diffusion of oxygen as oxymyoglobin. By applying Fick's law of diffusion, and using concentrations of ATP, ADP, CP and creatine at the myofibril surface as 5, 0.02, 20 and 8 mM and a K of 100 for CK, Meyer et al. (1984) calculated that over 99% of the flux of high energy phosphates can be carried by CP. These calculations are representative of a near equilibrium reaction. This is true even if the reaction is displaced from equilibrium,

as long as the ATP/ADP ratio is greater than 1.

Also, according to Meyer et al. (1984), the so-called intimate coupling between ATP and CK enzymes occurs to raise the local enzyme activity where the net flux is the greatest. By this mechanism, the overall reaction is maintained near equilibrium with less total enzyme activity than would be necessary with uniformly distributed enzyme. Perhaps the existence of different enzymes is to achieve this purpose, that is, the "coupling or so-called localization". Furthermore, Meyer et al. (1984) postulated that by maintaining the reaction at equilibrium, compartmentation of ATP does not occur.

In their opinion, the classic storage-buffer function of CP has been viewed to be the consequence of the equilibrium status of the CK reaction. Similarly, the transport function of CP is also thought to occur when the reaction is at equilibrium. Thus, according to Meyer et al. (1984) both the transport and storage function of CP are equivalent and inseparable functions of CP and the CK reaction responds instantaneously to any change in ATP, ADP and creatine, minimizing changes in their levels as the high energy phosphates are utilized in the cell. Therefore, a high ATP/ADP ratio is maintained as a consequence of the near equilibrium reaction of CK and the greater diffusability of CP is not the result of any functional compartmentation of ATP.

In a previous study, Meyer and Terjung (1979) observed that in rat soleus as in cardiac muscle only a 10% decrease in ATP was observed during stimulation to exhaustion. However, if the soleus muscle was stimulated after treatment with iodoacetate, blocking glycolysis, up to 75% of cellular ATP was hydrolyzed.

Similarly, in frog skeletal muscle, electrical stimulation to exhaustion with added caffeine caused over one-half of the remaining ATP to be utilized (Nasser-Genting et al. 1981). Therefore, ATP compartmentation may not be the explanation for failure of muscle contraction under ischemic, anoxic or exhaustive conditions. According to Meyer et al. (1984), in order to show that a compartment exists, experimental evidence must be provided based on quantitative analysis which cannot first be explained by facilitated diffusion.

Garfinkel and Kohn (1980) and Ach et al. (1979) believed that the purpose of the CP is to buffer the cell against sudden changes in available oxygen. The cell with its limited nucleotide transit may be more easily impaired by anoxia and CP can buffer these sudden changes. Garfinkel and Kohn (1980) argued that the heart must always maintain ATP level sufficiently high to support mechanical work, drive ion pumps and maintain substrate level phosphorylation. It must also adjust to physiological conditions including changes in workload that increase the use of ATP more rapidly than the rate at which ATP can be synthesized. Thus, the heart must have some readily mobilizable form of energy to meet transient falls in ATP.

The heart is afforded some metabolic reserve by the vascular resistance of coronary arteries and by the ability to increase oxygen extraction. At the onset of increased work, transient hypoxia occurs and coronary flow increases, possibly because of the release of adenosine or the decrease in partial pressure of oxygen (Rubio et al. 1972). The flow may be redistributed from nonexchanging vessels to exchanging vessels or the density of

open channels may increase (Friedman 1968). If the oxygen demand keeps increasing, an aerobic limit will be reached and the coronary reserve dilating capacity will be exceeded. With continual increase in myocardial contractility at the limit of myocardial oxygen capacity, hypoxia ensues and results in lactate production which in turn limits contractility (Braunwald 1971; Weber 1979).

In order to provide evidence to support the CP buffer theory a computer model of an isolated working pyruvate-perfused rat heart based on previous models (Kohn et al. 1979) was designed by Ach et al. (1979). Using the data from experimental work by Illingworth et al. (1975) of a pyruvate-perfused rat heart preparation, they reproduced the levels of metabolic substrates and their fluctuations with appropriate temporal profiles. The model showed that during the first 0.5 min of the simulated workjump, CP decreased by 50% while ATP levels remained constant. When oxidative phosphorylation increased in response to the increased workload, CP levels recovered. This suggested to these authors that even when oxidative metabolism was increased in response to the increased work of the heart, some buffering of ATP by CP did occur and was necessary in the transition step from low to high workloads.

A similar model based on experiments by Opie et al. (1971) was simulated by Garfinkel et al. (1979) in which a glucose-perfused rat heart was suddenly made to work hard. Bursts of glycogenolysis were accompanied by an increase in Mg^{+2} levels, which increased the activity of glycolytic enzymes. After a few seconds, coronary flow and hence oxygen delivery increased,

allowing increased oxidation of free fatty acids. During the period of adjustment, ATP fell by 9% and CP fell approximately by 55% but later increased, showing partial recovery as steady-state work was achieved. Thus when workload increases faster than oxygen supply, the CK equilibrium is shifted by the falling pH (during the glycolytic period) toward the production of ATP at the myofibrillar end of the cell. This experiment suggested to Garfinkel and Kohn (1980) that there is a great dependence on CP to maintain ATP levels. On the basis of these results they felt that ATP levels are buffered at the expense of CP, which supplements endogenous stores of fuels and oxygen to sustain ATP production and contractile function during periods of stress.

C) The Use of Analogues to Deplete Creatine and Creatine Phosphate in Muscle

To test the CP shuttle vs. buffer theory, CK inhibitors would permit the most direct experimentation however, no CK-specific inhibitors are presently available. Although Cain and Davis used FDNB to inhibit CK, it is not specific to CK isozymes only and may inhibit other enzymes and affect various proteins. There are, however, creatine analogues such as β-guanidinopropionic acid (GPA) and β-guanidinobutyric acid (GBA) which inhibit creatine uptake by muscle, permitting one to study the effects of creatine and CP depletion on muscle function. These structural analogues have been synthesized and fed to animals in attempts to study creatine metabolism in muscle (Fitch et al. 1968; 1974; 1975; 1978; Fitch and Chevli 1980; Mohanna et al. 1978; Petrofsky and Fitch 1980; Mainwood et al. 1982a,b). Depletion of total creatine (creatine and CP) by 65-90% in skeletal muscles was achieved by replacing creatine and CP with the analogue, GPA. This analogue as well as GBA is absorbed through the gastrointestinal tract, is transported to the muscle and competes for entry with creatine at the uptake sites of the sarcolemmic membrane (Fitch et al. 1968).

In order to determine the effectiveness of the method which replaces creatine by the analogues, Fitch and Shields (1966) first studied creatine movement across the rat extensor digitorum longus (EDL) muscle using creatine-C^{14}. They found that the entry process of creatine is energy-dependent and carrier-mediated. They also observed that creatine is retained intracellularly by its phosphorylation. How the unphosphorylated

form of creatine is normally retained in muscle is not completely understood.

In 1968, Fitch et al. investigated the substrate specificity and the inhibition of this transport system. A variety of compounds were tested, and those that were able to inhibit entry, such as N-ethylguanidinoacetic acid, guanidinoacetic acid and GPA possessed the amidine group. Fitch et al. (1968) concluded that creatine and its analogues enter the cell via a transport site specifically adapted to interact with an amidine group. From all of the analogues tested, GPA was the most effective and most competitive in inhibiting creatine entry into skeletal muscle. The Vmax of GPA is identical to that of creatine but the Km is less: 0.2 mM for GPA versus 0.5 mM for creatine (Fitch et al. 1968). Shields and Whitehair, (1973) used GPA in feeding trials and found that the analogue was a competitive inhibitor of creatine entry into skeletal muscle, without the appearance of any marked histologic or functional alterations. Since then, GPA has been used extensively to study the effects of creatine depletion in skeletal muscle.

Fitch et al. (1974) depleted rat gastrocnemius of CP by 93% (from 22.5 to 1.6 µmol/g of wet tissue) using GPA in 1% proportion to the standard chow, and a new phosphorylated guanidine compound was found in concentrations as high as 30 µmol/g of wet weight after 5 weeks of feeding the analogue. On examination by chromatography this new compound was indistinguishable from phosphorylated β-guanidinopropionic acid (GPAP) formed <u>in vitro</u>. These authors also found that the GPAP that is formed <u>in vitro</u> is relatively ineffective as a substrate

for CK. However, when the gastrocnemius muscles of the GPA-fed rats were stimulated under anoxic conditions, the GPAP decreased significantly (from 17.0 μmol/g to 13.3 μmol/g of wet weight), while GPA increased from 6.5 μmol/g to 10.5 μmol/g of wet weight. Adenosine triphosphate decreased negligibly, which suggested to the authors that under anoxic conditions GPAP is labile and may be able to serve as a regenerating system for ATP in the same manner as CP. Fitch et al. (1974) also suggested that metabolic adaptations could occur, since the animals appeared to be in good health despite the severe depletion of CP *in vivo*. They surmised that GPA might be able to serve as a substrate for CK but further study of this mechanism was warranted, particularly since *in vitro* GPAP was found to be ineffective as a substrate for CK.

Fitch et al. (1975) fed rats 1% GPA in their standard diet to determine the extent and effects of depletion in various organs. They found that GPA accumulated in skeletal muscle, heart, liver, kidney and spleen and depleted the muscle of creatine by 70-80%. Phosphorylated guanidinopropionic acid accumulated only in skeletal muscle and the heart, neither GPA nor GPAP was detected in the brain or testes.

In another study, Fitch et al. (1975) stimulated hypoxic rat anterior tibialis muscle to contract isometrically *in situ* until twitch tension fell to 25% of the peak value. Muscles of rats fed 1% GPA failed to exhibit a "staircase" phenomenon and developed 28% less tension than muscles of control rats. Creatine phosphate in the muscle of control decreased from 15.78 to 1.52 μmol/g of wet tissue while in the depleted muscle, CP decreased only from 0.73 to 0.30 μmol/g and GPAP decreased from

30.34 to 19.45 μmol/g of wet tissue. These researchers calculated that the use of high-energy phosphates was reduced by 32% in the GPA-fed rats. They concluded that GPAP can replace CP as a source of energy and may be able to sustain muscle contraction even though isometric twitch tension declined more rapidly in the GPA-fed rats than in the controls.

However, in a later study where rats were fed 1% GPA, Fitch et al. (1978) found that GPAP accumulated in both soleus and plantaris muscle in concentrations of 20-25 μmol/g and was not used significantly for muscle contraction. With fatiguing isometric contractions, the GPAP concentration decreased by less than 12%, while CP concentrations decreased by 68-70% in muscles of control rats. This difference in phosphogen consumption showed that skeletal muscle does not use large amounts of the phosphorylated analogue during contractile activity.

This finding was further substantiated by Mainwood et al. (1982a), who stimulated diaphragm strips from rats fed 1% GPA in the presence of inhibitors of respiration (NaCN) and glycolysis (iodoacetate). They postulated that if GPA was phosphorylated and used instead of CP during muscular activity, inhibition of respiration and glycolysis would test the use of any effective energy reserve that may be present. The muscles were stimulated every 10 s by a 0.2 second tetanic stimulus of 100 Hz. Control diaphragm strips gave 15 ± 2 contractions before contracture developed while the strips from GPA-fed rats gave only 4 ± 1 contractions. The authors concluded that GPAP was not contributing to any effective energy reserves of the muscles. They proposed that feeding analogues to decrease CP levels is an

effective method which may be used to study the function of CP in muscle.

To investigate the function of contractile characteristics of skeletal muscle, Petrofsky and Fitch (1980) fed rats 1% GPA for 10 weeks. They found that the maximal isometric tetanic tension was decreased by approximately 12% in the creatine depleted plantaris muscle and that the muscle weighed 30% less than control muscle. No other variables were affected in the plantaris muscle. On the other hand, the creatine depleted soleus muscle took 34% longer to reach maximal developed tension which was 37% lower. However, the frequency of synchronous stimulation required to obtain maximal isometric tension was only 30 Hz versus 50 Hz for the controls.

These authors also measured the isometric endurance of the soleus muscle by sequentially stimulating motor nerves at 10 Hz while the muscle was held at the optimal length (55% of the previously measured fatiguing isometric contractions). The length of time that these contractions could be held was called the isometric endurance of the muscle. Petrofsky and Fitch (1980) found that isometric endurance was almost three times as long in the creatine depleted muscles than in controls. Recovery of the depleted muscles was almost complete within 1 minute after the end of stimulation, the same amount of time it took the control muscles to recover.

Mainwood et al. (1982b), after feeding rats 1% GPA for 4-5 weeks, found that the diaphragm of the GPA-fed rats had longer time to peak tension and half-relaxation in single twitches and that maximum contraction tension during a 0.2 second tetanus at

100 Hz was only 8% less than in the controls. Time to half-relaxation however was reduced by about 24% in the creatine-depleted muscles. Recovery from a train of 0.2 second tetanus at 100 Hz was faster in the creatine depleted muscles than in controls and the depression of tension was less.

In contrast, high intensity activity appears to be affected in creatine depleted animals as was reported by Shields et al. (1975). They evaluated the muscle function of rats fed 1% GPA on a high intensity, short duration exercise program using the running wheel. The running performance of the rats, assessed by the number of revolutions performed on the running wheel, was decreased. The Type II (white) fibers from the gastrocnemius muscle were smaller in analogue fed rats than in controls and when the mean area of the fiber type (Type II) was divided by the body weights, the difference persisted. The Type I fibers in the soleus were not significantly different in the analogue fed rats than in the controls.

From these studies, it can be concluded that depletion of creatine and CP does not effect the Type II fibers (fast-twitch), but decreases the power output of the Type I fibers (slow-twitch). On the other hand, the endurance of Type I fibers at submaximal workloads appears to be improved and the recovery from these workloads is also enhanced. This may mean that the aerobic capacity of the Type I fibers is increased while the anaerobic capacity is reduced.

As demonstrated by the above experiments, GPA has been used extensively to study the effects of depletion of creatine and CP. This analogue has been used because it is a poor substrate for CK. But GPA can accumulate (at least in skeletal muscles) in its

phosphorylated form up to concentrations of 30 μmol/g of wet weight. Although the phosphorylated GPA is a poor substrate for CK, having a Vmax of 0.1% of that produced by CP, it can still substitute to a limited extent for CP (Fitch and Chevli 1980).

Another analogue, β-guanidinobutyric acid (GBA), has been studied by Fitch and Chevli (1980) and was also found to inhibit creatine transport into muscle. GBA does not serve as a substrate for CK, having a Vmax of 0.01% that of creatine and it does not inhibit CK activity. GBA has been shown to be as potent as GPA in depleting creatine but does not accumulate as a phosphorylated compound and has not been used as extensively as GPA.

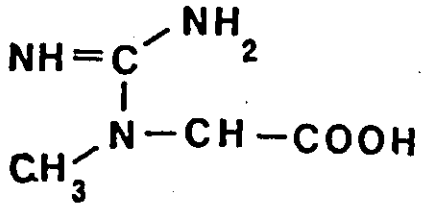
The structures of creatine, GPA and GBA are shown in Figure 2. Creatine has the same molecular formula as GPA but has a methyl group attached to the guanidine group. The two analogues have the guanidine group present in the position on the same chain, but GBA differs from GPA by having a methyl side-chain. Some of these differences among these compounds may account for the different affinity for the carrier and also for the ability of the phosphorylated analogue, (GPAP) to be potentially used as a substrate by CK (Fitch and Chevli, 1980). The similarities among the three compounds may explain their ability to bind to the same carrier.

GPA can also deplete myocytes of taurine. This has so far been demonstrated only when a heart is isolated and perfused with a perfusate containing GPA. Dietary regimes using GPA to deplete taurine have not been reported. When compared to several compounds that inhibit taurine entry, GPA was the least

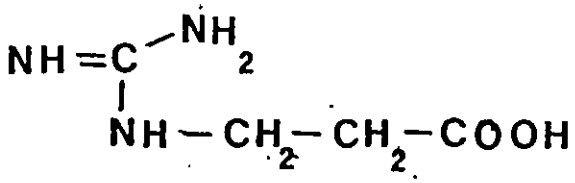
effective. GPA decreased taurine concentration by 21% while β-guanidinoethyl sulfonate (GES) decreased taurine concentration in the myocardium by 80% (Huxtable et al. 1979).

Taurine is found in the heart in high concentrations and comprises up to 60% of the total free amino acid pool. Although it is elevated above normal levels in congestive heart failure, and has shown to antagonize the negative inotropic effect brought about by low Ca^{++} concentrations, the depletion of taurine from normal levels by 80 to 90% by GES has no dramatic effect on the heart (Guidotti et al. 1971). Although GPA does decrease myocardial taurine, it does only by 21%. Therefore, effects of GPA on taurine metabolism and its consequences on heart function may be disregarded. GBA has not been investigated with respect to its effect on taurine displacement from the heart, and its effects on taurine are unknown.

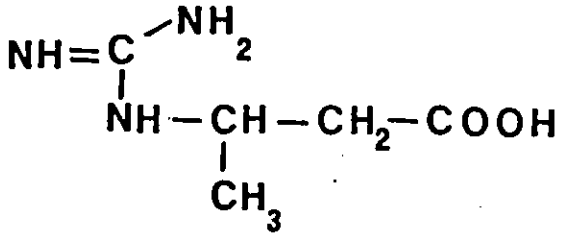
Figure 2: Molecular structures of creatine and β-guanidinopropionic acid (β-GPA) and β-guanidinobutyric acid (β-GBA).



CREATINE



β-GPA



β-GBA

D) The Use of P^{31}-NMR to Study the Role of Creatine Phosphate in the Myocardium

Over the past 10 years, a new tool has been devised and used in the study of the metabolism of phosphate-containing tissues such as muscles. Hault et al. (1974) were among the first researchers who demonstrated that nuclear magnetic resonance (NMR) can be applied to living tissues. Since then P^{31}-NMR has been used extensively to study the metabolism of isolated frog, rat and human skeletal and heart muscle as well as other organs (Meyer et al. 1984; 1985). P^{31}-NMR has measured noninvasively high energy phosphates such as CP, ATP and inorganic phosphate (Pi). Over the past 5 years P^{31}-NMR has detected changes in high energy phosphates during a cardiac cycle, enabling researchers to measure the flux of CP and ATP.

Also, attempts have been made to investigate the importance of CP in muscle function by studying the relationship between metabolic fluxes through the CK enzyme in Langendorff preparations and in isolated buffer-perfused working hearts. Mathews et al. (1981) found that the reaction mediated by CK does remain at equilibrium when steady-state work is performed by the perfused heart. However, Kupriyamov et al. (1984) observed that the rate of flux from CP to ATP was increased by 50% and that oxygen consumption correlated with the rise when the work of the heart was increased. Bittl and Ingwall (1985) who measured reaction rates from CP to ATP in a buffer-perfused working rat heart, reported an increase in flux when potassium arrested hearts underwent a transition to increased workloads. The reaction at that moment was closely coupled to oxygen consumption and ATP

production. Bittl and Ingwall (1985) argued that at times of such close coupling, the reaction could not be at equilibrium and that energy channeling had to occur through the CP shuttle. This hypothesis was supported by the results of Micelli et al. (1983), who performed a similar experiment.

However, in 1983, Ingwall et al. found that under normal steady-state conditions in glucose- or pyruvate-perfused hearts, the CK reaction was in equilibrium and that flux increased only when external work was increased, such as in a transition from potassium arrest to external workloads of 150 mmHg of systolic pressure. They stated that the CK reaction is in equilibrium over a range of normal workloads and that the flux through the reaction could increase with increasing workload. The CK reaction was activated only when an energy "emergency" occurred and was an energy reserve.

Meyer et al. (1984) investigated the effects of total creatine depletion using P^{31}-NMR on rat hearts using a Langendorff preparation. No differences were found in the measured oxygen consumption between the hearts of rats fed 2% GPA for 6 months and control hearts, even though the CP levels in the hearts of analogue-fed rats were less than 10% of control levels. Slow hydrolysis of GPAP occurred in ischemic preparations, but it was too slow for GPAP to substitute for CP in mediating a steady state ATP turnover.

From the above mentioned studies it can be seen that the investigators using NMR spectroscopy have attempted to prove the physiological importance of CP in the myocardium either as a buffer or a shuttle. However, these studies do not provide conclusive evidence about the role of CP in myocardial cells.

Summary

At present, the controversy about the role of CP in muscle function still exists. Several research groups still believe the classic theory that CP functions as an energy buffer, providing high energy phosphates to ATP, maintaining a high ATP/ADP ratio via a mechanism of facilitated diffusion of high energy phosphates. In contrast, another group of investigators support the theory that CP shuttles between the sites of energy production to the sites of utilization carrying essential quantities of energy phosphates.

Since direct measurement of the CP-CK interaction to date has not been possible, researchers have tried to study the importance of CP in energy transduction from the mitochondria to the sites of utilization, by depleting muscles of total creatine (creatine and CP). This has been achieved by substituting structural analogues of creatine in the cell. These analogues, GPA and GBA competetively inhibit the entry of creatine into the myocyte. Although both of these analogues are poor substrates for CK, GPA can be phosphorylated and accumulate to high concentrations in the cell. It can substitute for CP but to a limited extent, while GBA does neither. Nevertheless, GPA has been used extensively in studies trying to determine the effects of creatine depletion on muscle function but the results show that CP depleted skeletal muscles are not impaired by the depletion, in fact, Type I fibers appear to have their endurance capacity enhanced.

Over the past five years, NMR spectra on isolated non-working and working heart preparations have been used

to determine whether CP plays an important role in energy transport in the myocardial cell. The results from these studies have provided evidence both for and against the importance of CP in energy transport and they do not clarify the role of this phosphogen in the energy transduction in the myocardium.

Statement of the Purpose

The heart is primarily an aerobic organ which produces most of its energy (ATP) within the mitochondria. In view of the controversy regarding the role of CP in energy transduction in the myocardium, we wanted to gain more information about the physiological role of creatine and CP in the heart. To achieve this goal, we studied myocardial performance under normoxic and hypoxic conditions at various workloads in hearts of rats that were fed GPA or GBA. This was done at the tissue level, by assessing the contractile properties of the isolated ventricular papillary muscles; and, at the whole organ level, by investigating the hemodynamic properties of the isolated blood-perfused working heart preparation.

At each stage of experimentation the following questions were asked:

1. Does creatine depletion by analogue substitution affect the performance of the myocardium?

2. Do the two analogues have different effects on the performance of the myocardium?

3. Can depletion of creatine by these analogues provide a useful model to test the importance of CP in the myocardium?

METHODS AND MATERIALS

A) Treatment of Animals

Male Sprague Dawley rats (250-300 g) were purchased from Charles River Canada Inc., St. Constant, Que. On arrival, they were randomly divided into three groups. Group 1 was the control and received standard ground rat chow (Purina). Group 2 received 2% GBA in the ground rat chow, while group 3 received 1% GPA in the ground rat chow. All groups were fed 15 g of their diets for each of the first 3 days, after which time the amount was increased by 3 g every 3 days up to day 14. From then on, all groups received 25 g and water was provided ad libitum. At 5 weeks, biochemical analysis of the myocardium was done to determine the extent of depletion of creatine and CP. Since depletion to this level was not adequate (see results for values, Table 1), the rats were fed for 7 weeks.

After 7 weeks of analogue feeding, we depleted rats of total creatine to levels comparable to those obtained by other investigators (Fitch and Chevli 1980; Mohanna et al. 1980). However, we also wanted to determine the length of time it takes to maximally deplete the rats of myocardial total creatine. In order to determine that, we randomly distributed Sprague-Dawley rats (250-300 g) into one of the three groups (control, GBA or GPA) and fed them one of the three diets for up to 12 weeks. Five rats in each group were sacrificed after 2, 4, 6, 8, 10 and 12 weeks to determine total myocardial creatine and analogue concentrations.

B) Synthesis of Analogues

The synthesis of GBA was done according to Rowley et al. (1971) with minor modifications. The substrates for the synthesis were DL-β-aminobutyric acid (United States Biochemical Co.) and cyanamide (Sigma Chemicals). DL-β-aminobutyric acid (? M) and cyanamide (2.4 M) were dissolved in the smallest amount of water that would dissolve the compounds (in our case, it was 175 ml). Upon filtration of the mixture, 20 ml of concentrated ammonium hydroxide were added. After 10 days at room temperature, the crude product was filtered and the crystals were washed with hot water (85-90°C). Next, they were quickly filtered and kept at 4°C for 24 hours to maximize crystal formation. This hot water wash was repeated once more.

To the filtrate obtained from the initial crude product, 10-15 g of cyanamide were added. The filtrate then was kept at 4°C for 24 hours and the crystals that formed were washed as described above, as was the crude produce. Next, all formed crystals were dissolved in water (24°C) and filtered again. Ice cold acetone was added until no further crystal formation was evident. The mixture was filtered and the crystals were dried for 24 hours. The final yield obtained was 80 ± 10%.

For quality control, proton nuclear magnetic resonance (NMR) spectral analyses were run to identify the synthesized compound and to compare it to the reference sample provided by Dr. C. Fitch. The analogues synthesized in our laboratory and the reference samples were the same compound. Melting-poing (M.P.) analysis to determine the extent of impurities was performed on all of the samples and compared with the reference sample (M.P.

281°C). Any product that had a melting point which varied by more than 2% was discarded. Guanidinopropionic acid was purchased from Sigma Chemicals.

C) Investigation of Contractile Mechanics in Vitro

The concentrations of total creatine after 7 weeks of GBA or GPA feeding were comparable to those concentrations obtained by other investigators. However, maximal depletion of total creatine was attained after 10 weeks of analogue feeding. Therefore, we investigated contractile characteristics and hemodynamic parameters in rats fed GBA and GPA for 7 and 10 weeks.

1. Preparation of Papillary Muscles

After 7 and 10 weeks on the diet, the rats were stunned by a blow to the head and immediately decapitated. The chest cavity was opened, and the heart was dissected out and dropped into a beaker of cold saline. The atria were carefully trimmed, and the papillary muscles were exposed by cutting through the right ventricle and the septum. After the isolation and separation of the papillary muscle from the left ventricle, the muscles were placed in cold saline. The rest of the heart was quickly frozen between aluminum blocks in liquid nitrogen and stored for chemical analyses that were performed later. The excised papillary muscles were mounted between two acrylic clamps on duplicate Palmer muscle stands. The upper clamp was attached to a stainless steel wire that was connected to an isometric force transducer (Gould Statham UC2). Two platinum electrodes (2.2 x 0.8 cm) were arranged vertically on either side of the muscle (Fig. 3). The muscles were immersed in 120 ml of modified Krebs-Ringer solution of the following concentrations (in mM): NaCl 117.4, $CaCl_2$ 2.5, KCl 3.6, $MgSO_4$ 1.2, NaH_2PO_4 1.2, $NaHCO_3$ 25 and glucose 5.5. The pH was kept in the range of 7.38-7.45 by

aerating the solution with 95% O_2/5% CO_2. Temperature was kept at 25°C for all of the experiments except for one (in which it increased to 31°C) by a thermostatic bath (Radiometer Vts).

Upon immersion, the muscles were field stimulated through electrodes with a square-wave pulse of 5 ms duration at a supramaximal voltage (20% above threshold) by a Grass stimulator 588 and its slave through which the second muscle was stimulated . The output from the force transducer was amplified by a low level DC pre amplifier (Grass Model 7P1F) then by a driver amplifier (Grass Model 7DAG). The signal was simultaneously fed into a differentiator (Grass Model 7P20). The amplified output from both the force transducer and the differentiator was recorded on the Grass polygraph oscillograph (Model 7EWU16P) (Fig. 4). The one-half amplitude frequency was set at 75 Hz and the frequency response of the combined amplifier and oscillograph was linear within +5% and -10% from DC to 45 Hz. The length of the muscle was measured with a micrometer (Starrett, Mass, USA) attached in a fixed position relative to the adjustable arm of the Palmer stand.

