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METABOLIC STUDIES OF CREATINE ANALOGUES; ATP-SPARING DURING ISCHEMIA IN HEART AND SKELETAL MUSCLE OF CHICKS FED CYCLOCREATINE

BY

DAVID M. TURNER

A THESIS SUBMITTED IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE

DOCTOR OF PHILOSOPHY

APPROVED, THESIS COMMITTEE:

[Signatures and names]

HOUSTON, TEXAS

May 1986
ABSTRACT

METABOLIC STUDIES OF CREATINE ANALOGUES; ATP-SPARING DURING ISCHEMIA IN HEART AND SKELETAL MUSCLE OF CHICKS FED CYCLOCREATINE

BY

DAVID M. TURNER

Several embryonic tissues were utilized in the study of creatine analogues. Creatine analogues were taken up and phosphorylated by incubated chick embryo muscle, skin, and heart, in addition to the previously studied chick embryo brain. Accumulated phosphagen was utilized during insult by ischemia or by the addition of various inhibitory compounds to media containing chick embryonic tissues. Resynthesis of the phosphagen, upon cessation of insult and return of the tissues to the incubation media, usually occurred.

The enzyme arginine:glycine amidotransferase was partially purified for the first time from liver. Through the use of several chromatographic techniques a 160-fold purification of the enzyme was achieved from chick liver.

Hearts of chicks fed 1-carboxymethyl-2-iminoimidazol- idine (cyclocreatine) accumulated 15 µmol/g wet weight of cyclocreatine-P and had glycogen levels elevated three-fold relative to controls. During total ischemia in vitro, cyclocreatine-P was utilized by heart for resynthesis of ATP, glycolysis was greatly prolonged and depletion of both ATP and total adenylate pools was delayed two to five-fold relative to controls. Feeding of a second analogue,
1-carboxyethyl-2-iminoimidazolidine (homocyclocreatine) resulted in the accumulation of homocyclocreatine-P in the heart and a tripling of the glycogen levels. During ischemia, homocyclocreatine-P was only slightly utilized, glycolysis was prolonged, and most importantly, no delay in depletion of ATP levels was observed relative to controls. Feeding of creatine resulted in a doubling of creatine levels in the heart but no delay of ATP depletion during ischemia relative to controls. Maximal conservation of ATP and total adenylates in the heart during ischemia required, in addition to adequate glycogen reserves, a phosphagen which is both kinetically and thermodynamically competent to buffer ATP/ADP during the later stages of ischemia; that is, cyclocreatine-P.

Breast muscle of chicks fed cyclocreatine demonstrated a decreased rate of depletion of glycogen upon exposure to the β-adrenergic-agonist, isoproterenol, relative to controls. In addition, during ischemia following exposure to isoproterenol, breast muscle from cyclocreatine-fed chicks was protected against depletion of ATP, with ATP levels remaining 6-fold elevated after 2 hours of ischemia relative to controls. A correlation was demonstrated between total cyclocreatine accumulated and sparing of ATP during ischemia.
ACKNOWLEDGEMENTS

The research presented in this thesis has been greatly improved by the contributions of a number of people. In appreciation, I would like to acknowledge several of them.

My research advisor, J. B. Walker, is responsible for many of the ideas behind this research. His insight has greatly aided my understanding of a variety of concepts pertaining to this study.

My labmates, Jeff Roberts and Dennis Woznicki, helped to make the hours spent in the lab more enjoyable during the time in which our studies overlapped. A large portion of this work was based on research previously performed by Jeff and Dennis, and additionally, on that done by Tom Annesley.

My wife, Pam Turner, has displayed considerable patience and perseverance during the years I have pursued this goal. As well, she aided greatly in the preparation of this thesis manuscript.

My parents, Gene and Norma Turner, are responsible for laying the background which led to my undertaking of the attempt to obtain a Ph.D. As well, they are responsible for instilling many of the traits which aided me in staying the course.

Finally, to all the friends I have enjoyed good times with around the biochemistry department, a heartfelt thanks. Long live basketball!
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<td>AIS</td>
<td>N-acetimidoylsarcosine</td>
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<td>AMP</td>
<td>Adenosine-5’-monophosphate</td>
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<td>ATase</td>
<td>Arginine:glycine amidinotransferase (EC 2.1.4.1)</td>
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<td>ATP</td>
<td>Adenosine-5’-triphosphate</td>
</tr>
<tr>
<td>ATPase</td>
<td>Aderoine-5’-triphosphatase</td>
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<tr>
<td>BSA</td>
<td>Bovine serum albumin</td>
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<td>cCr</td>
<td>Cyclocreatinine (1-carboxymethyl-2-iminoimidazolidine)</td>
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<td>CHP</td>
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<td>CN</td>
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<td>DEAE-cellulose</td>
<td>Diethylaminoethyl-cellulose</td>
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<td>EDTA</td>
<td>Ethylenediaminetetraacetic Acid</td>
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<td>eGA</td>
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<tr>
<td>$K_{eq}$</td>
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<td>mGP</td>
<td>N-methyl-3-guanidinopropionate</td>
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<tr>
<td>NADH</td>
<td>Reduced nicotinamide adenine dinucleotide</td>
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<tr>
<td>NADP</td>
<td>Nicotinamide adenine dinucleotide phosphate</td>
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<tr>
<td>NH$_2$OH</td>
<td>Hydroxylamine</td>
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<td>$P_i$</td>
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Chapter I: Introduction

Creatine phosphate is a high-energy compound which serves as a buffer for adenosine triphosphate (ATP). When ATP is utilized and its terminal phosphate removed resulting in formation of adenosine diphosphate (ADP), creatine-P (by way of the enzyme creatine kinase) transfers its terminal phosphate group to (ADP) resulting in the resynthesis of ATP and the formation of creatine:

\[
\text{CKase} \quad \text{Creatine-P + ADP} \rightleftharpoons \text{creatine + ATP.} \quad (I-1)
\]

Creatine was crystallized from beef meat by Chevreul (1835) but its function was not determined for many years. As a result, it and its phosphorylated derivative creatine-P were included in a debate concerning the immediate energy source for muscular contraction (Hoyle, 1983 and Katz, 1977).

Fletcher and Hopkins (1907) published work on lactate accumulation in fatigued excised muscle, followed by the publication of Parnas and Wagner (1914) describing the accompanying loss of carbohydrate (glycogen). Following this work Meyerhof in the 1920's demonstrated that the amount of carbohydrate catabolized during muscular contraction was proportional to the amount of work done, and furthermore, was sufficient to supply the necessary energy for contraction. This resulted in the formation of a lactate theory of contraction by Meyerhof and also by A. V.
Hill:

Glycogen \( \rightarrow \) lactate + mechanical energy. (I-2)

However, Lohmann and Meyerhof (1927) found that more phosphate was released than lactate early in contraction. The lactate theory then collapsed when experiments by Lundsgaard (1930) demonstrated that contraction could continue in the presence of the inhibitor iodoacetate which blocks glycolysis, preventing lactate formation.

Eggleton and Eggleton (1927) discovered the presence of a phosphagen responsible for the released phosphate. Fiske and Subbarow (1929) identified this substance as creatine phosphate. With the finding that creatine-P depletion was proportional to the amount of work done during contraction a new theory of contraction was put forth by Lundsgaard (1931):

Creatine-P \( \rightarrow \) creatine + \( P_i \) + mechanical energy. (I-3)

However, this theory, too, was short-lived. Lohmann (1929) isolated a second compound from muscle which also released phosphate. Four years later the base was proved to be adenosine and adenosine triphosphate was identified. It was found that creatine-P could not be broken down to creatine and phosphate in cell-free extracts to produce energy unless adenine nucleotides were included. When it was furthermore demonstrated that the addition of creatine-P to a muscle extract in gel form could not bring about contraction, but that the addition of only ATP could, it was realized that ATP must be the immediate energy source for
muscular contraction and creatine-P was acting to rephosphorylate ADP to ATP:

\[ \text{Creatine-P} + \text{ADP} \rightleftharpoons \text{creatine} + \text{ATP} \]  \hspace{1cm} (I-4)

\[ \text{ATP} \rightarrow \text{ADP} + P_i + \text{mechanical energy}. \]  \hspace{1cm} (I-5)

Lehmann found that the second reaction actually exists as a reversible equilibrium:

\[ \text{ATP} \rightleftharpoons \text{ADP} + P_i. \]  \hspace{1cm} (I-6)

If indeed ATP was being broken down during contraction it was being reformed very quickly. It was not until many years later that Cain and Davies (1962) were able to demonstrate that ATP did actually decrease during muscular contraction. By utilizing fluorodinitrobenzene to selectively inhibit creatine kinase, they were able to prevent the rephosphorylation of ADP by creatine phosphate, and thus to observe the breakdown of ATP during contraction. It was found that the amount of ATP hydrolysis which occurred was indeed proportional to the amount of muscular work performed.

In 1941, although without the benefit of Cain and Davies' work, Fritz Lipmann proposed a general hypothesis for energy transfer in the living cell. He set forth the principle that ATP functions in a cyclic manner. The compound carries energy from catabolic reactions which produce energy, such as the breakdown of glucose or lipids, to cellular processes which consume energy. In this way ATP utilization drives such diverse events as muscular contraction, active transport and biosynthesis. Thus the
terminal phosphate of ATP is continually turned over with rephosphorylation accomplished by the degradation of various cellular fuels or buffering by the phosphagen, creatine-P.

The utilization of creatine-P as a buffer for ATP as well as the central role in the transfer of energy played by ATP can be best understood with a knowledge of thermodynamics. Hence, there follows a short review of the subject (Newsholme and Leech, 1983 and Lehninger, 1975).

Thermodynamics is the study of energy and its transformation from one type to another. The first law of thermodynamics states that energy can be neither created nor destroyed, it must be conserved. Thus, for a reaction where compound A goes to compound B and the pressure is kept constant the internal energy is equal to the amount of heat produced plus the work done by the reaction. In equation form:

$$\Delta H = q - w.$$  \hspace{1cm} (I-7)

$\Delta H$ is referred to as the change in enthalpy; $q$ is the heat absorbed and $w$ is the work performed. The maximal amount of energy available from a reaction is $\Delta H$.

The second law of thermodynamics states that the tendency of the total entropy of the environment and reactants to be maximized is the driving force of all chemical and physical processes. Entropy is a measure of the randomness of a system and is characterized as $\Delta S = q/T$. $\Delta S$ is the change in entropy and $T$ is temperature. However, it can be quite difficult, if not impossible, to measure the
change of entropy in both the environment and the system being analyzed. It is possible, however, to cause a chemical reaction to occur such that the only effect on the environment of the change in entropy is seen as the amount of heat taken up or produced ($\Delta H$). By combining this heat with a term involving the entropy change of the reactants a suitable equation is produced for the measurement of a term which delineates the spontaneity of a reaction. At constant temperature and pressure,

$$\Delta G = \Delta H - T \Delta S.$$  

(I-8)

If the free energy ($\Delta G$) of a reaction is negative the reaction occurs spontaneously and is said to be exergonic. If the free energy is positive the reaction does not occur spontaneously and is said to be endergonic. If the free energy is 0 the reaction is said to be in equilibrium, that is, the rate of the forward reaction is exactly equal to the rate of the reverse reaction. Thus, free energy is that portion of the total energy which can perform work as the system proceeds toward equilibrium.

For a reaction $A \rightleftharpoons B$ at equilibrium, the reaction can be displaced from equilibrium by the addition of compounds $A$ or $B$. Since this addition will cause the reaction to proceed to either the right or left and it has already been demonstrated that $\Delta G$ dictates the spontaneity of a reaction, then $\Delta G$ can be related to the concentration of the reactants and products. Such a relationship has been determined:
\[ \Delta G = \Delta G^0 + RT \ln \frac{[B]}{[A]} \]  
(I-9)

where \( \Delta G^0 \) = standard free energy change, \( R \) = the gas constant and \( T \) = temperature.

Since at equilibrium \( \frac{[B]}{[A]} = K_{eq} \) at \( \Delta G = 0 \), if the system is at equilibrium then \( \Delta G^0 = -RT \ln K_{eq} \) where \( \Delta G^0 \) is determined with the reaction being in the standard state with a pressure of 1 atm, a temperature of 25°C, a pH of 0.0 and the concentration of reactants and products being 1 M. If the standard free energy change is determined at pH 7.0 it is denoted as \( \Delta G^0' \).

If the \( \Delta G^0 \) for a reaction \( A \rightarrow B \) is large and positive, the reaction can still be made to occur spontaneously with a negative \( \Delta G \) through the coupling of an additional exergonic reaction \( X \rightarrow Y \):

\[ A + X \rightarrow B + Y. \]  
(I-10)

In this case \( \Delta G \) for \( A + X \rightarrow B + Y \) is equal to the sum of the \( \Delta G \) for the reaction \( A \rightarrow B \) and the \( \Delta G \) for the reaction \( X \rightarrow Y \).

The central compound most utilized by the cell to perform this function of providing energy to facilitate a reaction is ATP. As is true for all compounds, ATP contains a characteristic amount of free energy due to its molecular structure. Hydrolysis of ATP,

\[ ATP + H_2O \rightleftharpoons ADP + P_i \]  
(I-11)

results in a large amount of free energy, \( \Delta G^0' = -7.3 \) kcal/mole, being liberated as the less stable ATP breaks down.
to form its more stable products. There are four factors which contribute to this increase of stability. First is the increase in the number of resonance forms available to the products over that possible for the reagents. Second is the increase in hydration of the products. Third is the repulsion of negatively charged ADP and phosphate ions, preventing their recombination, and finally, charge repulsion occurring between the terminal phosphates of ATP results in bond strain in the molecule, which is released when it is hydrolyzed.

Conditions within the intact cell also affect $\Delta G$. In intact cells both ADP and ATP are complexed in a 1:1 ratio by Mg$^{2+}$. $\Delta G$ for the hydrolysis of ATP, which is altered by the presence of Mg$^{2+}$, varies furthermore, with varying pH. It has been estimated that $\Delta G_p$ for the reaction is approximately 14 kcal/mole in the cell cytosol (Veech, et al, 1979) where:

$$\Delta G_p = \Delta G'_{\text{ATP}} + RT \ln \frac{(ATP)}{(ADP_{\text{free}})(P_i)}.$$  \hspace{1cm} (I-12)

At this high phosphorylation potential creatine-P can buffer ATP quite well. As noted earlier this is accomplished by the creatine kinase reaction:

$$H^+ + Cr-P^2- + MgADP^{-} \rightleftharpoons Cr + MgATP^2-. \hspace{1cm} (I-13)$$

Because there is such an abundance of creatine kinase in the muscle cell and the forward and reverse fluxes of the reaction are faster than the total cellular ATPase activity or ATPase synthesis rate the reaction is at or near equili-
brium in the muscle cell (Kushmerick, 1985).

It has been determined that, with a (Mg$^{2+}$)$_{\text{free}}$ of 1 mM,

$$K_{eq} = \frac{(ATP)(C)}{(ADP_{\text{free}})(PC)(H^+)} = 1.66 \times 10^9 M^{-1} \quad (I-14)$$

for the creatine kinase reaction. Put another way,

$$\frac{(ATP)}{(ADP_{\text{free}})(H^+)(1.66 \times 10^9 M^{-1})} = \frac{(PC)}{(C)} \quad (I-15)$$

Because the free (ADP) in the cell is on the order of 1000-fold lower than (ATP), and (C) is only 3 to 4 fold less than (Cr-P) a large decrease can occur in (Cr-P) with only a minute change in (ATP) levels. Thus, because the ratio of ATP/ADP is proportional to the ratio Cr-P/Cr, an increase in the amount of phosphagen utilized will bring about a smaller relative decrease of ATP than of the Cr-P utilized.