Figure 3: Schematic representation of a papillary muscle in a thermostatically controlled bath

A- Grass 88 stimulator

B- Waterpump and thermostat

C- Outer water jacket with inflow and outflow outlets for a waterpump and thermometer

D- Clip stand

E- Platinum electrodes

F- Force transducer

G- Micrometer

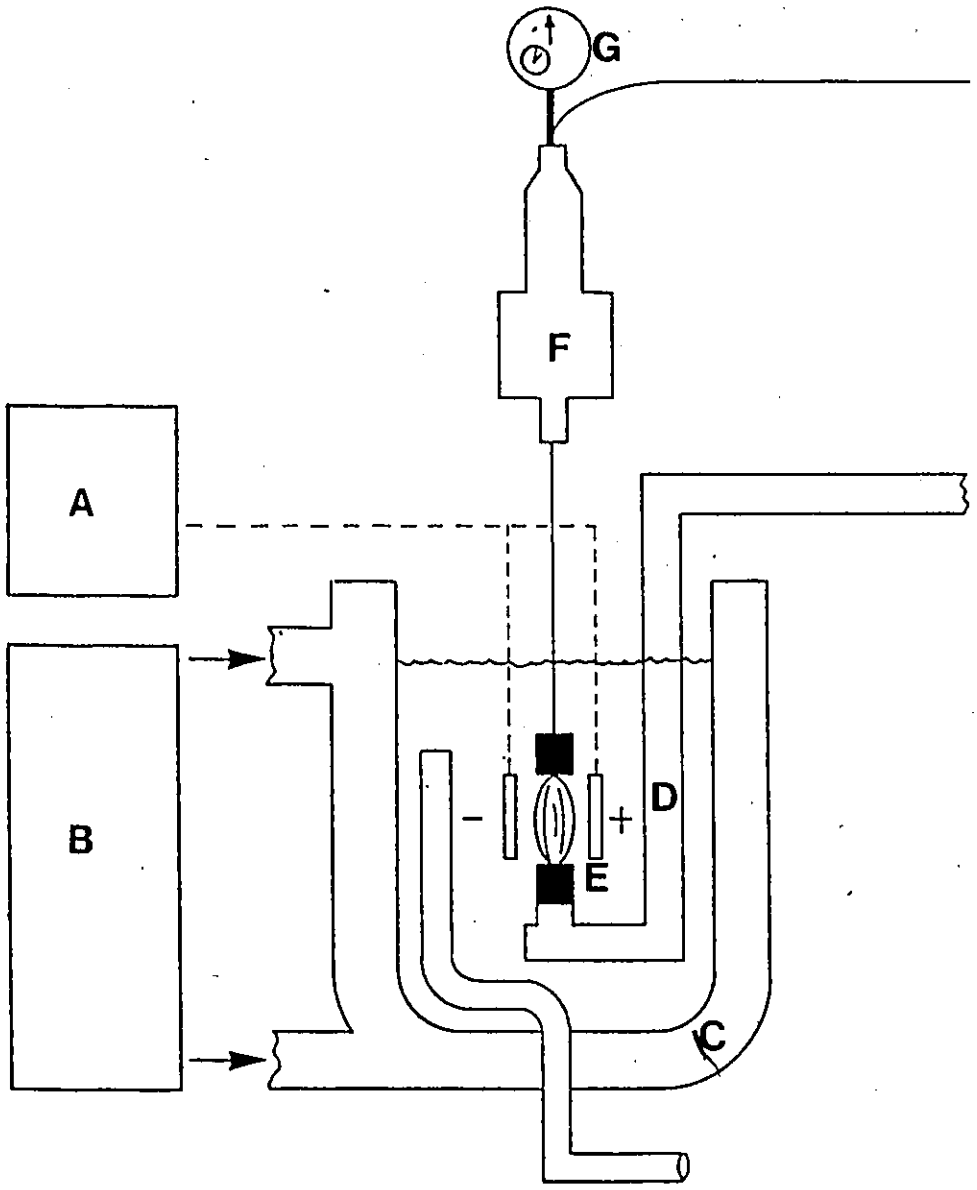

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Figure 4: Schematic diagram of papillary muscle monitoring apparatus for isometric contractions

A- Stimulator and slave

B- Force transducer

C- Low level DC pre-amplifier

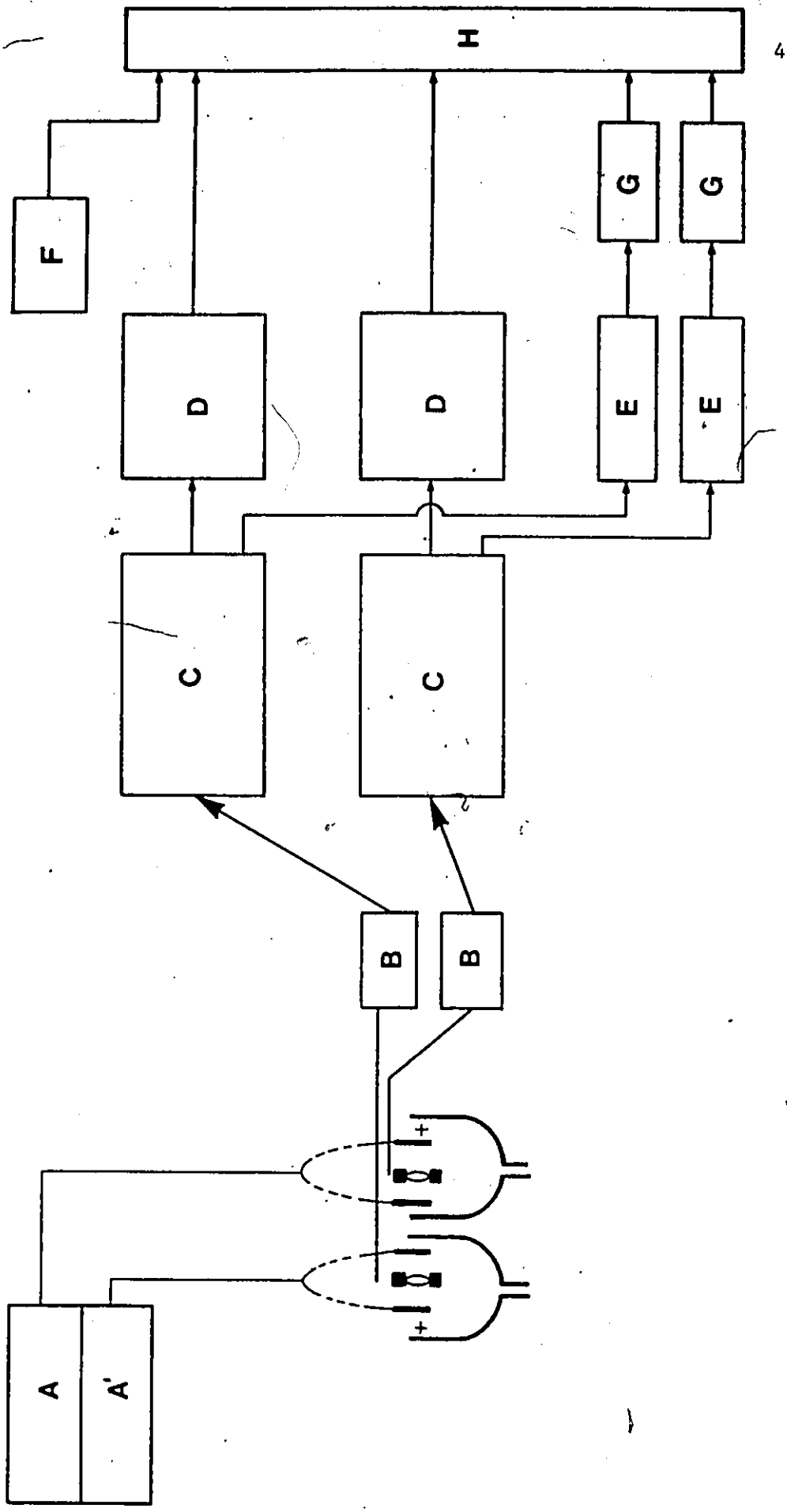
D- Driver amplifier

E- Differentiator

F- Timer

G- Driver amplifier

H- Oscillograph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2. Experimental Procedure

Several protocols were applied to the muscles, each designed to increase the workload of the muscle. This was achieved by 1) applying trains of progressively higher stimulation frequencies to the muscle, 2) by pair-pulsing the muscle with progressively decreasing intervals between the pair of stimuli, 3) by increasing the temperature of the fluid in which the muscles are bathed and simultaneously applying trains of stimulation at progressively increasing frequencies. We also attempted to measure the response of creatine depleted muscles to hypoxia, by gradually decreasing the partial pressure of oxygen in the bathing fluid of the muscles.

a) Frequency-Force Relationship at 25°C

The muscles of rats fed control, GBA and GPA diets for 7 and 10 weeks were left to stabilize in the bath at a temperature of 25°C for 30 min at a preload of 0.5 g and at a stimulation rate of 6 pulses/min. After 30 min the preload was reduced to 0 g, and the stimulation rate was decreased to 3 pulses/min. Upon stabilization a stepwise increase in preload of 0.25 g was begun. At each preload, after stabilization, the length of the muscle was noted and the isometric twitch was recorded at a paper speed of 100 mm/s. This loading of the muscle was repeated until no further increase in force development was observed. The muscle length at which maximum force development was attained was called maximal length (Lmax). At Lmax a frequency-force relationship was examined at a stimulation rate of 3, 6, 12, 24, 48, 96 and 192 pulses/min. At each frequency, the muscle was allowed to stabilize and a representative twitch was recorded at a paper

speed of 100 mm/s. At the end of each experiment, the muscles were removed, blotted dry and weighed.

b) Isometric Contractions During Paired Stimulation

The papillary muscles of rats fed control, GBA and GPA diets for 10 weeks were left to stabilize at 3 pulses/min and the Lmax was established as above. The muscles were kept at the rested-state stimulation rate (3 pulses/min) but superimposed on that were paired-pulses with decreasing intervals of 700, 500, 300, 200 ms. Then the paired-pulse intervals were decreased by 5 ms until fusion of the two twitches was observed and only one twitch was obtained.

The maximum developed force (DF) of each twitch during the paired-pulse stimulation was recorded as a percent of the DF obtained during single pulse stimulation (at 3 pulses/min) just before the onset of the paired-pulse protocol. Comparison of the percent DF of each twitch among the three groups was made at each paired stimulation frequency.

After the completion of this protocol, the muscles were stimulated at 3 pulses/min and then in order to deplete the potential energy supply of the cells, a train of single pulses at a rate of 192/min was applied for 20 s. Following a 5 s rest period, a single stimulus was given to assess the recovery.

c) Isometric Contractions During Hypoxia

A pilot study was performed on the muscles in section b) to determine if hypoxia has an effect on isometric contraction. Thus, when protocol b) was completed, the buffer was aerated with 95% nitrogen and 5% CO_2 while the stimulation rate of 24 pulses/min was applied. The partial pressure of oxygen and pH of

samples of the Krebs-Ringer solution were measured every 2 min for a total of 10 min on the IL 813 Blood Gas Analyzer and isometric twitches were recorded every 2 minutes at 100 mm/s. Upon completion of this protocol, the muscles were removed, blotted dry and weighed.

d) Frequency-force Relationship at 31°C

Muscles of rats fed control, GBA and GPA diets for 10 weeks were stabilized for 30 minutes at 3 pulses/min but at a bath temperature of 31°C. Upon establishment of Lmax, the muscle length was reduced to 75% of the Lmax and the papillary muscles were stimulated at 270, 300, 330, 360 and 390 pulses/min for 85 s. Between each frequency of stimulation, the muscles were allowed to stabilize at 3 pulses/min. At each frequency an isometric twitch was recorded at a high speed at 7 and 80 s after the onset of stimulation to assess the contractile mechanics. At the end of the experiment, the muscles were removed, blotted dry and weighed.

D) Investigation of Hemodynamic Parameters in the Isolated Blood-Perfused Rat

1. Preparation of Reconstituted Blood

Human red cell concentrate (RCC) preserved in citrate phosphate dextrose adenine solution (CPDA-1) was obtained from the Ottawa Chapter of the Canadian Red Cross. The RCC was washed twice with saline and sodium bicarbonate (30 mM) and once with Krebs-Henseleit bicarbonate solution consisting of (concentrations in mM): NaCl 120, KCl 4.7, $CaCl_2$ 2.5, $MgSO_4$ 1.2, K_2PO_4 1.2, pyruvate 2.0, $NaHCO_3$ 30.0, and glucose 11.0. For the final reconstitution of red blood cell (RBC) perfusate, 15 g/l of bovine albumin (Fraction V) was added to the Krebs-Henseleit solution, which was mixed with the washed RCC to obtain a final hematocrit of 25-27%.

The perfusate was warmed to 37°C in a large reservoir (1000 ml) placed in a water bath and homogeneity of the RBC perfusate was maintained by a magnetic stirring device that was placed under the reservoir. The RBC perfusate was continuously pumped by a peristaltic pump (Masterflex, 1-100 RPM) into a Bentley infusion blood filter (PFF 100) from which it passed into an artificial kidney (Cordis DOW C-Dak 1.8D) through the inner hollow fibers, while 95% O_2/5% CO_2 gas flushed the outer compartment. The RBC perfusate entered a small, preheated, water-jacketed, atrial reservoir (20 ml), from which it either entered the heart or was returned to the main reservoir (Fig. 5). Before the experiment, the RBC perfusate recirculated through the

circuit for 1 hour to equilibrate the perfusate at the above gas mixture and to maintain the pH of the perfusate between 7.38-7.42 (measured by IL 813 pH/Blood Gas Analyzer).

Figure 5: The perfusion apparatus for the isolated blood-perfused rat heart

A- Retrograde perfusion reservoir

B- Small atrial reservoir

C- Thermostatic bath for small atrial reservoir

D- Pressure transducer

E- Heart

F- Thermostatic bath for the heart

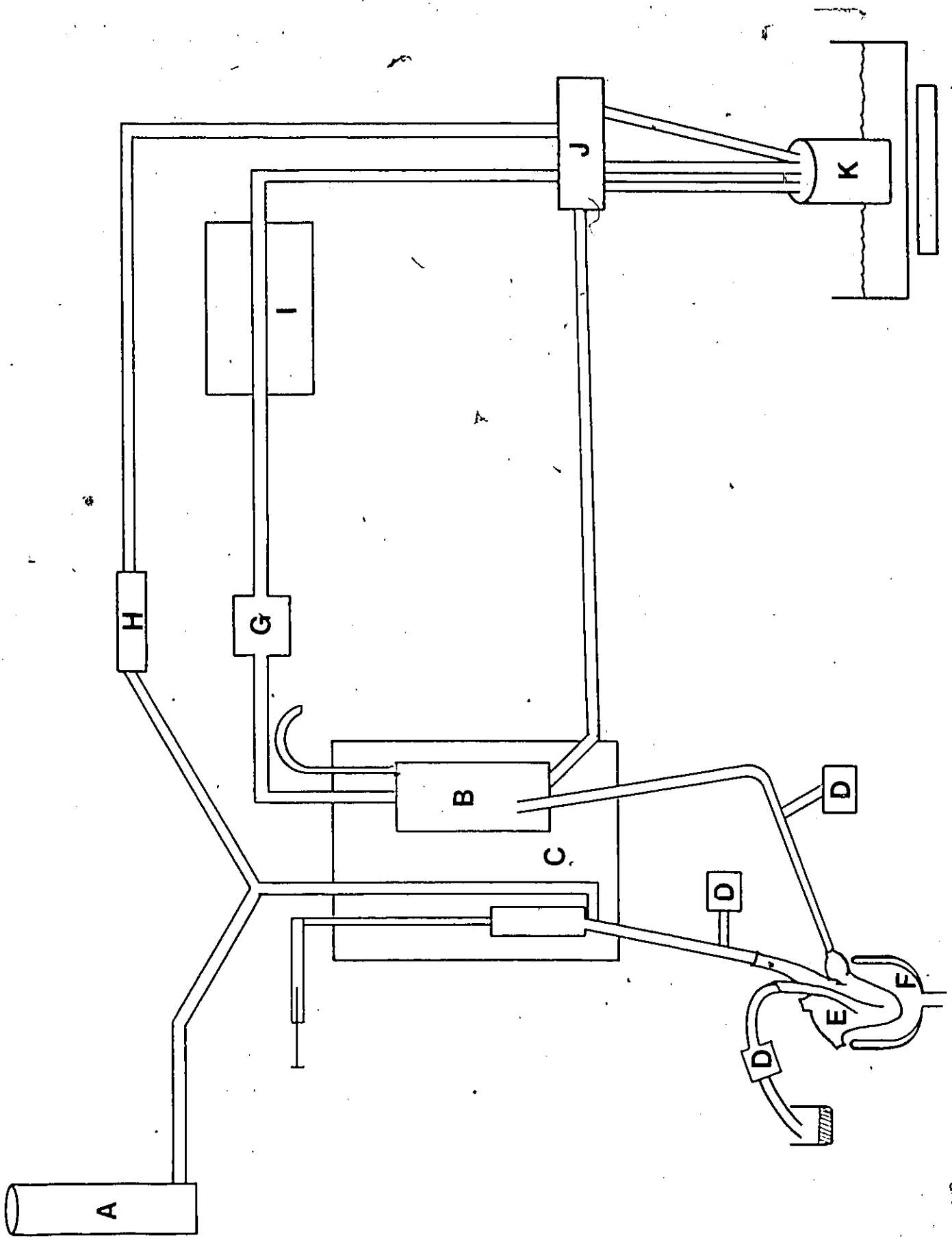
G- Gas oxygenator

H- Electromagnetic flow probe

I- Filter

J- Peristaltic pump

K- Large perfusate reservoir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2. Isolation of the Heart and its Connection to the Apparatus

Male Sprague-Dawley rats (350-400 g) were anesthetized with ether. A laparotomy was performed and heparin (200 IU) was injected into the abdominal vena cava. The rat was bled and a thoracotomy was performed. The thymus was removed, and the aortic arch and the pulmonary artery were isolated. The superior and inferior venae cavae were ligated and cut. The aorta was transected high in the aortic arch, and the heart was removed and quickly dropped into a small beaker filled with ice-cold saline.

The heart was fitted over a straight stainless steel cannula (inside/outside diameter, 2.15/3.2 mm), and retrograde perfusion with oxygenated Krebs-Henseleit buffer was begun from a column height of 100 cm H_2O. The pulmonary artery was cannulated with a plastic cannula (inside/outside diameter, 1.57/2.08 mm) to allow for the collection of the blood from the coronary sinus. Then the left atrium was cannulated through one of the pulmonary veins with a curved stainless steel cannula (inside/outside diameter, 2.15/3.25 mm). Once cannulation of the left atrium had been completed, the preparation was switched from non-working to the working mode, in which the RBC perfusate entered the heart from a small atrial reservoir (described in section 1). An overflow system was made above the small atrial reservoir which allowed the excess perfusate to return to the large reservoir. On a part of the overflow system, a stepladder was used to increase the hydrostatic column of RBC perfusate entering the heart when a step on the ladder was clamped. This allowed indirect control of

the end-diastolic volume by increasing or decreasing the column of perfusate entering the heart. The atrial perfusing pressure was measured immediately above the left atrial cannula by the Statham pressure transducer P23. The RBC perfusate was then ejected into the aorta against a hydrostatic pressure of 110 cm H_2O. The aortic output was measured by an electromagnetic flowmeter (Biotronex Model 610) by placing the flow probe (Biotronex-2032-F03, 1/8 inch) in the aortic line. After passing through the flowmeter probe, the perfusate passed through a small bubble trap and then returned to the large reservoir. Within the first 2-3 min, upon stabilization of the heart's hemodynamic variables, the heart was pierced through the left ventricle with a 22 gauge needed to measure intracavitary pressure (Statham pressure transducer P23) and its first derivative (dP/dt). The output from this transducer along with the left atrial and aortic output were amplified by a pressure amplifier (Grass Model 7P1F) then by a driver amplifier (Model 7DAG) and both signals were displayed on a Grass oscillograph (Model 7EWU16P).

3. Experimental Protocol

a) Normoxemia

In this series of experiments, hearts from rats fed control, GBA and GPA diets for 7 and 10 weeks were prepared as in section 2. Hearts were removed from the rats and retrogradely perfused in the non-working mode in less than 2.5 minutes. After stabilization of the hearts, the left atrial pressure was increased to values between 18 and 23 cm H_2O to obtain the maximum cardiac output of each heart. The left atrial pressure was then decreased to obtain cardiac output of 75 to 80% of the initial maximum. This was identified as the submaximum steady state cardiac output.

The left atrial and aortic pressure, aortic and coronary flows of the hearts were monitored continuously throughout the experiment. Partial pressures of oxygen, of carbon dioxide and the pH of the RBC perfusate were measured using the IL 813 pH/Blood Gas Analyzer. Oxygen content was calculated once the oxyhemoglobin percent saturation was determined on the IL 283 CO-Oximeter every 15 minutes until the cardiac output decreased to less than 10% of its submaximal steady-state. The variables listed in Fig. 6 were obtained. Upon completion of the experiment, the hearts were then quickly removed and frozen between precooled aluminum blocks, placed in liquid nitrogen and finally stored at -80°C until biochemical analyses were undertaken.

Figure 6: Measured and calculated variables of the isolated blood perfused rat heart.

MEASURED VARIABLES

Aortic flow

Coronary flow

Left atrial lateral pressure

Aortic lateral pressure

Intraventricular pressure

First derivative of intraventricular pressure

Atrial, aortic and coronary sinus* partial pressure of oxygen and carbon dioxide

Atrial, aortic and coronary sinus oxygen content (percent saturation)

Atrial, aortic and coronary sinus pH

CALCULATED VARIABLES**

Arterio-venous oxygen difference

Myocardial oxygen consumption

Work input, power input

Work output, power output

Efficiency

*Collection was done by placing a cannula in the pulmonary artery.

**(see Appendix 1 for calculations of values)

b) Hypoxemia

In these experiments, fresh (1-3 day old) human red cells preserved in heparin were used instead of the stored cells (3-4 weeks) preserved in citrate phosphate dextrose adenine solution (CPDA-1) used in section 3a).

The basic circuit in this experiment was set up as in section 1 (Fig. 5) however, some parts of it were duplicated so that the normoxemic phase could be followed by the hypoxemic phase. Therefore, an additional peristaltic pump, filter, oxygenator and a large RBC reservoir were added. The two large reservoirs were placed in the waterbath and maintained at 37°C. From the first reservoir the perfusate was pumped through a Bently filter and equilibrated with 95% O_2/5% CO_2 when it passed through the hollow fiber oxygenator. The perfusate entered the small atrial reservoir and returned to the first large reservoir in the waterbath through an atrial overflow outlet. From the second reservoir, perfusate was pumped through a second Bently filter, then passed into an oxygenator and was equilibrated with 95% N_2/5% CO_2. The perfusate then returned into the second large reservoir and recirculated through this system until pO_2 of the perfusate reached 30-40 mmHg. Since deoxygenation proceeded slowly, recirculation through the second circuit was begun 45 minutes before the onset of the experiment so that pO_2 of 30-40 mmHg was obtained at the start of the experiment. When the normoxemic perfusate was replaced with the hypoxemic one, two stopcocks were switched such that it allowed only one of the perfusates to enter the atrial reservoir and the heart. Upon switching, some mixing of normoxemic and hypoxemic perfusates

occurred in the small atrial reservoir, and the initial pO_2 entering the heart increased to 45–55 mmHg.

Samples of aortic and coronary sinus effuent (from the pulmonary artery) were taken every 10 minutes and the measurement of hemodynamic variables was continuous until failure (defined as 10% or less of the submaximum steady state cardiac output).

E) Biochemical Techniques

1. Freezing and Extraction

For the determination of the creatine and analogue concentrations at 2, 4, 6, 8, 10 and 12 weeks, rats at these 2 week intervals were stunned by a blow to the head and decapitated. The hearts were quickly exposed and frozen between precooled aluminum blocks, then weighed in a covered styrofoam weighing dish to keep the hearts from thawing during weighing. The rats from which papillary muscles were obtained were sacrificed in the same manner and the hearts were frozen immediately after the removal of the papillary muscles. All hearts were dropped into precooled (in liquid nitrogen) containers containing liquid nitrogen and stored at -80°C until analysis.

The extraction procedure (previously described by Mainwood et al. 1982a) was performed at 0°C. Approximately 100 mg of the heart was crushed into a fine powder in a precooled mortar and pestle. It was then transferred to a precooled test-tube kept on ice, and 0.8 ml of perchloric acid was added. The contents were mixed for 1 min and allowed to stand on ice for 20 min. After the second mixing the samples stood on ice for 10 min. Following the third mixing the contents were centrifuged at 10,000 g for 10 min. The supernatant was collected into a fresh, precooled test-tube and neutralized with 120 µl of 2.5 M potassium carbonate. The extracted sample was immediately used or stored at -80°C.

2. Acid Hydrolysis

To hydrolyze CP in the extracted sample, 150 µl of 0.5 N HCL was added to 0.5 ml of the sample. This mixture was then heated at 60°C for 10 min. Immediately after heating, the samples were cooled on ice and neutralized with 5-12 µl of 2.5 M K_2CO_3 (Mainwood et al. 1982a).

3. Determination of Total Creatine

An assay described by Ennor and Rosenberg (1952) was modified and used to determine the total creatine content in hydrolyzed heart extracts. The reaction mixture contained 1.0 ml of distilled water, 0.1 ml of muscle extract and 0.5 ml of 0.05% butane-2,3-dione. After letting the reaction mixture sit for 30 min, it was read spectrophotometrically at 510 nm at room temperature. A linear standard curve was made from 0 to 100 nM based on total creatine levels from previous studies, which ranged from 10 to 80 µmoles of creatine/g of heart wet weight.

Both GPA and GBA react to a small degree with butane-2,3-dione and give a positive colorimetric reaction when the creatine assay is performed. The method of Mainwood and Totosy de Zepetnek (1985) was followed. A known quantity of either GBA or GPA was added in the absence of creatine to the standard assay of creatine and read spectrophotometrically. A correction factor (F, ranging from 0.12 to 0.18) was obtained from the ratio of the analogue reading to the creatine reading from an equivalent amount of creatine. The corrected creatine (Crc) in each sample

was estimated according to the following formula:

$$Crc = Cra - F \times Gp \text{ or } Gb$$

where Cra is the apparent creatine concentration and Gp/Gb is the guanidine (propionic/butyric acid) concentration.

4. Determination of Total β-guanidinopropionic and β-guanidinobutyric acid

The color reaction, first described by Sakaguchi (1925) was used to estimate the guanidine levels as used by Bonas et al. (1965). The reaction mixture contained 1.0 ml of distilled water, 0.1 ml of hydrolyzed heart extract, 0.25 ml of thymine-α-napthol mixture, 0.1 ml of sodium hypochlorite, and 0.1 ml of 2% thiosulfate. This mixture stood on ice for 5 min and was read spectrophotometrically at 510 nM at 10°C. A linear standard curve was made from 0 to 60 nM based on total guanidine levels from previous studies, which ranged from 5 μmoles to 20 μmoles of guanidine compound/g of heart wet weight.

F) Electron Microscopy

Sprague-Dawley rats (n=4 in each group) fed control, GBA and GPA diets for 7 weeks were anesthetized with Somnotol, (50 mg/kg), heparinized (200 IU) and perfused through the carotid artery with a fixative containing: 0.045 M cacodylate buffer, 1.5% gluteraldehyde, 400 mOsmol/kg H_2O maintained at pH of 7.4. Upon fixation, the hearts were excised and cut into 2 mm pieces. The individual pieces of the ventricle were fixed for 2 h in 2% osmic acid and 150 mM cacodylate buffer and embedded in Araldite (520) for 12 h at 40°C, followed by 24 h at 60°C and then 18 h at 90°C. Semi-thin and thin section were cut with a diamond knife. The sections were stained with uranyl acetate (saturated in 50% methanol, pH 4.4) for 30 min at 25°C, counter-stained with Reynold's lead citrate (pH 12.0) for 20 min at 25°C.

Random samples of subendocardial, midwall and subepicardial sections were selected for analysis and were examined with a Philips Elu electron microscope.

G. Statistical Analysis

All data (means and standard errors of the mean) were analyzed using one-way analysis of variance to determine significant changes among the three treatment groups. Significant differences among the groups were analyzed using the post-hoc Scheffe test. Calculation of the percent survival was done according to Cutler and Ederer (1958) and Fleiss et al. (1976), modified for this study and Chi-square analysis was performed to obtain significance between the groups, control, GBA and GPA, and significant differences were analyzed using a post-hoc Chi-square test.

RESULTS

A) Preliminary Feeding Trials

Concentrations of total creatine (creatine and CP) and of total GBA and GPA were measured in hearts of rats on control, GBA and GPA diets for 5 weeks. As shown in Table 1, the levels of total creatine decreased to 33% in the GBA group and to 31% in the GPA group of the levels obtained in the control group at 5 weeks. Negligible amounts of guanidino-reactive compounds were found in control muscles (Table 1) when the guanidine-assay was used (Bonas et al. 1965) to analyze the control muscles.

Since decreases in creatine to 25-20% were desirable, rats were fed for up to 7 weeks, and the results are presented in Table 2. The myocardial concentrations of total creatine in rats fed the GBA diet decreased to 21% of concentrations obtained in the control rats at 7 weeks, and to 25% in the rats fed the GPA diet. The GBA analogue in the myocardium of the GBA fed rats, increased to 53% of the GPA analogue observed in the myocardium of the GPA fed rats. This was not anticipated, since in both groups, creatine was depleted to the same extent.

When we measured the levels of total creatine and of the analogues every two weeks for a total of 12 weeks, we observed that total creatine increased up to 10 weeks in the control group, but little change was observed thereafter (Table 3). Also, in the GBA and GPA groups, concentrations of total creatine decreased up to 10 weeks but negligible change was observed after that time.

The decrease in total creatine in the GBA group was to 11% and in the GPA group it was to 14%. This was the maximal

depletion obtained, even when we fed several animals up to 16 weeks in a pilot study.

The accumulation of the GBA and GPA in the control, GBA and GPA groups is shown in Table 4. The accumulation of GPA was 50% greater than that of GBA with little difference in creatine depletion between the two groups (Table 3).

The effects of the two analogues on body weight at 0, 2, 4, 6, 8, 10 and 12 weeks of feeding are given in Table 5. Body weight was not significantly different among the three groups at the start of the feeding trial, but retardation of growth in the GBA fed rats was noted by 8 weeks of feeding, and by 10 weeks the GBA group had significantly lower body weight ($p<0.01$, $F=13/4$) than the control group. Significant differences were obtained between the two experimental groups at 10 weeks ($p<0.05$, $F=5.7$) but further decreases were not observed thereafter. The difference in body weight occurred in spite of the effort to control the caloric intake (see methods section A) of the three groups to minimize differences in weight due to a differential food intake.

Table 1: Concentration of total creatine, GBA and GPA in μmol/g wet muscle weight in hearts of rats fed control, GBA and GPA diet for 5 weeks.

	Total Creatine	Total guanidino-reactive compound
Control	13.0 ± 1.1	0.25 ± 0.07

	Total Creatine	Total GBA	Total GPA
GBA-fed	4.3 ± 0.5	3.4 ± 0.2	---
GPA-fed	4.0 ± 0.8	---	7.6 ± 0.5

- all values are means and standard errors of the mean
- n is 5 in all groups

Table 2: Concentrations of total creatine and analogues, (GBA and GPA) in μmol/g of wet weight of hearts in rats fed control, GBA and GPA diets for 7 weeks.

	Total Creatine	Total guanidino-reactive compound
Control	13.8 ± 1.8	0.45 ± 0.03

	Total creatine	GBA	GPA
GBA	3.0 ± 0.8	4.7 ± 1.5	---
GPA	3.4 ± 0.7	---	8.9 ± 0.9

- all values are means and standard errors of the mean
- n equals five hearts in each group

Table 3: Total creatine levels in μmol/g wet muscle weight in hearts of rats fed control, GBA or GPA diets for 2, 4, 6, 8, 10 and 12 weeks.

WEEK	CONTROL	GBA-fed	GPA-fed
2	9.6 ± 0.6	5.0 ± 0.3	4.3 ± 0.2
4	10.2 ± 0.8	4.1 ± 0.8	4.0 ± 0.4
6	13.6 ± 0.9	3.3 ± 0.5	2.2 ± 0.2
8	13.8 ± 1.5	2.8 ± 0.5	2.5 ± 0.5
10	15.2 ± 0.4	1.5 ± 0.2	2.3 ± 0.1
12	15.3 ± 0.2	1.7 ± 0.1	2.2 ± 0.2

- all values are means and standard errors of the mean
- n is 5 in all groups

Table 4: Total analogue levels (GBA, GPA) in μmol/g of wet muscle weight in hearts of rats fed control, GBA or GPA diets for 2, 4, 6, 8, 10 and 12 weeks.

WEEK	Guanidine-Compound in Control	GBA in GBA-fed	GPA in GPA-fed
2	0.3 ± 0.04	2.4 ± 0.2	5.1 ± 0.9
4	0.4 ± 0.10	3.4 ± 0.3	6.8 ± 0.7
6	0.3 ± 0.09	4.2 ± 0.7	8.4 ± 1.1
8	0.4 ± 0.05	4.9 ± 0.7	9.4 ± 1.0
10	0.3 ± 0.06	5.1 ± 0.8	10.0 ± 0.5
12	0.2 ± 0.04	5.0 ± 0.8	10.7 ± 0.04

- all values are means and standard errors of the mean
- n is 5 in all groups

Table 5: Body weights in grams of rats fed control, GBA and GPA diets for up to 10 weeks

WEEK	CONTROL	GBA-fed	GPA-fed
0	262 ± 5	257 ± 8	250 ± 7
2	307 ± 6	304 ± 8	303 ± 3
4	339 ± 5	345 ± 5	332 ± 8
6	375 ± 6	373 ± 6	369 ± 7
8	389 ± 9	376 ± 7	383 ± 16
10	417 ± 5	381 ± 7*+	408 ± 8

- all values are means and standard errors of the mean

* $p < 0.01$, statistically different from control

\+ $p < 0.05$, statistically different from GPA

- n is 5 in each group

B) Mechanical Characteristics In Vitro

1. Contractile Characteristics of a Single Isometric Twitch

Initial experiments were done after 7 weeks, at which time the levels of total creatine in the heart declined to 21% in the GBA group and to 25% in the GPA group of the levels obtained in the control group. The experiments were repeated after 10 weeks at which time total creatine decreased to 11% in GBA group and to 14% in GPA group of the levels in the control group. The differences in concentrations of total creatine between GBA group and GPA group at 7 or at 10 weeks were not statistically significant.

Contractile characteristics were compared among the three groups at Lmax as defined in methods section C 2a. Representative sample of a single twitches developed at Lmax at a rate of 3 pulses/min are displayed in Figure 7. Preload, length and cross-sectional area of the papillary muscles are shown in Table 6 in the control, GBA and GPA groups after 7 weeks and 10 weeks. Cross-sectional area (mm^2) was calculated by first converting the weight of the muscles (mg) into volume using density of 1.063 and then dividing the volume by length (mm) of each muscle at Lmax. After 7 weeks the preload necessary to attain Lmax in the GBA group was significantly lower ($p<0.01$, $F=8.1$) than that in the control and GPA groups and no significant differences existed between control and GPA groups. This discrepancy is most likely due to the fact that a technician performed the 7 week experiments and the author the 10 week experiments.

The obtained force was expressed as tension by normalizing force per cross-sectional area (see Appendix II for concept of normalization). The maximum developed tension (DT), the rate of tension development (dT/dt), mean rate of tension development (mean $\Delta T/\Delta t$), time to maximal tension development (TMT) and time to half-relaxation (T $\frac{1}{2}$ R) are presented in Table 7. No significant differences were found in any of these variables.

During the preliminary feeding trial, we noted that the body weights of the rats differed among the three groups after 10 weeks on the three diets. In that trial, the hearts were immediately frozen, therefore left and right ventricular heart weights could not be obtained. Thus, during this experimental protocol, we weighed the hearts and recorded whole heart, left ventricular (plus septum) and the right ventricular weights in each group both after 7 and 10 weeks on the diet. These weights are presented in Table 8. No significant differences were observed among the three groups in whole heart, left or right ventricular weights after 7 weeks. After 10 weeks, the left ventricular weight in the GBA group was less than that in control and GPA groups, but the difference was not statistically significant. Upon normalization of heart weight per body weight, the difference was even less apparent (Table 9).

Figure 7: Recording of a single isometric twitch in a papillary muscle of a rat fed the control diet for 7 weeks. The upper tracing is force (F) and the lower tracing is the first derivative of developed force (dF/dt). Stimulation rate was 3/min, bath temperature was 25°C.