The standard free energy of hydrolysis of creatine-P is -10.3 kcal/mole at pH 7.0, thus the reaction:

$$H^+ + Cr-P^2- + MgADP \rightleftharpoons \text{CKase} \rightarrow Cr + MgATP^2- \quad (I-16)$$

proceeds to the right with a $\Delta G^0'$ of -3.0 kcal/mole. The creatine kinase reaction is the only enzymatic reaction in which creatine-P participates, thus ideally suiting the compound for its function as a buffer. During glycolysis the reaction is displaced to the right due to acidification. It then functions in a three-fold manner. First ADP is rephosphorylated; secondly, pH is buffered and finally, inorganic
phosphate is released which may then act to inhibit deamina-
tion of adenosine monophosphate (AMP), stimulate phosphofruc-
tokinase, increase glycogen breakdown and increase substrate
level and oxidative phosphorylation.

An additional function for creatine kinase and
creatine-P, the creatine phosphate shuttle, has been proposed
as a mechanism for intracellular transport of high-energy
phosphate (Bessman, 1972). According to this theory,
creatine is phosphorylated in the mitochondria and diffuses
to the myofibrils. Creatine kinase bound to the myofibril
then catalyzes transfer of the phosphate from creatine
phosphate to ADP, providing ATP for continued contraction.
Creatine then diffuses from the myofibrils to the
mitochondria, where it is rephosphorylated by mitochondrial
creatine kinase. The significance of this shuttle is not
clear at present. Some researchers believe that the shuttle
is not a necessary component of cellular metabolism (Meyer et
al, 1984, Shoubridge and Radda, 1984 and Shoubridge et al,
1985). Others steadfastly argue that a creatine phosphate
shuttle is important to the cellular function of certain
tissues (Jacobus, 1985 and Bessman and Carpenter, 1985).

The buffering action of creatine-P is not in doubt
however. At the high $\Delta G_p$ and pH characteristic of respiring
cells creatine-P functions quite well to buffer the ATP/ADP
ratio. At the lower $\Delta G_p$ and low pH characteristic of the
later stages of ischemia, however, a phosphagen with a lower
$\Delta G^0$ would function better in this capacity. Such a
compound has been found, 1-carboxymethyl-2-imino-3-phosphono-imidazolidine (cyclocreatinine-P), a cyclic analogue of creatine containing an extra methylene group.

Cyclocreatinine-P participates in the creatine kinase reaction in much the same manner as does creatine-P (Figure 1). However, because it is a unique compound, one would expect that its characteristics would differ from those of creatine-P. To examine these differences a first step might be to determine $K_{eq}$ for the reaction described in Figure 1 (Annesley and Walker, 1977). However, owing to the various equilibria existing with differing pHs and Mg$^{2+}$ concentrations, a second reaction was actually examined, a combination of two previously mentioned reactions.

\[
\begin{align*}
\text{Cr-P}^2^- + \text{ADP}^- + \text{H}^+ & \rightleftharpoons \text{Cr} + \text{MgATP}^2^- \quad (I-17) \\
\text{cCr} + \text{MgATP}^2^- & \rightleftharpoons \text{cCr-P}^2^- + \text{ADP}^- + \text{H}^+ \quad (I-18) \\
\text{Cr-P}^2^- + \text{cCr} & \rightleftharpoons \text{Cr} + \text{cCr-P}^2^- \quad (I-19)
\end{align*}
\]

Reaction I-19 is fairly independent of pH and Mg$^{2+}$ concentrations in the physiological range. The equilibrium constant for this reaction with creatine kinase from chick breast muscle was found to be 26. It was furthermore found that reaction (I-19) exists in near equilibrium in both in vitro loaded Ehrlich ascites tumor cells (Annesley and Walker, 1978) and in vivo in mouse brain (Woznicki and Walker, 1979).
Figure 1. Creatine kinase-catalyzed reaction of cyclocreatine and ATP.
From the above $K_{eq}$ value, the $\Delta G^0$ for cCr-P was determined to be 2 kcal/mole lower than that of Cr-P. Thus the free energy of hydrolysis of cCr-P is approximately -8.3 kcal/mole. For reaction (I-19):

$$\frac{(Cr-P)}{(Cr)} = \frac{(cCr-P)}{(26)(cCr)}.$$  \hspace{1cm} (I-20)

Combining this with the ATP/ADP ratio:

$$\frac{(ATP)}{(H^+)(ADP)(1.66 \times 10^9)} = \frac{(Cr-P)}{(Cr)} = \frac{(cCr-P)}{(26)(cCr)}.$$  \hspace{1cm} (I-21)

From this equation it can be seen that for a given decrease in the ATP/ADP ratio or in pH, the ratio cCr-P/cCr will decrease to a much smaller degree than will the Cr-P/Cr ratio. Thus, cyclocreatinine-P can buffer the ATP/ADP ratio at lower cytosolic phosphorylation potentials than can creatine-P.

**Creatine Analogues**

A growing body of research has been performed on cyclocreatinine and other analogues of creatine in an attempt to learn more about the functions of creatine and creatine-P (Figure 2). This work can be grouped into four major areas:

1) The study of an analogue, cyclocreatinine, which acts to increase levels of high-energy phosphate when taken up into muscle cells, and which provides a longer lasting response to insult with ischemia than does creatine.
Figure 2. Creatine Analogues.
Creatine (Cr), cyclocreatine (cCr), guanidinoacetate (GA),
N-ethylguanidinoacetate (eGA), β-guanidinopropionate (GPA),
N-methyl-3-guanidinopropionate (mGP), β-guanidinobutyrate
(GBA), N-acetimodylsarcosine (AIS), 1-carboxymethyl-2-
iminohexahydropyrimidine (CHP), homocyclocreatine (hcCr).
2) The study of an analogue, β-guanidinopropionate, which, when taken up in muscle cells, acts to deplete creatine serving as a model for several diseases of muscle. In the muscle cell a pool of guanidinopropionate-P is synthesized; this compound can then be utilized to rephosphorylate ADP, although much more slowly than creatine-P, thus complicating the use of this compound in studies involving creatine depletion.

3) The study of various other analogues of creatine, including homocyclocreatine, β-guanidinobutyrate, and N-ethylguanidinoacetate, among others, for a variety of purposes.

4) The use of a number of analogues in the study of the mechanism of action of creatine kinase.

Cyclocreatine

One of the most useful analogues to date has been cyclocreatine. The compound was first synthesized by Kenyon's group in 1971 (Rowley et al, 1971). Since that time most of the studies done on cyclocreatine have been performed in the laboratory of J. B. Walker.

Work with this analogue began during a study of liver amidinotransferase (Walker and Hannan, 1976). Various analogues of creatine were injected into chick eggs or were fed to young male chicks. After a period of time livers were analyzed for repression of amidinotransferase. It was found that creatine, cyclocreatine, and a second analogue,
N-acetimidoylsarcosine, were able to repress amidinotransferase. Results of the study included the finding that two precursors of creatine, arginine and guanidinacatate, could not themselves suppress amidinotransferase, but must be converted into creatine in order for the feedback mechanism to occur.

An improved synthesis of cyclocreatinine was devised from reactions involving chloroacetic acid, ethylenediamine, and cyanogen bromide in Walker's laboratory (Wang, 1974). Quantities of cyclocreatinine were now available for mass feeding of chicks (Griffiths and Walker, 1976). It was found that a phosphorylated derivative of cyclocreatinine could accumulate to high levels in chick breast muscle. This derivative, though initially thought to be phosphorylated on the primary nitrogen (Rowley, et al., 1971) was later found to be phosphorylated on the secondary nitrogen by Quirocho's laboratory as cited by Annesley and Walker (1977). This location was indirectly supported by the work of Struve and coworkers (1977), and established conclusively by Phillips and coworkers (1979). It was furthermore found that cyclocreatinine-P does not cyclize to creatinine as does creatine-P (Griffiths and Walker, 1976), and that cyclocreatinine uptake in the muscle of cyclocreatinine-fed chicks resulted in the displacement of creatine. Upon return to a control diet, levels of creatine returned towards normal and cyclocreatinine was lost at a rate similar to the rate of uptake.
In vitro studies by Kenyon revealed that cyclocreatinine was 6-fold less active than creatine in the phosphorylation of the substrate by creatine kinase and was the most active analogue found (McLaughlin, et al, 1972). The $K_m$ of creatine was found to be 5 mM while that of cyclocreatinine was found to be 25 mM (Table 1). Considering the $V_{max}$ of creatine as 100%, the relative $V_{max}$ of cyclocreatinine was found to be 90%. The reverse reaction catalyzed by creatine kinase (donation of the phosphate group to ADP) was found to have a $K_m$ of 3.2 mM for creatine-P and 3.6 mM for cyclocreatinine-P while the $V_{max}$ of cyclocreatinine-P was 0.7% relative to creatine-P, yielding a 160-fold difference between the two compounds for the reverse reaction (Annesley and Walker, 1977). As was noted above, the $\Delta G^0'$ for cyclocreatinine-P was found to be 2 kcal/mol lower than that of creatine-P (Annesley and Walker, 1977).

Ehrlich ascites tumor cells have been used to study the metabolism of cyclocreatinine (Annesley and Walker, 1978). It has been observed that when incubated in media containing cyclocreatinine, high levels of the compound are taken up and phosphorylated by the cells. This phosphagen can then be utilized during energy stress (addition of 2-deoxyglucose) and regenerated following cessation of this stress. This occurs in much the same manner as does utilization and regeneration of creatine-P by the tumor cells with the exception that cyclocreatinine is more completely phosphorylated (98% as compared to 70% for creatine) and its phos-
### Table 1. Kinetics of the Reaction of Creatine Analogues with Creatine Kinase

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Relative $V_{\text{max}}$ (%)</th>
<th>$K_m$ (mM)</th>
<th>Activity of Substrate as Compared to Creatine</th>
</tr>
</thead>
<tbody>
<tr>
<td>Creatine</td>
<td>100%</td>
<td>$5^{a,17,b,c}$</td>
<td>most active</td>
</tr>
<tr>
<td>Cyclocreatine</td>
<td>90%</td>
<td>$25^a$</td>
<td>6-fold less</td>
</tr>
<tr>
<td>N-Ethylguanidinoacetate</td>
<td>32%</td>
<td>$47^a$</td>
<td>30-fold less</td>
</tr>
<tr>
<td>Guanidinoacetate</td>
<td>10%</td>
<td>$72^a$</td>
<td>150-fold less</td>
</tr>
<tr>
<td>$\beta$-Guanidinopropionate</td>
<td>0.3%</td>
<td>$50^b$</td>
<td>1000-fold less</td>
</tr>
<tr>
<td>N-methyl-3-guanidinopropionate</td>
<td>0.16%</td>
<td>$48^c$</td>
<td>1500-fold less</td>
</tr>
<tr>
<td>Homocyclocreatine</td>
<td></td>
<td></td>
<td>10000-fold less</td>
</tr>
<tr>
<td>$\beta$-Guanidinobutyrate</td>
<td>0.01%</td>
<td>$^c$</td>
<td>less active than guanidinopropionate</td>
</tr>
<tr>
<td>1-carboxymethyl-2-imino-hexahydropyrimidine</td>
<td>n.d.$^{a,f}$</td>
<td></td>
<td>inactive</td>
</tr>
</tbody>
</table>

---

a. McLaughlin et al., 1972.
e. This substrate was found to be 15000-fold less active by McLaughlin and coworkers (1972). Roberts and Walker (1983) found that the substrate possessed an activity intermediate between that of guanidinoacetate and that of $\beta$-guanidinopropionate.
f. n.d. = not detectable.
g. as calculated from $V_{\text{max}}/K_m$. 
phorylated derivative is more slowly utilized during stress.

Cyclocreatine-P utilization by a variety of tissues has been studied; chief among these are studies on muscle, brain and heart. In all of these it has been found that cyclocreatine can be taken up from the bloodstream of animals fed the compound, phosphorylated, and utilized in response to stress. In both skeletal and cardiac muscle it has been shown that the prior loading of the tissue with cyclocreatine-P protects against loss of extensibility during ischemia following death. Onset of rigor was delayed two-fold in both cardiac and skeletal muscle (Annesley and Walker, 1980 and Roberts and Walker, 1982a). Glycogen metabolism was also affected in cyclocreatine-fed animals. In the rat heart glycogen levels were nearly doubled by cyclocreatine feeding. Rates of utilization during ischemia were similar to those of controls. In mouse skeletal muscle glycogen levels were not affected by feeding; following injection of epinephrine, glycogen levels dropped from 43 µmol/g to 20 µmol/g after 4 hours in control animals, while cyclocreatine animals suffered no loss of glycogen; levels remained at 41 µmol/g.

In the brain two approaches to cyclocreatine metabolism have been pursued; mice have been fed cyclocreatine resulting in its uptake into the brain, and chick embryo brains have been minced and incubated in media containing the analogue (Woznicki and Walker, 1979 and Woznicki and Walker, 1980). It was found, using these minces, that cyclocreatine could be
taken up, phosphorylated, depleted during incubation in potassium and then reporphosphorylated. Potassium elicits this effect by causing depolarization of the cell membrane, resulting in an energy drain on the brain minces. As noted earlier, it was also found that $K_{eq}$ for the creatine kinase reaction aforementioned (Equation I-19) was equal to 30, very near to the $K_{eq}$ of 26 found in vitro. This finding demonstrated for the first time that creatine kinase in the brain is in equilibrium or at least in near equilibrium.

Mouse brain has been found to take up large amounts of cyclocreatinine from the diet, 16.7 μmol/g as compared to the 7.7 μmol/g of total creatine normally found in the brain. Furthermore, a large percentage of the cyclocreatinine was phosphorylated. Cyclocreatinine-P caused the levels of ATP to be sustained for a longer period of time than the ATP levels in control brains following decapitation. Glycolysis was also extended in the phosphagen-loaded brains (Woznicki and Walker, 1980).

β-Guanidinopropionate

The analogue, β-guanidinopropionate, has been used to deplete creatine in an attempt to mimic certain muscle diseases.

It has been known for a number of years that creatininuria and reduced urinary excretion of creatine occur in patients with different types of muscle disease (Milhorat, 1953). These diseases may also present symptoms such as low
concentrations of creatine and creatine-P in skeletal muscle, found in both muscular dystrophy and in neurogenic muscular atrophy (Nevin, 1934, Reinhold and Kingsley, 1938, Ronzoni, et al, 1958 and Vignos and Wagner, 1962). Hence, the study of creatine uptake and phosphorylation in skeletal muscle is a vital one.

Fitch and colleagues performed studies on human patients suffering from muscle disease (Fitch, et al, 1968). By administering creatine-$1^{14}$C and later measuring radioactivity levels in urine, he found that the low creatine content found in skeletal muscle of patients suffering from neurogenic muscular atrophy was due to reduced transport of creatine into the muscle. On the other hand low creatine content of skeletal muscle in muscular dystrophy patients was caused by ineffective trapping of creatine in the muscle. Thus if one could find an analogue of creatine which affected transport into muscle cells or which lowered amounts of creatine phosphate in the cells, research on muscle diseases would be greatly facilitated. The analogue which met these qualifications was $\beta$-guanidinopropionate (GPA).

Early work by Fitch and Shields (1966) revealed that a special mechanism for creatine entry into muscle cells, a saturable process, existed. Upon investigation of the specificity of this entry mechanism with a number of analogues (Fitch et al, 1968) it was found that a reversible interaction of creatine or certain inhibitory guanidine compounds with transport sites of a single type occurred.
Analogues such as guanidinopropionate, guanidinoacetate, N-ethylguanidinoacetate, and β-guanidinobutyrate were able to interact with the transport site to inhibit creatine transport. Other compounds were not able to exhibit this inhibition. These included arginine, creatinine, glycine, guanidine and sarcosine among others. From these results it was concluded that the specificity of entry was due to an interaction between the transport site and an amidine group. Furthermore it was found that the optimal chain length for these compounds, monosubstituted guanidines with a terminal carboxyl group, was three. One analogue, guanidinopropionate, was best able to competitively inhibit the uptake of creatine. 1 mM guanidinopropionate was able to inhibit uptake of 0.1 mM creatine by 86%.