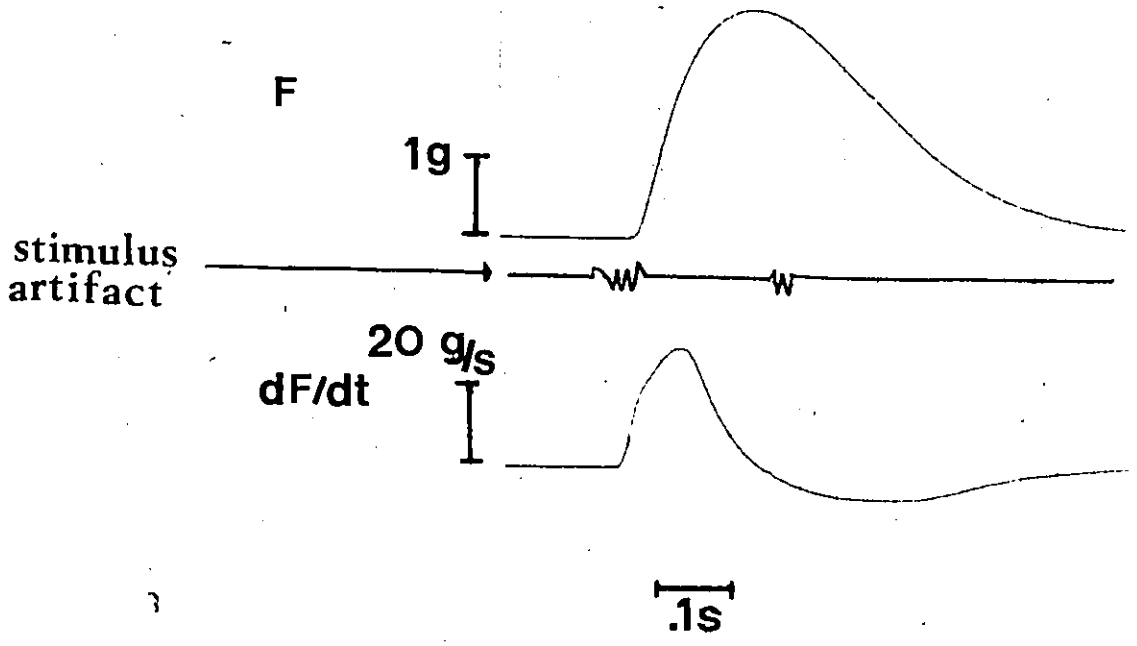

F
1g
stimulus
artifact
20 g/s
dF/dt
.1s

Table 6: Preload, length and cross-sectional area at Lmax in papillary muscles of rats fed control, GBA and GPA diets for 7 and 10 weeks

	n	Preload (g/mm^2)	Length (mm)	Cross-Sectional Area (mm^2)
			7 WK	
Control	9	1.6 ± 0.1	6.2 ± 0.9	1.2 ± 0.1
GBA-fed	10	1.1 ± 0.1*	7.2 ± 0.3	1.1 ± 0.1
GPA-fed	9	1.5 ± 0.1	7.1 ± 0.4	1.0 ± 0.1
			10 WK	
Control	10	1.5 ± 0.2	6.2 ± 0.4	1.0 ± 0.1
GBA-fed	10	1.5 ± 0.1	6.1 ± 0.2	1.1 ± 0.1
GPA-fed	8	1.7 ± 0.2	6.3 ± 0.5	1.3 ± 0.2

- all values are means and standard errors of the mean
- stimulation rate is 3/min
- temperature is 25°C
- five animals were used in each group
- n refers to the number of muscles

* $p < 0.01$ statistically different from control and GPA

Table 7: Contractile characteristics at Lmax of papillary muscles of rats fed control, GBA and GPA diets for 7 and 10 weeks

	n	DT (g/mm^2)	dT/dt ($g/s/mm^2$)	$\Delta T/\Delta t$ ($g/s/mm^2$)	TMT (ms)	T ½ R (ms)
				7 WK		
Control	9	3.7±0.3	37.8±2.0	23.3±0.7	179±8	153±18
GBA-fed	10	4.5±0.9	45.5±9.6	23.8±4.4	186±10	190±34
GPA-fed	9	4.1±0.5	43.0±5.0	24.0±2.2	173±9	174±23
				10 WK		
Control	10	3.6±0.7	33.5±3.8	19.5±2.3	182±8	164±20
GBA-fed	10	4.0±0.5	29.8±6.3	10.0±7.1	200±15	191±14
GPA-fed	8	3.7±1.0	34.6±8.9	18.7±8.0	198±8	192±8

DT - maximum developed tension

dT/dt - rate of tension development

$\Delta T/\Delta t$ - mean rate of tension development

TMT - time to maximum tension development

T ½ R - time to one-half relaxation

- all values are means and standard errors of the mean
- stimulation rate is 3 pulses/min
- temperature is 25°C
- n refers to the number of muscles
- five animals were used in each group

Table 8: Whole heart (WH), left (left ventricular and septum, LV) and right ventricular (RV) weights in grams of rats fed control, GBA and GPA diets for 7 and 10 weeks

7 WK	WH	LV	RV
Control	1.26 ± 0.07 (8)	0.86 ± 0.04 (9)	0.39 ± 0.04 (8)
GBA	1.13 ± 0.08 (8)	0.84 ± 0.06 (8)	0.29 ± 0.08 (8)
GPA	1.33 ± 0.07 (9)	0.89 ± 0.04 (9)	0.42 ± 0.08 (9)

10 WK	WH	LV	RV
Control	1.41 ± 0.08 (6)	0.90 ± 0.04 (6)	0.51 ± 0.07 (6)
GBA	1.30 ± 0.07 (6)	0.86 ± 0.06 (6)	0.44 ± 0.06 (6)
GPA	1.37 ± 0.11 (7)	0.94 ± 0.08 (7)	0.44 ± 0.06 (7)

- all values are means and standard errors of the mean
- numbers in brackets represent the number of animals

Table 9: Percentages of whole heart (WH), left (left ventricle and septum, LV) and right ventricular (RV) weights of body weights of rats fed control, GBA and GPA diets for 7 and 10 weeks.

7 WK	WH	LV	RV
Control	0.32 ± 0.02 (8)	0.22 ± 0.01 (9)	0.09 ± 0.02 (8)
GBA	0.30 ± 0.22 (9)	0.22 ± 0.02 (9)	0.08 ± 0.01 (9)
GPA	0.34 ± 0.02 (9)	0.23 ± 0.01 (9)	0.11 ± 0.01 (9)

10 WK	WH	LV	RV
Control	0.33 ± 0.01 (6)	0.22 ± 0.01 (6)	0.14 ± 0.02 (6)
GBA	0.35 ± 0.02 (6)	0.23 ± 0.01 (6)	0.12 ± 0.02 (6)
GPA	0.34 ± 0.02 (7)	0.23 ± 0.01 (7)	0.11 ± 0.01 (7)

- all values are means and standard errors of the mean
- numbers in brackets represent the number of animals

2) Frequency-Force Relationship at 25°C

Contractile mechanics of the papillary muscle are shown in Table 10. A negative inotropic response in the maximum developed force (DF) was seen in all three groups with an increase in the stimulation rate from 3 to 192 pulses/min (Table 10). The DF at 3 pulses/min was assigned the value of 100% in each muscle in each group and the DF attained at higher frequencies was taken as a percent of the DF obtained at 3 pulses/min. There were no significant differences in DF at any stimulation frequency among the groups.

A similar trend was observed in all of the contractile characteristics that were examined such as the rate of force development (dF/dt) (Table 11), mean rate of force development, ($\Delta F/\Delta t$) (Table 12), time to maximal force development (TMF) (Table 13), and in time to half relaxation (T $\frac{1}{2}$ R) (Table 14). No significant differences were observed at 7 or 10 weeks in any of these twitch characteristics among the three groups at any of the frequencies. (Actual values obtained are in Appendix VI, Tables 1 to 5).

Table 10: Frequency-force relationship in the papillary muscles of rats fed control, GBA and GPA diets for 7 and 10 weeks

Frequency of Stimulation (per min)	3	6	12	24	48	96	192
7 WK			% of Maximum Developed Force				
Control n = 9	100	89 ± 7	83 ± 6	76 ± 4.6	54 ± 4.5	38 ± 3	28 ± 3
GBA-fed n = 10	100	92 ± 7	82 ± 4	75 ± 6	53 ± 7	36 ± 9	23 ± 5
GPA-fed n = 9	100	86 ± 5	71 ± 5	60 ± 8	50 ± 4	36 ± 3	26 ± 3
10 WK							
Control n = 10	100	89 ± 8	78 ± 9	64 ± 5	56 ± 5	38 ± 7	28 ± 5
GBA-fed n = 10	100	94 ± 6	81 ± 4	75 ± 5	53 ± 5	36 ± 4	27 ± 7
GPA-fed n = 8	100	85 ± 3	72 ± 3	60 ± 7	50 ± 4	37 ± 8	26 ± 4

- all values are means and standard errors of the mean and are expressed as percent of the maximal force developed at 3 pulses/min
- n is the number of muscles
- five animals were used in each group
- bath temperature was 25°C

Table 11: Rate of force development (dF/dt) at increasing rates of stimulation in the papillary muscles of rats fed control, GBA and GPA diets for 7 and 10 weeks.

Frequency of Stimulation (per min)	3	6	12	24	48	96	192
				% of dF/dt			
7 WK							
Control n=9	100	90±3	87±4	68±5	63±3	46±2	39±3
GBA-fed n=10	100	97±3	86±6	73±5	52±3	43±4	38±4
GPA-fed n=9	100	71±3	76±3	65±4	56±3	43±3	36±3
10 WK							
Control n=10	100	95±3	75±14	73±14	73±12	61±7	48±7
GBA-fed n=10	100	79±10	67±9	64±10	51±7	49±8	41±6
GPA-fed n=8	100	79±10	62±8	60±7	50±8	46±8	38±8

- all values are means and standard errors of the mean and are taken as a percent of the value obtained at 3 pulses/min
- n is the number of muscles
- five animals were used in each group
- bath temperature was 25°C

Table 12: Mean rate of force development ($\Delta F/\Delta t$) at increasing rate of stimulation in the papillary muscles of rats fed control, GBA and GPA diets for 7 and 10 weeks.

Frequency of Stimulation (per min)	3	6	12	24	48	96	192
			% of maximum $\Delta F/\Delta t$				
7 WK							
Control n=9	100	80±2	76±2	60±3	53±3	44±2	22±2
GBA-fed n=10	100	92±3	76±3	65±6	44±7	46±3	39±3
GPA-fed n=9	100	87±2	80±3	72±3	64±4	56±3	32±2
10 WK							
Control n=10	100	95±4	90±5	90±4	85±7	60±8	42±8
GBA-fed n=10	100	95±2	85±5	85±3	60±4	50±4	36±6
GPA-fed n=8	100	85±3	91±8	65±5	70±6	66±2	38±5

- all values are means and standard errors of the mean and are taken as a percent of the maximal value obtained at 3 pulses/min
- n is the number of muscles
- five animals were used in each group
- bath temperature was 25°C

Table 13: Time to maximal developed force (TMF) at increasing rates of stimulation in the papillary muscles of rats fed control, GBA and GPA diets for 7 and 10 weeks.

Frequency of Stimulation (per min)	3	6	12	24	48	96	192
			% of TMF				
7 WK							
Control n=9	100	100±4	97±4	97±4	96±4	81±5	77±5
GBA-fed n=10	100	101±4	96±4	98±7	99±5	81±13	74±8
GPA-fed n=9	100	98±6	92±6	83±4	77±8	79±8	67±5
10 WK							
Control n=10	100	96±3	93±4	85±4	78±5	70±6	59±8
GBA-fed n=10	100	100±2	94±2	93±2	81±4	76±4	68±5
GPAOfed n=8	100	96±2	96±3	89±3	86±4	81±3	73±7

- all values are means and standard errors of the mean and are taken as a percent of the maximal value obtained at 3 pulses/min
- n is the number of muscles
- five animals were used in each group
- bath temperature was 25°C

Table 14: Time to half-relaxation (T ½ R) at increasing rates of stimulation in the papillary muscles of rats fed control, GBA and GPA diets for 7 and 10 weeks.

Frequency of Stimulation (per min)	3	6	12	24	48	96	192
				% of T ½ R			
7 WK							
Control n=9	100	80±8	70±9	67±10	61±6	51±8	48±12
GBA-fed n=10	100	81±7	80±11	73±12	62±9	55±7	44±6
GPA-fed n=9	100	79±12	70±8	65±13	55±6	51±10	48±8
10 WK							
Control n=10	100	100±7	99±5	82±8	70±4	62±5	49±6
GBA-fed n=10	100	75±3	89±3	67±5	58±4	53±1	37±6
GPA-fed n=8	100	98±2	90±3	87±4	86±4	63±6	43±10

- all values are means and standard errors of the mean and are taken as a percent of the maximal value obtained at 3 pulses/min
- n is the number of muscles
- five animals were used in each group
- bath temperature was 25°C

3) Effects of Paired Stimulation

The DF of each twitch during paired-pulse protocol was taken as percent of the DF obtained during single stimulation at 3 pulses/min. At any paired-pulse interval, the first twitch never decreased below 95% of that obtained at single stimulation rate of 3 pulses/min in any of the three groups.

The DF of the second twitch was less than that of the first twitch and no significant differences in DF of the second twitch among the three groups were observed at any of the paired-pulses intervals above 180 ms. But at 180 ms and at lower intervals, the DF in the GBA and GPA groups was significantly less ($p<0.05$, $F=8.4$) than in the control group (Table 15). Also, fusion of the first and second twitch occurred at a longer paired interval in the creatine-depleted muscles than in controls. In Fig. 8, we provide an example of the twitches in the GBA and GPA groups where the twitches are almost fused at an interval of 175 ms while the control muscles still manifest two separate twitches. The paired-pulse interval at which fusion occurred was significantly lower ($p<0.01$, $F=10.4$) for the control group, 144 ± 9 ms, than for the GBA (170 ± 11 ms) and for the GPA group (173 ± 3 ms). No significant differences were obtained in the time at which fusion occurred between the experimental groups.

After the last paired-pulse interval, the muscles were stimulated by a train of pulses of 192/min for 20 s. Stimulation was stopped and after 5 s rest period, a single pulse was given to assess the recovery. The DF at 192 pulses/min was taken as a percent of the DF obtained at 3 pulses/min and was 42.9 ± 1.9% in

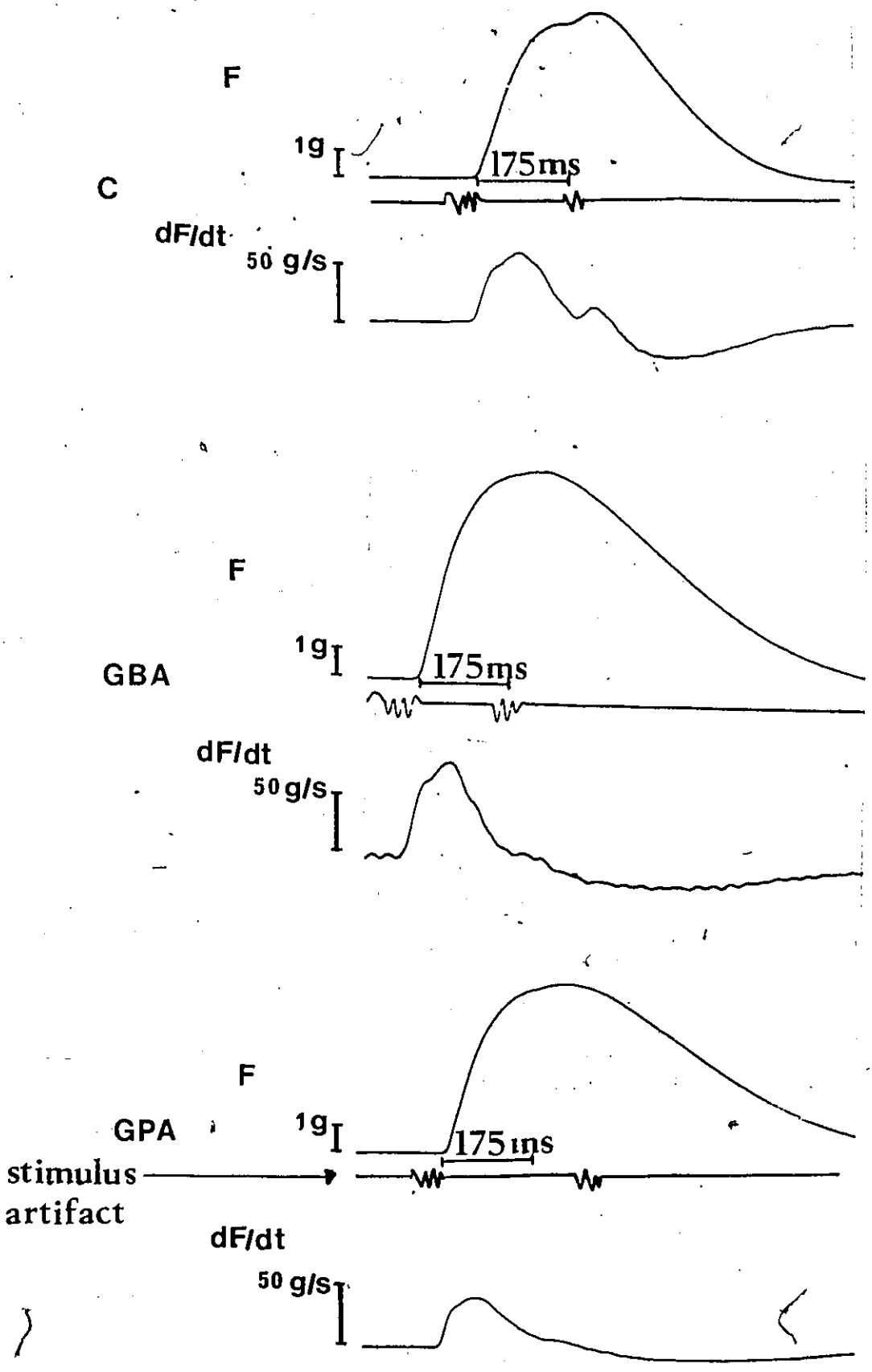
the control group, 47.5 ± 8.8% in the GBA group and 36.6 ± 8.3% in the GPA group. The DF obtained with a single stimulus was 66.4 ± 6.6% in control, 70.0 ± 5.8% in GBA and 58.7 ± 8.3% in the GPA group.

Table 15: Developed force of paired twitches in papillary muscles of rats fed control, GBA and GPA diets for 10 weeks.

		% OF MAXIMUM DEVELOPED FORCE		
		CONTROL	GBA	GPA
PAIRED PULSE INTERVAL (ms)	Twitch			
700	2	73.8 ± 3.1	69.1 ± 5.2	72.1 ± 2.5
500	2	71.1 ± 7.2	65.4 ± 6.9	60.6 ± 2.9
300	2	27.4 ± 7.8	26.3 ± 7.9	27.4 ± 8.3
200	2	20.5 ± 3.9	19.7 ± 7.3	22.9 ± 4.8
190	2	17.9 ± 2.9	17.6 ± 1.7	14.1 ± 3.1
180	2	17.6 ± 2.3	9.9 ± 2.8*	9.1 ± 2.6*
n		8	6	8

- the pulse interval between the twitches decreased from 700 to 500 to 300 to 200 ms to 190 and to 180
- values are means and standard errors of the mean and are expressed as percent of the values obtained at 3 pulses/min
- *$p<0.05$ statistical significance between control and experimental groups, no significant differences between experimental groups
- n the number of muscles
- four animals were used in each group
- ms refers to milliseconds
- bath temperature was 25°C

Figure 8: A recording of papillary muscle response to paired stimuli of control, GBA and GPA fed rats for 10 weeks. The upper tracing in each case is force (F) and the lower is the first derivative of the developed force (dF/dt). The paired-pulse interval in each case was 175 ms. Bath temperature was 25°C.


F
1g
175ms
C
dF/dt
50 g/s
F
GBA
1g
175ms
dF/dt
50 g/s
F
GPA
1g
175ms
stimulus
artifact
dF/dt
50 g/s

4) Effects of Hypoxia on Contractile Characteristics

A pilot experiment was performed to determine if hypoxia would affect DF differently in the creatine depleted muscle than in controls. Immediately following the protocol in section 3, the stimulation rate was increased from 3 to 24 pulses/min and a gas mixture of 95% N_2 and 5% CO_2 was introduced into the buffer. Maximum developed force was taken as a percent of the DF obtained at 24 pulses/min when the muscles were immersed in buffer with high pO_2 values.

The DF decreased from 100% (that was taken as a percent of the DF obtained before the onset of hypoxia at 24 pulses/min) as pO_2 decreased from 500 ± 50 mmHg to 30 ± 10 mmHg (Table 16). No significant differences were obtained in DF among the three groups. Similarly no significant differences were obtained in any other contractile characteristics among the 3 groups at any pO_2 measured.

When the rate was increased to 48 pulses/min, the papillary muscles in all 3 groups failed within several beats after the introduction of nitrogen/carbon dioxide gas mixture.

Table 16: Effects of hypoxia on maximum developed force (DF) which was expressed as a percent of DF at 24 pulses/min during normoxia in papillary muscles of rats fed control, GBA and GPA diets for 10 weeks.

	Control		GBA-fed		GPA-fed	
Time(min) of hypoxia	%DF	pO_2	%DF	pO_2	%DF	pO_2
0	100	465 ± 42	100	477 ± 37	100	490 ± 11
2	82.1 ± 5.6	80.1 ± 3.9	80.5 ± 8.6	87.6 ± 3.2	85.7 ± 9.0	79.1 ± 8.2
4	61.2 ± 5.5	71.1 ± 4.4	67.7 ± 1.5	65.8 ± 11.4	65.5 ± 9.2	63.5 ± 2.7
6	46.5 ± 6.4	56.8 ± 2.3	56.0 ± 4.3	51.5 ± 6.3	61.2 ± 8.5	54.6 ± 4.2
8	37.7 ± 4.4	48.5 ± 3.0	44.3 ± 5.6	50.5 ± 1.5	47.5 ± 9.3	44.5 ± 7.0
10	26.5 ± 3.0	39.2 ± 2.2	32.8 ± 7.9	43.8 ± 1.7	39.5 ± 9.0	34.5 ± 5.5
n	6	6	4	4	6	6

all values are means and standard errors of the mean

%DF - percent of developed force

pO_2 - partial pressure of oxygen in mmHg

n refers to the number of muscles used

- three animals were used in each group

5) Frequency-Force Relationship at 31°C

Contractile characteristics of rats fed control, GBA and GPA diets for 10 weeks were examined with trains of stimuli at 270, 300, 330, 360 and 390 pulses/min. The temperature of the bathing fluid in which the papillary muscles were kept was brought to 31°C to increase the metabolic rate of the muscle and to decrease the amount of dissolved oxygen.

No significant differences were observed in preload, length of cross-sectional area among the three groups at Lmax (Table 17). Also the contractile characteristics were very similar among the three groups when measured at Lmax (Table 18).

When we compared the contractile characteristics that we obtained at 25°C to those at 31°C, we found that the dT/dt and $\Delta T/\Delta t$ were greater at 31°C and TMT and T ½ R were shorter than at 25°C (Table 18). This was true for all three groups.

The isometric twitches were recorded at each stimulation rate for a period of 85 s and DF was measured at 7 and 80 s after the onset of each stimulation rate (Fig. 9). A fast recording at 100 mm/s is represented in Fig. 10. At most frequencies of stimulation, the DF was lower in muscles of the analogue fed rats than in those of control fed rats however the differences in DF among the three groups were not statistically significant (Table 19). Incomplete relaxation was observed in all of the papillary muscles stimulated at all frequencies, since the pulses were given at a faster rate than that required for complete relaxation.

After each train of high frequency stimuli lasting for 85 s, the rate was decreased to 3 pulses/min and the DF with each subsequent stimulus increased until a plateau was reached. Measurements of DF were done continuously for 35 min in each group, and the statistical analysis (one-way analysis of variance) of DF among the groups was done at that time. This DF of the control group was higher after each train of stimuli than the DF of the GBA and GPA groups. It was also significantly greater in the control group after the train of stimuli 360/min ($p<0.01$, $F=8.9$) and 390/min ($p<0.01$, $F=10.6$) than DF in the GBA and GPA groups. The DF of GPA group was significantly lower than the DF of the GBA group after each of these two trains of stimuli (Fig. 11).

The length of time at 3 pulses/min required to reach their maximal DF and to reach a plateau was significantly longer ($p<0.001$, $F=89$ at 270-300 pulses/min, $F=41$ at 300-330 pulses/min, $F=39$ at 330-360 pulses/min, $F=24$ at 260-390 pulses/min and $F=53$ after recovery from 390 pulses/min) in the creatine-depleted muscles than in controls after every train of stimuli (Fig. 12). It took significantly less time (12 min) for the control muscles to reach this plateau, while it took 31 min for the muscles in the GBA and GPA groups to reach the plateau after every frequency of stimulation.

Table 17: Preload, length and cross-sectional area of papillary muscles at Lmax at 31°C of rats fed control, GBA and GPA diets for 10 weeks

	Preload(g/mm^2)	Length (mm)	Cross-Sectional Area(mm^2)
Control n=10	1.6 ± 0.3	5.6 ± 0.6	1.0 ± 0.1
GBA-fed n=9	1.4 ± 0.1	6.5 ± 0.3	1.0 ± 0.5
GPA-fed n=10	1.5 ± 0.1	6.4 ± 0.5	1.1 ± 0.1

- all values are means and standard errors of the mean
- n refers to the number of muscles
- five animals were used in each group
- stimulation rate was 3 pulses/min

Table 18: Contractile characteristics at Lmax, 25°C and 31°C of papillary muscles of rats fed control, GBA- and GPA-fed diets for 10 weeks.

	n	DT (g/mm^2)	dT/dt ($g/s/mm^2$)	$\Delta T/\Delta t$ ($g/s/mm^2$)	TMT (ms)	T ½ R (ms)
			10 WK (25°C)			
Control	10	3.6±0.7	33.5±3.8	19.5±2.3	182±8	164±20
GBA-fed	10	4.0±0.5	29.8±6.3	10.0±7.1	200±15	191±14
GPA-fed	8	3.7±1.0	34.6±8.9	18.7±8.0	198±8	192±8
			10 WK (31°C)			
Control	10	4.2±0.8	57.7±11.2	43.6±9.8	112±6	86±9
GBA-fed	9	5.0±0.8	66.3±8.8	43.0±6.4	118±5	97±10
GPA-fed	9	4.7±0.7	66.6±11.3	36.5±5.6	125±5	93±6

DT - maximum developed tension

dT/dt - rate of tension development

$\Delta T/\Delta t$ - mean rate of tension development

TMT - time to maximum tension development

T ½ R - time to one-half relaxation

- all values are means and standard errors of the mean
- n is the number of muscles used
- five animals were used in each group
- stimulation rate was 3 pulses/min

Figure 9: Recording of papillary muscle twitches at a stimulation rate of 360 pulses/min of a rat fed the control diet for 10 weeks. The upper tracing is force (F) and the lower tracing is the first derivative of the developed force (dF/dt). Measurements of maximum F and dF/dt were done at 7 and 80 s after the onset of stimulation. Bath temperature was 31°C.

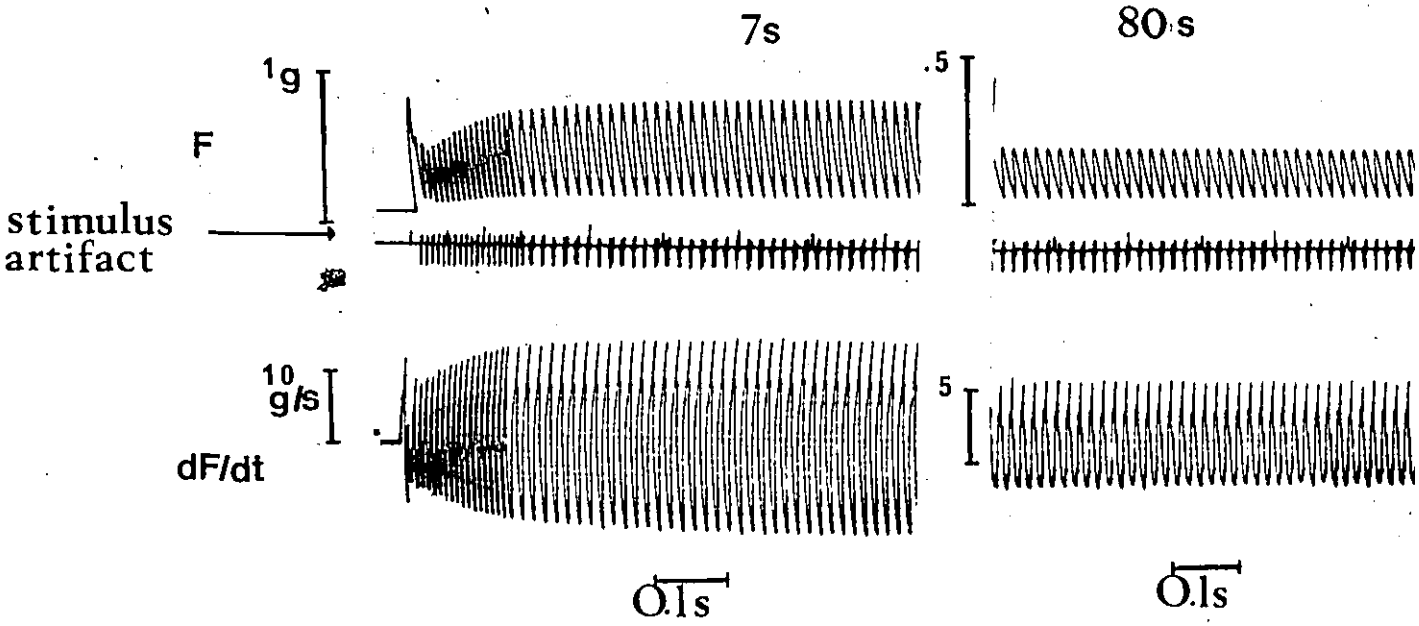

7s
80s
1g
.5
F
stimulus
artifact
10
g/s
dF/dt
5
0.1s
0.1s

Figure 10: Recordings of papillary muscle twitches of rats fed the control, GBA and GPA diets for 10 weeks. Measurements of maximal developed force (F) and its first derivative (dF/dt) at 100 mm/s were done at 7 and 80 s after the onset of stimulation at 330 pulses/min. The upper tracing is force (F) and the lower is the first derivative of developed force (dF/dt). Bath temperature was 31°C.

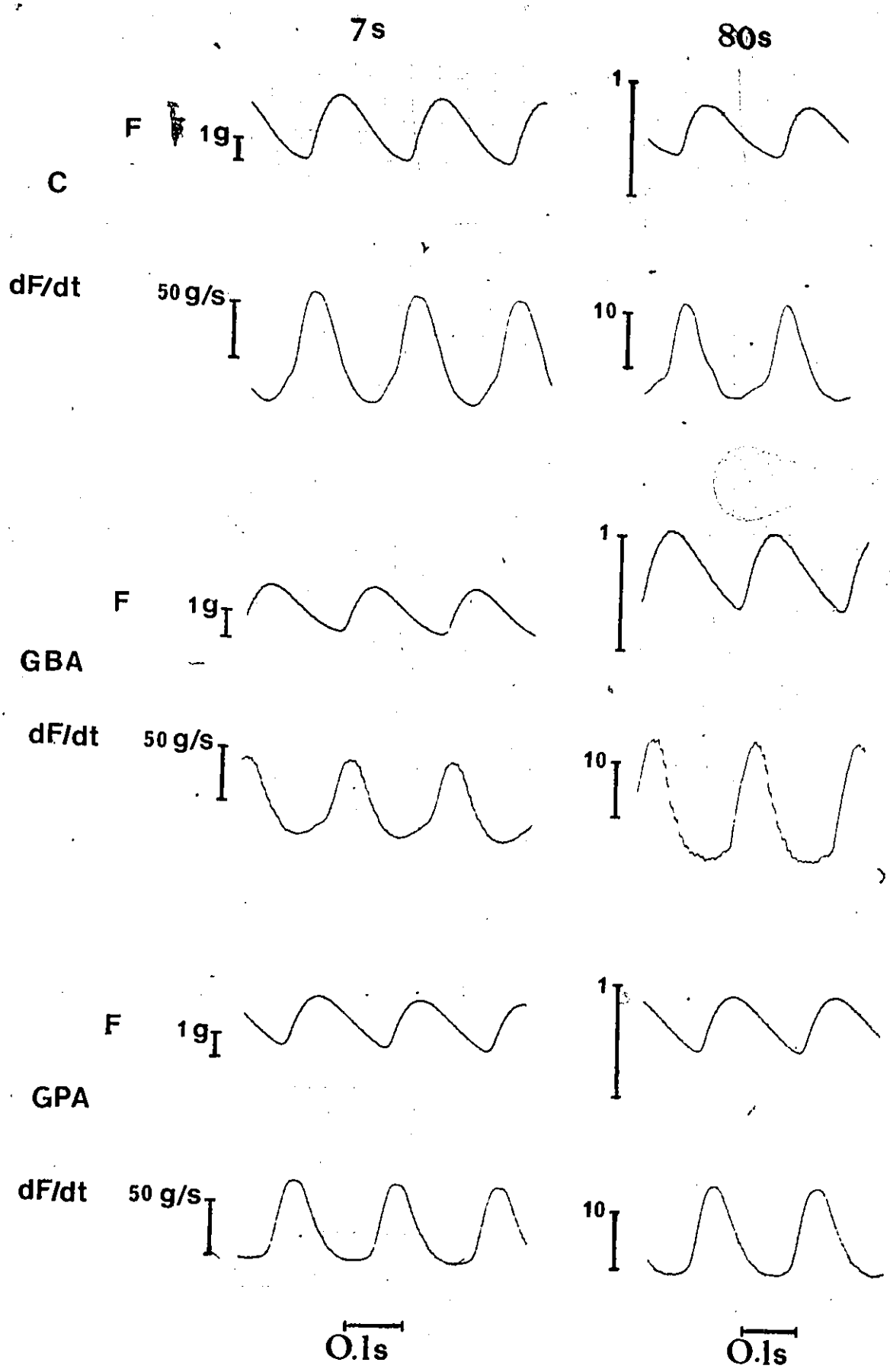

7s
80s
F
1g
C
dF/dt
50 g/s
10
1
F
1g
GBA
dF/dt
50 g/s
10
1
F
1g
GPA
dF/dt
50 g/s
10
1
0.1s
0.1s

Table 19: Maximum developed force in papillary muscles taken as a percent of maximum developed force (DF) obtained at 3 pulses/min of rats fed control, GBA and GPA diets for 10 weeks at stimulation rates of 270, 300, 330, 360 and 390 pulses/min

Stimulation Rate(pulses/min)	Percent of Maximum Developed Force (%)					
	Control		GBA-fed		GPA-fed	
	7s	80s	7s	80s	7s	80s
270	81.6 ± 6.5	24.8 ± 4.0	84.0 ± 8.4	18.0 ± 2.2	88.4 ± 9.8	28.2 ± 0.4
300	83.7 ± 7.4	27.1 ± 4.9	73.1 ± 6.3	15.1 ± 2.7	74.3 ± 12.1	18.8 ± 2.4
330	72.6 ± 8.7	19.7 ± 3.4	58.7 ± 5.9	18.1 ± 4.4	58.2 ± 7.5	18.8 ± 3.3
360	56.8 ± 5.3	17.4 ± 2.9	45.2 ± 6.9	15.8 ± 2.5	44.9 ± 3.6	15.6 ± 1.3
390	30.1 ± 13.1	24.2 ± 5.1	33.9 ± 8.4	19.9 ± 1.3	24.6 ± 9.0	16.5 ± 1.9
n	10	10	9	9	10	10

- all values are means and standard errors of the mean
- n refers to the number of muscles used
- five rats were used in each group
- bath temperature was 31°C

Figure 11: The maximum developed force (DF) in papillary muscles of rats fed control, GBA and GPA diets for 7 weeks. The DF was measured at 3 pulses/min after each stimulation rate of 270, 300, 330, 360, and 390 pulses/min until a plateau in DF was obtained. The number of muscles was 10 in Control and GPA and 9 in GBA. Five animals were used in each group. Bath temperature was 31°C.

*$p<0.01$, statistical differences between GBA and GPA groups

+$p<0.01$ statistical differences between the two analogue treated groups and the control group

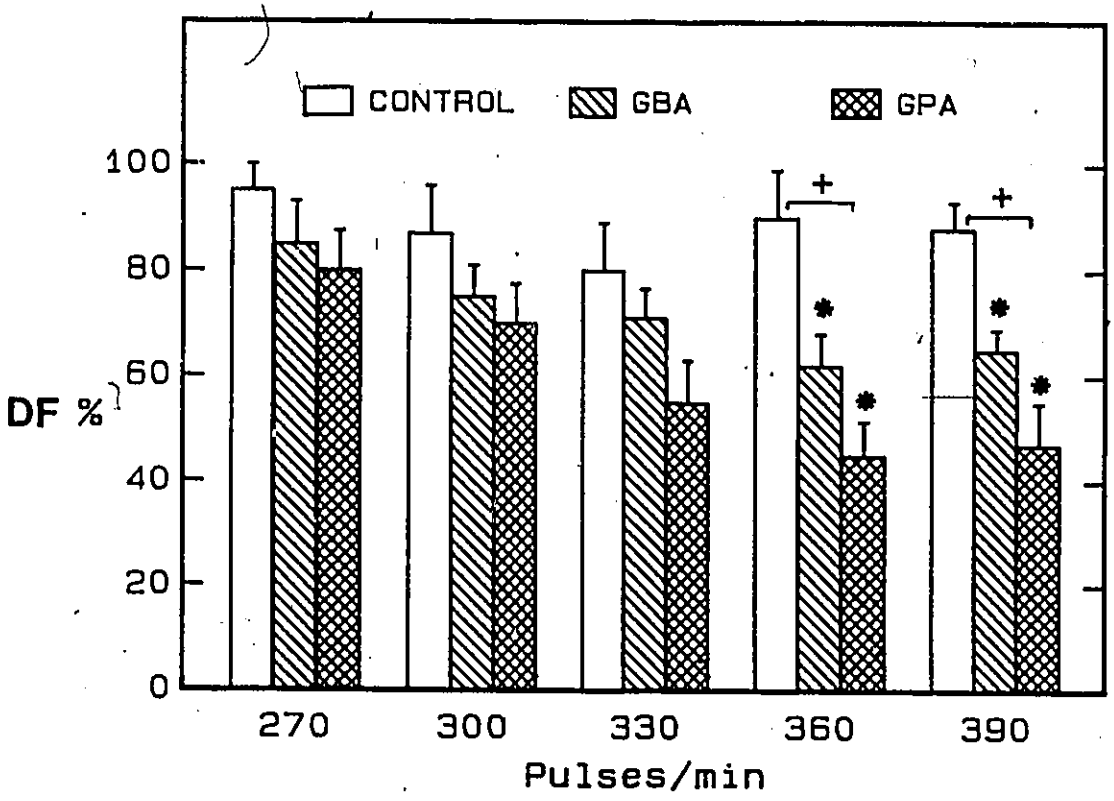
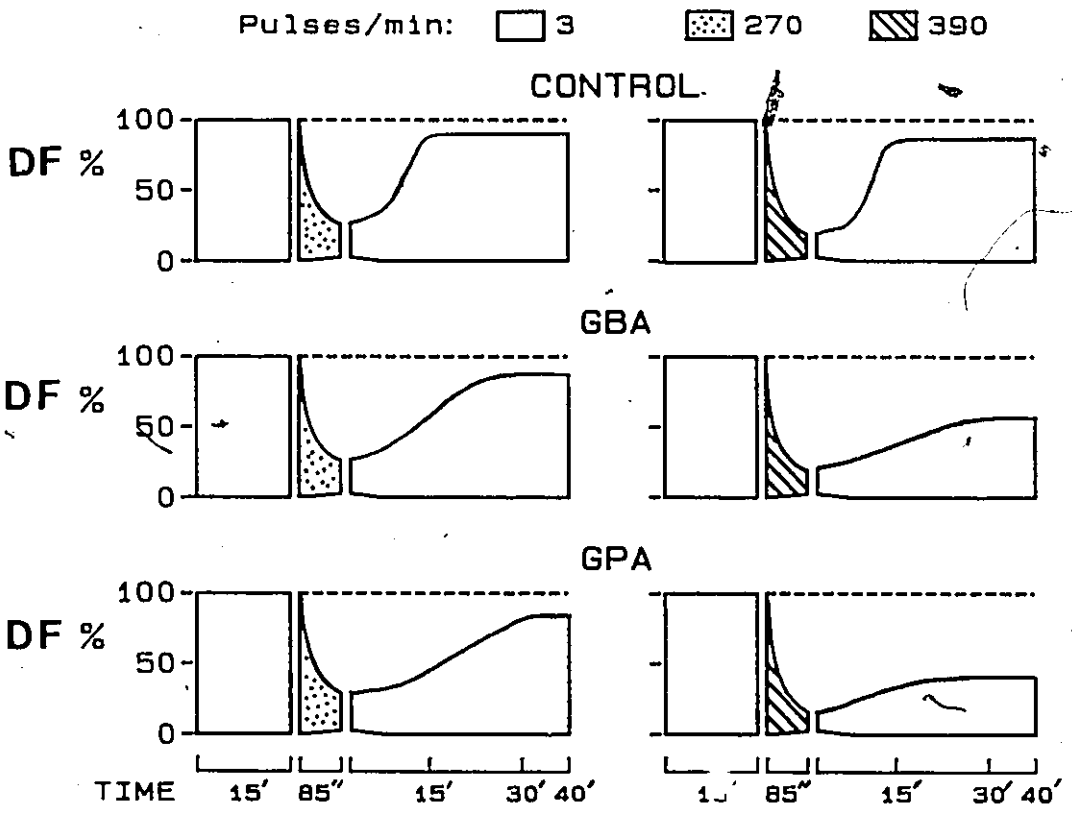

CONTROL
GBA
GPA
100
80
60
40
20
0
DF %
+
+
*
*
*
*
270
300
330
360
390
Pulses/min

Figure 12: This figure represents the average percent DF at stimulation rates of 3, 270 and 390 pulses/min over a 56 min period in the control, GBA and GPA groups. The temperature was 31°C. The number of muscles was 10 in control and GPA groups and 9 in GBA group. Five animals were used in each group.


Pulses/min: 3 270 390
CONTROL
DF %
100
50
0
GBA
DF %
100
50
0
GPA
DF %
100
50
0
TIME
15'
85"
15'
30'
40'
85"
15'
30'
40'

The major findings of these experiments are:

1) Contractile characteristics of single isometric twitches are not significantly altered by 75-90% depletion of total creatine.

2) Imposition of workloads up to 192 pulses/min at 25°C did not alter force development during recovery at stimulation of 3 pulses/min in creatine depleted papillary muscles when compared to controls.

3) In creatine depleted papillary muscles, the interval between paired-pulses at which fusion occurred was significantly longer than in controls.

4) Hypoxia did not alter contractile characteristics more adversely in creatine depleted papillary muscles than in controls at a stimulation rate of 24 pulses/min.

5) Imposition of high workloads of up to 390 pulses/min at 31°C did not alter actual force development in creatine depleted papillary muscles but the subsequent recovery at low frequency of stimulation took significantly longer and the recovered developed force was significantly lower than in controls.

C) Hemodynamic Performance of the Isolated Blood-Perfused Working Rat Heart

1. Hemodynamic Performance After 7 and 10 Weeks of Analogue Feeding

Cardiac performance was evaluated in hearts of rats fed control, GBA and GPA diets for 7 and 10 weeks. All variables (see methods for description) were obtained at times 0, 15, 30, 45, 60, 75, 90, 105 and 120 min of perfusion in control, GBA and GPA groups. Since no significant differences were obtained in any of the hemodynamic variables during the first 15 min of steady state among the three groups at 7 or 10 weeks or among the corresponding groups of rats fed the diet for 7 and 10 weeks, the data was summarized and is given in Table 20.

Of the recorded hemodynamic variables (Fig. 6) cardiac output (CO) was the first and most immediate indicator of the performance in each group. Figure 13 shows that after 7 weeks, all of the hearts were able to maintain the initial submaximal steady-state CO (as defined in methods) for 60 min. During the second hour, CO began to decline in all the hearts however the rate of decline was much faster in the GBA and GPA groups than in the control group. By 75 min, CO decreased by 50% in the GBA group and by 44% in GPA group, which was significantly lower ($p<0.05$, $F=6.4$) than the output of the control group (89%). By 120 min, the hearts of both the GBA and GPA groups had a CO of less than 20% while the control hearts had a CO of 54% of the initial submaximum steady-state CO. No significant differences were obtained between the hearts of the GBA and the GPA groups (see Appendix VI, Table 6 for actual values of CO).

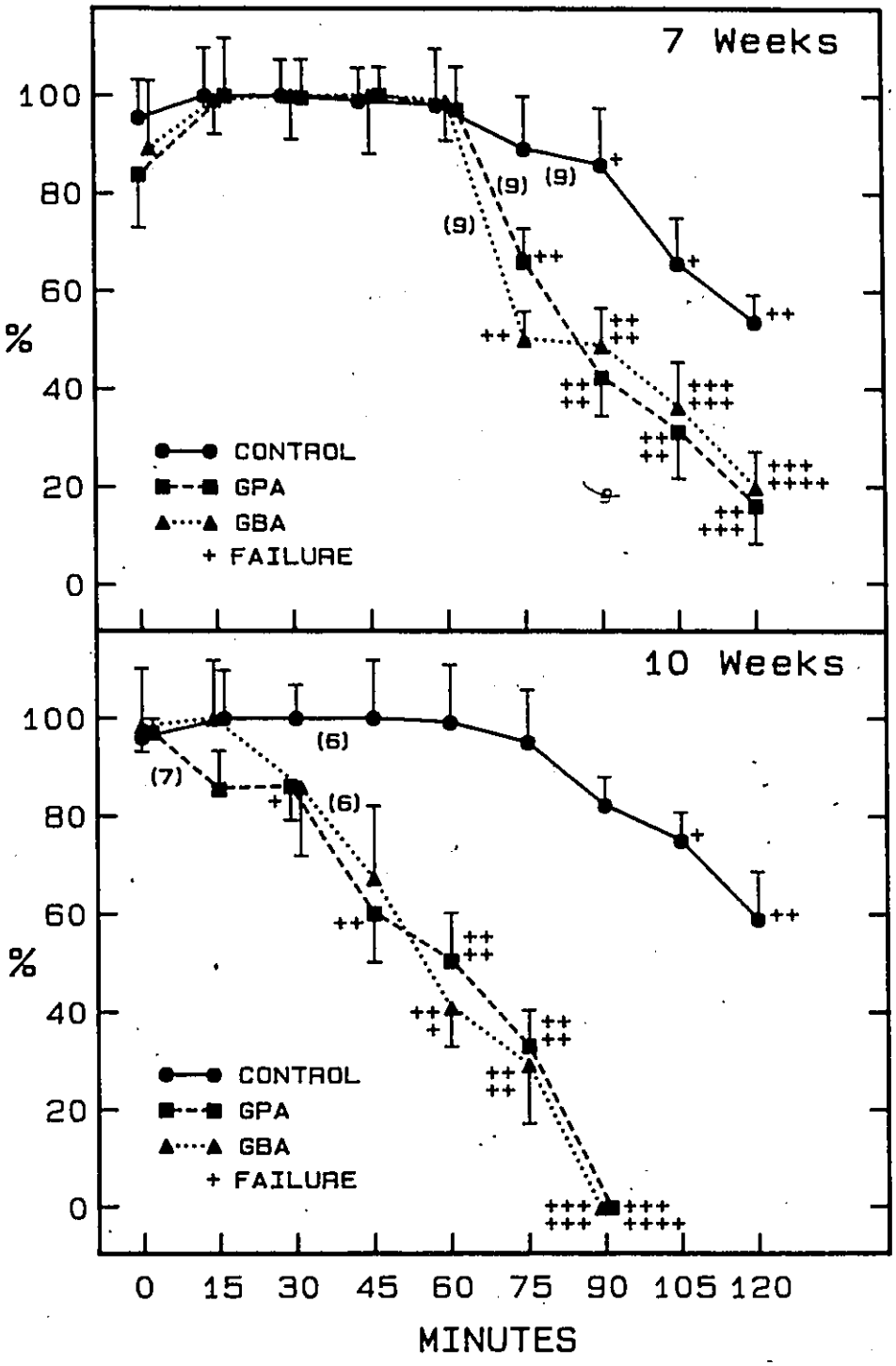
Table 20: Pooled hemodynamic data of isolated working rat hearts during the first 15 minutes of steady state function in control, GBA and GPA rats fed for 7 and 10 weeks perfused with red blood cell enriched perfusate.

	Control	GBA	GPA
n	15	15	15
Hematocrit %	27 ± 1	27 ± 1	26 ± 1
pH a	7.42 ± 0.03	7.41 ± 0.04	7.43 ± 0.02
pH c.s.	7.34 ± 0.02	7.32 ± 0.01	7.33 ± 0.02
PO_{2a} mmHg	305 ± 72	291 ± 50	344 ± 68
PO_{2cs} mmHg	31 ± 3	39 ± 5	32 ± 3
PCO_{2a} mmHg	40 ± 7	36 ± 9	40 ± 2
PCO_{2cs} mmHg	46 ± 2	43 ± 6	46 ± 4
C O_{2a} ml O_2/dl	13.1 ± 1.3	14.1 ± 1.1	14.1 ± 1.6
C O_{2cs} ml O_2/dl	9.2 ± 0.9	9.1 ± 2.0	9.8 ± 1.5
Hb g%	8.6 ± 0.9	9.7 ± 0.7	9.7 ± 0.4
Heart Rate bpm	290 ± 15	305 ± 20	275 ± 30
Aortic Flow ml/min/g	55 ± 6	51 ± 7	54 ± 5
Coronary Flow ml/min/g	6.6 ± 1.1	6.7 ± 0.9	5.2 ± 1.3
Myocardial Oxygen Consumption ml/min/g	0.32 ± 0.11	0.30 ± 0.13	0.27 ± 0.12
Power Input mW/g	117 ± 14	106 ± 19	104 ± 13
Total External Power mW/g	10.8 ± 1.8	10.1 ± 1.6	9.7 ± 1.1
Efficiency %	9 ± 1	10 ± 2	10 ± 2
LVP mmHg	125 ± 6	120 ± 10	119 ± 2
LVEDP mmHg	8.8 ± 1.5	11.8 ± 1.2	11.5 ± 1.6
+dP/dt mmHg/s	3558 ± 105	3636 ± 350	3296 ± 193
-dP/dt mmHg/s	3265 ± 286	2944 ± 241	3116 ± 207

a = aortic; c.s. = coronary sinus; PO_2, PCO_2 = partial pressure of oxygen, carbon dioxide; C O_2 = total oxygen content; Hb = hemoglobin; LVP = peak left ventricular pressure; LVEDP = left ventricular end-diastolic pressure.
- All data expressed per gram refer to left ventricular wet weight (free wall and septum) and n refers to the number of hearts.

Figure 13: This figure represents cardiac output during 120 min of perfusion and is expressed as percentage of the highest values of cardiac output of control, GBA and GPA fed rats for 7 and 10 weeks. The highest cardiac output was achieved within 15 minutes of stabilization, and was assigned the value of 100%. The numbers in brackets represent initial numbers of hearts in each group. + represents one heart that has failed (achieved cardiac output of less than 10% of the maximum). The mean values represent the average cardiac output of the surviving hearts.

CARDIAC OUTPUT



After 10 weeks on the diet, the CO attained during steady-state in all three groups was similar to that in the rats fed for 7 weeks. However, the onset of failure in the hearts of the GBA and GPA groups occurred sooner as seen in Fig. 13. By 45 min, the CO had declined by 33% in the GBA group and by 40% in the GPA group while the control group maintained their submaximum steady-state CO. The decline in CO observed at 45 minutes in GBA and GPA groups was significantly lower ($p<0.05$, $F=4.5$) from that of the control group but no significant differences were observed in CO between the two creatine depleted groups. By 90 min all of the hearts in the GBA and GPA groups had failed, while the control group maintained 82% of the CO obtained during steady-state.

After CO, the second most significant indicator of cardiac performance was the rate of survival. A survival curve was constructed (Appendix III) to describe cardiac performance since statistical analysis became invalid as an increasing number of hearts failed in the second hour of perfusion, in rats fed for 7 and for 10 weeks. Survival of the hearts was defined as maintenance of CO greater than 10% of the initial submaximal steady state CO. After 7 weeks of feeding, 56% of the GBA and GPA groups had survived by 90 min (Figure 14) while 89% of the hearts in the control group had survived. By 120 min, only 23% of the GBA group and 32% of the GPA group had survived as compared with 78% of the control group ($p<0.05$, $X^2=12.2$). After 10 weeks on the diet, 50% of the GBA group and 40% of the GPA group had survived by 60 min while hearts of the control group were still functioning at 100%. At 90 min all of the hearts of

the control group survived, while all hearts in the GBA and GPA groups failed.

The next most important indicator of performance was myocardial oxygen consumption, then stroke volume, power input and output, the rate of pressure development (+dP/dt) and the rate of relaxation (-dP/dt) and efficiency. These parameters were compared among the control, GBA and GPA groups (Appendix VI, Tables 7, 8, 9, 10, 11, 12 and 13) and a similar pattern of decline was obtained in the three groups. This pattern of decline resembles that of cardiac output in the 7 and 10 week fed rats during the submaximal steady-state cardiac performance. The only parameter that did not vary was heart rate (Appendix VI, Table 14).

Left ventricular end-diastolic pressure (LVEDP) was compared among the three groups at 15, 90 adn 105 min in the surviving hearts of rats fed for 7 weeks and at 15, 45 and 75 min in surviving hearts of 10 weeks. In Fig. 15 representative examples of intraventricular pressure at 15 and 75 min are given for hearts of rats fed control, GBA and GPA diets for 10 weeks.

After 7 weeks, LVEDP was significantly higher in the GBA group than in the control and the GPA groups at 90 and 105 min ($p<0.01$, $F=9.20$) (Table 21). After 10 weeks LVEDP was higher in both the GBA and GPA groups than in the control group at 75 min however; statistical analysis was not performed since only two hearts were functioning in the experimental groups at that time.

Figure 14: Percentage of surviving hearts of rats fed control, GBA and GPA diets for 7 and 10 weeks, whose cardiac output remained above 10% of the initial value. The initial n value was 9 in all three groups fed for 7 weeks. Initial n was 6 in the control and GBA groups and 7 in the GPA group fed for 10 weeks.

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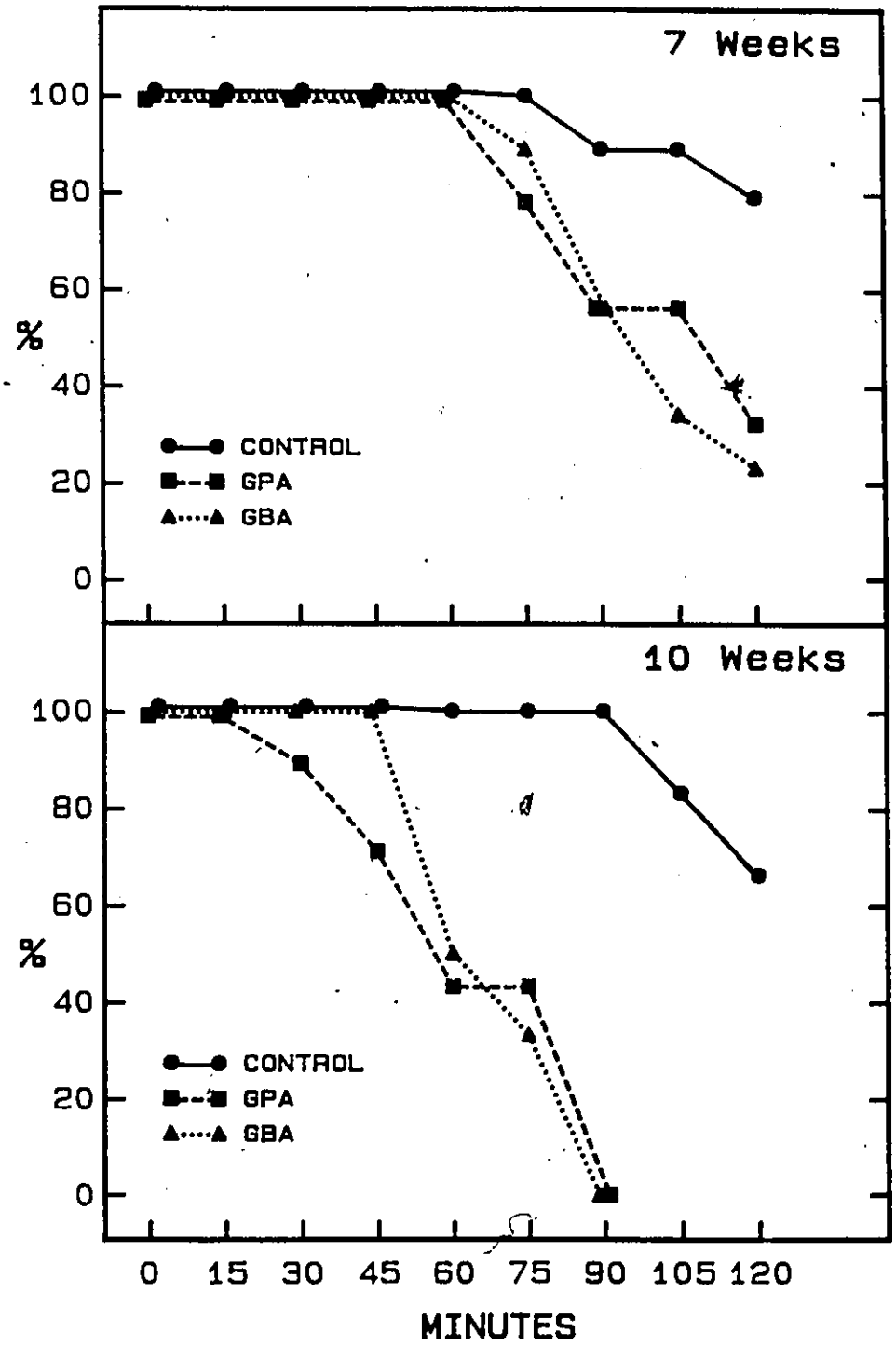

7 Weeks
100
80
60
%
40
20
0
CONTROL
GPA
GBA
10 Weeks
100
80
60
%
40
20
0
CONTROL
GPA
GBA
0 15 30 45 60 75 90 105 120
MINUTES

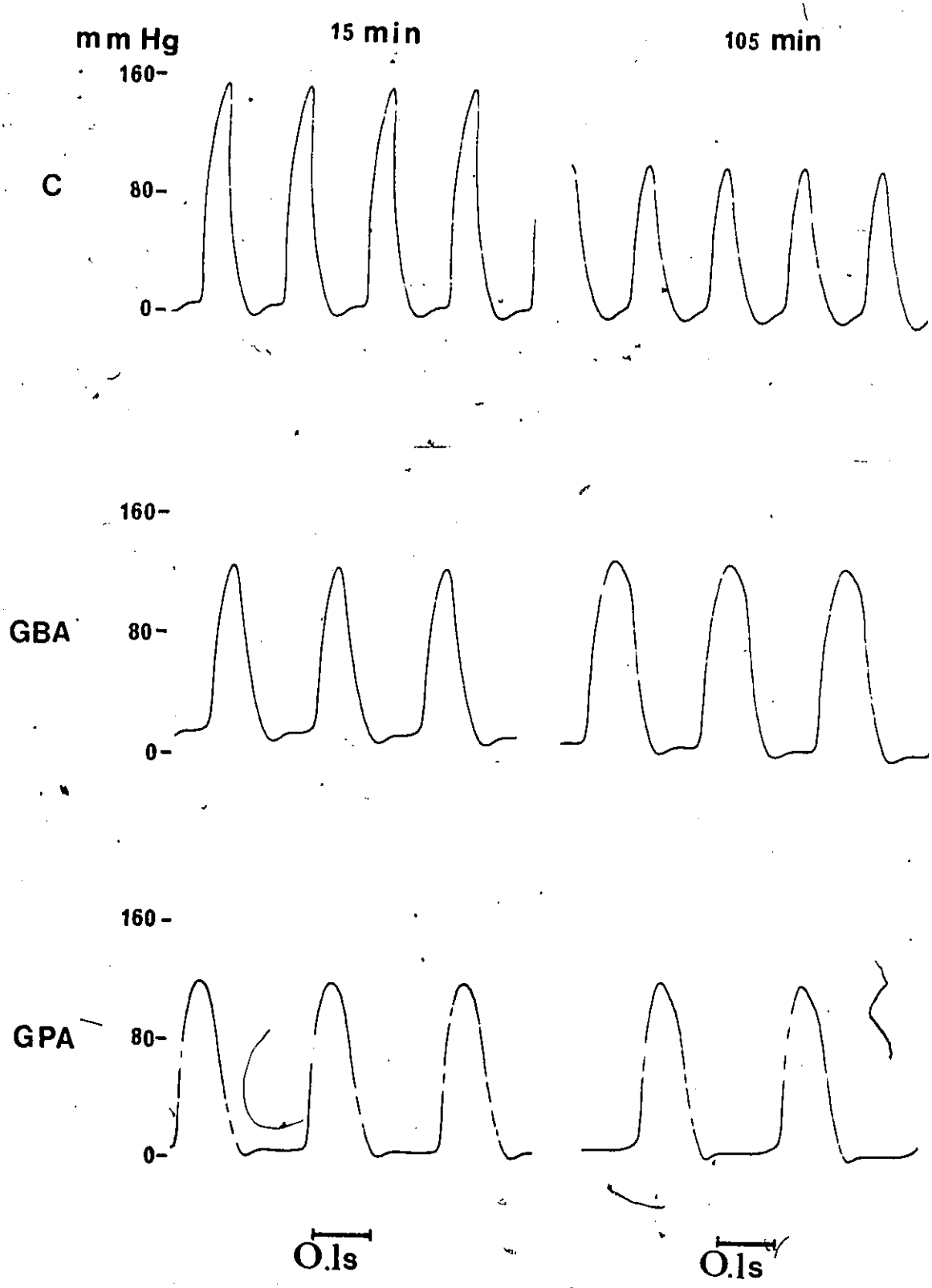
Table 21: Left ventricular end-diastolic pressure (mmHg) in hearts of control, GBA and GPA-fed rats for 7 and 10 weeks.

7 WEEK	15 min	90 min	105 min
Control	9.7 ± 1.7(9)	10.5 ± 1.6(9)	12.3 ± 1.7(9)
GBA	13.8 ± 1.2(9)	17.6 ± 1.7(6)*	21.4 ± 2.7(6)*
GPA	10.7 ± 1.9(9)	12.7 ± 2.0(4)	12.8 ± 2.2(4)
10 WEEK	**15 min**	**45 min**	**75 min**
Control	7.9 ± 1.0(6)	8.0 ± 1.0(6)	11.8 ± 1.4(6)
GBA	9.7 ± 0.8(6)	9.7 ± 0.3(6)	14.7 ± 0 (2)
GPA	10.3 ± 1.2(7)	12.0 ± 1.8(5)	18.3 ± 0 (2)

- the numbers in brackets represent the numbers of surviving hearts -

* $p<0.01$ statistically different from control and GPA

Figure 15: Recordings of intraventricular pressure obtained in hearts of rats fed control, GBA and GPA diets for 10 weeks.


mm Hg
15 min
105 min
160-
C
80-
0-
160-
GBA
80-
0-
160-
GPA
80-
0-
0.1s
0.1s

In summary it can be stated that creatine depletion does not appear to have an effect on the initial performance of isolated blood perfused heart at the maximum cardiac output at constant arterial pressure (110 cm H_2O). However, the length of time that the creatine depleted hearts were able to maintain initial submaximal (75%) cardiac output decreased with decreasing levels of myocardial creatine. A similar pattern of decrease was observed in all other parameters measured, except heart rate which did not change significantly from its initial rate. The survival time of the creatine depleted hearts under the above experimental conditions was significantly shorter than that of the control hearts. A significant increase in LVEDP with time was also observed in the creatine-depleted isolated hearts.

2. Hemodynamic Performance in Hypoxia

Hearts of rats fed control, GBA and GPA diets for 7 weeks were perfused with normoxic perfusate (pO_2 350-400 mmHg) for 10 minutes and were stabilized at 75-80% of their maximum CO (submaximal steady-state CO). No significant differences in any of the hemodynamic variables were observed among the three groups during the initial normoxemic period, and the hemodynamic data are given in Table 22. After 10 min, the perfusates were switched, and the hearts received hypoxic RBC perfusate with a starting pO_2 of 39 ± 5 mmHg in control group, 45 ± 6 mmHg in the GBA group and 45 ± 4 mmHg in the GPA group.