It was found by Shields and Whitehair (1973) that with feeding of guanidinopropionate to rats, muscle levels of creatine were decreased by 75%, and excessive creatininuria along with reduced creatinine excretion occurred - some of the symptoms of muscle disease. Fitch and coworkers (1974) validated these findings observing that in gastrocnemius muscle, P-creatine levels were depleted over a six week period from 22.5 μmol/g to 1.6 μmol/g when rats were fed 1% guanidinopropionate. He also observed that large amounts of guanidinopropionate (37 μmol/g) were taken up and that a large percentage (80%) of this was phosphorylated. In addition, feeding of guanidinopropionate was further noted to cause depletion of ATP levels. It was found that upon
stimulation of the muscle this phosphorylated derivative was depleted as was phosphocreatine in stimulated control muscle.

The kinetics of the phosphorylation and dephosphorylation of guanidinopropionate by creatine kinase have been examined (Fitch and Chevli, 1979).

\[
\text{Guanidinopropionate} + \text{MgATP} \xleftrightarrow{\text{CKase}} \text{Guanidinopropionate-P + MgADP + H}^+ \quad (I-22)
\]

Guanidinopropionate was found to be a 1000-fold poorer substrate of creatine kinase than was creatine. Guanidinopropionate-P was found to be a 2500-fold poorer substrate than was creatine-P. Although guanidinopropionate is a substrate for creatine kinase, it does appear to be a quite poor one.

A number of experiments have been performed to examine the effect of guanidinopropionate feeding on the dynamic aspects of muscle action. One of the earliest of these by Shields and coworkers (1975) found that running performance in rats fed guanidinopropionate was less than that of rats fed a control diet. Fitch and coworkers (1975) found that the initial twitch tension in tibialis anterior muscle from rats fed guanidinopropionate was the same as that from muscle of control animals. However, upon stimulation, muscles from control rats exhibited the staircase phenomena (in which tension actually increases prior to its decline) while muscle from guanidinopropionate-fed rats exhibited an immediate decrease in tension. There was a 28% greater reduction in
total tension in the guanidinopropionate muscles as compared to the controls. This correlated closely with a 32% lower utilization of high energy phosphate in the muscles of guanidinopropionate-fed rats.

Further studies (Fitch et al, 1978) showed that creatine-depleted soleus muscle from guanidinopropionate-fed rats exhibited sustained isometric contractions during fatiguing. Thus the adaptation of these red fibers to phosphocreatine depletion increased their endurance. This did not appear to be due to protection by guanidinopropionate utilization as the guanidiopropionate-P decreased by only 12% as compared to a 70% decrease of phosphocreatine in the controls.

It was found by Shields and coworkers (1975) that the size of white fast-twitch fibers was decreased by a substantial amount in rats from the litters of females fed guanidinopropionate during pregnancy and during the weaning period. Except for this reduction in size and a slight decrease in strength, plantaris muscle from guanidinopropionate-fed rats showed no abnormalities. Soleus muscle from guanidinopropionate-fed rats exhibited a lower initial strength, greater isometric endurance, even slower twitch, lower maximal velocity of shortening, and the requirement of a lower frequency of stimulation to elicit maximal isometric tetanic tension compared to control muscle (Petrofsky and Fitch, 1980). A possible explanation, as stated by Fitch, is that the decrease in size of plantaris fibers and the altered
contractile characteristics of soleus muscle could be due to an abnormality in the control of myosin biosynthesis.

Work by Mainwood and coworkers (1982a) with diaphragm muscle from rats fed guanidinopropionate has given results similar to those of Fitch in his work with the soleus muscle. Mainwood found that over time periods of several minutes the single twitch response in diaphragm shows characteristics of slowing, including increased time to peak tension and prolonged relaxation. This was interpreted as a transition of some fibers from the fast to the slow type upon guanidinopropionate feeding.

As concluded by Mainwood from the results of both Fitch and Mainwood, it appears that factors other than creatine phosphate levels determine the ability of muscles to maintain contractions for long time periods. On the other hand, the creatine-depleted fibers from guanidinopropionate-fed rats demonstrate a much more rapid decline in contractile characteristics elicited by stimulation for short periods of time. Mainwood believes that this decline is most probably caused by an increase in ADP.

Further work by Mainwood and coworkers (1982b) revealed that inorganic phosphate generated during stimulation to exhaustion with accompanying inhibition of glycolysis and respiration was less in guanidinopropionate-fed rats than in controls. Additionally, it was found that under these conditions, muscles from guanidinopropionate-fed rats were able to contract only 4 times in response to stimulation
while muscles from control rats were able to contract 15 times.

Studies in frog muscle were carried out by De Saedeleer and Marechal (1984). After frogs had been injected with guanidinopropionate for a period of several weeks, their sartorius muscles were excised. It was found that a small amount of guanidinopropionate had been taken up and a portion of this phosphorylated without depleting total creatine content. However, the amount of creatine present in the phosphorylated form was halved by this treatment. Upon electrical stimulation of the muscle to rigor, it was observed that guanidinopropionate-P was not split during contraction. Additionally, the number of twitches performed by the muscle prior to rigor was decreased.

Roberts and Walker (1982a) studied the effects of guanidinopropionate feeding on rat heart. Hearts of rats fed 1% guanidinopropionate accumulated 16 μmol/g of guanidinopropionate derivatives and displayed a reduction of total creatine content to 2.4 μmol/g as compared to the 12 μmol/g present in control hearts. In these experiments in which cyclocreatine feeding was found to protect hearts against rigor and depletion of ATP during ischemia, no protective effect was conferred by prefeeding of guanidinopropionate. Annesley and Walker (1980) demonstrated that while prefeeding of cyclocreatine delayed rigor in mouse skeletal muscle relative to controls, prefeeding of guanidinopropionate resulted in no such protective effect.
Most of the recent studies involving guanidinopropionate have utilized the technique of $^{31}$P-NMR (nuclear magnetic resonance). Shoubridge and colleagues (1985) studied the adaptation of rat skeletal muscle to the depletion of creatine caused by the feeding of guanidinopropionate. While verifying the differential effects on fast and slow-twitch muscles, they concluded that changes in (ATP) and (creatine-P) may by themselves be sufficient to alter the expression of two types of proteins - enzymes and contractile proteins. They also observed a nearly two-fold increase in glycogen content of skeletal muscle, verifying the previous finding by Annesley and Walker (1980) of increased glycogen in mouse skeletal muscle.

Meyer and colleagues (1985) depleted creatine in rat skeletal muscle with guanidinopropionate. It was found that glycogenolysis was decreased greatly in stimulated muscle from those rats fed the analogue relative to stimulated control muscle. In addition guanidinopropionate-P hydrolysis was much slower than the hydrolysis of creatine-P observed in controls, resulting in phosphate levels in stimulated muscles containing guanidinopropionate which were less than half the phosphate levels in control muscles. The conclusion was drawn that the phosphate released by hydrolysis of creatine-P during stimulation of muscle is a contributing factor to the activation of glycogenolysis.

Studies with $^{31}$P NMR of muscles depleted of creatine by guanidinopropionate have also shed light on the creatine
shuttle controversy (Shoubridge and Radda, 1984 and Shoubridge et al, 1985). Through determinations of creatine kinase flux and ATP turnover rates in creatine-depleted skeletal muscle and heart, it was concluded by these researchers that the operation of a creatine-P shuttle is not a necessary component of aerobic metabolism.

Other Creatine Analogues

A number of other creatine analogues have been studied. A survey of these analogues for their ability to inhibit accumulation of $^{14}$Ccreatine in vivo and to also serve as substrates in vitro was performed by Fitch and Chevli (1980).

It was found that a number of analogues, when injected intraperitoneally along with $^{14}$Ccreatine, could inhibit uptake of this radioactive creatine. Among the best inhibitors were 1-carboxymethyl-2-iminohexahydropyrimidine, and $\beta$-guanidinopropionate. Second only to creatine in preventing uptake of $^{14}$Ccreatine was DL-$\beta$-guanidinobutyrate. It was furthermore found that guanidinobutyrate was a very poor substrate of creatine kinase, much poorer than guanidinopropionate. Guanidinobutyrate was found to have a $V_{\text{max}}$ of only 0.01% that of creatine while the $V_{\text{max}}$ of guanidinopropionate was 0.3%. Thus, it appeared that, for the study of muscular disease, guanidinobutyrate could possibly function to deplete creatine without being phosphorylated to act as an energy buffer in the muscle as does guanidinopropionate.
Feeding experiments with 6% β-guanidinobutyrate included in the diet revealed that in gastrocnemius muscle (Fitch and Chevli, 1980) creatine was depleted by 50% (from 21.1 μmol/g to 10.2 μmol/g). With feeding of 2% guanidinobutyrate, creatine phosphate was depleted by 40% in tibialis anterior muscle and 20% in pectoralis muscle (Fitch and Chevli, 1980 and Laskowski et al, 1981). In the gastrocnemius study about 15 μmol/g of guanidinobutyrate were taken up with only 20% of this phosphorylated. In the tibialis anterior about 12 μmol/g were taken up and only about 5% was phosphorylated. This compares with findings that about 80% of the guanidino-propionate that is taken up by skeletal muscle is phosphorylated.

In a histological study (Laskowski, et al, 1981) it was found that guanidinobutyrate feeding and the resulting depletion of creatine resulted in damage to a small number of the fibers of the pectoralis and gastrocnemius muscles. Mitochondria were dilated as was the sarcoplasmic reticulum. In one fiber "streaming" occurred, Z bands were totally disrupted as well as was the entire organization of the sarcomeres.

Another analogue of creatine which has been studied is its precursor, guanidinoacetate (James and Morrison, 1966). In vitro kinetics revealed that it was a substrate of creatine kinase, although a poor one. In the study it was found that the $V_{max}$ of guanidinoacetate-P was less than 0.2% that of creatine-P while the $K_m$ was 5.3 mM as compared to a
$K_m$ of 2.9 mM for creatine-P.

A closely related analogue is N-ethylguanidinoacetate. Its synthesis from N-ethylglycine and cyanamide was published by Armstrong (1956). It was found that ethylguanidinoacetate could be taken up by rats fed a diet containing the compound (Armstrong, 1953). The analogue was found to be a substrate of creatine kinase (Ennor et al, 1958). Recent work by Roberts and Walker (1985) revealed that ethylguanidinoacetate acts to repress amidinotransferase in chick and chick embryo liver. In another study it was shown that the analogue could be taken up and phosphorylated in Ehrlich cells, utilized during stress, and then rephosphorylated following removal of the stress (Roberts and Walker, 1982b). Through use of dually phosphagen-loaded Ehrlich cells the analogue was determined to have a Gibbs free energy of hydrolysis similar to that of creatine-P (about \(-10.3\) kcal/mole). Feeding experiments demonstrated that ethylguanidinoacetate could be accumulated and phosphorylated in the heart, brain and skeletal muscle of both chicks and rats, replacing creatine-P on an equimolar basis without significantly affecting muscle levels of ATP, glucose-6-phosphate or glycogen.

Another analogue of creatine, 1-carboxyethyl-2-iminoimidazolidine (homocyclocreatine), has been found to be accumulated and phosphorylated in several tissues upon feeding of the compound to growing chicks (Roberts and Walker, 1983). It was found that during ischemia in muscle homocyclocreatine-P was utilized at a much slower rate than
was creatine-P or cyclocreatine-P. Further studies revealed that the phosphoryl group transfer potential ($\Delta G^\circ$) of the compound was quite similar to that of cyclocreatine-P. Homocyclocreatine and its phosphorylated derivative were found to be much poorer substrates of creatine kinase than were cyclocreatine or creatine and their phosphorylated derivatives; homocyclocreatine reacts with creatine kinase 1500 fold more slowly than does cyclocreatine and 10,000-fold more slowly than creatine, while homocyclocreatine-P reacts with creatine kinase 1000-fold more slowly than does cyclocreatine-P and 200,000-fold more slowly than creatine-P (Roberts and Walker, 1983).

A number of other analogues have been studied including N-methyl-3-guanidinopropionate and 1-carboxymethyl-2-iminohexahydropyrimidine. Chicks fed N-methyl-3-guanidinopropionate accumulated large amounts of free and phosphorylated N-methyl-3-guanidinopropionate in brain, heart, and muscle (Roberts and Walker, 1985). In brain, creatine levels were only slightly lowered by accumulation of N-methyl-3-guanidinopropionate, thus resulting in a greatly enlarged total phosphagen reservoir. 1-carboxymethyl-2-iminohexahydropyrimidine was found to be inactive as a substrate for creatine kinase by McLaughlin and coworkers (1972).

Other studies of creatine which included the use of two of its analogues, 1-carboxymethyl-2-iminohexahydropyrimidine and cyclocreatine, were reported by Ingwall and coworkers (1972) and Ingwall and coworkers (1974). They found, that by
adding creatine to cultured chick embryo cells, myosin heavy chain biosynthesis could be increased two-fold with essentially no increase in total protein synthesis; synthesis of actin was also increased two-fold by the addition of creatine to embryo cultures. It was reported that creatine analogues, including the two mentioned above, 1-carboxymethyl-2-iminohexahydropyrimidine and cyclocreatine, could also cause increased myosin synthesis.

This work, however, was rebutted several years later by one of the original authors (Fry and Morales, 1980). Also working with chick embryo cultures, they were unable to repeat the work of Ingwall's group. They found that alterations of cellular creatine had no effect on synthesis of myosin heavy chain protein. Even more in disagreement with the previous work was the finding that 1-carboxymethyl-2-iminohexahydropyrimidine not only did not stimulate myosin synthesis, it inhibited it. Thus, at this point, it still is not known whether creatine functions in the control of muscle protein biosynthesis.

A number of creatine analogues show promise as useful tools in the study of creatine metabolism. Ethylguanidinoacetate-P appears in many ways to be the most creatine-P-like analogue thus far, exhibiting an identical free energy of hydrolysis and the highest reactivity with creatine kinase of any known creatine-P analogue. β-guanidinobutyrate seems to be the best candidate as a model for certain muscular diseases. Additional work with 1-carboxymethyl-2-iminohexa-
hydropyrimidine might reveal its possible usefulness as a depleter of creatine phosphate in the study of the diseases of muscle. Homocyclocreatine, with its exceedingly slow kinetics, shows promise as a useful probe in the study of high-energy phosphate utilization.

**Creatine Kinase Studies**

The original reason for the synthesis of a large number of the creatine analogues was to study the mechanism of catalysis of creatine kinase. This work has been led by G. L. Kenyon.

Finding that the enzyme much preferred to interact with five-membered rings (for example, cyclocreatine) as compared to six-membered rings (1-carboxymethyl-2-iminohexahydropyrimidine), Kenyon deduced that only relatively flat, or compact structures were able to readily access portions of the active site of the enzyme (Rowley et al., 1971). Working with a number of other analogues he found that the active site of the enzyme had a very tight steric requirement in the region near the carboxylate moiety. Further studies (McLaughlin et al., 1972) with a homologous series of amidino glycines revealed that creatine kinase functions best when the alkyl side chain group length is one methyl group. \( V_{\text{max}} \) was maximal and \( K_{m} \) was minimal at this length.

From knowledge gathered from a variety of studies, including the utilization of creatine analogues, Cook and coworkers (1981) were able to postulate a structure for the
transition state of the creatine kinase reaction. Creatine kinase itself, is a dimer with a molecular weight of 82,600. At pH 8.0 the catalyzed reaction is random bimolecular, bimolecular, with phosphoryl transfer being the rate-limiting step (Kenyon and Reed, 1983).

Summary

Two main approaches have been pursued in the study of the functions of creatine in tissues utilizing various synthetic analogues of creatine. The first has been to replace creatine in tissues with certain analogues, mainly cyclocreatinine, which result in both positive and negative effects. Second has been the depletion of creatine, largely with guanidinopropionate, and the study of the ensuing negative effects. The research performed by this author and related in this thesis deals primarily with the first approach. The accumulation and utilization of cyclocreatinine-P and other analogues and effects engendered by this accumulation have been studied in a number of tissues and under a variety of conditions.