The maximum steady-state CO attained during the normoxemic period was not significantly different among the three groups and was 64.7 ± 4.9 ml/min/g in the control group, 65.0 ± 5.2 ml/min/g in the GBA group, and 59.2 ± 6.9 ml/min/g in the GPA group. When cardiac output was expressed as a percent of the initial submaximal steady-state CO and related to decreasing pO_2, significant differences between the control and experimental groups were observed (Fig. 16). The pO_2 at which CO began to decline from normoxic steady-state CO, (onset of decline was chosen as the point when CO decreased to 90% of the submaximal steady-state CO) was significantly higher (p 0.01, $F=25.5$) in the GBA and GPA groups (43 ± 2 and 43 ± 3 mmHg respectively) than in the control groups (33 ± 1 mmHg). As the pO_2 was decreasing from 40 to 30 mmHg, the CO of GBA and GPA groups was lower than that of control group. For example at pO_2 of 33 mmHg when the CO of the control group was 87% the CO in GBA and GPA groups had declined to 42% and 39% respectively.

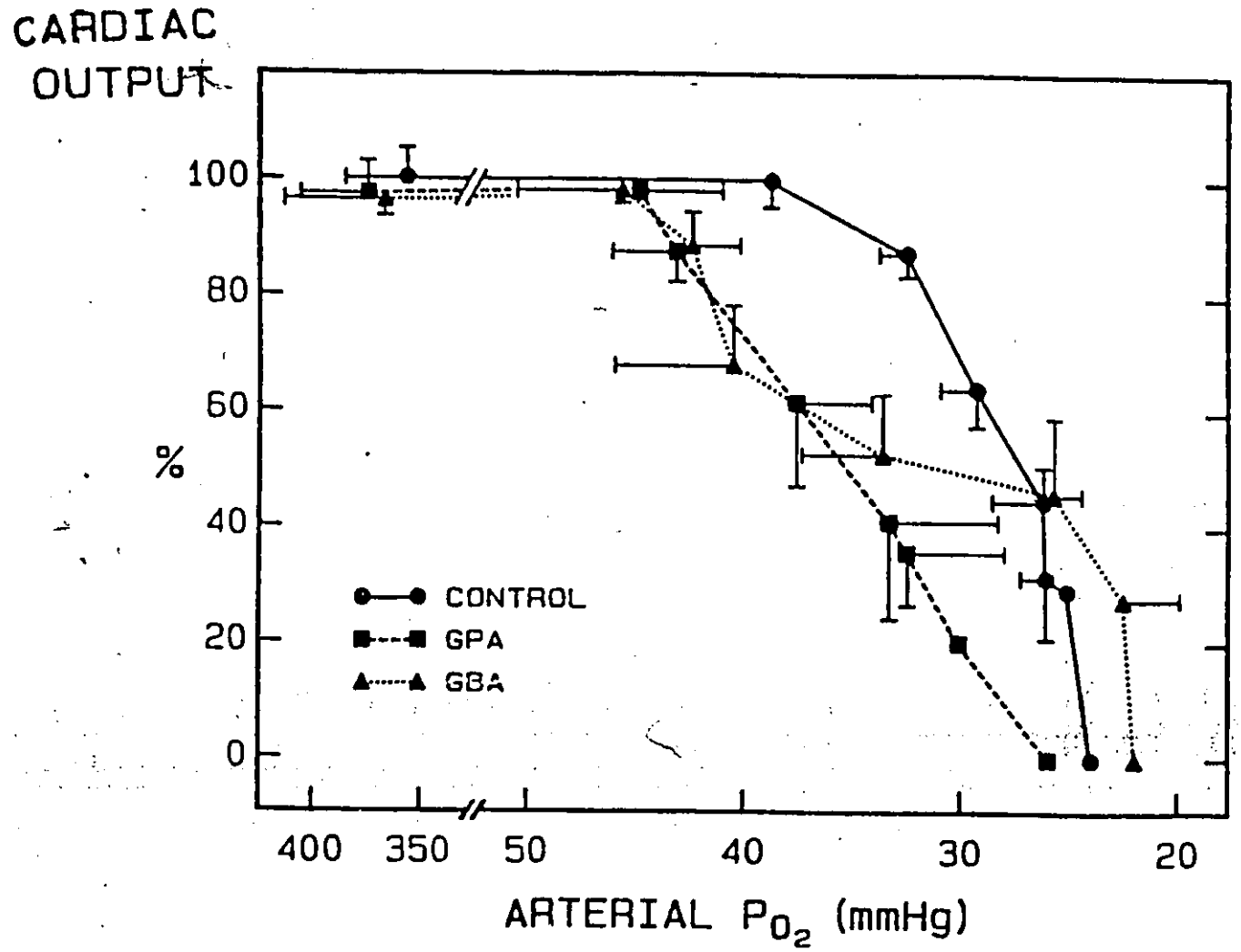
This pattern remained in both analogue treated groups, until the pO_2 decreased to 25 mmHg. At that pO_2, all of the hearts in the GPA group had failed while the hearts in the GBA group were maintaining 40% of their initial CO (Fig. 16). Also, the number of hearts surviving in this group was greater than in the control (Fig. 17).

Table 22: Hemodynamic data at the end of the first 10 min of normoxemia of isolated blood perfused working hearts of control, GBA and GPA rats fed for 7 weeks.

	Control	GBA	GPA
n	5	5	5
Hematocrit %	26.0 ± 1.0	27 ± 1.0	27 ± 1.0
pH a	7.46 ± 0.07	7.44 ± 0.01	7.44 ± 0.01
pH v	7.44 ± 0.02	7.44 ± 0.03	7.42 ± 0.03
PO_{2a} mmHg	350 ± 51	385 ± 25	344 ± 48
PO_{2cs} mmHg	34 ± 4	38 ± 3	35 ± 4
PCO_{2a} mmHg	41 ± 6	40 ± 9	38 ± 6
PCO_{2cs} mmHg	46 ± 2	43 ± 6	46 ± 4
C O_{2a} ml O_2/dl	13.2 ± 1.4	12.9 ± 1.6	13.5 ± 1.8
C O_{2cs} ml O_2/dl	8.8 ± 0.5	8.2 ± 1.1	9.0 ± 0.7
Hb g%	8.4 ± 0.8	9.0 ± 0.7	8.2 ± 0.6
Heart Rate bpm	320 ± 20	305 ± 15	310 ± 25
Aortic Flow ml/min/g	59.2 ± 4.4	59.5 ± 5.2	53.2 ± 6.9
Coronary Flow ml/min/g	5.5 ± 2.1	5.5 ± 2.1	6.0 ± 3.2
Myocardial Oxygen Consumption ml/min/g	0.30 ± 0.1	0.27 ± 0.09	0.29 ± 0.12
Power Input mW/g	115.0 ± 10	105 ± 8	111 ± 13
Total External Power mW/g	11.4 ± 1.9	12.1 ± 2.8	10.5 ± 3.6
Efficiency %	10.1 ± 1.1	11.7 ± 1.0	9.8 ± 1.6
LVP mmHg	129 ± 4	130 ± 4	126 ± 7
LVEDP mmHg	6.3 ± 1.7	10.0 ± 1.9	10.4 ± 2.0
+dP/dt mmHg/sec	3750 ± 200	3985 ± 155	3280 ± 190
-dP/dt mmHg/sec	3250 ± 245	3150 ± 210	2850 ± 155

a = aortic; c.s. = coronary sinus; PO_2, PCO_2 = partial pressure oxygen, carbon dioxide; C O_2 = total oxygen content; Hb = hemoglobin; LVP = peak left ventricular pressure; LVEDP = left ventricular end-diastolic pressure. Data were expressed per gram of left ventricular wet weight (free wall and septum). n refers to the number of hearts.

Figure 16: The effects of decreasing partial pressure of oxygen on cardiac output. Cardiac output was expressed as a percentage of the highest value of cardiac output of control, GBA and GPA fed rats for 7 weeks. The highest value achieved within ten minutes was taken as 100%. The number of hearts in each group was 5.


CARDIAC
OUTPUT
100
80
60
%
40
20
0
CONTROL
GPA
GBA
400
350
50
40
30
20
ARTERIAL P_{O_2} (mmHg)

The survival of isolated hearts as related to decreasing pO_2 is shown in Fig. 17. The number of hearts that survived in the GPA group at pO_2 of 34 ± 3 mmHg was 80% while all hearts in the control and GBA groups survived. At pO_2 of 32 ± 4 mmHg, only 40% of the hearts survived in the GPA group ($p<0.01$, $x^2=11.4$) while all of the hearts in the GBA and control groups were still surviving. At pO_2 of 25 ± 3 mmHg 60% of the hearts in the GBA and control groups were surviving and all hearts failed in GPA group.

Coronary flow rates in the all three groups were not significantly different during normoxemia. However, when the hearts began receiving the hypoxemic perfusate the increase in coronary flow was significantly lower ($p<0.05$, $F=3.9$) in the GBA group than in control and GPA groups. The maximal coronary flow obtained in the GBA group was 12.9 ± 1.0 ml/min/g, while in the control group it was 15.9 ± 1.3 ml/min/g and in the GPA group it was 16.3 ± 1.2 ml/min/g (Fig. 18). The maximum increase in coronary flow occurred at significantly lower ($p<0.05$, $F=7.1$) pO_2 in the control group than in the GBA and GPA groups and the pO_2 was 33 ± 1, 43 ± 2 and 43 ± 3 mmHg in the control, GBA and GPA groups respectively.

The end-diastolic left ventricular pressure was higher in both the GBA and GPA groups than in the control group in normoxemia (Fig. 19). During hypoxemia, as the pO_2 decreased to about 38 ± 2 mmHg, LVEDP increased significantly ($p<0.05$, $F=5.7$) in both GBA (15.2 ± 2.7 mmHg) and GPA groups (16.2 ± 2.0 mmHg) from that of the control group (7.6 ± 1.0 mmHg). Although LVEDP increased in all three groups as pO_2 decreased, it remained significantly higher in the creatine depleted hearts.

Figure 17: Percentage of surviving hearts of control, GBA and GPA fed rats for 7 weeks at decreasing partial pressures of oxygen. The percentage of hearts that survived was based on the total number of hearts that maintained cardiac output above 10% of the initial value. The number of hearts at the onset was 5.

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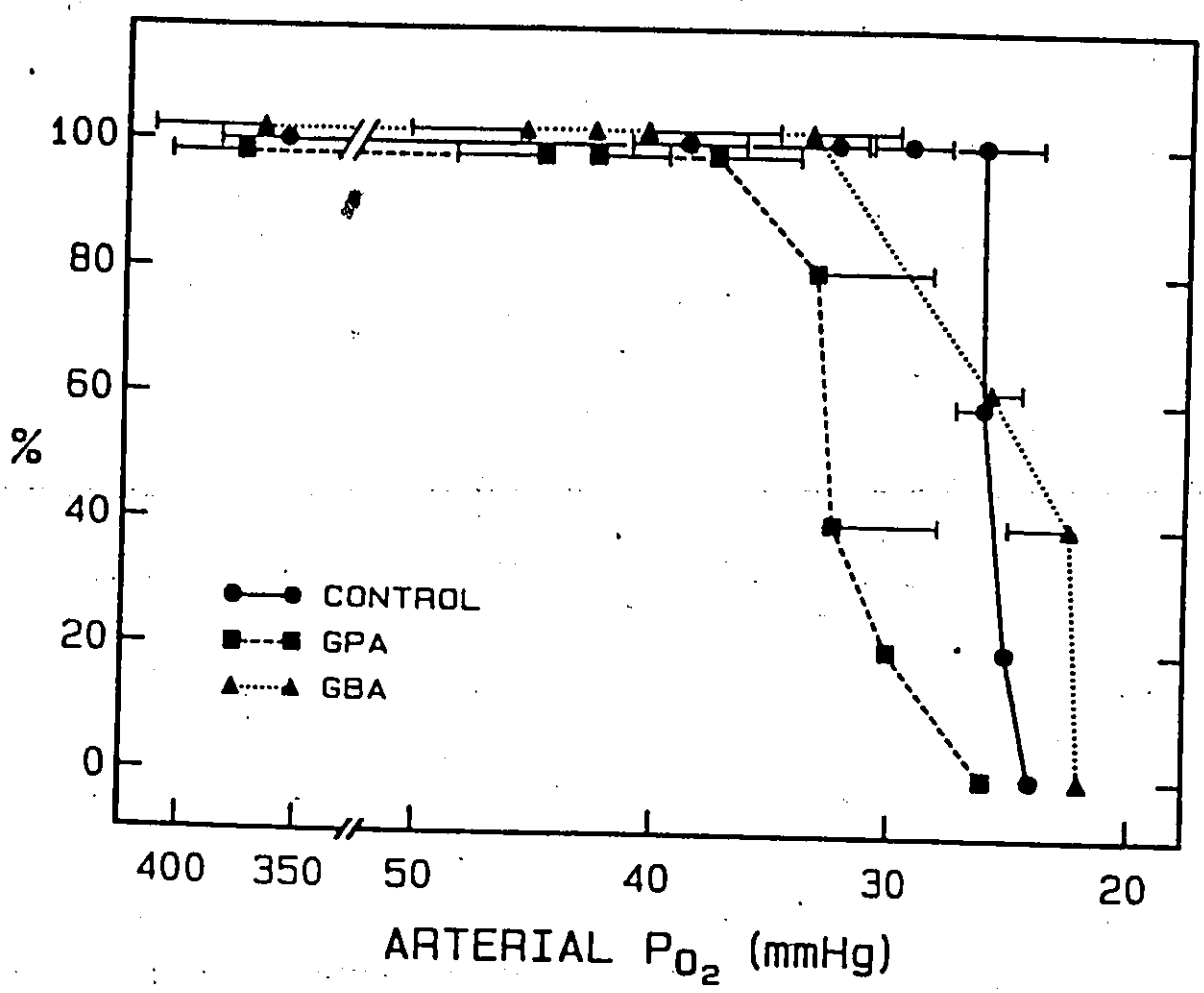

100
80
60
%
40
20
0
CONTROL
GPA
GBA
400
350
50
40
30
20
ARTERIAL P_{O_2} (mmHg)

Figure 18: The effect of decreasing partial pressure of oxygen on coronary flow in control, GBA and GPA fed rats for 7 weeks. The number of hearts in each group was 5.

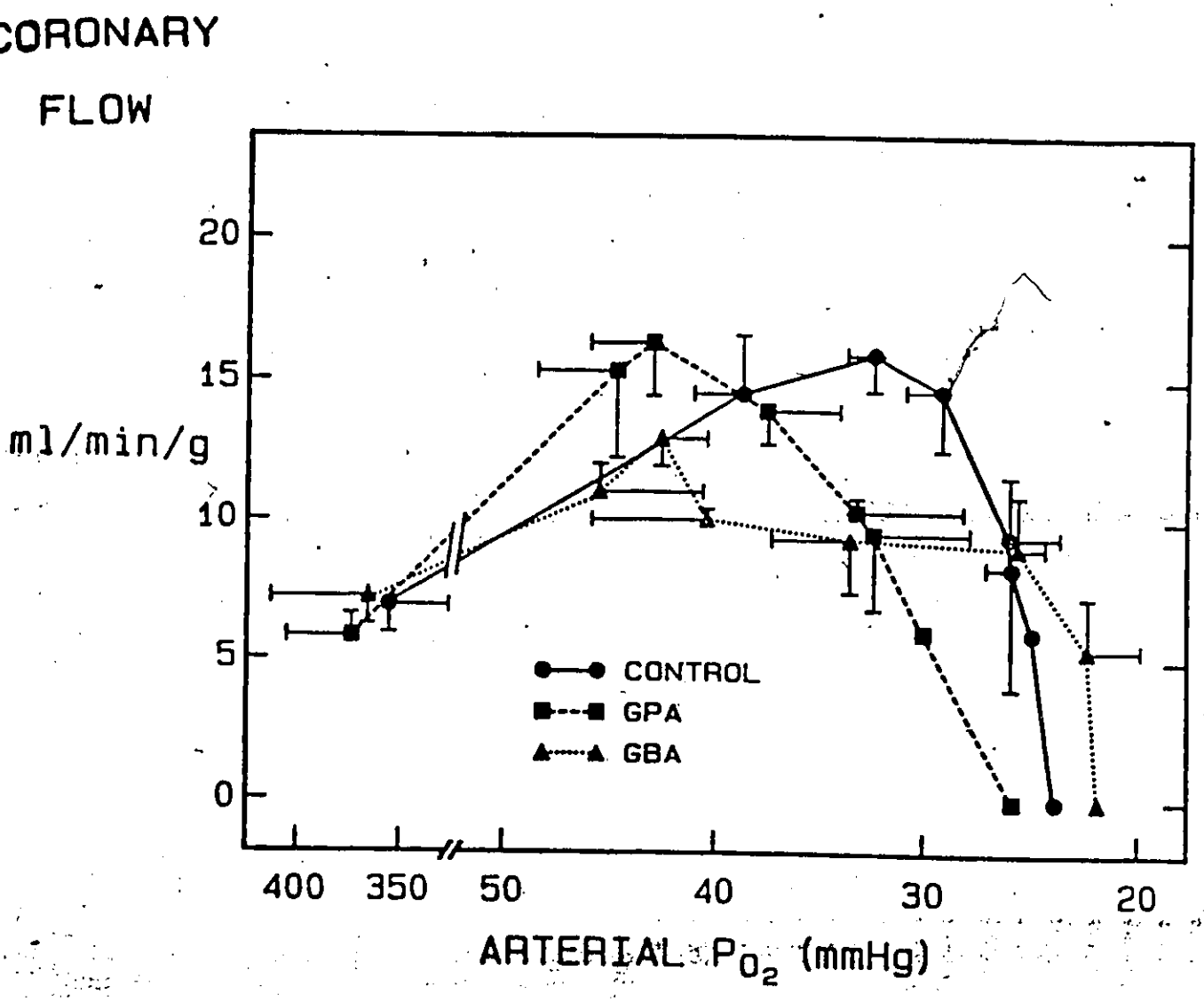
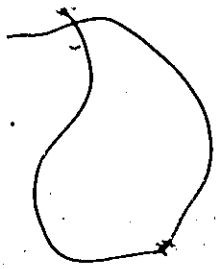
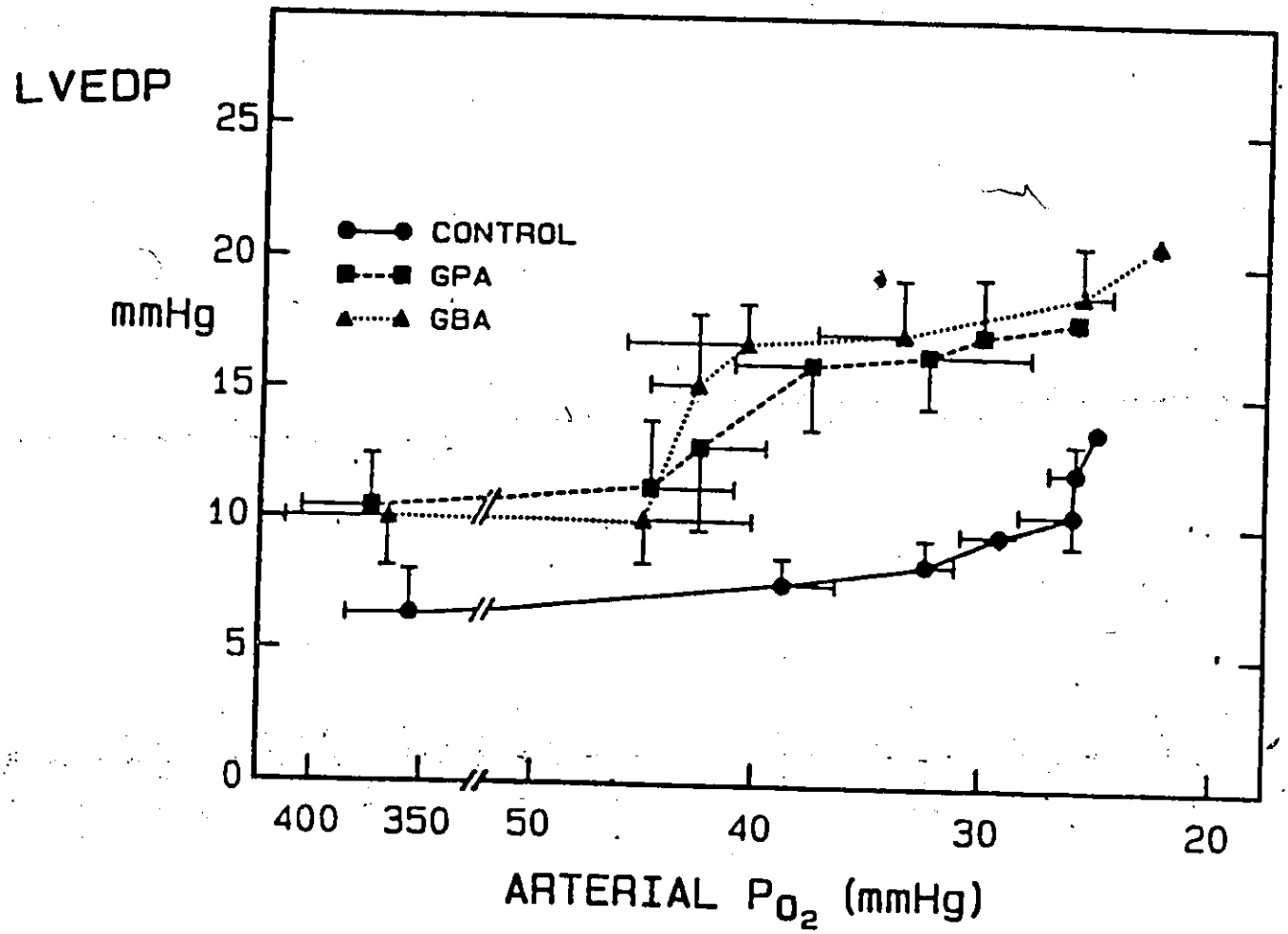

CORONARY
FLOW
ml/min/g
20
15
10
5
0
CONTROL
GPA
GBA
400
350
50
40
30
20
ARTERIAL P_{O_2} (mmHg)

Figure 19: The effect of decreasing partial pressure of oxygen on the left ventricular end-diastolic pressure in hearts of control, GBA and GPA fed rats for 7 weeks. The number of hearts in each group was 5.




LVEDP
mmHg
25
20
15
10
5
0
CONTROL
GPA
GBA
400
350
50
40
30
20
ARTERIAL P_{O_2} (mmHg)

Myocardial oxygen consumption (MVO_2) was not significantly different among the three groups at normoxemic pO_2 values. A similar pattern of decline to that observed with CO was observed in MVO_2 as pO_2 decreased and is shown in Table 23.

During steady-state under normoxemic conditions, no significant differences were observed in the heart rate, power input and output, or efficiency (Appendix VI, Tables 15, 16, 17 and 18). Power input was lower and efficiency was higher in GBA group than in the other two groups. These differences were not statistically significant due to intragroup variability, and a steady decline in the above variables was observed in all three groups, again similar to the pattern observed in CO (Fig. 16).

The rate of pressure development (dP/dt) (Table 24) , and more so the rate of relaxation, (-dP/dt) (Table 25), was lower in the creatine-depleted rats at pO_2 values between 40 to 30 mmHg when compared to controls. The variability among the hearts in each group was too large and thus these values were not statistically significant.

Table 23: The effects of decreasing partial pressure of oxygen on myocardial oxygen consumption in hearts of rats fed control, GBA and GPA diets for 7 weeks. Hearts were perfused on the isolated blood-perfused working rat heart preparation for 60 min.

Control		GBA		GPA	
PO_2 (mmHg)	MVO_2 (ml/min/g)	PO_2 (mmHg)	MVO_2 (ml/min/g)	PO_2 (mmHg)	MVO_2 (ml/min/g)
356±29	0.24±0.06(5)	366±48	0.21±0.02(5)	374±32	0.20±0.03(5)
38.8±2.1	0.30±0.07(5)	45.1±5.0	0.27±0.05(5)	44.8±4.0	0.35±0.07(5)
32.5±1.3	0.37±0.08(5)	40.7±2.2	0.26±0.04(5)	44.8±4.0	0.35±0.07(5)
29.3±1.7	0.34±0.06(5)	33.5±6.0	0.19±0.04(5)	37.6±3.5	0.32±0.04(5)
26.1±2.4	0.16±0.07(3)	26.6±4.2	0.14±0.02(3)	32.5±4.5	0.15±0.10(4)
25.1±1.2	0.15±0 (1)	25.9±1.0	0.10±0 (2)	30.1±1.5	0.15±0 (1)
24.0±0		22.0±0	0	26.0±0	0

- all values are means and standard errors of the mean
- the numbers in parentheses represent the number of surviving hearts at that partial pressure of oxygen

Table 24: The effects of decreasing partial pressure of oxygen on the rate of pressure development (+dP/dt) in hearts of rats fed control, GBA and GPA diets for 7 weeks. Hearts were perfused on the isolated blood-perfused working rat heart preparation for 60 min.

Control		GBA		GPA	
PO_2 (mmHg)	+dP/dt (mmHg/s)	PO_2 (mmHg)	+dP/dt (mmHg/s)	PO_2 (mmHg)	+dP/dt (mmHg/s)
356±29	4340±227(5)	366±48	4290±371(5)	374±32	3640±654(5)
38.8±2.1	4060±154(5)	45.1±5.0	4120±392(5)	44.8±4.0	3810±337(5)
32.5±1.3	4020±521(5)	42.7±2.2	4340±400(5)	42.6±3.0	3862±511(5)
29.3±1.7	3440±624(5)	33.5±6.0	3840±449(5)	37.6±3.5	3540±854(5)
26.1±2.4	2960±457(3)	26.6±4.2	2825±630(3)	32.5±4.5	2810±283(4)
25.1±1.2	1900±0 (1)	25.9±1.0	1200± 0(2)	30.1±1.5	1416± 0(1)
24.0±0	0	22.0±0	0	26.0±0	0

- all values are means and standard errors of the mean
- the numbers in parentheses represent the number of surviving hearts at that partial pressure of oxygen

Table 25: The effects of decreasing partial pressure of oxygen on the rate of relaxation (-dP/dt) in hearts of rats fed control, GBA and GPA diets for 7 weeks. Hearts were perfused on the isolated blood-perfused working rat heart preparation for 60 min.

Control		GBA		GPA	
PO_2 (mmHg)	-dP/dt (mmHg/s)	PO_2 (mmHg)	-dP/dt (mmHg/s)	PO_2 (mmHg)	-dP/dt (mmHg/s)
356±29	4180±254(5)	366±48	3860±509(5)	374±32	3850±495(5)
38.8±2.1	3780±246(5)	45.1±5.0	4380±549(5)	44.8±4.0	3450±247(5)
32.5±1.3	3740±446(5)	42.7±2.2	4160±447(5)	42.6±3.0	3330±704(5)
29.3±1.7	3240±605(5)	33.5±6.0	3410±243(5)	37.6±3.5	3260±711(5)
26.1±2.4	2660±595(3)	26.6±4.2	2950±378(3)	32.5±4.5	2580±437(4)
25.1±1.2	1567±0 (1)	25.9±1.0	2000±0 (2)	30:1±1.5	2233±0 (1)
24.0±0	0	22.0±0	0	26.0±0	0

- all values are means and standard errors of the mean
- the numbers in parentheses represent the number of surviving hearts at that partial pressure of oxygen

In summary, the performance of creatine-depleted hearts, on the isolated blood-perfused preparation, under hypoxic conditions, began to decline from submaximum steady-state performance at higher pO_2 than that of the control hearts. A decline in the number of surviving hearts at higher pO_2 was observed only in the GPA group. The LVEDP increased significantly in GBA and GPA groups at the same pO_2 at which the onset of decline from maximum steady-state CO occurred, however, an increase in LVEDP was observed in all three groups as pO_2 decreased. Coronary flow increased significantly as pO_2 decreased, but the increase was significantly lower in the GBA group than in the control and GPA groups. Although the rate of pressure development and the rate of relaxation were lower in the creatine depleted hearts than in controls after the onset of hypoxemia, due to intragroup variability, the differences were not statistically significant. Similarly, the pattern of decline in MVO_2, power input and output of the GBA, GPA and control groups were similar to that of cardiac output. That is, the MVO_2, power input and output at pO_2 above 45 mmHg in the creatine depleted groups was similar to that of the control group which maintained its maximal MVO_2, power input and output to down to 38 mmHg. Decline in these parameters in all three groups occurred below 45 mmHg in the GBA and GPA groups and below 38 mmHg in the control group.

E) Electron Microscopic Analysis of Hearts from Control and GBA- and GPA-fed Rats

Electron microscopic analysis was performed in the control, GBA and GPA rats fed for 7 weeks. The transmission electron micrograph in Fig. 20 is representative of the control rat left ventricular midwall myocardium in longitudinal section. The cardiocytes are well preserved, with clearly visible T-tubules, myofilaments and mitochondria. Similarly, the ventricular wall (either subendocardial, midwall or subepicardial) in the analogue treated rats resembled that of the control rats as shown in Figure 20.

Some focal changes were observed and are described in Figure 21. This figure shows an electron micrograph of the left ventricular midwall longitudinal section of a GBA-fed rat. The cardiocyte has some formation of myofibrillar strips and lysis. A few dilated mitochondria with deranged cristae are evident. However, these changes were very rare, extremely focal and cannot be taken as definitive signs of degeneration. Degeneration is usually manifested by a high number of lipofuscin granules which are rather scarce in these micrographs. On the whole, most of the myocardium of both analogue-fed groups looks very similar to that of the control myocardium in Fig. 20.

Figure 20: This transmission electron micrograph shows a portion of the left ventricular midwall myocardium of a control rat. Clearly visible are the rows of mitochondria (M) lining the sarcomeres. The T-tubules (T) and sarcoplasmic reticulum (SR) are clearly seen in the lower right hand corner. A portion of the nucleus (N) is seen on the left hand side of the micrograph. Magnification is 13,800X.

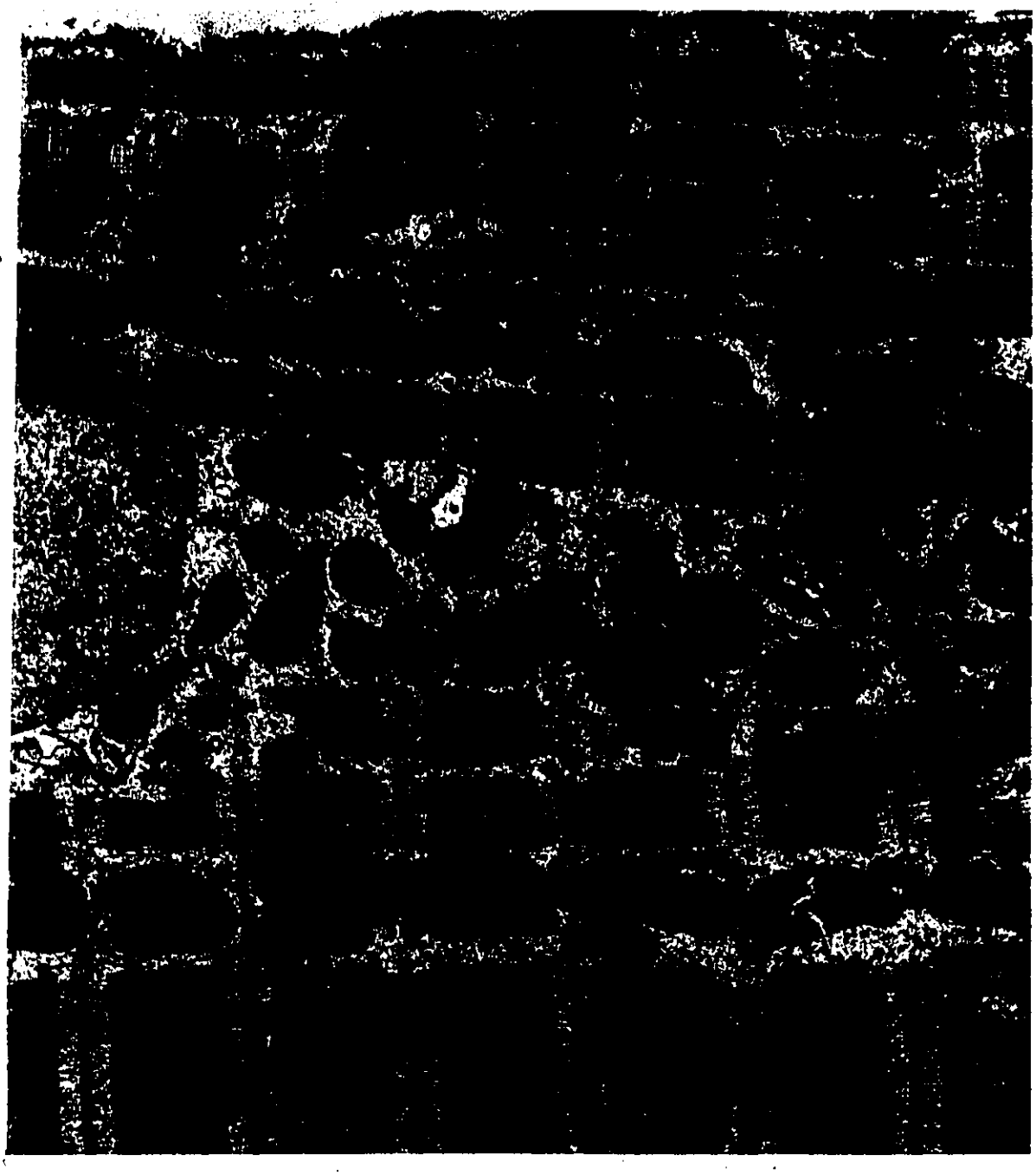


Figure 21: This transmission electron micrograph shows the left ventricular myocardial midwall from a heart of a GBA fed rat for 7 weeks. Some myofibrillar strips and lysis (MTS) are evident along with several dilated mitochondria (M). Magnification ix 21,000X.

DISCUSSION

The discussion is divided into 2 major sections. The first deals with the effects of depletion of total creatine on the animal. The second is divided into three subsections and each deals with one of the questions asked on page 34. The first deals with the effects of creatine depletion by analogue substitution on myocardial performance. The second deals with the differences the two analogues have on myocardial performance and the third deals with the usefulness of analogue feeding in testing the essentiality of creatine and CP in the heart.

A) General Effects of a Diet Containing Creatine Analogues on the Animal

In order to investigate the effects of various chemicals on animal function, researchers in many instances have added chemicals to the animal's diet. The effects of such supplemented diets may result in body or organ weight gain or loss, depending upon the chemical in question. Weight loss in the test animal may be due to a direct effect of the chemicals on the body and/or on specific organs, or a decreased consumption of the modified diet. Unfavorable taste or smell of the chemical additives may lead to decreased food intake by the animal if it is fed ad libitum. To preclude the effects of decreased food intake, so-called "pair-feeding" has been implemented (AIN Guide 1977). Pair-feeding is accomplished by measuring the amount of food consumed by the animals in the treated group and alloting a similar amount to the non-treated control group. Only when such

controlled feeding trials are conducted can differences in body or organ weight be objectively assessed and attributed to the chemical intervention.

1. Effects on Body Weight

In our experiments, the body weights of rats on control and analogue (GBA and GPA) supplemented diets were similar for up to 8 weeks. However by 10 weeks, the GBA group had significantly lower body weight than did those of the other two groups (Table 5).

The lower body weights that were observed in the GBA rats, could be due in part to the amount of chow that was displaced by the GBA analogue. For example, by day 14, the rats eating the 2% GBA diet, were receiving 25 g of chow per day of which 0.5 g was GBA. This means that these rats were receiving only 24.5 g of chow per day. While such a small deficit in chow may not lead to significant changes in daily caloric intake, over 10 weeks the total amount of chow that these rats received was 35 g less than the controls. This amount may be large enough to account at least in part for lower body weight in the GBA group.

A similar argument applies to the GPA fed rats, whose body weights were also decreased but not to the same extent since they were fed only 1% of the GPA analogue.

Mainwood et al. (1982a) who also pair-fed control and GPA rats for 5 weeks did not find any significant differences in body weight between the two groups. Nonetheless, the GPA fed rats did weigh less than the controls. Another investigator (Mohanna et

al. 1980) found differences in body weights between control mice and those fed 1% GPA for up to 13 months. Since food was provided ad libitum in their study, it is difficult to determine whether the decrease in body weight was due to the analogue itself or to a decrease in caloric intake. Therefore, pair-feeding should be employed when feeding trials with either GBA or GPA analogues are planned.

2. Effects on Heart Weight

The heart supplies blood to all of the active organs in the body in order to meet their metabolic demands, so it follows that the growth of the heart must correlate with that of the body (for review see Korecky et al. 1966). Therefore, heart weight is often expressed as a percentage of the body weight and is called the heart ratio (Clark 1927). For rats of body weight 400-500 g, the heart ratio has been shown to be 0.32 to 0.35 (Clark 1927; Korecky et al. 1966).

The heart ratios obtained in our study are comparable to those obtained by Clark (1927) (Table 9). We conclude that the lower heart weights in the GBA group were due to the smaller size of the animals and do not appear to be the result of a direct effect of GBA on the heart.

3. Effects on Structure of the Heart

The observed changes in the myocardium of rats fed the GBA and GPA diets that we noted were very focal and cannot be taken as signs of overall degeneration. The loss of myofibrils as well as the cristal disfigurement, was very infrequent and highly localized. Usually signs of degeneration in ultrastructure are manifested by increased number of lipofuscin granules which we did not observe in the hearts of either GBA or GPA fed rats. It is possible that any observed changes were the result of inadequate fixation since no major histological changes in any tissues of rats fed 1% GPA diet have been observed by other investigators (Shields et al. 1975; Mohanna et al. 1980). The only species where damage was observed was in cornish chicks (Laskowski and Fitch 1980). They fed the chicks a diet of 2% GBA and observed ultrastructural changes in gastrocnemius and pectoralis muscles. However, the observed changes were reversed when 6% creatine was added to the 2% GBA diet into the chow.

Other experimentation on the effects of creatine and muscle growth were done by Ingwall et al. (1972; 1974) who showed that creatine stimulates actin and myosin synthesis in differentiating cultured skeletal muscles. However, this work was later challenged by Fry and Morales (1980) who presented evidence to show that creatine had no effect on contractile protein synthesis in developing muscle.

4. Effects on Total Myocardial Creatine Levels

Fitch and Chevli (1980) showed decreases in total creatine by 75% in 5 weeks, perhaps because they began to feed their rats when they were very young and had a faster metabolic rate. We did not measure a comparable decline in total creatine until 7 weeks of feeding in our rats, who were older and probably had a slower metabolic rate which may have contributed to lower cellular uptake of the analogue by the myocardium. We also fed our rats for 12 weeks, and found that maximal depletion occurred by 10 weeks, with no further change in the level of total creatine observed thereafter.

Slight differences in the rate of decline of creatine were observed between the two groups, that is the level of total creatine decreased fairly steadily over the 10 weeks in the GBA group, while in the GPA group, creatine levels reached a plateau after 6 weeks (Table 3). The levels of total creatine initially decreased somewhat faster in the GPA group while the levels in the GBA group continued to decrease more slowly but steadily. Nonetheless, the levels of total creatine obtained at 10 weeks were not significantly different between the two experimental groups.

B. Does Creatine Depletion by Analogue Substitution have an Effect on Myocardial Function?

1. Contractile Characteristics

a. Rested State Contraction

The rested state contraction has been defined as a contraction that is separated by a rest interval long enough that each subsequent contraction is not affected by the previous one (Koch-Weser and Blinks 1963). In our experiments, rested state contractions in the creatine depleted papillary muscles exhibited similar developed tension, rate of tension development and mean rate of tension development as in that of the controls. However, time to maximal tension development and to half relaxation were slightly but not significantly longer in the GBA and GPA groups than in the control group (Table 7). Similarly, in experiments on skeletal muscle by Mainwood et al. (1982b), contractile characteristics were found to be altered in creatine depleted rats. The time to maximal tension development and to half relaxation in single twitches of the diaphram muscle from rats fed 1% GPA were longer than those from controls. Mainwood et al. (1982b) put forward an explanation to account for the longer muscle relaxation based on observations that lower ATP and higher ADP levels were present in creatine-depleted muscles. Such changes in the adenine nucleotides decrease the free chemical energy available to do work ($-dG/d\xi$, Curtin et al. 1974), which in turn affects the mechanism associated with contractile and

relaxation cycles (Hasselbach and Oetliker 1983). It has also been demonstrated by Dantzing et al. (1984) that elevated ADP levels have an inhibitory influence on cross-bridge detachment leading to reduction of the relaxation rate. Shoubridge et al. (1985a) reported that in rats fed 1% GPA, the myocardial ADP concentration was 2 to 2.6 times higher than that observed in control rats. Similarly, Korecky and Jacobus (unpublished data) obtained myocardial ADP levels 2.8 times higher in rats fed 2% GBA than those of controls as measured by P^{31}-NMR. It is therefore possible that elevations in ADP levels in the heart may play a role in increasing the time to maximal tension development as well as to half-relaxation.

The possibility also exists that an increase in action potential duration could play a role in increasing the time to maximal tension development and to half-relaxation. To date, no such increase in action potential duration has been reported with chronic feeding of GPA or GBA. Also, with longer duration in the action potential, we would expect that the developed force would also be augmented and in our experiments no such increase was observed.

It has also been proposed by Petrofsky and Fitch (1980), Shoubridge et al. (1985b) and Mainwood et al. (1982b) that chronic analogue feeding may slow the myosin ATPase activity in the myocardium. Mainwood and Ingwall (personal communication) have analyzed skeletal muscles from rats fed GPA and found no significant differences between the creatine depleted and control muscles in myosin ATPase activity. We suspect that if the myosin

ATPase activity was decreased, then rate of force development would be diminished. We did not observe any such decreases in the creatine depleted papillary muscles nor did Mainwood et al. (1982b) in creatine depleted diaphram muscles.

In conclusion, the evidence suggests that the elevated myocardial ADP level alone may account for the slight increase in time to maximal tension and half-relaxation in the creatine depleted papillary preparations.

b. Frequency Force Relationship

The frequency-force relationship is one of the simplest ways to test the inotropic response in the myocardium. In the mammalian heart, the strength of a contraction depends on the interval between stimuli and changes in this interval alter the degree of activation (Abbott and Mommaerts 1959; Sonnenblick 1962a; 1962b). As the frequency of stimulation increases, the strength of each subsequent contraction is affected by the previous one. In most mammals a positive inotropic response predominates at extracellular calcium concentrations of 2.5 mM, however, in the rat heart a negative inotropic response has been observed (Koch-Weser and Blinks 1963; Forester and Mainwood 1974; Korecky and Michael 1974).

Several theories have been proposed to explain the negative response in the rat. One of the earliest theories deals with calcium movements in and out of various cellular fractions (Hoffman et al. 1956; Manring and Holander 1971). This theory states that each action potential liberates small fractions of intracellular bound calcium (Cr), also referred to as the releasable fraction of calcium (Lee et al. 1970; Forester and Mainwood 1974). During recovery the calcium is taken up into another intracellular fraction which recycles calcium to Cr but at a slower rate and has been referred to as Cs (Mainwood and Lee 1969). In the rat myocardium, as the number of stimuli increase, the amount of calcium that is taken up into the second fraction Cs, also increases and is taken up more effectively than in other species. Its return to Cr is longer, thus the amount of calcium

available as free calcium for excitation contraction is decreased (Forester and Mainwood 1974).

It has also been proposed that transsarcolemmal calcium currents (fast and slow) induce the release of calcium from the sarcoplasmic reticulum (SR) (Fabiato 1983). Fabiato (1983) feels that the fast calcium current which is dependent on the number of calcium channels that are open on the sarcolemmal membrane controls the release of calcium from the SR. The opening of the fast channels is stimulated by Mg^{++}ATP and free ATP and is inactivated by increased myoplasmic calcium concentration. The slow calcium current enters the SR and "tops off" the calcium levels within the SR, which is released during subsequent action potentials. The entry of the slow current into the SR is dependent on the activity of the calcium ATPase. If the filling of the SR is slowed due to decreased calcium ATPase activity, then the free calcium will be mopped up by binding proteins, and taken up by subsarcolemmal stores such as the mitochondria. Also, less calcium will be present in the SR for subsequent contractions. The magnitude of the subsequent fast calcium current will be diminished and less calcium will be released (Fabiato 1983).

More recently, it has been proposed by Ruegg (1987) as well as by Schouter and ter Keurs (1986) that with increasing frequency of stimulation, core hypoxia develops in papillary preparations whose diameter is greater than 0.20 mm. During this hypoxic period, CP is broken down to maintain constant levels and inorganic phosphate (Pi) levels increase.

Kentish et al. (1986) and Allen et al. (1986) have shown that inorganic phosphate (Pi) alters the force development in muscle. They provided evidence to show that as Pi levels increase, the relationship between force and calcium concentrations [Ca^{2+}] are shifted to higher concentrations of calcium. Therefore, as the frequency of stimulation increases in the rat heart it is possible that the Pi levels temporarily rise and shift the force-[Ca^{2+}] curve to the right, suggesting that a loss in calcium sensitivity occurs with increasing Pi concentrations (Ruegg 1987).

Blinks and Endoh (1986) point out that developed force is also increased by phosphorylation of troponin I. Eventhough the mechanism is not yet defined, it is postulated by these two authors that decreased ATP concentration in the vicinity of the myofibrils can result in decreased phosphorylation of troponin I, diminishing the sensitivity of troponin C for calcium.

Although one or more of these mechanisms may be the cause of the negative treppe during high frequency stimulation, we did not observe significant differences in developed tension between control and the creatine depleted muscles at stimulation rates of 3, 6, 12, 24, 48, 96 and 192 at a bath temperature of 25°C. Similarly, when the papillary muscles were stimulated at 192 pulses/min at 25°C, followed by a single stimulus after a 5 s rest, no significant differences in developed tension among the three groups were observed.

We postulated that if CP is the only transporter of high energy phosphates from the mitochondria to the areas of high energy demand in the cell, then total creatine depletion of 85-90% in muscles of analogue fed rats would decrease the ability

of these muscles to develop force with increasing rates of stimulation more than in the control muscles. Since that did not occur, we propose that either the amount of CP left in the myocytes of creatine depleted muscles was sufficient to supply high energy phosphates at a rate to maintain ATP at similar levels as in control muscles at the stimulation rates we applied. Alternatively, perhaps ATP is not compartmentalized and is able to freely diffuse to the sites of utilization within the myocyte to maintain contractile activity. This latter proposal is supported by calculations performed by Jacobus (1985) of fluxes of ATP and CP within a myocyte. He found that there is no limitation on ATP delivery from the source to the sink and concludes that there appears to be no compartmentation of ATP.

To determine if limitations of ATP utilization can occur in creatine depleted papillary muscles, we increased the temperature from 25°C to 31°C and also increased the stimulation rate. Again, creatine depletion did not have any significant effects on the developed force of papillary muscles at frequencies ranging between 270 to 390 pulses/min (Table 19). However, we observed that the maximal developed force obtained after each train was lower in the creatine depleted papillary muscles when compared to controls, particularly after 360 and 390 pulses/min (Fig. 11). Also, it took three times as long for the creatine depleted papillary muscles to attain their maximal developed force after each train of high frequency of stimulation (this period we termed the recovery period). There are a number of possibilities which may account for the depressed and prolonged recovery of

the twitch in the creatine depleted muscles.

One, if CP levels in creatine depleted rats decrease by 75-90% as has been shown previously (Saks et al. 1986; Kapelko et al. 1986), then the amount of total ATP present may decrease after a burst of high frequency stimulation. This decrease in ATP levels may lead to a reduction in $(-dG/d\xi)$ which slows down the uptake of calcium by the SR (Hasselbach and Oetliker 1983), resulting in a decrease in the activation of subsequent contractions as was proposed by Fabiato (1983).

Two, during periods of high stimulation, CP and ATP levels may transiently decline and a simultaneous transient increase in Pi will occur (Kentish et al. 1986; Allen et al. 1986). Increases in Pi, as discussed earlier, can decrease the sensitivity of calcium to troponin C, leading to a suppression in developed force (Ruegg 1987; Allen et al. 1986; Kentish et al. 1986).

Three, Alberty (1969) has shown that hydrogen ions are generated during ATP hydrolysis, and are consumed during CP hydrolysis (Curtin and Woledge 1978). In normal muscles, as ATP is recycled during activity, CP buffers the hydrogen ions produced. In creatine depleted hearts CP levels can be very low and the ability of CP to buffer hydrogen ions may be greatly diminished. The transient increase in acidity around the myofibrils may have decreased the reverse Lohman reaction, decreasing ATP formation. The decreased regeneration of ATP in creatine depleted muscles may have been further exaggerated by the initial low CP levels in these muscles. Such decreases in

ATP levels would result in a fall in $-dG/d\xi$ which may decrease the removal of calcium from the cytosol allowing calcium to be taken up by binding proteins, resulting in a reduced releasable fraction available for force development.

Even though all three possibilities may play a role in the slow recovery of the creatine depleted papillary muscles, the second is more likely to be the mechanism accounting for the slow recovery. The first is less likely since the resting tension during stimulation did not rise, and an increase in resting tension during the recovery period (rested state stimulation) was not elevated more than in the control muscles. The third is also less likely to play a large role in the long-term recovery of the creatine depleted muscle, since the buffering time of hydrogen ions, although longer in the creatine depleted muscle, most probably takes less than one minute. Although we do not have proof for number two, this mechanism seems more plausible than one or three.

In the heart, energy production is oxidative, but in skeletal muscles both oxidative and glycolytic energy production may occur. The energy production can be predominantly oxidative, such as in the soleus muscles which is categorized as slow oxidative or it can be glycolytic such as in the plantaris, which is referred to as fast glycolytic. It can also be a combination of both energy systems, such as the EDL (extensor digitorum longus) which is 38% fast glycolytic, 59% fast oxidative glycolytic and 3% slow oxidative. In this muscle, the twitch contractile characteristics are not altered by creatine depletion

of 80%. However, after 1-second tetanus, the post-tetanic potentiated twitch was depressed by 40% in the depleted muscles, while it was potentiated by 80% in the control muscles (Mainwood and Totosy de Zepetnek 1985). Also, it took considerably longer for the depleted EDL muscle to recover and exhibit a comparable single twitch as in controls.

Mainwood and Totosy de Zepetnek (1985) postulated that regeneration of ATP during tetanus by the CK reaction may be decreased, leading to the post-tetanic twitch suppression. This suppression, they believe, could be the result of a transient fall in $-dG/d\xi$, which is considered to be critical in the uptake and release of calcium from SR.

In contrast, in creatine depleted diaphram muscles, Mainwood et al. (1982b) found that the rate of relaxation and the velocity of tension development was decreased during brief bursts of activity. However, during less intense fatiguing stimuli, less depression of developed tension was observed in the creatine depleted diaphrams than in controls and the recovery of the depleted diaphrams was faster than that of the control diaphrams.

These results in skeletal muscles along with our results on the papillary muscles suggest that creatine depletion is not essential for energy transport from the sources of energy production to their sinks in a myocyte at submaximal workloads, in a variety of skeletal muscles or the heart. But CP may be important as an energy reserve to buffer changes in concentrations of ATP and ADP during high intensity stimulation in most types of muscles.

c. Paired Stimulation

The fusion of developed force during paired stimulation was first investigated by Lee et al. (1970). These authors found that mechanical and electrical uncoupling occurred when the paired pulses were given within 80 to 140 ms of each other. They provided two possible interpretations to explain this phenomenon.

The first is that the actin-myosin system is fully activated by a single action potential. This would mean that the rise in intracellular free calcium level from one impulse saturates the sites responsible for activating a concentration and no further increase in developed force is possible for a certain period of time. This is most probably not so, since recently, Ruegg (1987) reported that during a contraction, only one-half of all the calcium binding sites are occupied, allowing for up or down regulation.

The second interpretation is that the availability of calcium is the limiting factor for a second contraction if it occurs within a short period of time. That is, an action potential triggers off a rapidly releasable pool of calcium which is completely released during an action potential and no further release can occur until this fraction is recharged. Recharging of this rapidly releasable pool of calcium is dependent on the cycling of calcium from other stores such as the mitochondria back to the SR.

This latter interpretation fits in with the more recent proposal of calcium cycling in the cell, described by Fabiato (1983). His theory of the fast and slow calcium current

activating the release of calcium from the SR was discussed earlier in section B2. At paired stimulation rates of 140 ms or less, it is possible that the concentration of ATP is not kept constant and a transient decline in the ATP/ADP ratio can decrease calcium uptake by the SR. The transiently elevated calcium in the myoplasm can inactivate the fast calcium current which would prevent further release of calcium from the SR and the myoplasmic free calcium can be mopped up by binding proteins. This scenario may be occurring in the creatine-depleted muscles also, but at longer intervals. In these muscles, the ATP/ADP ratio may be declining at longer pulse intervals since CP is not present in high enough concentrations to maintain the ATP at levels to keep a high ATP/ADP ratio.

d. Hypoxia

In our pilot study, we wanted to determine if hypoxia would affect twitch characteristics in creatine depleted capillary muscles more than in controls. We did not observe any differences in the developed force of the creatine depleted muscles when compared to controls.

When we increased the stimulation rate from 24 to 48 pulses/min and maintained the hypoxia, the muscles stopped developing force within the next two to three twitches. Also, it was difficult to control the pO_2, so that a smooth progressive decline in pO_2 could be obtained. Therefore, we did not pursue these hypoxic experiments using this preparation.

e. Limitations of the Papillary Muscle Preparation

In any in vitro system, there are a few technical shortcomings. In the papillary muscle preparation, the muscles must be isolated from the ventricular wall and clamped at the ends when attached to the preparation. This causes a number of fibers on the excised side and at each end of the muscle to deteriorate. The damaged area may develop abnormal properties acting in isometric contractions as an additional "series elastic component" (Reichel 1976).

Also, when the muscles are suspended between the two clamps, one must be very careful to obtain and to maintain a parallel orientation of the fibers so that as many fibers as possible will contribute to each contraction. If twisting or overstretching occurs, the fibers will not be contributing to the development of tension, and falsely low tension may be obtained. Since it is difficult to perform excision and mounting exactly the same way each time, some degree of variability may be seen in the responses obtained from a number of muscles in one experiment, and this may account for some of the variability we observed in our experiments.

The preparation is oxygen diffusion limited, since blood supply to the papillary muscles has been eliminated by excision. The preparation is dependent on oxygen delivery through diffusion and the pO_2 in the buffering solution in which the muscles are bathed. Thus, to minimize differences between energy supply and demand, the preparation is often operated at 25°C. Similarly, the elimination of hydrogen ions, Pi, lactate and other metabolic

wastes is dependent on diffusion and on energy dependent ion pumps. Therefore, during protocols such as those that call for increasing frequencies of stimulation, there may be a transient accumulation of any of these ions which may contribute to the negative inotropic treppe.

Nonetheless, this preparation allows us to measure contractile characteristics of the myocardial tissue. It also provides us with valuable information of myocardial mechanical properties under altered conditions, such as high rates of stimulation, increased or decreased preload and temperature. It also gives us information about the effects various pharmacological agents may have on contractile properties. Such direct information cannot be obtained from an intact heart from which it can only be extrapolated.

On the other hand, studies using an intact ex vivo heart preparation provide us with pertinent information regarding hemodynamic function and cardiac performance that cannot be obtained with papillary muscle preparations. Therefore, experimentation using the two preparations is necessary when testing the effects of altered cellular composition or various drugs on myocardial performance.

2. Hemodynamic Function

The isolated perfused rat heart has been used extensively to study cardiac function. The classical Langendorf preparation was the first to be used and is a non-working heart preparation. In this preparation, the aorta is cannulated and the coronary vessels are perfused by introducing the perfusate into the aorta. This preparation does not perform external mechanical work but does develop ventricular pressure.

A more sophisticated preparation is the working heart preparation, in which the aorta and the left appendage are cannulated, the perfusate is introduced into the left atrium and the left ventricle pumps the perfusate into the aorta. In this system, ventricular pressure development and cardiac output can be controlled by varying the left atrial filling pressure and/or aortic resistance (Morgan et al. 1961). The standard perfusion fluid consists of a Tyrode, Ringer or Krebs-Henseleit solution. The perfusion pressure is usually raised to a relatively high level but the oncotic pressure is low and net movement of water across the capillary is into the interstitium and not out. This situation usually leads to increased interstitial and intracellular water content, which is indicated by a dry to wet weight ratio of less than 0.20.

In our isolated working heart preparation, we used 1.5% albumin to increase the oncotic pressure within capillaries. We also used RBC which increased the oxygen carrying capacity of our perfusate over the previously used electrolyte solution (Gamble et al. 1967; Neely et al. 1967). Parameters such as maximum

cardiac output, total work output and myocardial oxygen consumption were higher, and the coronary flow was lower, in hearts perfused with RBC-enriched solution than in those perfused with Krebs-Henseleit only (Neely et al. 1967; Opie et al. 1972).

The hemodynamic values obtained in this study were in agreement with those of Duvelleroy et al. (1976), who also used RBC enriched perfusate and are similar to values obtained in vivo (Lee et al. 1970). It is generally accepted that in vivo, the fraction of coronary flow is usually 5% of cardiac output. In our blood-perfused hearts it was 7%, while in hearts perfused with an electrolyte solution it was 26% (Neely et al. 1967). Therefore, the use of RBC perfusate in the isolated working heart is superior since it is more physiological and provides better oxygenation at high cardiac output levels. However, there was some variability in the performance observed in our hearts, and this could be controlled only to a degree. The variability was due to differences in the time it took to isolate and perfuse the hearts. This became one of the most critical components of determining optimal steady-state function, and when isolation and perfusion was performed in less than 3 min steady-state performance was maintained for a minimum of 1 hour.

The optimal length of time to isolate and perfuse the hearts was 2 min ± 30 s. Hearts which took longer than that to retrogradely perfuse were not used in the following studies.

a. Maximum Volume and Pressure Work

We initially perfused the isolated hearts in a non-working mode and upon cannulation of the atrial appendage, we switched them into the working mode. Upon stabilization, to obtain maximal volume work, the left atrial pressure was increased to values between 18 to 23 cm H_2O (and the resistance pressure was maintained at 110 cm H_2O) which made the heart pump at maximum cardiac output. No significant differences in the maximal cardiac output, oxygen consumption or total external power output were observed among the three groups either after 7 or 10 weeks of feeding and this data was pooled for each group (Table 20).

Similarly, Kapelko et al. (1986), and Saks et al. (1986) also observed that maximal cardiac output of creatine depleted hearts (who were fed 1% GPA) was similar to that of controls. Both groups were able to deplete hearts of total creatine by 80% and CP by 90%. In both of these studies, the maximal filling pressure was increased from 5 to 20 cm H_2O while the resistance pressure was kept constant at 80 cm H_2O.

In our experiments we maintained the same resistance pressure (110 cm H_2O) in all of the experimental protocols. We did not measure pressure work, since it was difficult to maintain a stable working preparation at high resistance (120-140) in all three groups. However, other investigators altered the resistance pressure and measured the pressure work of the creatine depleted hearts. Shoubridge et al. (1985) used an isolated, perfused working heart to study the effects of creatine depletion after feeding 1% GPA to rats for 6-10 weeks. They

showed that with a 80% reduction in total creatine and a 90% reduction in CP there were no differences in cardiac output, rate-pressure product or oxygen consumption observed at steady-state performance at low or high workloads (at a left atrial filling pressure of 12 cm H_2O and resistance pressure of 70 cm H_2O or at atrial filling pressure of 15 cm H_2O and resistance pressure of 140 cm H_2O).

Kapelko et al. (1986) argued that the work of the heart and oxygen consumption at these workloads used by Shoubridge et al. (1985) was below the maximum reported for isolated hearts, and thus maximal work was not evoked in this preparation. Kapelko et al. (1986) decided to repeat the experiments on creatine depleted rats and induced both high pressure and volume work on the hearts. These investigators elevated the filling pressure to the heart to 15 cm H_2O and increased the aortic resistance to a maximum of 120 cm H_2O. Then they increaseed the heart rate and clamped off the outflow tract. Under these conditions, these hearts were not able to eject a cardiac output as large as the controls due to an insufficient stroke volume. A similar experiment was performed by Saks et al. (1986) inducing only high aortic resistances on the hearts and similar observations were noted. It is well known that an increment in pressure has greater oxygen consumption than the same increment in volume work (Sonnenblick et al. 1968). Therefore, CP may be more important in maintaining myocardial function at high pressure loads than at high volume loads.

b. Submaximum Steady-State Performance

In our experiments, once the maximal cardiac output was established in each heart, the left atrial filling pressure was reduced to a pressure which maintained the cardiac output at 75-80% of the initial maximum. This was then called the submaximal steady-state performance. The submaximal steady-state performance was similar in the GBA, GPA and control groups (Fig. 13). The myocardial oxygen uptake (MVO_2), which is an indicator of the heart's ability to use oxygen, was similar in all three groups, showing that energy production was not altered in the creatine depleted hearts. However, a significant difference in the duration of the submaximal steady-state performance was observed between the hearts of the analogue groups and the control hearts after feeding the rats for 7 and 10 weeks. As the length of the dietary regime was increased and as creatine concentrations in the hearts decreased, the submaximal steady-state performance declined. This again was manifested in all parameters measured in the creatine depleted hearts, except in heart rate, which did not change significantly from its initial rate.

Saks et al. (1986) and Kapelko et al. (1986) observed an increased LVEDP in creatine depleted rats similar to what we observed in all three groups by the end of the experiment (Table 29). Increased LVEDP is usually due to the increased left-ventricular end diastolic volume (LVEDV) and is a manifestation of a hypodynamic heart. The initial response of the heart is to increase the resting length of the ventricular fibers to put into

effect the Frank-Starling compensator mechanism, increasing the stroke work as the end-diastolic volume increases. However, if the heart cannot contract against the load, then it continues to dilate until the fibers become overstretched and cardiac function deteriorates (Leyton 1974).

Although the increase in LVEDP can be due to LVEDV as described above, it can also increase as a result of increased stiffness of the ventricles (Saks et al. 1986). Saks et al. (1986) and Kapelko et al. (1986) also found that LVEDP was increased in isolated buffer perfused working hearts of GPA fed rats. These authors measured concentrations of CP and ATP using P^{31}-NMR spectra and obtained CP decreases of 4.5 times when compared to controls while ATP levels remained constant. They postulated that such severe decrements of CP may lead to an eventual depletion of ATP in the compartment around the myofibrils during intense ATP turnover (for example, when the heart works against an increased resistance load). If CP cannot maintain the ATP pool under such conditions, the the ATP/ADP ratio decreases and according to Saks et al. (1986), may increase left ventricular diastolic stiffness.

c. Hemodynamic Performance in Hypoxemic Conditions

In these experiments we wanted to determine how the hearts of creatine depleted rats would perform in an isolated working preparation exposed to hypoxemia. The purpose of these experiments was to determine if CP is important as a buffer under conditions of reduced ATP production by the mitochondria resulting from hypoxemia, when the only pathway producing ATP is glycolysis.

Kohn et al. (1979), Ach et al. (1979) and Dhalla et al. (1972) stated that CP is important in buffering ATP levels during states of reduced oxygen production, such as hypoxia. In this situation, reduced mitochondrial production of ATP is supplemented by glycolytic production of ATP. No restriction on diffusion of ATP occurs during glycolysis since it takes place in the cytoplasm, but the diffusion coefficient of ATP is 30% smaller than that of CP (Jacobus 1985b; Mainwood and Rakusan 1982). Therefore, in hypoxia CP may be important in buffering ATP levels because of its ability to diffuse faster to the sites of utilization. If this is true, and if CP is depleted by 80-90% as in our hearts, then the myocardial performance of these hearts should be affected sooner than in controls.

One way to examine the effects of hypoxemia in an isolated working preparation would be to decrease the pO_2 of the perfusate to a set value and see if any differences in cardiac performance would occur in time. It is also possible to gradually decrease the pO_2 to determine if at a particular pO_2, cardiac performance differed in the creatine-depleted rats from controls. We chose

the latter method, since maintaining stable hypoxemia at a set of predetermined levels was very difficult in the RBC enriched perfusate and the earlier decay in performance observed in the hearts of creatine-depleted rats in normoxemia could have interfered with the effects of hypoxemia. Upon the introduction of RBC perfusate with low and gradually decreasing pO_2, we observed that CO declined at a higher pO_2 in the creatine-depleted hearts when compared to controls at all pO_2's above 35 mmHg. However, at pO_2 of 35 mmHg to 22 mmHg, the GBA group maintained a slightly higher CO than the control group (Fig. 16), while the GPA group had the lowest CO at each pO_2.

A similar pattern was also observed in the survival rates (Fig. 17) of the three groups. The difference in survival may have been related to the following factors. In rats fed GPA, the phosphorylation of GPA (GPAP) occurs to a higher level than the corresponding phosphorylation of GBA (GBAP) in GBA fed rats. GPAP can be used by creatine kinase to a limited extent, while GBAP can not (Fitch and Chevli 1980). Also, in hypoxia, as CP is quickly hydrolyzed Pi can accumulate (Allen 1986). High levels of Pi have been shown to decrease the sensitivity of the myofibrils to calcium, thus, decreasing maximum force development and contributing to contractile failure in hypoxia (Kentish 1986). Since the GPAP could be used as a substrate for CK to a limited extent, we postulate that in extreme hypoxemia, GPAP may have been hydrolyzed and the Pi produced in the reaction may have contributed to the earlier failure observed in the GPA group.