Metabolite levels are expressed as μmol/g of wet weight. Error bars in figures reflect the standard error of the mean for the value presented. Some values presented in tables are represented as ± the standard deviation. Where P values are expressed, calculations were performed with Student's t test.
Chapter II: Chick Embryo Tissue

The first body of work executed and now presented by this investigator includes the study of the uptake and depletion of various creatine analogues in minced tissues from chick embryos.

Animal tissues can be studied in two ways: in vivo and in vitro. In vivo, that is, in the intact organism, studies allow one to view the mechanisms in question as a function of the whole. In vitro, that is, in an artificial environment outside of the organism, studies allow one to study the particulars of a specific functional unit unencumbered by the influences of the remainder of the organism. In vitro techniques furthermore have the capability of allowing ease of modification of experimental parameters and permit control of external influencing factors.

A variety of in vitro techniques have been described in the last century. Cell and tissue culture have become increasingly important tools in biochemical research. Tissues are disaggregated by mechanical dispersion (usually in embryonic tissue) or separated by the use of proteolytic enzymes such as trypsin. Following separation the cells are immersed in a growth medium and experiments are performed at a later date. Cell culture has permitted advancement of understanding in a number of areas of research.

The usage of tissue slices has proved of benefit in the study of metabolism. The study of brain through the use of
the slicing technique has become a growing area of research. The procedure consists of the following - the brain of an animal is exposed and the desired region of the brain removed. This region is then sliced, either with a vibratome, a tissue chopper, or by hand to a very fine thickness (Alger et al, 1984). The slices are placed in a superfusion chamber, where they are either totally submerged in artificial cerebrospinal fluid or placed on a gas-liquid interface. These slices, in addition to being able to be maintained for many hours, retain relatively intact synapses, allowing a variety of experimental approaches.

A third type of in vitro technique is that utilizing minced tissue. This technique has been used by a number of researchers. Undem and Buckner (1984) used it to study inhibition of antigen-induced histamine release in guinea pig lung. Carroll and Benishin (1984) used minced mouse forebrains to study the release and/or hydrolysis of acetylcholine from the cytoplasm. In work performed in the author's laboratory and upon which the author's work is based, Woznicki and Walker (1979) studied uptake and depletion of a phosphagen in chick embryo brain. The brain was removed from the embryo, minced and placed in media containing the creatine analogue and a number of salts, coupled with an energy source and a buffering system. These compounds are: NaCl, KCl, CaCl₂, MgSO₄, NaH₂PO₄, HEPES, glucose, bovine serum albumin (BSA) and the creatine analogue. The author has utilized these techniques for the
study of brain and expanded them to include the study of chick embryo heart, skin and muscle. The study has, for the most part, been a preliminary one, with points on most graphs representing a single sample containing multiple tissues.

**Methods**

**Materials**

Creatine and guanidinoacetate were purchased from Sigma Chemical Company. Cyclocreatine was synthesized in the author's laboratory from chloroacetic acid, ethylenediamine and cyanogen bromide as described by Wang (1974) and Griffiths and Walker (1976) and was recrystallized 2 or 3 times from water. Trisodium pentocyanaminoferate ($\text{Na}_3[\text{Fe(CN)}_5\text{NH}_3]$), utilized for assay of cyclocreatine, was purchased from Fisher Scientific. Another salt of this compound, $\text{Na}_2\text{NH}_4[\text{Fe(CN)}_5\text{NH}_3]\cdot5\text{H}_2\text{O}$, can be substituted in the assay and was obtained from Aldrich Chemical Company. The enzymes glucose-6-phosphate dehydrogenase and hexokinase were obtained from Sigma Chemical Company, as were the compounds nicotinamide adenine dinucleotide phosphate, isoproterenol bitartrate, iodoacetate, veratrine, ouabain octahydrate, and bovine serum albumin. The fraction V powder of bovine serum albumin, which contained 96-99% albumin, was used. White Leghorn eggs were obtained from Albers Hatchery in LaGrange, Texas.
Media Composition

40 mM Cyclocreatine Uptake Medium: 80 mM NaCl, 4.8 mM KCl, 1.2 mM MgSO₄, 1.1 mM CaCl₂, 10 mM NaH₂PO₄, 25 mM HEPES, 10 mM glucose, 1% BSA, 40 mM cyclocreatine, pH 7.4. If cyclocreatine concentration was increased, NaCl concentration was decreased by half of this change. For example, if 60 mM cyclocreatine was placed in the media, the concentration of NaCl was adjusted to 70 mM. Creatine Uptake Medium: 70 mM NaCl, 4.8 mM KCl, 1.2 mM MgSO₄, 1.1 mM CaCl₂, 10 mM NaH₂PO₄, 25 mM HEPES, 10 mM glucose, 1% BSA, 60 mM creatine, pH 7.4.

Guanidinoacetate Uptake Medium: 85 mM NaCl, 4.8 mM KCl, 1.2 mM MgSO₄, 1.1 mM CaCl₂, 10 mM NaH₂PO₄, 25 mM HEPES, 10 mM glucose, 1% BSA, 30 mM guanidinoacetate, pH 7.4. Control Medium: 100 mM NaCl, 4.8 mM KCl, 1.2 mM MgSO₄, 1.1 mM CaCl₂, 10 mM NaH₂PO₄, 25 mM HEPES, 10 mM glucose, 1% BSA, pH 7.4.

Rinse Medium: 100 mM NaCl, 4.8 mM KCl, 1.2 mM MgSO₄, 1.1 mM CaCl₂, 10 mM NaH₂PO₄, 25 mM HEPES, 10 mM glucose, pH 7.4.

Rinse Medium (no glucose): 100-110 mM NaCl, 4.8 mM KCl, 1.2 mM MgSO₄, 1.1 mM CaCl₂, 10 mM NaH₂PO₄, 25 mM HEPES, pH 7.4.

Rinse Medium (no phosphate): 100 mM NaCl, 4.8 mM KCl 1.2 mM MgSO₄, 1.1 mM CaCl₂, 35 mM HEPES, 10 mM glucose, pH 7.4.

Rinse Medium (no glucose, no phosphate): 100-110 mM NaCl, 4.8 mM KCl, 1.2 mM MgSO₄, 1.1 mM CaCl₂, 35 mM HEPES, pH 7.4.

1% BSA was added to rinse media utilized during rephosphorylation following depletion.

Three compounds, ouabain, iodoacetate or cyanide were individually added to one of the rinse medium variants to
bring about phosphagen depletion. Concentrations employed were: Ouabain (0.25 mM), iodoacetate (2 mM) and cyanide (3 mM). 5 mM 3-hydroxybutyrate was typically substituted for glucose in incubations utilizing chick embryo hearts.

**Tissue Incubation:**

The procedure given here is that performed on chick embryonic skin tissue. Modifications of the procedure for incubations of muscle, brain and heart will be discussed afterward.

Fertilized chicken eggs were incubated at 37°C for a period of 10-15 days. On the experimental day, eggs were removed from the incubator, cracked, and the embryo extricated from the shell and amniotic sac. The neck of the embryo was severed and the head was rinsed with distilled water and placed on a paper towel. Grasping the head with a small pair of tweezers, a second set of tweezers was utilized to remove the scalp. This was most easily accomplished by pinching the skin at the rear of the head and pulling it forward to the eyes. Remaining portions of skin were then picked from the top of the head. The skin was placed in a glass petri dish containing 10 ml of rinse medium and was setting on ice in a larger petri dish. Following the collection of skin from a suitable number of embryos (usually 6-10), the tissue was transferred to a precooled petri dish. The tissue was then minced for a period of 10-15 seconds with a pair of razor blades held together. After the addition of
2-5 ml of uptake media, mincing was continued for an additional 15-20 seconds. The suspension was then transferred into a 50 ml Erlenmeyer flask which contained additional media to yield a total of 10 ml.

The flask and its contents were placed into a 37°C Dubnoff water bath and shaken at 150 strokes/minute. After the desired incubation time the flask was removed and its contents transferred to a preweighed 50 ml centrifuge tube. The tube was then centrifuged at 600 x g at room temperature for 30 seconds, resulting in a pelleting of the tissue. The tube was removed, the liquid poured off and the tube wiped with Kimwipes. Following the resuspension of the tissue in 10 ml of rinse medium, the tube was again centrifuged at 600 x g for 30 seconds at room temperature, the liquid poured off, and the tube wiped. Once again the tissue was resuspended in 10 ml of rinse medium and centrifuged, this time at 2000 x g at room temperature for 45 seconds. The tube was removed, the liquid poured off and the tube wiped and weighed. 5 ml of 0.4 M HClO₄ was added to the tube and the tube was placed on ice and its contents homogenized for one minute with a Tekmar Tissuemizer set at high speed. The tube was then centrifuged with a Sorvall refrigerated centrifuge at 17000 x g and -4°C for eight to ten minutes. The supernatant was poured off and a portion of this was neutralized with 4 M KOH, cooled on ice, centrifuged, the supernatant poured away from the precipitated KC10₄ and later assayed for metabolites. The remaining portion was heated at 65°C and
neutralized with KOH, followed by centrifugation to remove KC104.

Adjustments were made to this protocol if the tissue was to be stressed by some means, or resuspended in media for continued incubation. If the tissue was to be resuspended the third centrifugation was done at 600 x g after which the pellet was resuspended in 10 ml of the desired media and incubation continued on the shaking 37°C water bath. If the tissue was to undergo nitrogen anoxia no liquid was added after the final centrifugation, and the tube containing the pellet was flushed six to ten times with nitrogen, sealed and placed in a nonshaking water bath.

In addition to skin, three other embryonic tissues were studied - brain, muscle and heart, requiring slight modifications of procedure where necessary. The study of brain required the removal of the tissue from the skull beginning to form around it. The brain was removed (usually intact) with a pair of tweezers, and the lower portion of the brainstem removed. The brains from 2-4 embryos, 11 to 13 days old, were then placed in media and minced for 30-45 seconds and the remainder of the protocol carried out.

Chick embryo muscle was acquired both from the breast and legs of 12-day-old embryos. Tissue from 2 embryos was pooled and minced in uptake media for 45 seconds and the above protocol followed.

The study of chick embryo heart required several different manipulations. The chest was opened and the heart
removed and placed in ice cold media. After 3 to 5 hearts were accumulated, the hearts were placed in a precooled Pyrex dish and sliced into quarters with a pair of razor blades. The quartered sections were placed into a 50 ml flask containing 10 ml of uptake media which contained 5 mM 3-hydroxybutyrate instead of 10 mM glucose. The flask was then flushed with 100% O₂ for 30 seconds, sealed with a rubber stopper and placed in the shaking water bath and the incubation carried out as described above. Bovine serum albumin, necessary to prevent aggregation of minces, was sometimes omitted from the incubation media for quartered hearts; no effect was noted. Except for experiments involving the study of accumulation of cyclocreatine-P with embryo age, in which 10 to 18-day-old embryos were used, hearts were obtained from 13 or 14-day-old embryos.

Assays

Creatine was assayed by the α-naphthol/diacetyl method (Dubnoff, 1957). To 1 ml of neutralized sample plus water is added 1 ml of 2% alpha-naphthol dissolved in stock base containing 1.5 M NaOH, 1.5 M Na₂CO₃. One ml of diacetyl, diluted 1:2000 in water is added, the solution vortexed and allowed to stand for 15 minutes. Following addition of 7 ml of water, the absorbance is read at 525 nm. To obtain creatine-P measurements, two samples from each perchloric acid extract were measured. One sample was the cold, neutralized portion, while the other was a portion which was
heated at 65°C for fifteen minutes prior to neutralization. Heating resulted in hydrolysis of the labile phosphate bond, allowing the liberated creatine to be measured with the assay. As a result the value obtained for the heated sample represented free creatine plus creatine-P. The nonheated sample represented only the free creatine. Hence, subtracting the value obtained for the cold sample from that obtained for the heated one yielded the amount of creatine-P present.

Guanidinoacetate was assayed by the Sakaguchi method (Van Pilsum and Carlson, 1970). One ml of neutralized sample plus water is put in a test tube and placed on ice. To this was added 1 ml of a solution containing 10% NaOH and 2% thymine, followed by vortexing and the addition of 1 ml of 0.1% α-naphthol in 95% ethanol. After vortexing, the test tube was allowed to set on ice for 5 minutes. One ml of 50% Chlorox was then added and the solution again vortexed. Following the addition of 4 ml of water, the absorbance was read at 525 nm. Guanidinoacetate-P was determined by the differential treatment of two portions of a sample, as in the case of creatine-P (see above).

Cyclocreatine was assayed by the pentocyanoaminoferrate (PCAF) method devised by Griffiths and Walker (1976). To 1 ml of neutralized sample plus water was added 2 ml of 30 mM Na₂CO₃, followed by vortexing. Following the addition of 0.5 ml of a 1% aqueous solution of PCAF aged at least 2 days and subsequent vortexing, the solution was allowed to stand for
10-20 minutes. The absorbance was then read at 605 nm. Cyclocreatinine-P can be determined by the differential treatment of two portions of a sample, one neutralized portion kept cold and the other heated in 0.4 M HCl at 65°C for 45 to 60 minutes prior to neutralization and assay.

Adenosine triphosphate was assayed by a coupled enzyme method (Lamprecht and Trautschold, 1974). In a 1 ml cuvette was added 0.4 ml of sample plus water, 0.5 ml of buffer containing 0.6 M triethanolamine-HCl, 6 mM glucose and 14 mM MgSO₄, neutralized to pH 7.5 with NaOH, 0.1 ml of 4 mM nicotinamide adenine dinucleotide phosphate and 3 units of the enzyme glucose-6-phosphate dehydrogenase. After incubation at 30°C for 2.5 minutes the absorbance of the mixture was read at 340 nm. Following the addition of 2 units of hexokinase to yield a final total volume of 1.02 ml, the sample mixture was allowed to react for an additional 7.5 minutes. The absorbance was again read at 340 nm and the ATP calculated from the differential absorbance.

Results

Chick embryo brain work begun by Dennis Woznicki was continued by this author. Rapid accumulation of creatine analogues accompanied by phosphorylation of these analogues was accomplished by incubating chick embryo brain in vitro in a solution containing the analogue in uptake media.

Cyclocreatinine was loaded into chick embryo brain minces. When the amount of cyclocreatinine in the uptake media was
varied, the amount of cyclocreatine taken up and phosphorylated by the brain minces was altered correspondingly (Figure 3). When incubated with 3 mM cyclocreatine the minces took up and phosphorylated in 2 hours 4 μmol/g of cyclocreatine. By increasing the concentration of cyclocreatine in the incubation media to 10 mM the amount of cyclocreatine-P accumulated by the tissue could be nearly doubled. Increasing the concentration of cyclocreatine in the media to 150 mM resulted in a second doubling to a level of 16 μmol/g of cyclocreatine-P. As the amount of cyclocreatine in the media was increased the brain minces were not able to continue phosphorylation of the incoming cyclocreatine at the same rate as was possible with low cyclocreatine levels in the media. Thus, in increasing the concentration of cyclocreatine from 3 mM to 150 mM the percentage of cyclocreatine phosphorylated in the tissue dropped from 68% to 36%.

In addition, it can be seen in Figure 4 that levels of ATP decline as cyclocreatine concentration is increased. Even though an energy source, glucose, was provided in the media, ATP production could not keep pace with its utilization to phosphorylate the incoming cyclocreatine between 2 and 4 hours of incubation for all concentrations. Ionic displacement by the accumulated cyclocreatine-P may also contribute to this effect. The trauma of excision and mincing of the brain causes a loss of initial high-energy in phosphate levels (Woznicki, 1981). Thus, upon incubation in
Figure 3. Accumulation of cyclocreatine-P by chick embryonic brain minces incubated in uptake media containing various concentrations of cyclocreatine. Numbers in parentheses indicate the percentage of the total cyclocreatine pool which is phosphorylated, in this and succeeding figures in this chapter.
**Figure 4.** Alteration of ATP levels of chick embryonic brain minces elicited by incubation of the minces in uptake media containing various concentrations of cyclocreatine.
media containing glucose, levels of ATP actually increased in brains after 2 hours in media containing lower concentrations of cyclocreatine. ATP levels decreased from a high of almost 2 \( \mu \text{mol/g} \) following 2 hours of incubation in media containing no cyclocreatine to only 0.8 \( \mu \text{mol/g} \) when 150 mM cyclocreatine was present in the minces.