Similarly, in the hearts of control fed rats, the Pi levels could have risen higher than in those of GBA fed rats, since the CP concentration in the controls is much higher. It may also be possible that GBA itself has an effect on the biochemical composition of the cell, allowing the hearts of the GBA group to survive longer under severe oxygen restrictions.

We also observed that in all three groups, the coronary flow increased to compensate for the lower oxygen tension in the perfusate. However, the increase in coronary flow in the GBA group was significantly less than the increase in GPA group (Fig. 18). Why this occurred is unexplainable at this time, except perhaps a lower release of vasodilator such as adenosine or Pi may have occurred. It may be that the myocardium in the GBA group was not as hypoxic as were the other two groups, particularly since the percent survival in that group was greater at any pO_2 than in the other two groups.

Again, we cannot rule out the fact that the GBA compound itself may have a direct effect on the coronary vasculature preventing a full dilation under hypoxemic conditions. How? Perhaps by interacting intracellularly with adenosine causing a lower release of this vasodilator, by inactivating its transcellular transport or by inhibiting its actual production.

Of the other parameters measured, only LVEDP was equally elevated in both GBA and GPA groups in normoxemia (Fig. 19). All other parameters decreased as hypoxemia increased. As discussed in the section preceding this one, the rise in LVEDP in our experimental hearts may have been due to either an increased

LVEDV, or due to increased stiffness of the ventricle.

Based on this experiment, we propose that CP probably acts as a buffer under hypoxemic conditions. In hypoxemia, less oxygen diffuses from capillaries to various parts of the cell and oxidative phosphorylation in mitochondria may be limited to those that are in close proximity to the capillaries. The amount of ATP produced by these mitochondria is most probably reduced and may not be able to diffuse to the sites of utilization quickly enough to meet the energy demand of the cell. Since CP is able to diffuse faster throughout the cell, (Jacobus 1985b; Meyer et al. 1984) it probably is able to regenerate ATP faster, thus maintain a higher ATP/ADP ratio longer.

When the concentration of total creatine is reduced by about 85-90%, as in our experiment, the amount of ATP regenerated by CP is reduced as pO_2 decreases, thus -dG/dE decreases to levels at which force development cannot be maintained, resulting in an accelerated decline in force. This may explain the observed reduction in CO and survival in the creatine-depleted hearts at higher pO_2.

3. Possible Role of CP in the Myocardium

In this study, we observed that depletion of total creatine had three major effects. The first was the significantly longer recovery of papillary muscles from high rate of stimulation (360 and 390 pulses/min, at 31°C). The second effect was the fusion of developed force in papillary muscles which occurred at significantly longer paired-pulse intervals and the third was a decline in the hemodynamic performance of the isolated blood-perfused working rat heart both under normoxemic and hypoxemic conditions.

Even though it has been proposed by some investigators (Petrofsky and Fitch 1980; Mainwood et al. 1982b; Shoubridge et al. 1985b) that the altered function of creatine depleted muscles may be due to structural changes in contractile proteins, no experimental evidence to date has yet supported this proposal. Moreover, in the heart, no biochemical alterations either in glycolytic, aerobic or creatine kinase enzymes have been shown to occur (Shoubridge et al. 1985b) after chronic (6-10 wk) feeding of 1% GPA to young or mature rats.

As stated earlier, the only observation made by Shoubridge et al. (1985a), Meyer (1985b) and Korecky and Jacobus (unpublished data) is that rats fed either 1% GPA or 2% GBA for 6-10 weeks had myocardial ADP concentrations 2 to 2.8 times higher than controls. This increase in ADP may increase the time to half-relaxation as we showed in single twitches of papillary muscles. However, a greater effect resulting from a rise in ADP is the decrease in the ATP/ADP ratio. This alters the free

energy change of ATP hydrolysis -dG/dE (Curtin et al. 1974; Kammermeier et al. 1982) which is a measure of the chemical energy available to do work:

$$-dG/dE = -G_{obs} + R\ T\ \ln \frac{[ADP]\ [Pi]}{[ATP]}$$

where G_{obs} = -30.5 kJ/mol, [Pi] = 4×10^{-1}M, according to Kammermeier et al 1982.

As stated by Dawson et al. (1980) and Kammermeier et al. (1982, 1987), small changes in ATP hydrolysis can have very large effects on all processes requiring ATP. According to Kammermeier et al. (1982), a decrease in the ATP/ADP ratio is considered to be the main causal factor of contractile failure. Since the levels of ATP, ADP and CP that we obtained were relatively low (Appendix IV) in comparison to others (Wilkman-Coffelt et al. 1983) who measured them, we used values obtained by Korecky and Jacobus (unpublished data). Korecky and Jacobus used hearts of rats fed 2% GBA and measured with P^{31}-NMR the concentrations of CP, free creatine, ATP, ADP, Pi and hydrogen ions. Based on their data, we calculated that the free energy of ATP hydrolysis was 63.4 kJ/mol in the controls and 56.4 kJ/mol in the hearts of the rats fed GBA. This means that -dG/dE was reduced by 14% in the creatine depleted hearts. Since Shoubridge et al. (1985a) reported similar levels of CP, Pi, hydrogen ions and adenine nucleotides in the myocardium of rats fed 1% GPA for 6-10 weeks, we can presume that the same decreases in -dG/dE can be obtained

in the hearts of rats fed the GPA diet. It has been calculated by Kammermeier et al. (1982) that if -dG/dE is maintained above 50 kJ/mol of ATP, contractile performance will not be altered. If the initial -dG/dE was 56.4 kJ/mol in our creatine depleted hearts, it may explain why the initial steady state performance of these hearts was not reduced from that of controls.

This finding is in direct contrast to that of Gudbjarnason et al. (1970) who found that contractile performance ceased when high levels of ATP were still present. Gudbjarnason et al. (1970) induced local ischemia in dog hearts by completely occluding the left main descending artery and took punch biopsies every 30 seconds. They observed that the heart ceased beating within one minute of the occlusion at which time the ATP levels were decreased by only 20% from the initial levels before the occlusion. Similarly results were observed by Dhalla et al. (1971) who induced hypoxemia in isolated working rat hearts by gassing the perfusion medium with 95% N_2 and 5% CO_2 for 10 minutes. These early results provided the basis for the idea of compartmentation from which the creatine shuttle was developed (see review of literature).

Under ischemic conditions, it is possible that ATP compartmentation occurs due to the accumulation of fatty acids (Mila-Reiker 1985) which inhibit the adenine nucleotide translocase. But under normoxemic and even hypoxemic conditions, ATP does not appear to be compartmentalized nor is its diffusion restricted in any way (Meyer et al. 1984; Jacobus 1985a; Bittl et al. 1987). Moreover, our data show that under severe total

creatine depletion, the initial myocardial function is not impaired, implying that ATP must be able to diffuse the myofibrils from the mitochondria.

Although this theory is hypothetical, the function of CP in the myocardium may be to maintain the ATP/ADP ratio thus, -dG/dE in a range above 50 to 60 kJ/mol. This means that CP could be buffering any change in the adenine nucleotide pool at least in in vitro heart preparations, during transitions from low to high workloads (as pointed out by Kohn et al. 1970) and during submaximal steady state work.

One major limitation to any in vitro preparation is that in time it fails. There are many reasons that can account for this failure, such as inconsistent oxygen delivery, exhaustion of endogenous substrates (Reisser 1979) and inadequate exogenous substrated delivery (Balaban et al. 1986; Bittl et al. 1987). If endogenous glycogen and triglyceride stores are exhausted during prolonged perfusion and if exogenous glucose cannot entirely support the workload of the muscle, mitochondrial respiration may become limiting and the supply of ATP from oxidative phosphorylation may decrease. This may lead to a decline in the ATP/ADP ratio, leading to a lower -dG/dE, which may reach 50 kJ/mol or less and the cessation of myocardial contractility may ensue.

Moreover, de novo synthesis of adenine nucleotides is slow (several hours) and during one-two hours of extracorporeal perfusion, adenylate kinase converts the increasing levels of ADP to AMP (2 ADP $\rightleftharpoons$ AMP + ATP). The AMP is eventually broken down to

adenosine which is lost to the circulation (Rubio et al. 1972). This eventually leads to the loss of the total adenine nucleotide pool (Turner and Walker 1985). Creatine phosphate may not only play a role in maintaining ATP at adequate levels to maintain high -dG/dE (above 50 kJ/mol), it may also prevent the loss of the adenylate pool, delaying myocardial failure in in vitro preparations such as ours.

C. Do the Two Analogues Have Different Effects on Myocardial Performance?

In all of the experiments performed in this study, we noted very similar responses in both the contractile characteristics of the papillary muscles and the hemodynamic parameters of the isolated blood-perfused working hearts, both in normoxemia and hypoxemia of rats fed the GBA and GPA diets. However, there were a few minor differences that we found in myocardial function as a result of the analogue feeding.

We observed that the papillary muscles of the rats fed the GPA diet both for 7 and 10 weeks had lower developed force than the muscles of the rats fed the GBA diet. This was noted during the frequency-force protocols both at 25°C and 31°C. However, these differences in developed force were not statistically significant. The difference in developed force was also noted during the recovery period from high frequencies of stimulation and was significantly lower in the GPA group after 360 and 390 stimuli/min.

Similarly, the GPA group had lower cardiac output and survival rate on the isolated blood perfused preparation, particularly during hypoxemic perfusion. As explained in section B 2c, we believe that these differences between the GBA and GPA groups may be related to the different concentration of Pi ions (Kentish et al. 1986; Ruegg 1987). As shown by Fitch and Chevli (1980), GPA can be phosphorylated and may be used to some extent. This was demonstrated by Roberts and Walker (1982) who excised hearts from GPA fed rats and observed that GPAP was used during

the first 20 min of ischemia. Simultaneously, Pi ions rose significantly during the initial 20 minutes and continued to rise for the next 80 minutes of ischemia. Since Pi ions play a role in force development (Kentish et al. 1986; Ruegg 1987), then the difference in myocardial performance between the GBA and GPA groups can be accounted for by this phenomenon.

We also noted that the coronary blood flow in the GBA group under hypoxemic conditions in the isolated blood-perfused hearts was lower than in the GPA group. At present, there is little known about the effects of GBA on the myocardium, smooth muscle or skeletal muscle function, other than its ability to deplete these tissues of creatine and CP. As we proposed in section B 2b, it is possible that GBA may have less of a vasodilatory effect on smooth muscle cells of coronary vessels, interfere with the release of adenosine or some other vasodilating substance. It is also possible that the hearts of the rats fed the GBA diet did not become as hypoxic as those of the GPA rats or that the amount of Pi released in the hearts of the GPA and control groups played a role in vasodilation.

Also, we cannot disregard the possibility that some minor impurities from our synthesis of the GBA crystals were part of the food given to the GBA fed rats. The impurities could have played a role in some of the differences we observed between the GBA and GPA groups. However, the primary impurity is a derivative of cyanide (NH_3CN) and therefore, it probably did not effect myocardial function since any substantial quantity of cyanide would result in death of the animal.

In conclusion, we do not feel that major differences exist between GBA and GPA in their effects on myocardial performance except in the ability of GPA to become phosphorylated. Nonetheless, further studies should be carried out to determine the effects of GBA on smooth muscle function, particularly under hypoxic conditions.

D. Is the Depletion of Total Creatine Using Creatine Analogues (GBA and GPA) a Useful Model to Test the Essentiality of Creatine and CP in the Myocardium?

Any pharmacological agent has both desirable and undesirable effects. In our case, the desirable effects of GBA and GPA are the severe depletion of creatine and CP which was achieved equally well with both analogues. As a result of such depletion, the effect on myocardial function was similar. Simultaneously intracellular glycogen levels were not altered by either analogue (pilot study performed by the author; Roberts and Walker 1982), nor were the activities of glycolytic, aerobic and CK enzymes (Shoubridge et al. 1985b).

The undesirable effects were that the depletion of total creatine was not 100% and that the analogues did accumulate in the heart. Therefore, optimally, we do suggest that for future studies testing the essentiality of CP in the myocardium should be with CK-specific inhibitors. At present, however, there are none available (FDNB used by Cain and Davies 1962, was not specific only to CK enzymes) thus, creatine analogues are the only way to test this theory.

Even though both analogues deplete the myocardium of total creatine to the same extent, we would prefer the use of GBA over GPA for future studies since its chances of becoming phosphorylated are less, and its intracellular accumulation in comparison to GPA is also less.

E. Proposal for Future Studies

To further clarify the role of CP in the myocardium, we recommend that the future investigator uses the GBA analogue for a 10 week feeding trial with small animals such as rats. Rats thus depleted of CP and creatine could be used in an open-chested, ventilated state and placed in P^{31}-NMR, with the NMR coil laid over the left ventricle. In this way, relevant information pertaining to CP and adenine nucleotide levels could be obtained over extended periods of time. This preparation could also be manipulated to induce changes in workload on the heart by: cardiac denervation and pacing, potassium arrest, volume loading of the animal and perhaps even temporary aortic banding to induce high pressure conditions.

Such information could be compared to an isolated-blood perfused working rat heart also from a GBA fed rat analyzed spectroscopically under similar conditions as listed above. This would provide comparative information about *in vivo* and *ex vivo* preparations and allow the investigator to determine what happens to myocardial energetics and function (that is the loss of it) in an isolated working preparation.

GENERAL FINDINGS

1. In creatine depleted papillary muscles, contractile characteristics were not altered at rested state stimulation rate. At high workloads, force development was not altered in the creatine depleted rats when compared to controls, however, the subsequent recovery of the creatine depleted muscles at low frequencies was significantly longer. During paired stimulation, the maximum developed force was significantly lower in the creatine depleted muscles at higher intervals than in controls and the contractions fused at significantly longer intervals.

2. Creatine depletion does not affect the initial maximal and submaximal steady-state performance of the isolated blood-perfused rat heart under normoxemic conditions. However, prolonged steady state work is decreased as the depletion of total creatine increases.

3. Under hypoxemic conditions, the creatine depleted hearts were not able to maintain steady state at the same pO_2 values as controls.

4. Although there are differences in the analogues with respect to their ability to become phosphorylated in the cell and be used as substrates, their ability to deplete the cell of creatine does not differ. Also, they have overall similar effects on myocardial performance in normoxemic conditions, but under hypoxemic conditions, the hearts that were depleted of

creating with the analogue GPA, had somewhat faster deterioration of myocardial performance. Despite this difference creatine depletion by these analogues provides a useful model to study the importance of CP in the myocardium.

APPENDICES

APPENDIX I

Samples for the measurement of oxygen content were taken from the outflow tract of the aorta and from the coronary sinus effluent. The oxygen content was obtained upon analysis of oxyhemoglobin saturation by the IL 232 CO-Oximeter at the Children's Hospital of Eastern Ontario, Ottawa. Myocardial oxygen consumption (MVO_2) was calculated as a product of coronary flow times the arterio-venous O_2 content difference. Cardiac efficiency was derived from the ratio of total external cardiac work produced to total energy expended. Total external cardiac work was expressed as external power (Wo) and was derived from pressure power (Wp) and kinetic power (Wk). Since power is an instantaneous quantity, thus Wp is a product of the instantaneous arterial pressure (P) and volume flow rate (Q). Since instantaneous volume flow rate is difficult to measure in an isolated heart, average power was approximated from the data assuming that flow was constant during ejection. Thus average pressure power ($\dot{W}p$) was approximated from mean pressure ($\bar{P}$) times the mean flow rate ($\dot{Q}$). Flow in an isolated heart is the cardiac output (CO) (aortic and coronary flow), and pressure is the average systolic pressure. In our case, it was the mean aortic pressure, measured in the isolated heart thus:

$$\dot{W}p = \bar{P}_{aortic} \times CO$$

$$= \frac{\bar{P}_{aortic\ mmHg}}{760\ mmHg} \times (101325\,P_a) \times (CO\ ml/min)^3 \times \left(min/_{603}\right) \times \left(100 mW/_{W}\right)$$

P_a = Pascal

Kinetic power by definition is $1/2 mv^2$, where m is the mass of the fluid, and v is the velocity. By substituting $m=\rho v$, where ρ is the density of the fluid and $v=Q/A$, where Q is the volume flow rate and A is the cross sectional area, we obtain:

$$W_k = 1/2\ \rho\, v\, Q^2/A^2$$

to calculate instantaneous kinetic power, we differentiate the equation and obtain:

$$W_k = \rho/2A^2[Q^3 + V\,Q\,\dot{Q}]$$

Since volume flow rate in our case is CO and time averaging of instantaneous kinetic power was not available to us, a simplification was made by treating Q as a constant, therefore $\dot{Q}=0$ and,

$$\dot{W}_k = \rho \frac{CO^3}{2A^2}$$

$$= \frac{\left(\rho\ \frac{kg}{l}\right) \times \left(\frac{CO\ ml}{min}\right)}{2(A\,mm^2)^2} \times \frac{\left(\frac{10\,dm}{m}\right)^3 \left(\frac{m}{100\,cm}\right)^9 \left(\frac{min}{60\,s}\right)^3 \left(\frac{1000\,mW}{W}\right)}{\left(\frac{m}{10^3\,mm}\right)^4}$$

and,

$$\dot{W}_o = \dot{W}_\rho + \dot{W}_k$$

Power input ($\dot{W}i$) was calculated as the product of myocardial oxygen consumption per minute, times the caloric equivalent of the substrate in the buffer. Since glucose was the primary substrate, and is calculated to be 5.5 cal/ml and one cal is 4.18 J.

$$\dot{W}_i = (5.5\ cal/ml\,O_2) \times (4.18) \times (MVO_2) \times (min/60s) \times (10^3 mW/W)$$

Mechanical efficiency was determined by the dividing of total external power produced by the power input.

Computation formulas:

$$MVO_2 (ml/min) = (CO_a - CO_{cs}\ ml O_2 / 100\ ml) \times (Q_{cor}\ ml/min)$$

$$\dot{W}i\ (mW) = (383.56\ mW \times min/ml) \times (MVO_2\ ml/min)$$

$$\dot{W}k\ (mW) = \left[\left(\frac{\rho\ kg/l}{2(60)^3 (A\ mm^2)^2}\right) \times \left(\frac{mm^4 \times mW}{kg/l\ (ml/min)^3}\right)\right] \times (CO\ ml/min)^3$$

$$\dot{W}p\ (mW) = (0.00222\ mW \times min/mmHg \times ml) \times (P_a\ mmHg) \times (CO\ ml/min)$$

Efficiency = $\dot{W}o/\dot{W}i$

- a = aortic
- c.s. = coronary sinus
- cor = coronary

These computational formulae were modified from Kannengiesser et al. (1978) by Dr. David Antecol.

APPENDIX II

Normalization

In order to be able to equate the mechanical performance of two muscles, it was necessary to correct for differences in size and weight of the muscles by normalization per cross-sectional area. This was first done by Ramsey and Street in 1940 and then by Helandar and Thulin in 1960. These researchers based their calculations on the cross-sectional area of a cylinder, where the volume was $\pi r^2 h$ and the density of the muscle (mass/volume) was taken as 1.063. The formula used today for the calculation of cross-sectional area is:

$$\frac{\text{muscle mass (mg)}}{1.063(\text{mg/mm}^3) \times \text{length of the muscle (mm)}}$$

This formula is meaningful if the following assumptions are made:

1) uniform geometry of the muscle bundle
2) parallel arrangement of the fibers
3) equal number of myofibrils/unit of cross-sectional area

Since any two muscles are usually uneven in size and weight, normalizing according to the above formula allows comparison of developed tension based on the cross-sectional area which reflects the presumptive number of force generating sites of muscle fibers lying in parallel. The developed tension is primarily dependent on these force generating components which consist of thick and thin filaments within a sarcomere. Thus,

the arrangement of the fibers in a tissue sample under investigation will affect the developed tension. The sample is attached to a fixed clamp at one end and at the other end it is attached to a clamp that is joined to a transducer. The developed tension then reflects the force generated by the sum of the muscle fibers when the fibers are lying in parallel.

APPENDIX III

Calculation of survival of hearts on the isolated blood-perfused preparation.

The method of Life Tables is used to measure patient survival in evaluation of treatment of fatally ill patients. This statistical method is effective in the computation of survival of a group of patients who are exposed to a disease. In the computation of the Life Tables, the estimated survival is not only based on the number of patients at the onset of the study, but also on the entry of patients at a later time, or the loss of patients due to death, or withdrawal due to other reasons than death at different times during the course of the study. Since we did not have other reasons but "death" or failure of the heart to change the survival in our study, we did not use this method, instead we calculated the cumulative survival of each group.

In our experiments the calculation of percent survival was done as follows:

$$(E_1 x E_2 \ldots x E_1 x 100)$$

			(C/B)	(1-D)	
A	B	C	D	E	F(%)
Time Interval of Survival	Alive at Beginning	Died During Interval	Proportion of dying	Proportion of Surviving	Cumulative Proportion of surviving

Percent survival of hearts from rats fed control, GBA and GPA diets for 7 weeks while the hearts were perfused for 120 min under normoxemic conditions.

A	B	C	(C/B) D	(1-D) E	($E_1 x E_2 ... x E_i x 100$) F(%)
Control					
0-15	9	0	0	1.00	100
15-30	9	0	0	1.00	100
30-45	9	0	0	1.00	100
45-60	9	0	0	1.00	100
60-75	9	0	0	1.00	100
75-90	9	1	0.11	0.89	89
90-105	8	0	0	1.00	89
105-120	8	1	0.13	0.87	76
GBA					
0-15	9	0	0	1.00	100
15-30	9	0	0	1.00	100
30-45	9	0	0	1.00	100
45-60	9	0	0	1.00	100
60-75	9	1	0.11	0.89	89
75-90	8	3	0.38	0.62	55
90-105	5	2	0.40	0.60	36
105-120	3	1	0.33	0.67	24
GPA					
0-15	9	0	0	1.00	100
15-30	9	0	0	1.00	100
30-45	9	0	0	1.00	100
45-60	9	0	0	1.00	100
60-75	9	2	0.20	0.78	78
75-90	7	2	0.28	0.72	56
90-105	5	0	0	0	56
105-120	5	1	0.17	0.83	46

A is the time interval in minutes of hearts' survival on the isolated blood-perfused preparation.

Percent survival of hearts of rats fed control, GBA and GPA diets for 10 weeks while the hearts were perfused for 120 min under normoxemic conditions.

A	B	C	(C/B) D	(1-D) E	(E_1xE_2...xE_ix100) F(%)
Control					
0-15	6	0	0	1.00	100
15-30	6	0	0	1.00	100
30-45	6	0	0	1.00	100
45-60	6	0	0	1.00	100
60-75	6	0	0	1.00	100
75-90	6	0	0	1.00	100
90-105	6	1	0.16	0.84	84
105-120	5	1	0.20	0.80	67
GBA					
0-15	6	0	0	1.00	100
15-30	6	0	0	1.00	100
30-45	6	0	0	1.00	100
45-60	6	3	0.50	0.50	50
60-75	3	1	0.33	0.66	33
75-90	2	2	0	0	0
90-105					
105-125					
GPA					
0-15	7	0	0	1.00	100
15-30	7	1	0.14	0.86	86
30-45	6	1	0.16	0.84	72
45-60	5	2	0.40	0.60	43
60-75	5	0	0.40	0.60	43
75-90	3	3	0	0	0
90-105					
105-120					

A is the time interval in minutes of hearts' survival on the isolated blood-perfused preparation.

Percent survival of hearts from rats fed control, GBA and GPA diets for 7 weeks while the hearts were perfused under hypoxemic conditions.

A	B	C	D	E	F
Control					
365 ± 29.0	5	0	0	1.00	100
38.8 ± 2.0	5	0	0	1.00	100
32.4 ± 1.0	5	0	0	1.00	100
29.3 ± 1.0	5	0	0	1.00	100
26.1 ± 1.0	5	2	0.40	0.60	60
25.1 ± 1.0	3	2	0.66	0.34	20
24.0 ± 0	1	1	0	0	0
GBA					
366 ± 68.0	5	0	0	1.00	100
45.1 ± 5.0	5	0	0	1.00	100
42.7 ± 2.3	5	0	0	1.00	100
40.5 ± 6.1	5	0	0	1.00	100
33.6 ± 4.6	5	0	0	1.00	100
25.9 ± 1.4	5	2	0.40	0.60	60
22.1 ± 1	2	1	0.33	0.67	40
20.0 ± 0	2	2	0	0	0
GPA					
374 ± 31.4	5	0	0	1.00	100
44.8 ± 3.8	5	0	0	1.00	100
42.6 ± 3.0	5	0	0	1.00	100
37.6 ± 4.0	5	1	0.20	0.80	80
32.5 ± 5.0	4	2	0.50	0.50	40
30.1 ± 1.0	2	1	0.50	0.50	20
26.0 ± 0	1	1	0	0	0

A = pO_2 of the perfusate entering the left atrium of the rat hearts.

APPENDIX IV

The Use of the Clamp-freezing Technique for the Analysis of Adenine Nucleotide, Creatine and Creatine Phosphate Levels

The hearts from rats fed control, GBA and GPA diets for 10 weeks, were perfused with RBC-enriched perfusate in the working mode for a preselected time, at the end of which the hearts were quickly switched into the non-working mode (see methods for the definitions of working and non-working modes) and perfused with oxygenated Krebs-Henseleit to wash out the RBC and immediately frozen. The hearts were frozen at the following three different time points: at 15 min in hearts that maintained 100% of the submaximal steady-state CO in the three groups; at 45 min in hearts of the GBA and GPA groups that maintained 60% of the submaximal steady-state and in the control group that maintained the submaximal steady-state and at 90 min in GBA and GPA groups that failed and in the control group that maintained 80% of the submaximal steady-state. The levels of adenine nucleotides, CP and creatine that we obtained in the control group at 15 min were taken as a reference and all subsequent measurements were compared with these levels since submaximal steady-state CO was obtained at that time. Analysis of the tissue for ATP, ADP, AMP, adenosine, CP and creatine concentrations was done by Mr. G. Bedford at the Department of Medicine, Queens University, Kingston, Ontario.

The method of Bedford and Chiong (1984) was applied, using the HPLC (Beckman 332 Model).

Extraction of the hearts was performed by homogenization

with a Polytron sonicator/homogenizer (Brinkman, Westbury, NY, USA) in 2.0 ml of 0.9 M perchloric acid. The pH was adjusted to 5 with 3.7 M potassium carbonate. A 100 µl aliquot of the extract was then tested in the HPLC system. Peaks were analyzed by their retention times. This method allowed determination of energy stores in tissue samples as small as 10-15 mg. The lower limits of detection (in nM) were 2.27 for CP, 0.343 for ATP; 0.213 for ADP and 0.07 for AMP.

The levels of adenine nucleotides and CP that we obtained were lower than those previously reported (Fossel et al. 1980; Saks et al. 1986; Kapelko et al. 1986). The hearts used in our study weighed up to one gram and took approximately 2 s to freeze completely. Meyer et al. (1985) compared clamp-frozen tissue samples that were extracted and analyzed with a chemical analysis versus ^{31}P-NMR spectra analysis and found that the amount of hydrolysis that can occur in 1 s particularly in CP is up to 4 µmol/g of tissue (wet weight). The freezing of large tissues such as a one gram heart may take several seconds to freeze entirely, thus large decreases in CP, ATP and ADP levels may be observed. To avoid extensive hydrolysis of CP, ATP and ADP, freezing of the tissue should occur in several milliseconds. In order to achieve that, sophisticated equipment such as a high speed crusher which can "smash" the heart while still on the perfusion apparatus in 5 ms and drop the temperature at the center of the heart from 37 to -80°C during that time, has been described by Wikman-Coffelt et al. (1983) and should optimally be used.

The levels of adenine nucleotides, creatine and CP that we obtained are shown in Tables 1 and 2. The CP concentrations in Table 1 shows that at 15 min, the CP concentration had decreased significantly by 76% and by 80% in the GBA and GPA groups from the control group levles. Adenosine triphosphate, ADP, AMP and adenosine levels are shown in Table 2; they were similar in all three groups.

At 45 min, CP concentrations were significantly lower in the GBA and GPA groups than control concentrations at 15 min and at 45 min and were 93% lower in both the GBA and GPA groups than control concentrations at 15 min. ATP concentrations were also significantly lower than control levels at 15 min and at 45 min were 56% lower in GBA group, 45% lower in the GPA group and 29% lower in the control group than the control levels at 15 min. No significant differences among the three groups were observed in ADP, AMP, or adenosine at 45 min. However, the adenosine concentrations were increased significantly in all three groups from control levels at 15 minutes.

At 90 min, the CP concentrations were decreased significantly from control levels at 90 min and were decreased by 96% in the GBA group and by 94% in the GPA group from control at 15 min. ATP concentrations were decreased by 74% in the GBA group and by 69% in the GPA group, by 62% in the control group from the control levels at 15 min. Differences in ATP concentrations at 90 min among the three groups at 90 min were not significantly different. Adenosine levels were significantly elevated by 285% in the GBA group, by 231% in the GPA group and

by 184% in the control group from the control levels at 15 min. The adenosine levels in the control group at 90 min were significantly lower than those in the GBA and GPA groups.

Since the levels of adenine nucleotides and CP that we obtained during the 15 min of steady-state performance were lower than what Fossel et al. (1980) and Wikman-Coffelt et al. (1983) obtained, conclusions relating to hemodynamic performance based on these adenine nucleotide levels cannot be made as a result of the low concentrations of ATP and CP obtained during the steady-state performance. We did, however, see that the CP levels in all three groups decreased during the 90 min of perfusion and that the percent decrease in CP concentration was greater than the percent decrease in ATP concentration at 90 min when compared to 15 min concentrations in all three groups.

Table 1: Myocardial concentrations of total creatine (C + CP), creatine, and creatine phosphate in the isolated blood perfused rat heart at 15, 45 and 90 minutes, in control, GBA and GPA fed rats for 10 weeks.

	TOTAL CREATINE	CREATINE	CREATINE PHOSPHATE
15 min			
C	66.6 ± 1.5	51.0 ± 0.7	15.6 ± 2.1
GBA	9.9 ± 1.0*	6.2 ± 1.1*	3.7 ± 0.1*
GBA	10.4 ± 0.6*	7.3 ± 1.8*	3.1 ± 0.1*
45 min			
C	58.7 ± 0.8	54.3 ± 0.6	4.4 ± 0.1+
GBA	8.4 ± 0.7*+	7.1 ± 1.2*+	1.3 ± 0.1*+
GPA	8.5 ± 1.1*+	7.4 ± 1.2*+	1.1 ± 0.3*+
90 min			
C	59.8 ± 1.6	56.0 ± 2.6	2.9 ± 0.5+
GBA	7.6 ± 1.2*+	7.0 ± 1.6*+	0.6 ± 0.4*+
GPA	8.6 ± 1.9*+	7.7 ± 2.3*+	0.9 ± 0.4*+
n	3	3	3

- all values are means and standard errors of the mean
- units are μmoles g/dry weight, dry wt/wet wt = 0.20
* $p<0.001$, statistically different from control
+ $p<0.001$, significant difference between control at 15 and experimental at 45 or at 90

Table 2: Myocardial concentrations of ATP, ADP, AMP and adenosine in the isolated blood perfused rat heart 15, 45 and 90 minutes in control, GBA and GPA fed rats for 10 weeks.