In the experiment presented in Figure 5 embryonic brains were not minced but were incubated whole. During shaking while incubating, the brains usually broke apart into their 5 component lobes. It was a point of interest to see how much effect mincing or not mincing would have on the uptake properties of the brain. The whole brain acquires cyclocreatine at a slower rate, presumably due to the fact that there is less surface area exposed to the incubation mixture. The cyclocreatine taken up was phosphorylated to a lesser degree than in minced tissue.

Woznicki and Walker (1979) found that the cyclocreatinine-P formed in embryonic brain minces could be depleted by further incubation in a high potassium medium or in medium containing the potassium ionophore, valinomycin. In Figure 6 a third method of depleting cyclocreatine-P stores is described. Following 90 minutes of incubation in media containing cyclocreatine, the minces were washed and 2 mM iodoacetate was added. It can be seen that iodoacetate appeared to bring about a slight depletion of the cyclocreatine-P. Iodoacetate alkylates thiol groups and as such acts to inhibit the enzyme glyceraldehyde-3-phosphate
Figure 5. Time course of accumulation of cyclocreatinine-P by whole (nonminced) chick embryonic brains. Brains were incubated in 60 mM cyclocreatinine uptake medium.
Figure 6. Depletion of cyclocreatinine-P and ATP in chick embryonic brain minces by iodoacetate. Minces were incubated in uptake media with 30 mM cyclocreatinine (●) or without cyclocreatinine (■) for 90 minutes. At time X minces were washed and then incubated in rinse medium containing 2 mM iodoacetate, no glucose, no phosphate, and no cyclocreatinine (●,■), or in rinse medium containing no glucose, no phosphate, and no cyclocreatinine (Ο,□).

A) Depletion of cyclocreatinine-P by iodoacetate.

B) Corresponding depletion of ATP by iodoacetate.
dehydrogenase. In doing so iodoacetate blocks the glycolytic pathway resulting in depression of ATP production. In brain minces inhibited with iodoacetate, ATP levels decreased to zero within 2 hours. This occurred in both cyclocreatine-loaded minces and control (unloaded) brain minces.

After initial experimentation with brain minces, it was decided to perform studies with other embryonic tissues; with the expectation that these might prove to be interesting models for further creatine analogue study. The first tissue to be experimented with was chick embryo muscle, which was obtained from both the breast and the legs of 12-day-old embryos and was minced for about 45 seconds prior to incubation in uptake medium containing cyclocreatine. In Figure 7 it is seen that cyclocreatine was taken up and phosphorylated by the muscle mince, with accumulation increasing with the amount of incubation time. However, only small amounts of cyclocreatine-P were accumulated by the mince. In two hours of incubation only 3 µmol/g were accumulated, compared with 13 µmol/g in embryonic brain minces. In light of this finding it was decided to continue experimentation with other tissues, with the expectation that a more active one might be found.

The next tissue studied was chick embryo skin, which was also minced. Tissue from 10 to 15 day-old embryos was obtained from the head of the embryo and was, essentially, the scalp. Cyclocreatine was readily taken up by these embryonic skin minces (Figure 8). Additional work indicated
Figure 7. Time course of accumulation of cyclocreatine-P by chick embryonic skeletal muscle minces. Minces were incubated in 60 mM cyclocreatine uptake medium.
Figure 8. Time course of accumulation of cyclocreatine-P by chick embryonic skin minces. Minces were incubated in 60 mM cyclocreatine uptake medium.
that within an hour almost maximal uptake was achieved, around 20 μmol/g. With continued incubation phosphorylation of the cyclocreatinine increases, achieving a maximum of 14-15 μmol/g of cyclocreatinine-P.

The age of the embryo had a distinct effect on its ability to uptake and phosphorylate cyclocreatinine. Figure 9 reveals a sharp profile of this age related function. On day 10 less than 1 μmol/g of cyclocreatinine-P was accumulated by the skin minces. The ability to acquire cyclocreatinine-P increased rapidly with age; it appeared to peak on days 13 and 14 of age. It is possible that in epidermal skin cells of embryos younger than 10 days old creatine kinase function may be insufficient to bring about synthesis of much cyclocreatinine-P, in addition to a decreased ability of the cells to take up cyclocreatinine, and that after day 14 functional integrity begins to be lost when the tissues are minced.

Subjecting minced embryonic skin to anoxia resulted in utilization of the acquired cyclocreatinine-P. As seen in Figure 10 the minces were first preloaded with cyclocreatinine and after two hours of uptake the cells were washed, pelleted, flushed with nitrogen, and incubated for an additional hour. If, as in this experiment, the cells were then removed from the nitrogen and supplied with media containing an energy source once again, the previously acquired cyclocreatinine was rephosphorylated.
Figure 9. Effect of age of embryo on accumulation of cyclocreatine-P by chick embryonic skin minces. Minces were incubated for 4 hours in 60 mM cyclocreatine uptake medium. Tissues on day 15 were incubated under O₂; tissues from other days were incubated with air.
Figure 10. Reversible depletion of cyclocreatinine-P in chick embryonic skin minces by nitrogen anoxia. Minces were incubated for 2 hours in 60 mM cyclocreatinine uptake medium. At time X minces were washed with medium containing no glucose or cyclocreatinine, pelleted, flushed with nitrogen and incubated for 1 hour. At time Y minces were resuspended in rinse medium containing 10 mM glucose and incubated under air for an additional hour.
Two compounds were utilized in a further attempt to deplete cyclocreatinine-P stores in embryonic skin minces. Neither of these, deoxyglucose nor histamine, appeared to result in much depletion.

As a final point of interest, cyclocreatinine was assayed in the skin of chicks which had been fed 1% cyclocreatinine for 16 days. The skin was found to contain 6.4 µmol/g of total cyclocreatinine, 71% of which was phosphorylated to yield a total of 4.5 µmol/g of cyclocreatinine-P.

The final embryonic tissue studied was heart. Early experiments revealed that glucose did not appear to be the optimal energy source. Accumulation of cyclocreatinine-P by embryonic heart minces appeared to be enhanced by the substitution of 3-hydroxybutyrate for glucose in the media when the tissue was incubated under O₂ (Figure 11). While results of other independent experiments also indicated increased accumulation of the phosphagen with usage of 3-hydroxybutyrate, additional experiments are required to verify the effect. It is known that under normal circumstances the heart metabolizes fatty acids as its major fuel, using glycolysis as an emergency energy source. It appears that the embryonic heart is able to utilize the ketone body 3-hydroxybutyrate, which can be converted to acetoacetate and then on to form two acetyl-CoA molecules and oxidized via the Krebs cycle, more readily than it can utilize glucose. Two other possible energy substrates, acetate and pyruvate, were utilized in an experiment to
Figure 11. Effect of age of embryo and exogenous energy source on accumulation of cyclocreatine-P by chick embryonic heart minces. Minces were incubated under O_2 for 3 hours in 40mM cyclocreatine uptake medium containing either 10 mM glucose (■) or 5 mM 3-hydroxybutyrate (●).
determine accumulation of creatine-P. Neither of these resulted in as much accumulation of creatine-P as did 3-hydroxybutyrate.

It was further found in early experiments utilizing glucose that mincing the heart tissue did not appear to be the best protocol possible (Table 2). Incubating the whole heart in media appeared to result in a greater accumulation of cyclocreatine-P. Cutting the hearts in half appeared to result in an even greater accumulation, while quartering the hearts appeared to result in maximum performance. In Figure 12 it is seen that the superiority of accumulation of quartered hearts to minced hearts also appeared to occur when 3-hydroxybutyrate was utilized as the energy source. This milder treatment resulted in a near doubling of acquisition of the phosphorylated compound. It was also seen that embryonic heart was not as sensitive to the age of embryo as embryonic skin in terms of phosphorylation ability.

Figure 13 illustrates the uptake and phosphorylation of creatine or cyclocreatine over a period of time in separate incubations. While the amounts of total creatine and total cyclocreatine taken up were approximately equal, a greater percentage of cyclocreatine was phosphorylated than was creatine, illustrating the lower thermodynamic potential of cyclocreatine-P.

Quartered embryonic hearts rapidly depleted preaccumulated phosphorylated creatine analogues when subjected to anoxia. When, following anoxia, the hearts were incubated
**TABLE 2. EFFECT OF VARIOUS CHOPPING TECHNIQUES ON ACCUMULATION OF CYCLOCREATINE-P BY CHICK EMBRYO HEART**

<table>
<thead>
<tr>
<th>CHOPPING&lt;sup&gt;a&lt;/sup&gt;</th>
<th>CYCLOCREATINE-P (µmol/g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>NONE</td>
<td>6.58</td>
</tr>
<tr>
<td>HALVED</td>
<td>7.25</td>
</tr>
<tr>
<td>QUARTERED</td>
<td>9.25</td>
</tr>
<tr>
<td>30 SECOND MINCE</td>
<td>4.71</td>
</tr>
<tr>
<td>60 SECOND MINCE</td>
<td>4.22</td>
</tr>
</tbody>
</table>

<sup>a</sup>Hearts were removed from 15-day-old chick embryos, chopped into the fractions described or minced for a period of time and incubated in 40 mM cycocreatine uptake medium containing 10 mM glucose for a period of 3 hours. Samples were then assayed for accumulation of cycocreatine-P.
**Figure 12.** Effect of age of embryo and degree of chopping on accumulation of cyclocreatine-P by chick embryonic hearts. Hearts were quartered (●) or minced (■) and incubated under \( O_2 \) for 3 hours in 40 mM cyclocreatine uptake medium containing 5 mM 3-hydroxybutyrate.
Figure 13. Time course of phosphagen accumulation by quartered chick embryonic hearts. Hearts were incubated under $O_2$ in either 60 mM creatine uptake medium (■) or 40 mM cyclocreatine uptake medium (●), both containing 5 mM 3-hydroxybutyrate.
once again in media containing an energy source, accumulated analogues were rephosphorylated. Figures 14, 15, and 16 illustrate this roller coaster treatment of embryonic heart incubated with creatine, cyclocreatine or guanidinoacetate. First the tissue was preloaded with the analogue for a period of two hours, allowing a substantial uptake and phosphorylation of the analogue. The hearts were then washed, pelleted and subjected to anoxia under nitrogen for an hour followed by an additional hour of incubation with media containing 3-hydroxybutyrate.

After the initial two hours of incubation more than half of the accumulated cyclocreatine was phosphorylated while only about a third of the accumulated guanidinoacetate and creatine were phosphorylated. Subjecting the tissues to anoxia resulted in a rapid depletion of creatine-P until none remained after 30 minutes. Cyclocreatine-P and guanidinoacetate-P were also depleted by anoxia but to a lesser extent than creatine-P. In each case some of the phosphorylated analogue remained following an hour of anoxia. Resuspension of these tissues in media for an hour resulted in a rephosphorylation of the accumulated analogues to a degree approximately equal to preanoxic levels, illustrating that each of these compounds is able to serve as a functional phosphagen in embryonic heart tissue. Paired with the analogue analysis are curves depicting the effects of the treatment on endogenous ATP. It was observed that ATP levels responded in much the same way in each of the three
Figure 14. Reversible depletion of guanidinoacetate-P and ATP in quartered chick embryonic hearts by nitrogen anoxia. Hearts were incubated under O₂ for 2 hours in 30 mM guanidinoacetate uptake medium containing 5 mM 3-hydroxybutyrate. At time X hearts were washed with medium containing no guanidinoacetate, no 3-hydroxybutyrate and no phosphate, or guanidinoacetate, pelleted, flushed with nitrogen and incubated for 1 hour (●). At time Y hearts were resuspended in rinse medium containing 5 mM 3-hydroxybutyrate but no phosphate and incubated under O₂ for an additional hour. An additional sample was incubated in the initial uptake medium for 3 hours (O). A) Reversible depletion of guanidinoacetate-P by anoxia. B) Corresponding reversible depletion of ATP by anoxia.
Figure 15. Reversible depletion of creatine-P and ATP in quartered chick embryonic hearts by nitrogen anoxia. Hearts were incubated under $O_2$ for 2 hours in 60 mM creatine uptake medium containing 5 mM 3-hydroxybutyrate. At time X hearts were washed with medium containing no creatine, no 3-hydroxybutyrate and no phosphate, pelleted, flushed with nitrogen and incubated for 1 hour (●). At time Y hearts were resuspended in rinse medium containing 5 mM 3-hydroxybutyrate but no phosphate or creatine, and incubated under $O_2$ for an additional hour. An additional sample was incubated in the initial uptake medium for 3 hours (○). A) Reversible depletion of creatine by anoxia. B) Corresponding reversible depletion of ATP by anoxia.
**Figure 16.** Reversible depletion of cyclocreatine-P and ATP in quartered chick embryonic hearts by nitrogen anoxia. Following a 2 hour incubation carried out under $O_2$ in 40 mM cyclocreatine uptake medium, containing 5 mM 3-hydroxybutyrate, hearts were washed with medium containing no cyclocreatine, no 3-hydroxybutyrate and no phosphate, pelleted, flushed with nitrogen and incubated for 1 hour (●). Hearts were then resuspended in rinse medium containing 5 mM 3-hydroxybutyrate but no phosphate and incubated under $O_2$ for an additional hour. An additional sample was incubated in the initial uptake medium for 3 hours (○). A) Reversible depletion of cyclocreatine-P by anoxia. B) Corresponding depletion of ATP by anoxia.
analogue-loaded tissues. During uptake ATP stayed constant, decreased during anoxia, and then returned to preanoxia levels within an hour following reincubation in 3-hydroxybutyrate-containing media. Other experiments in which nitrogen depletion was continued for three hours in quartered hearts which were incubated in media containing no analogue (unloaded) or loaded with creatine, cyclocreatine or guanidinoacetate were performed. The results indicated that in each tissue ATP levels had decreased to less than 0.3 \( \mu \text{mol/g} \) within two hours, accompanied by a decrease in amount of phosphorylation of accumulated analogue to a level of 5% or less.

Figure 17 illustrates an experiment in which cyanide, instead of anoxia, was used to insult preloaded tissue. Cyanide is an inhibitor of the respiratory chain, blocking electron transport from cytochrome aa\(_3\) to oxygen, resulting in an inability of the pathway to generate ATP. This effect was observed as ATP levels appeared to decrease slightly and previously accumulated creatine-P was depleted by the embryonic heart upon incubation with cyanide. Additional experimentation is necessary to verify the decrease in ATP.

A second compound, ouabain, was also utilized to place an energy drain on the system (Figure 18). Ouabain is a cardiac glycoside which inhibits Na\(^+\)-K\(^+\) ATPase and brings about a positive inotropic response in cardiac tissue. Application of ouabain to embryonic heart preloaded with creatine or cyclocreatine resulted in a depletion of
Figure 17. Depletion of creatine-P and ATP in quartered chick embryonic hearts by cyanide. Hearts were incubated under $O_2$ for 2 hours in 60 mM creatine uptake medium containing 5 mM 3-hydroxybutyrate. At time $X$ hearts were washed and then incubated under $O_2$ in rinse medium containing 3 mM sodium cyanide and 5 mM 3-hydroxybutyrate but no creatine ($\bullet$) or in rinse medium containing 5 mM 3-hydroxybutyrate but no creatine ($\circ$). A) Depletion of creatine-P by cyanide. B) Corresponding depletion of ATP by cyanide.
Figure 18. Depletion of creatine-P, cyclocreatine-P or ATP in quartered embryonic hearts by ouabain. Hearts were first incubated under $O_2$ for 2 hours in either 60 mM creatine uptake medium (■) or 40 mM cyclocreatine uptake medium (●), both containing 5 mM 3-hydroxybutyrate. At time X hearts were washed and incubated under $O_2$ either in rinse medium containing 0.25 mM ouabain, no creatine, or cyclocreatine and no phosphate or 3-hydroxybutyrate (●,■), or in rinse medium containing no creatine or cyclocreatine, and no phosphate or 3-hydroxybutyrate (○,□). A) Depletion of cyclocreatine-P or creatine-P by ouabain. B) Corresponding depletion of ATP by ouabain. Points represent 1-3 samples ± SEM for points representing 3 samples.
creatine-P or cyclocreatine-P accompanied by a decrease in the ATP levels of both preloaded tissues.

Two other compounds, isoproterenol and veratrine, were examined to determine if they brought about depletion of accumulated phosphorylated creatine analogues in embryonic hearts. The first of these, isoproterenol, a β-adrenergic-agonist, appeared to result in only a slight depletion, if any, of accumulated cyclocreatine-P. The second, veratrine, a mixture of several alkaloids, brought about depletion of accumulated creatine-P in embryonic heart tissue.

In additional work done on embryonic tissues, the effect of the addition of ATP to uptake media on the accumulation of creatine-P or cyclocreatine-P in brain or heart yielded surprising results (Figures 19 and 20). When embryonic hearts were incubated with cyclocreatine in the presence of ATP, cyclocreatine-P accumulation appeared to increase upon the addition of increasingly larger amounts of ATP to the media. Embryonic brain tissue, on the other hand, accumulated the same amount of cyclocreatine-P, whether or not ATP was included in the incubation media. The accumulation of creatine-P by embryonic brain showed the same type of behavior; accumulation was not correlated with increased ATP in the media. Embryonic heart tissue, however, appeared to demonstrate a correlation of creatine-P accumulation with increased ATP in the media, with creatine-P appearing to double in the presence of 20 mM ATP. ATP levels in heart and brain incubated in media containing ATP are also
Figure 19. Effect of the addition of various concentrations of ATP to the incubation medium on the levels of accumulated cyclocreatine-P or ATP in quartered chick embryonic hearts or chick embryonic brain minces. Brain minces (■) and quartered hearts (●) were incubated for 2 hours under \( O_2 \) in 40 mM cyclocreatine uptake medium containing various concentrations of ATP. Media for brain minces also contained 10 mM glucose, while media for quartered hearts contained 5 mM 3-hydroxybutyrate. A) Accumulation of cyclocreatine-P with addition of various ATP concentrations to the medium. B) Levels of ATP in tissue with addition of various ATP concentrations to the medium.
Figure 20. Effect of the addition of various concentrations of ATP to the incubation medium on the levels of accumulated creatine-P or ATP in quartered chick embryonic hearts or chick embryonic brain minces. Brain minces (■) and quartered hearts (●) were incubated for 2 hours under O<sub>2</sub> in 60 mM creatine uptake media containing varied concentrations of ATP. Media for brain minces also contained 10 mM glucose, while media for quartered hearts contained 5 mM 3-hydroxybutyrate. A) Accumulation of creatine-P with addition of various ATP concentrations to the medium. B) Levels of ATP in tissue with addition of various ATP concentrations to the medium.
shown in Figures 19 and 20.

Further work was also performed on three of the tissues - embryonic brain, skin and heart. Cyclocreatine uptake was studied in each of these tissues in the presence of 10 mM phosphate, and in the absence of phosphate. The results of preliminary experiments appeared to indicate that skin was the most dependent of the tissues on the presence of phosphate in the media for cyclocreatine-P accumulation; brain was slightly less dependent while heart demonstrated only a slight increase in accumulation upon addition of phosphate. Based on the analysis of two samples per tissue, skin contained less endogenous inorganic phosphate than heart or brain. Skin contained about 1.7 \( \mu \text{mol/g} \) of inorganic phosphate, while heart contained 4.0 \( \mu \text{mol/g} \) and brain 4.4 \( \mu \text{mol/g} \).

**Summary**

It was found that several embryonic tissues took up and phosphorylated creatine analogues when these analogues were present in specified media incubated with the separated tissues. Chick embryo heart, skin, and muscle tissue, in addition to brain were able to do so with heart, skin and brain able to accumulate relatively large amounts of phosphorylated analogues. Heart and skin uptake were characterized with respect to embryo age and duration of incubation time. In an attempt to optimize accumulation of phosphagens by heart, various incubation techniques were
utilized; including the quartering of hearts, instead of mincing, prior to incubation and the inclusion of 3-hydroxybutyrate in the media as an energy source, instead of glucose. It has further been found that the addition of ATP to media appeared to increase the accumulative ability of embryonic heart but not of embryonic brain.

It has been established that in embryonic heart and skin previously accumulated phosphorylated analogues can serve as functional phosphagens when subjected to nitrogen anoxia followed by incubation in media containing an energy source. The utilization of three compounds, iodoacetate, ouabain and cyanide to insult brain or heart tissue and apparently cause a depletion of previously acquired phosphagens has been described. The work presented has demonstrated the ability of several different chick embryonic tissues to serve as model systems for in vitro study of creatine analogues.
Chapter III: Chick Liver Amidinotransferase

Creatine can be synthesized by a wide variety of species. Biosynthesis is carried out through the actions of two enzymes: L-arginine:glycine amidinotransferase (EC 2.1.4.1) and S-adenosylmethionine:guanidinoacetate N-methyltransferase. Amidinotransferase catalyzes the initial reaction:

Arginine + glycine $\rightleftharpoons$ guanidinoacetate + ornithine (III-1),
while the second reaction is catalyzed by methyltransferase:

Guanidinoacetate + S-adenosylmethionine $\rightarrow$ Creatine + S-adenosylhomocysteine.

Amidinotransferase is found in a number of tissues with some variance in tissue localization between species. It is found in a number of mammalian livers including man, monkey and hog; but is not found in the livers of other mammals - rabbit, mouse, rat and dog. Amidinotransferase is also found to a large extent in mammalian pancreas and kidney. The second enzyme of the pair, methyltransferase, is found in large amounts in mammalian liver and pancreas and to a lesser degree in kidney.

Amidinotransferase has been localized in the mitochondria both in chick liver and in rat kidney. It is found in the mitochondrial matrix space in chick liver (Grazi et al., 1975) while being localized in the inner mitochondrial membrane in rat kidney (Mauri et al., 1975).
The apparent molecular weight of amidinotransferase has been found to be about 100,000 in hog kidney (Conconi and Grazi, 1965) and 83,000 in rat kidney, with the rat kidney enzyme appearing to be a dimer (McGuire et al, 1980). Transamidination has been found to occur by a double-displacement mechanism. First suggested by Walker, (1956) this mechanism was verified by Grazi and coworkers (1965) as follows:

\[
\text{Amidinotransferase + arginine} \quad \underset{\text{amidinotransferase-amidine + ornithine}}{\longrightarrow} \quad (\text{III-3})
\]

\[
\text{Amidinotransferase-amidine + glycine} \quad \underset{\text{amidinotransferase + guanidinoacetate}}{\longrightarrow} \quad (\text{III-4})
\]

A number of different compounds can act as substrates for the enzyme. Possible amidine donors include canavanine, 3-guanidinopropionate and 4-guanidinobutyrate while canaline and hydroxylamine can act as amidine acceptors (Walker, 1979). Along with other possible substrates, the coupling of any of these acceptors with donors results in a large number of possible assays for amidinotransferase.

In the first observations of endproduct repression in higher animals, it was found by Walker (1959) in rat kidney and more markedly by Walker (1960) in chick liver that amidinotransferase levels are subject to feedback repression by creatine and furthermore in chick liver by certain analogs of creatine (Walker and Hannan, 1976). McGuire and coworkers (1984) found that creatine exerted its regulatory action on amidinotransferase at the level of cellular
concentration of amidinotransferase mRNA. Several other factors also alter amidinotransferase levels. These include starvation, which causes a rapid decrease in amounts of amidinotransferase in chick liver, (Walker, 1961) and growth hormone and thyroxine, which are believed to be essential in maintaining the levels of amidinotransferase protein in rat kidney (McGuire et al, 1980).

Amidinotransferase has been purified from two tissue sources - hog kidney and rat kidney. Conconi and Grazi (1965) used a seven-step procedure to purify hog kidney 900-fold. Kidneys were homogenized and extracted with acetone followed by treatment with ammonium sulfate and a 15 minute period of heating. After adsorption to calcium phosphate gel the enzymic fraction was passed through three columns-phosphocellulose, DEAE-cellulose and Sephadex G-100 to yield the final purified enzyme. The rat kidney enzyme was purified by McGuire and coworkers (1980). Following homogenization of the kidney the supernatant solution was applied to a DEAE-cellulose column yielding two peaks of amidinotransferase labelled by the researchers as α and β. These were purified on a phenyl-sepharose column to result in a 138-fold and 157-fold purification respectively for the α and β fractions. In further studies the α and β fractions were found to have similar characteristics for all properties studied.

This researcher attempted to purify amidinotransferase from a different tissue, chick liver. A 160-fold purifica-
tion was obtained, using an eight step procedure which with further refinement could be reduced to a six step method.

**Methods**

 Amidinotransferase was assayed by the method of Walker (1960) in which the amount of hydroxyguanidine formed from arginine plus hydroxylamine is determined.

Arginine + NH$_2$OH $\rightleftharpoons$ ornithine + hydroxyguanidine (III-5)

First, an incubation mixture was prepared, consisting of 0.1 ml of 1 M arginine-HCl, 0.1 ml of 1 M potassium phosphate buffer, pH 7.4, 0.3 ml of 2 M NH$_2$OH-HCl dissolved in 2 M KOH and 0.5 ml of enzyme solution plus water. The mixture was incubated at 37°C for one hour and then the reaction stopped with 0.4 ml of 30% trichloroacetic acid. Next 1 ml of water was added and the mixture was allowed to stand for 10 minutes. Following centrifugation a 1.5 ml aliquot was removed. To this was added the following solutions: 0.5 ml of water, 2.0 ml of 1 M potassium phosphate buffer, pH 7.0, 3 ml of acetone and 0.3 ml of an aged 1% pentacyanoaminoferate solution. After mixing, the solution was allowed to stand for 10 minutes and absorbance read at 480 nm. A blank was prepared by substituting 0.6 M ornithine-HCl for arginine-HCl while a standard was prepared from hydroxyguanidine-1/2 H$_2$SO$_4$. A unit of amidinotransferase was defined as the amount of enzyme that catalyzes the formation of 1 μmole of hydroxyguanidine per hour in the assay described above.
Protein concentration was determined from absorbance at 280 nm where $A_{280} = 1.5$ for 1 mg/ml (Conconi and Grazi, 1965) for calculation of specific activity (units per milligram of protein).

Results - Procedure for Partial Purification

Chicks were anaesthetized with ether and decapitated eleven to twelve days after hatching. Livers were removed, diced with scissors and placed in a precooled Waring blender. Four volumes of cold distilled water were added and the mixture was homogenized for thirty seconds. The resulting homogenate was then frozen and stored for several months.

Supernatant - Homogenate was removed from the freezer, thawed and centrifuged for 30 minutes at 35000 x g and the supernatant was pipetted off.

Ammonium Sulfate Fractionation - To 84 ml of supernatant was added 28 ml of 160 mM triethanolamine (TEA), 2 mM $\text{Na}_4\text{EDTA}$, pH 7.4. To this was added 31 g of ammonium sulfate to yield a 45% saturated solution. After 20 minutes of stirring at 5°C the solution was centrifuged at 10000 x g at 2°C for 10 minutes. 112 ml of supernatant were collected and 14.8 g of ammonium sulfate was added to yield a 65% saturated solution. The solution was again stirred and centrifuged. The resulting precipitate was dissolved in 20 ml of 40 mM TEA, 0.5 mM $\text{Na}_4\text{EDTA}$. This 45%-65% ammonium sulfate cut was placed in cellulose dialysis tubing and dialyzed against 40 mM TEA, 0.5 mM $\text{Na}_4\text{EDTA}$. 
DEAE-cellulose Chromatography, first - To a DEAE-cellulose, medium mesh, column (1.5 cm x 42 cm) prewashed with NaOH, HCl and H₂O and preequilibrated with 40 mM TEA, 0.5 mM Na₄EDTA, was added 31 ml of the 45-65% ammonium sulfate fraction. The column was washed with the same buffer and approximately 60 fractions collected. At this point a 0 to 0.15 M NaCl gradient was begun and another 60 fractions totaling approximately 330 ml were collected. The enzyme came off the column in two fractions (Figure 21). The first, and larger fraction, came off shortly after the solution was placed on the column. The second fraction came off as the salt gradient was being run. Fractions from both peaks containing enzyme were pooled and ammonium sulfate was added to yield an 80% saturated solution. After stirring and centrifugation, the precipitate was dissolved in 15 ml of 40 mM TEA, 0.5 mM Na₄EDTA buffer. The solution was placed in cellulose tubing and dialyzed against the same buffer for six hours.

DEAE-cellulose Chromatography, second - The same procedure as above was utilized. After adding 22 ml of the dialyzed solution, 60 fractions totaling approximately 320 ml were collected and a 0 M NaCl to 0.2 M NaCl gradient was begun followed by collection of 50 more fractions totaling approximately 310 ml. The enzyme once again came off the column in two fractions which were pooled. Ammonium sulfate was added to yield an 80% saturated solution which was stirred and centrifuged. To the resulting precipitate was
Figure 21. Chromatography of chick liver amidinotransferase on DEAE-cellulose, first column. Enzyme activity (●) and amount of protein (○) are shown. A 0 to 0.15 M NaCl gradient was begun after the collection of 60 fractions.
added 10 ml of 20 mM potassium phosphate, pH 6.8. This solution was placed in cellulose tubing and dialyzed against the same buffer for five hours.

**Hydroxylapatite Chromatography** - To a hydroxylapatite column (1.5 cm x 44 cm) preequilibrated with 20 mM potassium phosphate, pH 6.8 was added 13.5 ml of the dialyzed DEAE-cellulose fraction. The column was washed with the same buffer and approximately 20 fractions totaling 80 ml were collected. At this point a 0.02 to 0.8 M potassium phosphate concentration gradient was begun and another 75 fractions totaling approximately 340 ml were collected. Amidinotransferase activity was found in the fractions collected following the initiation of the potassium phosphate gradient (Figure 22). Tubes containing a high amidinotransferase/protein ratio were selected and pooled. To this solution was added enough ammonium sulfate to yield an 80% saturated solution. After stirring and centrifugation the precipitate was dissolved in 3 ml of 40 mM TEA, 0.5 mM Na₄EDTA, pH 7.4. This solution was placed in cellulose tubing and dialyzed against the same buffer for five hours.

**Phenyl-agarose Chromatography** - A phenyl-agarose column (1 cm x 8 cm) was prepared from a solution containing phenyl-agarose, 40 mM TEA, 0.5 mM Na₄EDTA and a trace of NaCl and arginine. After preequilibration with the 40 mM TEA, 0.5 mM Na₄EDTA solution, 3.2 ml of the dialyzed hydroxylapatite fraction was applied. The column was washed with the same buffer. All of the amidinotransferase activity washed
Figure 22. Chromatography of chick liver amidinotransferase on hydroxylapatite. Enzyme activity (●) and amount of protein (○) are shown. A 0.02 M to 0.8 M potassium phosphate gradient was begun after the collection of 20 fractions.
PROTEIN (mg)

K_M (M)

PROTEIN

ATase

ENZYME ACTIVITY (units)

HYDROXYLAPATITE FRACTIONS
straight through the column. Two tubes containing the most activity were pooled.

Arginine-agarose Chromatography, first - An arginine-agarose column (0.7 cm x 9 cm) was prepared from a solution containing arginine-agarose, 40 mM TEA, 0.5 mM Na₄EDTA and a trace of stabilizing lactose. After preequilibration with the 40 mM TEA, 0.5 mM Na₄EDTA solution, 4.7 ml of the phenyl-agarose fraction was applied and the column washed with the same buffer. The amidinotransferase activity came off the column quite quickly and the fractions containing the enzyme were combined.