	ATP	ADP	AMP	ADENOSINE
15 min				
C	9.1 ± 0.7	4.0 ± 0.5	3.6 ± 0.6	3.2 ± 1.0
GBA	7.9 ± 0.8	5.8 ± 2.1	3.7 ± 0.4	3.7 ± 0.4
GPA	8.6 ± 1.3	3.9 ± 0.7	3.3 ± 0.1	3.3 ± 1.0
45 min				
C	6.4 ± 0.2	3.2 ± 0.4	3.4 ± 0.9	12.6 ± 1.5++
GBA	4.0 ± 0.5*+	6.5 ± 1.8	3.9 ± 0.5	9.5 ± 2.0+
GPA	5.0 ± 0.4*+	4.6 ± 1.3	3.7 ± 1.2	13.8 ± 1.8+
90 min				
C	3.5 ± 0.4++	5.0 ± 0.5	5.1 ± 0.7++	5.9 ± 0.3++
GBA	2.3 ± 0.4+	5.6 ± 0.6	7.2 ± 0.5+	9.1 ± 0.8*+
GPA	2.8 ± 2.3+	5.4 ± 0.7	5.9 ± 0.7+	7.4 ± 0.2*+
n	3	3	3	3

- all values are means and standard errors of the mean
- units are μmoles g/dry weight, dry wt/wet wt - 0.20
* $p<0.05$, statistically different from control
\+ $p<0.01$, significantly different from control at 15, experimental at 45, 90
++ $p<0.01$, significant difference between control at 15 and control at 45 or 90

APPENDIX V

The Use of Reconstituted Blood in Isolated Heart Perfusion Preparations

The red cell concentrate used in the isolated heart experiments was obtained from the Red Cross, Ottawa Chapter in transfusion bags that contained the anticoagulant citrate phosphate dextrose adenine solution (CPDA-1). The volume of the concentrate was 450 ml and that of the anticoagulant was 63 ml. Negligible hemolysis of the red cells during the experiment was observed, if used within 40 days after collection.

However, CPDA-1 solution along with ongoing cellular metabolism has an effect on the affinity of hemoglobin for oxygen. After 40 days of storage, we found that with the increased acidity of the blood and the fall in the 2,3-DPG concentration, the p50 (pO_2 at 50% oxygen saturation) was 9-15 mmHg rather than the normal p50 of 27 mmHg. The function of the hearts on the isolated preparation was not affected by the leftward shift during normoxemia, however, during hypoxemia, high saturation of oxygen was observed (80-90%) at pO_2 of 20-30 mmHg.

We tried to increase the p50 of the blood cell suspension to obtain p50 values comparable to those found in rat blood (35.5 mmHg, Baumann et al. 1971) by incubating the red blood cells in a medium containing inosine, pyruvate and phosphate which would increase the levels of 2,3-diphosphoglycerate (2,3-DPG) within the cells. The accumulation of 2,3-DPG proceeds by the following reaction: inosine is converted to hypoxanthine and ribose-1-phosphate (R-1-P) by nucleoside phosphorylase. Ribose-5-

phosphate (R-5-P) arising from the R-1-P is metabolized to fructose-6-phosphate and glyceraldehyde-3-phosphate (GA-3-P) via the pentose phosphate cycle. This sequence of reactions bypasses the two enzymes that limit substrate phosphorylation in the normal glycolytic chain (hexokinase and phosphofructokinase). Consequently, large amounts of sugar phosphates (eg. fructose-1-phosphate) as well as triose phosphates can be formed (Deuticke et al. 1971). These authors reported an increase in 2,3-DPG of more than five times normal values when incubation is done for 3 hr at 37°C. The blood that we used was always at least 14 days old and we observed that it hemolyzed after almost every attempt.

Alternatively, we tried to increase the p50 independently of 2,3-DPG using sodium benzoate derivatives (Lever et al. 1977). Lever et al. (1977) used it successfully in adult human blood, in sheep and cow blood, increasing the p50 from 24 to 32 mmHg in the human blood. In the sheep blood, the increase was from 37.8 to 52.7 mmHg, and in both cases the concentration of 2-iodosodium benzoate (IOSB) used was 32 mM.

Neither the mechanism by which benzoates decrease the affinity of hemoglobin for oxygen, (thus increasing p50), nor the manner in which benzoates interact with hemoglobin have been determined (Lever et al. 1977). However, these authors found that the technique did not produce hemolysis and that equilibrium of IOSB between the extracellular and intracellular compartments was rapid and reversible, when the RBC's were washed with saline. They concluded that the IOSB passes into the RBC rapidly, with no apparent protein denaturation or change in the properties of the

erythrocyte membrane.

We therefore tried this method to increase the p50 since our p50 was usually 9-15 mmHg. When we used low doses such as 13 mM IOSB, the p50 increased to 18-19 mmHg, when determined by an oxyhemoglobin curve as described by Rakusan and Marcinek (1975). Since this was a negligible increase in p50 a dose of 20 mM IOSB was used, which increased the p50 to 25-27 mmHg. However, this latter concentration had severe effects on the heart rate, decreasing it from 300-360 to 90-120 beats/min. Arrhythmias were also observed frequently along with periods of arrest even at the dose of 13 mM ISOB.

Since none of these above mentioned methods were able to increase the p50 to satisfactory levels and resulted in hemolysis of the RBC or induced arrhythmias in the hearts, the only alternative was to use freshly packed RBC's collected in heparinized bags. The p50 was 23-25 mmHg for the first 5 days after collection, and only after 5-7 days was there a decrease in p50. Clotting also became a problem and to prevent it, 2200 units of sodium heparin were introduced into the bag every 2 days.

APPENDIX VI

Table 1: Rate of force development (dF/dt) at increasing stimulation frequency in the papillary muscles of rats fed control, GBA and GPA diets for 7 and 10 weeks.

Frequency of Stimulation (per min)	3	6	12	24	48	96	192
7 WK				dF/dt (g/s)			
Control n=9	41.1±3.5	37.0±3.9	36.2±3.0	28.0±3.2	26.±12.4	19.3±1.3	16.4±2.5
GBA-fed n=10	42.1±8.7	41.5±8.6	36.0±7.0	31.1±7.2	22.0±6.0	18.7±1.4	16.2±5.5
GPA-fed n=9	44.2±5.7	33.2±6.0	35.0±4.1	80.6±4.3	26.4±4.0	20.1±2.6	17.1±2.5
10 WK							
Control n=10	41.1±4.3	39.4±2.9	31.6±1.7	30.0±3.9	30.1±3.2	25.0±3.8	20.1±2.2
GBA-fed n=10	39.1±3.9	31.1±3.6	26.7±2.7	25.5±5.2	20.4±3.6	19.2±5.1	16.4±1.7
GPA-fed n=8	48.1±5.2	38.4±4.4	30.1±4.8	29.4±3.9	24.4±1.9	22.4±4.0	18.4±1.9

- all values are means and standard errors of the mean
- n is the number of muscles
- five animals were used in each group
- bath temperature was 25°C

Table 2: Mean rate of force development at increasing stimulation frequency in the papillary muscles of rats fed control, GBA and GPA diets for 7 and 10 weeks.

Frequency of Stimulation (per min	3	6	12	24	48	96	192
			$\Delta F/\Delta t$ (g/s)				
7 WK							
Control n=9	24.9±3.1	20.0±1.9	19.1±2.2	15.0±5.1	13.3±2.1	11.0±4.1	8.2±4.8
GBA-fed n=10	26.1±2.4	24.4±3.6	20.0±4.1	17.0±3.2	11.0±1.1	12.5±3.2	14.5±0.9
GPA-fed n=9	25.2±3.9	22.3±2.2	20.1±1.8	18.0±3.0	16.1±2.1	12.0±2.1	13.0±1.0
10 WK							
Control n=10	20.4±3.2	19.4±1.6	18.4±1.7	18.0±3.3	17.3±4.3	12.1±3.1	8.5±3.3
GBA-fed n=10	20.0±4.1	19.3±2.2	17.3±2.4	17.3±3.2	12.2±2.4	10.0±2.9	1.2±2.7
GPA-fed n=8	24.2±2.8	23.4±3.4	22.3±4.1	17.4±1.9	16.1±3.2	17.1±2.6	9.0±2.8

- all values are means and standard errors of the mean
- n is the number of muscles
- five animals were used in each group
- bath temperature was 25°C

Table 3: Time to maximum developed force (TMF) at increasing stimulation frequencies in the papillary muscles of rats fed control, GBA and GPA diets for 7 and 10 weeks.

Frequency of Stimulation (per min)	3	6	12	24	48	96	192
				TMF (ms)			
7 WK							
Control n=9	172±7.0	176±4.0	163±11	166±7	165±10	140±11	133±10
GBA-fed n=10	177±12.0	179±11.0	171±8.0	174±14	175±10	143±9	131±9
GPA-fed n=9	184±8.0	181±11.0	170±9	154±6	142±6	145±9	123±11
10 WK							
Control n=10	219±18	211±15	203±14	186±11	172±14	154±10	129±13
GBA-fed n=10	187±16	187±16	176±16	174±17	167±14	145±10	128±14
GPA-fed n=8	189±7	181±10	183±9	170±8	163±8	154±8	138±11

- all values are means and standard errors of the mean
- n is the number of muscles
- five animals were used in each group
- bath temperature was 25°C

Table 4: Time to half-relaxation (T ½ R) at increasing stimulation frequencies in the papillary muscles of rats fed control, GBA and GPA diets for 7 and 10 weeks.

Frequency of Stimulation (per min)	3	6	12	24	48	96	192
			T ½ R (ms)				
7 WK							
Control n=9	178±18	140±12	121±12	118±11	108±12	90±7	86±8
GBA-fed n=10	229±30	186±32	183±25	167±27	143±20	125±15	101±14
GPA-fed n=9	183±13	145±7	127±8	119±7.0	101±5	93±8	88±29
10 WK							
Control n=10	185±23	192±24	190±19	156±22	134±21	117±17	93±18
GBA-fed n=10	209±26	187±27	168±23	139±23	121±15	110±14	78±9
GPA-fed n=8	167±22	165±19	151±18	146±15	143±14	106±10	72±9

- all values are means and standard errors of the mean
- n is the number of msucles
- five animals were used in each group
- bath temperature was 25°C

Table 5: Maximum developed force (DF) at increasing stimulation frequencies in the papillary muscles of rats fed control, GBA and GPA diets for 7 and 10 weeks.

Frequency of Stimulation (per min)	3	6	12	24	48	96	192
				DF (g)			
7 WK							
Control n=9	4.0±0.3	3.5±0.7	3.1±0.6	2.4±0.4	2.2±0.1	1.5±0.1	1.1±0.2
GBA-fed n=10	4.6±0.9	4.3±0.9	3.5±0.8	3.2±0.8	2.0±0.4	1.5±0.3	1.9±0.2
GPA-fed n=9	4.6±0.6	4.0±0.5	3.4±0.4	2.8±0.3	2.3±0.3	1.7±0.1	1.6±0.3
10 WK							
Control n=10	4.4±0.6	4.0±4.0	3.3±0.4	3.3±0.6	3.0±0.7	2.4±0.6	1.3±0.4
GBA-fed n=10	3.8±0.4	3.5±0.3	2.9±0.3	3.0±0.4	2.0±0.5	1.5±0.5	0.9±0.4
GPA-fed n=8	4.5±0.9	4.1±0.7	2.8±0.6	2.2±0.3	2.8±0.4	2.3±0.6	1.2±0.6

- all values are means and standard errors of the mean
- n is the number of muscles
- five animals were used in each group
- bath temperature was 25°C

Table 6: Cardiac output of hearts of control, GBA and GPA rats fed for 7 and 10 weeks that were perfused for 120 min on the isolated blood-perfused working heart preparation.

Time (min)	0	15	30	45	60	75	90	105	120
				Cardiac output (ml/min/g)					
7 WK									
Control	52.3±6.1	63.2±6.2	62.4±4.7	59.8±4.4	54.2±7.5	56.3±7.1	49.0±3.3	42.9±3.9	33.0±6.5
	(9)	(9)	(9)	(9)	(9)	(9)	(8)	(8)	(7)
GBA-fed	55.6±8.3	59.2±8.8	59.9±7.4	54.4±7.7	37.8±5.9	23.8±5.7	23.8±14.8	22.9±5.1	26.1±0
	(9)	(9)	(9)	(9)	(9)	(7)	(5)	(3)	(2)
GPA-fed	52.8±5.8	59.0±6.3	58.1±9.2	45.8±13.0	47.8±6.6	38.8±2.9	25.3±4.4	21.6±4.4	20.5±5.3
	(9)	(9)	(9)	(9)	(9)	(7)	(5)	(5)	(4)
10 WK									
Control	62.1±7.3	67.4±8.6	61.5±9.2	52.9±12.8	53.6±6.5	48.5±8.1	31.5±8.1	31.9±8.0	30.9±8.3
	(6)	(6)	(6)	(6)	(6)	(6)	(6)	(5)	(4)
GBA-fed	62.5±5.6	63.5±9.6	54.5±11.2	42.7±14.8	25.9±9.8	18.5±0	0	0	0
	(6)	(6)	(6)	(6)	(3)	(2)	(0)		
GPA-fed	50.2±7.2	51.7±6.5	41.8±5.5	35.2±5.8	28.3±8.2	24.1±8.2	0	0	0
	(7)	(7)	(6)	(5)	(3)	(4)	(0)		

- all values are means and standard errors of the mean
- numbers in parentheses represent the number of hearts that were maintaining cardiac output of more than 10% of the maximum cardiac output.

Table 7: Myocardial oxygen consumption (MVO_2) of hearts of control, GBA and GPA fed rats for 7 and 10 weeks that were perfused for 120 min on an isolated blood-perfused working heart preparation.

Time(min)	0	15	30	45	60	75	90	105	120
				MVO_2 (ml/min/g)					
7 WK									
Control	0.22±0.01	0.25±0.03	0.23±0.02	0.23±0.03	0.22±0.02	0.20±0.02	0.13±0.02	0.09±0.02	0.08±0.06
	(9)	(9)	(9)	(9)	(9)	(9)	(8)	(8)	(7)
GBA-fed	0.26±0.02	0.23±0.02	0.27±0.04	0.26±0.03	0.16±0.02	0.15±0.04	0.11±0.01	0.10±0.06	0.09±0
	(9)	(9)	(9)	(9)	(9)	(7)	(5)	(3)	(2)
GPA-fed	0.22±0.03	0.24±0.01	0.21±0.03	0.19±0.03	0.23±0.03	0.23±0.03	0.20±0.09	0.17±0.02	0.16±0.04
	(9)	(9)	(9)	(9)	(9)	(7)	(5)	(5)	(4)
10 WK									
Control	0.27±0.02	0.29±0.04	0.30±0.02	0.29±0.05	0.25±0.04	0.23±0.04	0.24±0.01	0.19±0.01	0.20±0.01
	(6)	(6)	(6)	(6)	(6)	(6)	(6)	(5)	(4)
GBA-fed	0.27±0.06	0.27±0.02	0.21±0.03	0.19±0.02	0.12±0.05	0.11±0	0	0	0
	(6)	(6)	(6)	(6)	(3)	(2)	(0)		
GPA-fed	0.21±0.05	0.27±0.03	0.20±0.02	0.17±0.03	0.14±0.03	0.10±0.02	0	0	0
	(7)	(7)	(6)	(5)	(3)	(4)	(0)		

- all values are means and standard errors of the mean
- numbers in parentheses represent the number of hearts that were maintaining cardiac output of more than 10% of the maximum cardiac output.

Table 8: Stroke volume of hearts of control, GBA and GPA rats fed for 7 and 10 weeks that were perfused for 120 min on the isolated blood-perfused working heart preparation.

Time (min)	0	15	30	45	60	75	90	105	120
				Stroke Volume (ml/g)					
7 WK									
Control	0.22±0.02	0.25±0.01	0.25±0.01	0.22±0.02	0.19±0.02	0.19±0.03	0.17±0.04	0.16±0.01	0.12±0.01
	(9)	(9)	(9)	(9)	(9)	(9)	(8)	(8)	(7)
GBA	0.21±0.02	0.22±0.02	0.20±0.03	0.19±0.04	0.12±0.04	0.10±0.04	0.10±0.01	0.09±0.01	0.09±0
	(9)	(9)	(9)	(9)	(9)	(7)	(5)	(3)	(2)
GPA	0.20±0.01	0.20±0.01	0.17±0.02	0.17±0.03	0.17±0.04	0.14±0.03	.08±0.01	0.07±0.01	0.06±0.04
	(9)	(9)	(9)	(9)	(9)	(7)	(5)	(5)	(4)
10 WK									
Control	0.23±0.03	0.23±0.03	0.21±0.01	0.19±0.02	0.18±0.02	0.17±0.01	0.13±0.01	0.13±0.09	0.10±0.04
	(6)	(6)	(6)	(6)	(6)	(6)	(6)	(5)	(4)
GBA	0.20±0.02	0.20±0.04	0.17±0.03	0.12±0.03	0.08±0.09	0.08±0	0	0	0
	(6)	(6)	(6)	(6)	(3)	(2)	(0)		
GPA	0.16±0.04	0.17±0.03	0.15±0.03	0.12±0.02	0.08±0.01	0.09±0.01	0	0	0
	(7)	(7)	(6)	(5)	(3)	(4)	(0)		

- all values are means and standard errors of the mean
- numbers in parentheses represent the number of hearts that were maintaining cardiac output of more than 10T of the maximum cardiac output.

Table 9: Power input of hearts of control, GBA and GPA rats fed for 7 and 10 weeks and were perfused for 120 min on the isolated blood-perfused working heart preparations.

Time (min)	0	15	30	45	60	75	90	105	120
				Power Input (mW/g)					
7 WK									
Control	96.9±6.2	89.5±7.8	91.5±6.8	82.5±11.8	76.4±6.8	75.1±11.2	61.1±10.0	55.0±6.0	50.1±10.6
	(9)	(9)	(9)	(9)	(9)	(9)	(8)	(8)	(7)
GBA-fed	118.3±11.9	110.2±10.7	108.4±9.9	98.1±40.8	87.6±14.0	71.1±10.6	70.2±10.2	60.0±2.6	40.3±0
	(9)	(9)	(9)	(9)	(9)	(7)	(5)	(3)	(2)
GPA-fed	89.2±12.0	89.0±8.4	85.2±10.7	81.7±5.6	80.2±10.2	73.6±15.8	68.5±9.2	64.5±9.6	51.4±7.8
	(9)	(9)	(9)	(9)	(9)	(7)	(5)	(5)	(4)
10 WK									
Control	114 ±17.1	123.1±15.4	114 ±14.3	115 ±12.1	94.0±19.2	91.0±15.9	90.5±28.5	82.3±19.1	86.5±23.5
	(6)	(6)	(6)	(6)	(6)	(6)	(6)	(5)	(4)
GBA-fed	104 ±19.9	105.6±19.1	97.5±15.5	92.8±12.1	61.8±7.1	51.0±0	0	0	0
	(6)	(6)	(6)	(6)	(3)	(2)	(0)		
GPA-fed	96.6±18.4	80.2±91	85.0±10.1	81.2±17.2	59 ±10.6	40.5±15.6	0	0	0
	(7)	(7)	(6)	(5)	(3)	(4)	(0)		

- all values are means and standard errors of the mean
- numbers in parentheses represent the number of hearts that were maintaining cardiac output of more than 10% of the maximum cardiac output.

Table 10: Power output of hearts of control, GBA and GPA rats fed for 7 and 10 weeks that were perfused for 120 min on the isolated blood-perfused working heart preparation.

Time (min)	0	15	30	45	60	75	90	105	120
				Power Output (mW/g)					
7 WK									
Control	9.3±1.3	11.3±1.3	12.5±1.2	12.0±1.4	9.7±1.7	9.1±1.3	7.0±0.96	6.0±0.96	6.2±1.1
	(9)	(9)	(9)	(9)	(9)	(9)	(8)	(8)	(7)
GBA-fed	10.7±1.4	10.4±1.4	10.4±1.3	8.6±1.1	8.1±1.0	5.5±0.9	4.4±1.0	3.5±1.2	2.7±0
	(9)	(9)	(9)	(9)	(9)	(7)	(5)	(3)	(2)
GPA-fed	8.9±1.2	8.3±1.3	8.6±1.7	7.1±2.5	8.1±2.5	6.4±1.5	4.5±0.97	4.0±0.93	4.1±1.3
	(9)	(9)	(9)	(9)	(9)	(7)	(5)	(5)	(4)
10 WK									
Control	9.9±1.2	10.8±1.6	9.8±1.9	10.0±1.9	8.2±1.8	7.2±9.7	7.1±1.1	6.8±1.0	6.0±1.3
	(6)	(6)	(6)	(6)	(6)	(6)	(6)	(5)	(4)
GBA-fed	10.9±1.6	10.1±1.5	8.6±1.7	7.3±1.95	6.5±2.3	3.3±0	0	0	0
	(6)	(6)	(6)	(6)	(3)	(2)	(0)		
GPA-fed	8.1±0.97	7.9±1.0	6.1±0.83	5.8±0.94	5.9±1.6	3.3±1.06	0	0	0
	(7)	(7)	(6)	(5)	(3)	(4)	(0)		

- all values are means and standard errors of the mean
- numbers in parentheses represent the number of hearts that were maintaining cardiac output of more than 10% of the maximum cardiac output.

Table 11: Rate of force development (+dP/dt) of hearts of control, GBA and GPA rats fed for 7 and 10 weeks that were perfused for 120 min on the isolated blood-perfused working heart preparation.

Time (min)	0	15	30	45	60	75	90	105	120
					+dP/dt (mmHg/s)				
7 WK									
Control	3406±388	3287±282	2875±476	3000±215	3075±233	2760±259	2640±264	2271±280	2100±197
	(9)	(9)	(9)	(9)	(9)	(9)	(8)	(8)	(7)
GBA-fed	2980±245	2963±283	2960±277	2750±298	2438±304	2075±329	2038±227	2133±228	1350±0
	(9)	(9)	(9)	(9)	(9)	(7)	(5)	(3)	(2)
GPA-fed	3033±242	3036±288	2933±300	2783±324	2600±267	2460±251	1920±305	1867±226	1700±122
	(9)	(9)	(9)	(9)	(9)	(7)	(5)	(5)	(4)
10 WK									
Control	3033±242	3558±429	3843±56.0	3392±51.0	3033±392	2960±490	3000±82	3100±175	2900±20
	(6)	(6)	(6)	(6)	(6)	(6)	(6)	(5)	(4)
GBA-fed	2958±461	3618±143	2840±301	2660±257.6	2600±218	2550±0	0	0	0
	(6)	(6)	(6)	(6)	(3)	(2)	(0)		
GPA-fed	2860±404	3296±54.0	2971±170	2943±64.0	3188±59	2833±373	0	0	0
	(7)	(7)	(6)	(5)	(3)	(4)	(0)		

- all values are means and standard errors of the mean
- numbers in parentheses represent the number of hearts that were maintaining cardiac output of more than 10% of the maximum cardiac output.

Table 12: Rate of relaxation (-dP/dt) of hearts of control, GBA and GPA rats fed for 7 and 10 weeks that were perfused for 120 min on the isolated blood-perfused working heart preparation.

Time (min)	0	15	30	45	60	75	90	105	120
				-dP/dt (mmHg/s)					
7 WK									
Control	3406±381	3287±281	2875±467	3000±215	3075±333	2760±259	2640±294	2271±286	2100±197
	(9)	(9)	(9)	(9)	(9)	(9)	(8)	(8)	(7)
GBA-fed	2988±227	2836±289	2662±288	2550±286	1975±114	2300±374	2000±374	1800±251	1600±0
	(9)	(9)	(9)	(9)	(9)	(7)	(5)	(3)	(2)
GPA-fed	3050±167	2941±220	2350±256	2417±195	2150±160	1880±255	1880±351	1650±310	1415±167
	(9)	(9)	(9)	(9)	(9)	(7)	(5)	(5)	(4)
10 WK									
Control	3133±311	3267±334	3142±369	3000±377	2560±449	2350±105	2300±261	3100±100	2567±249
	(6)	(6)	(6)	(6)	(6)	(6)	(6)	(5)	(4)
GBA-fed	3550±231	3319±211	2569±371	2356±280	2210±210	1980±0	0	0	0
	(6)	(6)	(6)	(6)	(3)	(2)	(0)		
GPA-fed	2936±340	2990±412	2851±267	2490±412	2267±218	1815±275	0	0	0
	(7)	(7)	(6)	(5)	(3)	(4)	(0)		

- all values are means and standard errors of the mean
- numbers in parentheses represent the number of hearts that were maintaining cardiac output of more than 10% of the maximum cardiac output.

Table 13: Efficiency of hearts of control, GBA and GPA rats fed for 7 and 10 weeks that were perfused for 120 min on the isolated blood-perfused working heart preparation.

Time (min)	0	15	30	45	60	75	90	105	120
					Efficiency (%)				
7 WK									
Control	9.7±1.7	12.7±1.0	13.7±1.0	14.5±1.3	12.6±1.7	8.5±1.4	11.5±1.7	11.0±1.8	12.3±1.3
	(9)	(9)	(9)	(9)	(9)	(9)	(8)	(8)	(7)
GBA-fed	9.0±2.6	9.5±2.5	9.6±1.2	10.4±1.1	9.2±1.1	7.7±2.1	6.2±1.4	5.8±0.8	6.8±0
	(9)	(9)	(9)	(9)	(9)	(7)	(5)	(3)	(2)
GPA-fed	10.0±1.9	9.3±1.9	10.1±1.5	8.6±1.7	10.1±1.3	8.8±1.4	6.1±1.3	6.2±1.3	11.7±1.9
	(9)	(9)	(9)	(9)	(9)	(7)	(5)	(5)	(4)
10 WK									
Control	8.7±1.6	8.9±1.1	8.6±1.2	8.7±2.2	7.9±2.7	7.8±2.7	8.3±2.7	6.9±2.7	6.2±1.2
	(6)	(6)	(6)	(6)	(6)	(6)	(6)	(5)	(4)
GBA-fed	10.5±2.2	9.6±1.6	8.8±1.5	10.5±1.3	6.5±1.2	7.8±0	0	0	0
	(6)	(6)	(6)	(6)	(3)	(2)	(0)		
GPA-fed	8.7±2.6	9.8±1.8	8.5±3.1	9.4±3.1	10.0±1.1	8.1±1.2	0	0	0
	(7)	(7)	(6)	(5)	(3)	(4)	(0)		

- all values are means and standard errors of the mean
- numbers in parentheses represent the number of hearts that were maintaining cardiac output of more than 10% of the maximum cardiac output.

Table 14: Heart rate of hearts of control, GBA and GPA rats fed for 7 and 10 weeks that were perfused for 120 min on the isolated blood-perfused working heart preparation.

Time (min)	0	15	30	45	60	75	90	105	120
					Heart Rate (bpm)				
7 WK									
Control	243±14	258±8.5	250±19	278±22	280±16	296±10	282±8	267±9	208±9
	(9)	(9)	(9)	(9)	(9)	(9)	(8)	(8)	(7)
GBA-fed	260±22	274±17	294±17	293±18	298±20	281±19	315±16	297±18	260±0
	(9)	(9)	(9)	(9)	(9)	(7)	(5)	(3)	(2)
GPA-fed	252±21	253±13	296±18	275±17	284±15	286±14	303±10	304±12	230±12
	(9)	(9)	(9)	(9)	(9)	(7)	(5)	(5)	(4)
10 WK									
Control	268±26	294±17	291±15	280±9	301±13	290±19	283±6	280±7	275±7
	(6)	(6)	(6)	(6)	(6)	(6)	(6)	(5)	(4)
GBA-fed	304±19	315±16	320±12	330±7	324±10	300±0	0	0	0
	(6)	(6)	(6)	(6)	(3)	(2)	(0)		
GPA-fed	329±19	284±12	282±19	284±20	300±11	308±20	0	0	0
	(7)	(7)	(6)	(5)	(3)	(4)	(0)		

- all values are means and standard errors of the mean
- numbers in parentheses represent the number of hearts that were maintaining cardiac output of more than 10% of the maximum cardiac output.

Table 15: The effects of decreasing partial pressure of oxygen on heart rate in hearts of rat fed control, GBA and GPA diets for 7 weeks. Hearts were perfused on the isolated blood-perfused working rat heart preparation for 60 min.

Control		GBA		GPA	
PO_2 (mmHg)	Heart Rate (bpm)	PO_2 (mmHg)	Heart Rate (bpm)	PO_2 (mmHg)	Heart Rate (bpm)
356±29	324±31(5)	366±48	308±13.5(5)	374±32	312±22(5)
38.8±2.1	318±12(5)	45.1±5.0	348±22 (5)	44.8±4.0	292±14(5)
32.5±1.3	324±16(5)	40.7±2.2	336±31 (5)	42.6±3.0	316±22(5)
29.3±1.7	328±14(5)	33.5±6.0	324±15 (5)	37.6±3.5	300±21(5)
26.1±2.4	292±20(3)	26.6±4.2	330±19 (3)	32.5±4.5	330±30(4)
25.1±1.2	300±0 (1)	25.9±1.0	248±0 (2)	30.1±1.5	300±0 (1)
24.0±0	0	22.0±0	0	26.0±0	0

- all values are means and standard errors of the mean
- the numbers in parentheses represent the number of surviving hearts at that partial pressure of oxygen

Table 16: The effects of decreasing partial pressure of oxygen on power input (W_i) in hearts of rats fed control, GBA and GPA diets for 7 weeks. Hearts were perfused on the isolated blood-perfused working rat heart preparation for 60 min.

Control		GBA		GPA	
PO_2 (mmHg)	W_i (mW/g)	PO_2 (mmHg)	(W_i) ((mW/g)	PO_2 (mmHg)	(W_i) (mW/g)
356±29	93.6±8.4 (5)	366±48	113.7±6.0 (5)	374±32	98.6±20.3(5)
38.8±2.1	116.6±25.3(5)	45.1±5.0	93.3±17.0(5)	44.8±4.0	138±23.8(5)
32.5±1.3	126.6±25.3(5)	32.7±2.2	98.5±10.5(5)	42.6±3.0	94.5±26 (5)
29.3±1.7	137.5±23.4(5)	33.5±6.0	83.5±16.2(5)	37.6±3.5	92.7±13.3(5)
26.1±2.4	66.2±12.4(3)	26.6±4.2	69.5±4.9 (3)	32.5±4.5	54.2±33 (4)
25.1±1.2	57.5±0 (1)	25.9±1.0	58.8±0 (2)	30.1±1.5	48.9±0 (1)
24.0±0	0	22.0±0	0	26.0±0	0

- all values are means and standard errors of the mean
- the numbers in parentheses represent the number of surviving hearts at that partial pressure of oxygen

Table 17: The effects of decreasing partial pressure of oxygen on power output in hearts of rats fed control, GBA and GPA diets for 7 weeks. Hearts were perfused on the isolated blood-perfused working rat heart preparation for 60 min.

Control		GBA		GPA	
PO_2 (mmHg)	Power Output (mw/g)	PO_2 (mmHg)	Power Output (mw/g)	PO_2 (mmHg)	Power output (mw/g)
356±29	11.5±1.8 (5)	366±48	14.3±2.3(5)	374±32	8.8±2.1(5)
38.8±2.1	10.6±1.4 (5)	45.1±5.0	13.4±1.8(5)	44.8±4.0	10.4±1.6(5)
32.5±1.3	10.8±1.4 (5)	40.7±2.2	10.3±1.7(5)	42.6±3.0	10.9±1.8(5)
29.3±1.7	9.3±2.6 (3)	33.5±6.0	7.7±1.7(5)	37.6±3.5	7.5±1.5(5)
26.1±2.4	3.7±0.97(3)	26.6±4.2	5.8±1.2(3)	32.5±4.5	4.3±0.4(4)
25.1±1.2	2.3±0 (1)	25.9±1.0	2.4±0 (2)	30.1±1.6	2.3±0 (1)
24.0±0	0	22.0±0	0	26.0±0	0

- all values are means and standard errors of the mean
- the numbers in parentheses represent the number of surviving hearts at that partial pressure of oxygen

Table 18: The effects of decreasing partial pressure of oxygen on efficiency in hearts of rats fed control, GBA and GPA diets for 7 weeks. Hearts were perfused on the isolated blood-perfused working rat heart preparation for 60 min.

Control		GBA		GPA	
PO_2 (mmHg)	Efficiency (%)	PO_2 (mmHg)	Efficiency (%)	PO_2 (mmHg)	Efficiency (%)
356±29	13.3±28 (5)	366±48	17.7±2.5(5)	374±32	16.5±3.1(5)
38.8±2.1	10.8±3.2(5)	45.1±5.0	15.4±3.1(5)	44.8±4.0	8.3±2.7(5)
32.5±1.3	7.6±1.8(5)	40.7±2.2	10.5±2.6(5)	42.6±3.0	8.2±1.9(5)
29.3±1.7	8.4±2.2(5)	33.5±6.0	9.6±0.9(5)	37.6±3.5	6.3±2.1(5)
26.1±2.4	7.1±2.2(3)	26.6±4.2	9.9±0.2(3)	32.5±4.5	7.1±2.8(4)
25.1±1.2	4.6±0 (1)	25.9±1.0	3.4±0 (2)	30.1±1.5	6.0±0 (1)
24.0±0	0	22.0±0	0	26.0±0	0

- all values are means and standard errors of the mean
- the numbers in parentheses represent the number of surviving hearts at that partial pressure of oxygen

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