Arginine-Agarose, second - The arginine-agarose column (0.7 cm x 9 cm) was repoured and preequilibrated with 40 mM TEA, 0.5 mM Na₄EDTA buffer. 4.9 ml of the arginine-agarose fraction was applied and the column was washed with the same buffer.

A 160-fold purification of chick liver amidinotransferase by the method illustrated was achieved (Table 3). Comparing the purification of the chick liver enzyme with previous purification schemes, it is seen that the DEAE-cellulose step utilized in both the rat kidney and the hog kidney (Conconi and Grazi, 1965 and McGuire et al, 1980) amidinotransferase purifications was also useful for the purification from chick liver. The phenyl-agarose used to purify rat kidney enzyme did not result in much purification of the chick liver enzyme.
### TABLE 3. PURIFICATION OF ARGinine:GLyCINE LIVER AMIDINOTRANSFERASE FROM CHICK LIVER

<table>
<thead>
<tr>
<th></th>
<th>ENZYME ACTIVITY (units)</th>
<th>PROTEIN (mg)</th>
<th>SPECIFIC ACTIVITY</th>
<th>FOLD PURIFICATION</th>
<th>YIELD %</th>
</tr>
</thead>
<tbody>
<tr>
<td>HOMOGENATE</td>
<td>541</td>
<td>5686</td>
<td>0.10</td>
<td>1</td>
<td>100</td>
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<tr>
<td>SUPERNATANT</td>
<td>394</td>
<td>2996</td>
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<tr>
<td>45-65% (NH₄)₂SO₄</td>
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<td>461</td>
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<tr>
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<td>1.9</td>
<td>9.1</td>
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<tr>
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<tr>
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<td>0.06</td>
<td>15.2</td>
<td>160</td>
<td>0.2</td>
</tr>
</tbody>
</table>

---

a A unit of amidinotransferase was defined as the amount that catalyzes the formation of 1 μmol of hydroxyguanidine in the assay described in the methods section.

b Units per mg of protein.
Summary

Amidinotransferase has been partially purified from liver for the first time. A 160-fold purification has been achieved for the chick liver enzyme. A new scheme of purification has been devised - with critical steps being ammonium sulfate fractionation, DEAE-cellulose chromatography, hydroxylapatite chromatography and arginine-agarose chromatography.

After a review of the steps presented in the purification table it can be seen that two of the procedures could be omitted - the second DEAE-cellulose column and the phenylagarose column. Neither of these steps resulted in much purification of the enzyme. While the work presented needs further refinement prior to establishment of the best purification scheme, it is hoped that this first partial purification of the liver enzyme will be of benefit to anyone wishing to pursue further research on amidinotransferase.
Chapter IV: Chick Heart

A study of the metabolism of cardiac tissue preloaded with creatine analogues was conducted. The bulk of this work consisted of the feeding of cyclocreatine or homocyclocreatine to growing chicks, followed by the subjection of the heart to ischemia.

The heart functions normally as a mechanical pump, propelling blood in a repetitive loop through the circulatory system of the body. The major portion of the volume of the heart is comprised of myocytes, the force-generating contractile tissue. Microscopic examination of the tissue reveals characteristic dark-staining striations, a single nucleus per cell and the presence of intercalated discs. The disc is a cell to cell junction composed of the plasma membranes (referred to as the sarcolemmas) of two abutting cells. The striations are actually composed of myofibrils, the contractile apparatus of the heart. Enveloping the collection of myofibrils and the nucleus is the sarcolemma, which delimits the cellular contents. Invaginations in the sarcolemma open into T-tubules, which pass into the interior of the cell, permit increased intracellular-extracellular communication. Also present in the cell are the sarcoplasmic reticulum and a large number of mitochondria, exhibiting the aerobic predisposition of the cardiac cell.

Myofibrils are composed of the contractile proteins actin and myosin, in addition to the regulatory proteins
troponin and tropomyosin. Actin, troponin and tropomyosin combine to form thin filaments, while thick filaments are composed mainly of myosin. Myofibrils display an ordered arrangement of these filaments. Two main bands are formed. The first of these, the I band, is composed entirely of thin filaments. The second, the A band, is made up of interspersed thick and thin filaments, with a small H band in the middle composed of only thick filaments. Contraction of the myofibril and thus the tissue occurs when thin filaments are pulled further into the A band causing the I band to narrow.

Contraction of the cardiac tissue originates with the pacemaker cells of the sinoatrial node, located in the right atrium near the junction with the superior vena cava. These cells initiate a wave of depolarization which is conducted through the atria and to the ventricles through specialized tissues. Upon reaching the working myocardial cell, depolarization of the sarcolemma occurs allowing calcium ions to pass through the membrane into the cell. Once inside, the Ca\(^{2+}\) stimulates the release of additional Ca\(^{2+}\) from the sarcoplasmic reticulum. Ca\(^{2+}\) binds to troponin resulting in a conformational change in which tropomyosin is removed from blocking the interaction of actin and myosin. At the myosin head ATP is hydrolyzed providing energy for the formation of actin-myosin crossbridges. Thin filaments (actin) are thus pulled further along the thick filaments, resulting in contraction. Relaxation occurs when ATP replaces the bound ADP
and Ca$^{2+}$ is removed from the troponin and sequestered in the sarcoplasmic reticulum.

Despite its elaborate design, the heart does not always function flawlessly. Diseases of the heart have been the major cause of death in the United States for more than forty years (Levy and Moskowitz, 1982). A large number of these deaths have been due to myocardial infarction; a condition which occurs when coronary flow to a region of the myocardium is abruptly reduced resulting in necrosis of the ischemic tissue. Ischemia, by definition, is the absence of oxygen, coupled with the inability to supply substrates or remove degradation products due to lack of blood flow. A short while, 30-40 seconds, after ischemia occurs, contraction of the affected region ceases in an apparent attempt to conserve ATP. This effect has been considered to be due to a variety of causes. It has been postulated that either the phosphate liberated during phosphocreatine and ATP breakdown or modulatory effects of ATP on ion fluxes could cause this decrease in contractility (Kubler and Katz, 1977).

Kammermeier and coworkers (1982) proposed that a main factor of early hypoxic failure of myocardium could be the low level of free energy change of ATP hydrolysis. Most recently it has been hypothesized that this loss of contractility is two-fold (Prinzen et al, 1986). Early loss of endocardial shortening was proposed to be due to hydrolysis of creatine phosphate or acidosis; the decrease in function of epicardial fibers would then be caused by the decrease in fiber
shortening in endocardium. Whatever the cause, this attempt to conserve ATP is necessary because ATP is required for many cellular tasks. In addition to contraction, ATP is necessary for ion metabolism, regulation of cellular volume and the activation of various substrates prior to reaction. As ischemia progresses, endogenous energy stores are depleted in the absence of exogenous supplies brought by the blood. Creatine-P is rapidly utilized and due to the absence of oxygen the heart shifts from oxidation of fatty acids to the production of ATP through anaerobic glycolysis of glycogen stores. As ATP utilization continues less of the ADP formed can be rephosphorylated as energy stores are depleted. Consequently, as the concentration of ADP increases myokinase acts to cause transfer of the terminal phosphate from one ADP molecule to a second one resulting in the formation of a molecule of ATP and one of adenosine monophosphate (AMP). AMP is rapidly degraded to adenosine by 5'-nucleotidase. This adenosine can then diffuse across the sarcolemma resulting in a loss of total adenine nucleotides for the heart tissue.

Loss of adenine nucleotides is thought to be an excellent marker for irreversible heart failure (Vary et al., 1979). In dogs, if ischemic cardiac tissue is reperfused within the first 15 minutes of insult injury can be reversed. However, within 40 minutes of total ischemia irreversible injury to most myocytes occurs. (Jennings et al., 1978). This loss of cellular integrity is thought to be due to the
loss of adequate membrane function resulting from the depletion of ATP to levels too low to maintain performance of ion pumps. Hypothermia has been found to demonstrate a protective effect on cardiac tissue during ischemia by slowing energy production and utilization (Jones et al., 1982). Pretreatment of hearts with the Ca²⁺ blockers verapamil or nifedipine was found to protect the tissue against loss of function and structure caused by ischemia and reperfusion (Naylor, 1980).

When ATP levels are exhausted, myofibrils enter an irreversible contracted state known as rigor. Since no ATP is present to bind to the myosin ATPase to displace ADP and P₅, the myofibril is unable to relax.

Within the author's laboratory, the rigor state of excised chick hearts was observed by Griffiths. It was found that rigor was delayed in hearts removed from chicks that had been fed a diet containing cyclocreatine (Annesley et al., 1978). This work was followed up by Roberts and Walker (1982a), who performed a study on rigor and ischemia in the rat heart, finding that prefeeding of cyclocreatine conferred a protective effect on the heart during insult. It is this work upon which the author's further investigation of ischemic chick heart was based.

Chicks were chosen for this research for several reasons. In addition to examining the effect of prior cyclocreatine feeding on heart subjected to ischemia, it was hoped that this effect could be compared to the effect of
prior feeding of a closely related compound, homocyclocrea-
tine, on similarly treated hearts. Rats and mice do not
accumulate sufficiently large quantities of this compound,
while hearts of rapidly growing chicks will accumulate and
phosphorylate homocyclocreatine quite readily (Roberts and
Walker, 1983). Furthermore glycogen and creatine stores
could be manipulated to a greater extent in chicks as
compared to rats.

Several other incidental differences between chicks and
mammals are also known to exist. Chick hearts contain BB
isozyme of creatine kinase instead of the MB isozyme commonly
found in mammalian species (Wallimann et al, 1977). There is
no M line visible in the H band of myofibrils of chick heart,
as is normally seen. Finally it has been found that as a
whole, birds have larger hearts and lower heart rates than do
similarly sized mammals (Grubb, 1983).

A portion of the work presented in this chapter has been
previously published (Turner and Walker, 1985).

Methods

Materials

Creatine was bought from biochemical supply firms.
Cyclocreatine was synthesized as noted in Chapter II while
homocyclocreatine was synthesized from ethylenediamine, ethyl
acrylate and cyanogen bromide (Roberts and Walker, 1983) and
was recrystallized 2 or 3 times from water. Elemental
analysis and NMR spectroscopy of homocyclocreatine were
previously performed, producing results consistent with the assigned structure (Roberts and Walker, 1983). The enzymes lactic dehydrogenase, amyloglucosidase, pyruvate kinase and myokinase were purchased from Sigma Chemical Company as were the compounds adenosine triphosphate, phosphoenolpyruvate, nicotinamide adenine dinucleotide and reduced nicotinamide adenine dinucleotide. Other compounds were obtained as noted in Chapter II.

Protocol

One-day-old male New Hampshire Red chicks were obtained from Hendrick Grain Co. and fed ground Purina chick chow. At five days of age some of the chicks were removed from the control diet, and placed on diets containing, by weight, 5% creatine, 5% homocyclocreatine or a level of cyclocreatine up to 1%. After up to 18 days of feeding, chicks were anaesthetized with ether, the chest opened and the heart excised and blotted on paper towels. (If in situ glycogen was to be determined, the heart was fast-frozen in situ with aluminum tongs precooled with liquid nitrogen.) Following excision the heart was incubated in a covered beaker in a 37°C water bath for the desired period of time. At the conclusion of ischemia the heart was fast-frozen with aluminum tongs and immersed in liquid nitrogen. The heart was then powdered in a nitrogen-cooled steel percussion mortar and transferred to a preweighed 50 ml plastic centrifuge tube. The tube and
contents were then weighed, 10-20 ml of cold 0.4 M HClO₄ added, and homogenized on ice for one minute with a Tekmar Tissuemizer set at 20,000 RPM. If glycogen was to be determined a portion of the whole homogenate was removed, neutralized with 0.7 M KHCO₃ and the mixture put on ice to await treatment with amyloglucosidase. The remainder of the acidified homogenate was centrifuged at 17000 x g for 8-10 minutes. A portion of the supernatant was neutralized with 4 M KOH and later assayed for metabolites. Samples were sometimes frozen and stored prior to assay. A second portion of the acidic supernatant was heated at 65°C for up to 60 minutes, then neutralized and assayed for creatine analogue content.

Assays

Adenosine triphosphate, creatine and cyclocreatine were assayed as described in Chapter II. Homocyclocreatine was assayed by the same pentcyanoaminoferate method as was used for cyclocreatine (Roberts and Walker, 1983). To 1 ml of neutralized sample plus water was added 2 ml of 30 mM Na₂CO₃ followed by vortexing. Following the addition of 0.5 ml of aged pentacyanoaminoferate solution and consequent vortexing, the solution was allowed to stand for 10-20 minutes. The absorbance was then read at 605 nm. Homocyclocreatine-P were determined by the differential treatment of two portions of a acid tissue extract, one neutralized and stored cold and the other acidic portion heated at 65°C for
60 minutes followed by neutralization and storage in the cold.

Inorganic phosphate was determined with the use of a method employing ammonium molybdate, polyvinylpyrrolidone and hydroxylamine sulfate (Ohnishi et al., 1975). To 1 ml of neutralized sample plus water placed on ice was added with stirring 0.5 ml of a freshly prepared ice cold solution consisting of one part of 4% ammonium molybdate, two parts of 16 mM Na\textsubscript{4}EDTA, and three parts of reductant catalyst composed of 1 mM polyvinylpyrrolidone, 86 mM hydroxylamine sulfate and 87.5 mM H\textsubscript{2}SO\textsubscript{4}. Thirty seconds after this addition 0.5 ml of color developer consisting of 6.5 M NaOH and 0.05 M Na\textsubscript{2}CO\textsubscript{3} was added and the solution vortexed. After allowing the solution to warm at room temperature for 10-20 minutes the absorbance was read at 720 nm and the amount of phosphate calculated.

Lactic acid was assayed with lactate dehydrogenase, following the reduction of nicotinamide adenine dinucleotide, with hydrazine added to pull the reaction to completion (Gutmann and Wahlfield, 1974). To a 3 ml cuvette was added 0.2 ml of neutralized sample plus water and 2.5 ml of a buffer consisting of 0.5 M glycine and 0.4 M hydrazine sulfate, pH 9.0. Nicotinamide adenine dinucleotide was then added and a short time later the absorbance at 340 nm was recorded. Following the addition of 200 units of lactate dehydrogenase, the mixture was allowed to react at room temperature for one hour. Absorbance at 340 nm was measured
once again and the amount of lactate calculated.

Glycogen was measured as glucose units, thus it must first be broken down to glucose. This was accomplished with amylglucosidase (Keppler and Decker, 1974). As noted earlier, a sample of the homogenized extract was removed prior to centrifuging. Usually, 0.5 ml was removed, placed in a 50 ml Erlenmeyer flask and neutralized with 0.25 ml of 0.7 M KHC03. Five ml of a solution containing 5 mg/ml amylglucosidase suspended in 0.2 M sodium acetate buffer, pH 4.8, was then added and the mixture incubated at 40\(^\circ\)C in a shaking water bath for 2 1/2 to 3 1/2 hours. The flask was then removed, 5 ml of 0.8 M HClO\(_4\) added and the mixture transferred to a 50 ml centrifuge tube. Following centrifugation at 8000 x \(g\) at 2-4\(^\circ\)C for eight minutes, 2 ml of supernatant was removed and neutralized with 0.2 ml of 4 M KOH. It was found necessary to also incubate a blank containing only neutralized HClO\(_4\) with amylglucosidase when it was discovered that the amylglucosidase contained small amounts of glucose or glycogen. This value could then be subtracted from samples later.

To assay for glucose-6-phosphate or free glucose in the neutralized supernatant, or glucose liberated from glycogen by amylglucosidase in the treated sample, a coupled enzyme assay was utilized (Keppler and Decker, 1974). To a 1 ml cuvette was added 0.3 ml of neutralized sample + water, 0.1 ml of 4 mM nicotinamide adenine dinucleotide phosphate, 0.1 ml of 10 mM adenosine triphosphate and 0.5 ml of buffer
containing 600 mM triethanolamine-HCl, 8.0 mM MgSO₄, pH 7.5, and the succeeding incubation was performed at 30°C. After two minutes the absorbance at 340 nm was read. Glucose-6-phosphate dehydrogenase was added, the mixture allowed to react for 2.5 minutes and the absorbance at 340 nm read again. Hexokinase was then added, the mixture allowed to react for 7.5 minutes, and the absorbance at 340 nm determined a final time. The difference between the first two readings was used to determine glucose-6-phosphate, while free glucose or glycogen (as units of glucose) was calculated from the difference between the final two readings.

Adenosine diphosphate (ADP) and adenosine monophosphate (AMP) were assayed with a procedure utilizing three enzymes - lactate dehydrogenase, pyruvate kinase and myokinase (Jaworek et al., 1974). At room temperature, to 1 ml of neutralized sample plus water, was added 1 ml of 600 mM triethanolamine-HCl, pH 7.6, 0.15 ml of a solution containing 14 mM phosphoenolpyruvate, 0.5 mM MgSO₄, and 1.8 mM KCl, and finally 0.03 ml of 14 mM reduced nicotinamide adenine dinucleotide dissolved in 5% NaHCO₃. Lactate dehydrogenase was then added, the mixture allowed to react for five minutes, and the absorbance at 340 nm read. Following this, pyruvate kinase was added, the mixture allowed to react a further 5 minutes and the absorbance at 340 nm determined once again. Finally, myokinase was added, the solution allowed to react for 10 minutes and the absorbance read at 340 nm one more time. The difference between the first two readings was used to
determine ADP concentration, while AMP was calculated from the difference between the final two readings.

**Results**

Chick hearts subjected to total ischemia show a steady decline in ATP as ischemia progresses. It was found that hearts from chicks fed cyclocreatine demonstrated a greatly decreased decline in ATP relative to controls (Figure 23). Control heart ATP reserves were depleted to their lowest level within 30 minutes while hearts from cyclocreatine-fed animals maintained elevated ATP for nearly 60 minutes. Loss of ATP in hearts from control animals occurred with initiation of ischemia; however, the decrease in ATP from initial levels did not occur in hearts from cyclocreatine-fed chicks until 20 minutes after ischemia was begun. ATP levels in cyclocreatine-loaded hearts actually increased during ischemia, peaking ten minutes after initiation. While the increase in ATP in cyclocreatine-loaded hearts was not significant at 5 minutes of ischemia, it was significant \( p < .01 \) at 10 minutes of ischemia and at 15 minutes of ischemia \( p < .05 \). The protective effect of cyclocreatine feeding reached its maximum after 30 minutes of ischemia. A 6-fold greater amount of ATP remained in cyclocreatine-fed hearts relative to controls after 30 minutes.

A second analogue of creatine, homocyclocreatine, was also fed to chicks and hearts from these chicks subjected to ischemia. ATP was depleted at approximately the same rate in
Figure 23. Time courses of ATP depletion in chick heart during ischemia. Chicks were previously fed a control diet (▲), 1% cyclocreatine (cCr) (●), or 5% homocyclocreatine (hcCr) (■) for 10-14 days. Each point represents mean ± SEM for 3 to 5 chicks.
these hearts as in controls, demonstrating the absence of a protective effect.

In an effort to examine the relationship of cyclocreatinine feeding to the protection of heart against ATP depletion during ischemia, an experiment involving duration of feeding was undertaken (Figures 24 and 25). Chicks were fed a 1% cyclocreatinine diet for up to 18 days. At selected intervals cyclocreatinine-fed chicks and matched controls were sacrificed, and the hearts subjected to a period of 30 minutes of ischemia. Some protection against depletion appeared to occur as early as two days after feeding had begun. As feeding progressed ATP levels remaining after ischemia increased, reaching a maximal level by day 10. Although not all points on the ATP-sparing curve were significant, ATP levels remaining in cyclocreatinine-fed hearts were elevated at each point on the curve. Points which were significant included the 6 day and 8 day points (p < .001), the 10 day and 13 day points (p < .002) and the 15 day point (p < .005). Interestingly, the protective effect lagged behind the accumulation of cyclocreatinine, which maximized at about 16 μmol/g by day 6. In hearts of chicks removed from the cyclocreatinine diet and returned to a control diet, the accumulated cyclocreatinine declined with continued feeding of the control diet. This loss was accompanied by an apparent corresponding loss of the ATP protective effect (Figure 25).

Accompanying the accumulation of cyclocreatinine was the depletion of existing creatine stores (Figure 26). Control
Figure 24. Accumulation of total cyclocreatine (free + phosphorylated) by hearts of chicks fed 1% cyclocreatine (●). After 12 days of feeding one group of chicks was removed from the diet and returned to a control diet (○), resulting in a decline in total cyclocreatine. Each point represents mean ± SEM for 3 or 4 chicks.
Figure 25. Development of ATP-sparing ability after 30 minutes of ischemia in hearts of chicks fed 1% cyclocreatine (●), relative to control hearts (▲). After 12 days of feeding one group of chicks was removed from the diet and returned to a control diet (○), resulting in a decline in ATP-sparing ability. Each point represents mean ± SEM for 3 or 4 chicks.
ATP AFTER 30 MIN ISCHEMIA (μmol/g)

DAYS ON DIET

CONTROLS

CCR-FED
Figure 26. Depletion of creatine in hearts of chicks fed 1% cyclocreatine (●), relative to control hearts (■). After 12 days of feeding one group of chicks was removed from the diet and returned to a control diet (○), resulting in an increase in creatine concentration. Each point represents mean ± SEM for 3 or 4 chicks.
chick hearts contained slightly less than 7 μmol/g of total creatine; creatine levels in cyclocreatine-fed chick hearts decreased to 2 μmol/g before leveling off. Upon cessation of cyclocreatine feeding, creatine levels increased as corresponding cyclocreatine levels decreased.

Cyclocreatine-P accumulated by chick heart was utilized during ischemia (Figure 27). By means of the creatine kinase reaction this synthetic phosphagen was able to rephosphorylate ADP formed upon hydrolysis. Levels of the synthetic phosphagen decreased rapidly for the first 20 minutes of ischemia, followed by a slowing of depletion for the remainder of the measured ischemic event. Homocyclocreatine-P was utilized only very slowly during ischemia, providing almost no buffering effect for the ATP pool. Homocyclocreatine-P, which is a close structural analogue of cyclocreatine-P and as noted earlier, also has similar thermodynamic properties, is a poor substrate of creatine kinase than is cyclocreatine-P, reacting 1000-fold more slowly than cyclocreatine-P and 200,000-fold more slowly than creatine-P (Roberts and Walker, 1983).

In Figure 27 it can be seen that initial homocyclocreatine-P levels were significantly below that of cyclocreatine-P. To ensure that this was not the cause of the differing ATP effects observed during ischemia, feeding experiments were pursued in an attempt to achieve cyclocreatine-P levels matching those of homocyclocreatine-P. After a number of trials it was found that the feeding of
Figure 27. Time courses of the utilization of accumulated cyclocreatinine-P (PcCr) (●) and homocyclocreatinine-P (PhcCr) (■) during ischemia in hearts of chicks previously fed 1% cyclocreatinine or 5% homocyclocreatinine for 10-14 days. Numbers in parentheses indicate percentages remaining in the phosphorylated form. Each point represents mean ± SEM for 3 to 5 chicks.
0.5% and 0.7% cyclocreatinine resulted in the accumulation of amounts of total cyclocreatinine bracketing the amount of total homocyclocreatinine accumulated in hearts of chicks (Table 4). Although hearts from homocyclocreatinine-fed chicks were not protected against ATP depletion, hearts from chicks fed either 0.5% or 0.7% cyclocreatinine had elevated ATP levels following 30 minutes of ischemia relative to controls and the homocyclocreatinine-fed group. Thus it was determined that with equivalent analogue accumulation, cyclocreatinine-P, unlike homocyclocreatinine-P, was able to function to protect ATP levels against depletion during ischemia.

An increased release of inorganic phosphate was observed in cyclocreatinine-fed animals relative to controls during ischemia (Figure 28). Although initial phosphate totals were several μmol/g lower during ischemia in hearts from cyclocreatinine-fed animals, within 60 minutes of ischemia inorganic phosphate present in the cyclocreatinine-fed hearts exceeded that in controls, increasing through the remainder of the ischemic period while the inorganic phosphate present in control hearts levelled off. It was also found that phosphate release in hearts of chicks fed homocyclocreatinine was only a little more than half that of hearts from chicks fed cyclocreatinine. This is quite possibly due to the much greater utilization of cyclocreatinine-P than homocyclocreatinine-P during ischemia, resulting in liberation of the terminal phosphate. Inorganic phosphate formed from phosphagen by way of ATP during ischemia can act as a
### TABLE 4. SPARING OF ATP AFTER 30 MINUTES ISCHEMIA
 IN CHICK HEARTS CONTAINING COMPARABLE
 AMOUNTS OF CREATINE ANALOGUES

<table>
<thead>
<tr>
<th>DIET&lt;sup&gt;a&lt;/sup&gt;</th>
<th>TOTAL ANALOGUE&lt;sup&gt;b&lt;/sup&gt; (μmol/g)</th>
<th>ATP REMAINING (μmol/g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CONTROL</td>
<td>0</td>
<td>0.36 ± 0.11</td>
</tr>
<tr>
<td>0.7% CYCLOCREATINE</td>
<td>13.8 ± 1.5</td>
<td>1.58 ± 0.46*</td>
</tr>
<tr>
<td>5% HOMOCYCLOCREATINE</td>
<td>12.8 ± 1.8</td>
<td>0.46 ± 0.12</td>
</tr>
<tr>
<td>0.5% CYCLOCREATINE</td>
<td>11.2 ± 1.1</td>
<td>1.11 ± 0.45*</td>
</tr>
</tbody>
</table>

<sup>a</sup> Five-day-old chicks were fed diets for 10-14 days. Following anaesthetization hearts were excised and incubated at 37°C for 30 minutes, fast-frozen, and assayed for metabolites. Means ± SD for 12 chicks.

<sup>b</sup> Free plus phosphorylated derivatives of analogues accumulated by heart.

*P < .001 vs control-fed group.
Figure 28. Time courses of the changes in inorganic phosphate levels in chick heart during ischemia. Chicks were previously fed a control diet (▲), 1% cyclocreatine (cCr) (●), or 5% homocyclocreatine (hcCr) (■) for 10-14 days. Each point represents mean ± SEM for 3 to 5 chicks.
substrate for both glycolysis and glycogenolysis. A second interaction between these systems also occurs in that protons released during glycolysis help drive the creatine kinase reaction resulting in the formation of ATP from the phosphagen and ADP.

Lactate production was greatly elevated in hearts from cyclocreatine-fed animals relative to those of controls, indicating an increased amount of glycolysis (Figure 29). Twice the amount of lactate was generated in cyclocreatine-fed hearts during 60 minutes of ischemia. Hearts from homocyclocreatine-fed animals also contained elevated lactate levels, intermediate between those in control and cyclocreatinine hearts.

Inasmuch as lactate is the product of the anaerobic breakdown of glucose and glycogen, it was suspected that glucose and glycogen stores were elevated in homocyclocreatinine and cyclocreatinine-fed chick hearts. This was indeed found to be the case (Table 5). While free glucose levels in hearts from animals fed cyclocreatinine were the same as those in controls, an increase of glucose was observed in homocyclocreatinine-fed chick hearts. It was furthermore found that glycogen stores were tripled in both cyclocreatinine-fed and homocyclocreatinine-fed chick hearts relative to controls. The finding that homocyclocreatinine-fed chick hearts also accumulated large quantities of glycogen, yet were still unable to demonstrate an ATP-sparing effect during ischemia, indicates that the glycogen increase is not the only factor
Figure 29. Time courses of lactate accumulation in chick heart during ischemia. Chicks were previously fed a control diet (▲), 1% cyclocreatine (cCr) (●), or 5% homocyclocreatine (hcCr) (■) for 10-14 days. Each point represents mean ± SEM for 3 to 5 chicks.
Table 5. ENERGY RESERVES IN HEARTS OF CHICKS FED CREATINE ANALOGUES

<table>
<thead>
<tr>
<th>DIET(^a)</th>
<th>GLYCOGEN ((\mu)mol/g)</th>
<th>GLUCOSE ((\mu)mol/g)</th>
<th>TOTAL HIGH-ENERGY PHOSPHATE RESERVES(^b) ((\mu)mol/g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CONTROL (10)</td>
<td>7.0 ± 2.6</td>
<td>4.4 ± 0.5</td>
<td>35</td>
</tr>
<tr>
<td>1% CYCLOCREATINE (3)</td>
<td>20.1 ± 4.5(^*)</td>
<td>4.5 ± 0.4</td>
<td>86</td>
</tr>
<tr>
<td>5% HOMOCYCLOCREATINE (5)</td>
<td>20.3 ± 5.7(^*)</td>
<td>6.3 ± 1.3(^@)</td>
<td>83</td>
</tr>
</tbody>
</table>

\(^a\) Five-day-old chicks were fed diets for 16 days. Following anaesthesia, hearts were fast-frozen in situ and assayed for metabolites. Numbers in parentheses denote chicks for each diet. Means ± SD are given.

\(^b\) Potential high-energy phosphate reserves available under anaerobic conditions at time of first tissue sampling were calculated as 3 (glycogen) + 2 (glucose) + 2 (ATP) + (creatine-P) + (analogue-P).

\(^*\) P < .001 vs. control-fed group.

\(^@\) P < .01 vs. control-fed group.
involved in the protective effect exhibited by cyclocreatinine. Additionally, calculation of total high-energy phosphates reserves indicated that these levels were elevated to the same extent in homocyclocreatinine-fed chick hearts as in those fed cyclocreatinine (Table 5).

The depletion of glycogen in control and cyclocreatinine-fed hearts is illustrated in Figure 30. Glycogenolysis occurred to a greater extent in cyclocreatinine-fed chick hearts. Whereas essentially all glycogen stores were depleted within 30 minutes in control hearts, a substantial amount of glycogen remained after 60 minutes of ischemia in hearts of chicks fed cyclocreatinine.

In addition to the glucose obtained from glycogenolysis, free glucose is available for glycolysis at the initiation of ischemia. As is seen in Figure 31, glucose depletion occurred at the same rate in both control and cyclocreatinine-fed hearts. Within 20 minutes of initiation of ischemia glucose concentrations had decreased to only 20% of their original values. After 40 minutes glucose concentrations reached their lowest value and levelled off for the remainder of ischemia.

The compound glucose-6-phosphate is an intermediate metabolite of glycolysis. Both free glucose and glucose-1-phosphate formed during the breakdown of glycogen are metabolized to this product, which is then isomerized to fructose-6-phosphate and continues through the glycolytic pathway. In Figure 32 glucose-6-phosphate concentrations in
Figure 30. Time courses of depletion of glycogen in chick heart during ischemia. Chicks were previously fed a control diet (□) or 1% cyclocreatine (cCr) (●), for 10-14 days. Each point represents the mean for 2 to 5 chicks. For points representing 3 to 5 chicks, ± SEM is presented.
Figure 31. Time courses of depletion of glucose in chick heart during ischemia. Chicks were previously fed a control diet (■), or 1% cyclocr eamine (cCr) (●) for 11-13 days. Each point represents mean ± SEM for 3 or 4 chicks.
Figure 32. Time courses of changes in glucose-6-phosphate levels in chick heart during ischemia. Chicks were previously fed a control diet (■) or 1% cyclocreatine (cCr) (●) for 11-13 days. Each point represents mean ± SEM for 3 or 4 chicks.
control and cyclocreatinine-fed chick hearts during ischemia are compared. Glucose-6-phosphate levels remained constant in control hearts, while a dramatic elevation of levels of this compound was seen in cyclocreatinine-fed hearts. After 100 minutes of ischemia glucose-6-phosphate levels in cyclocreatinine-fed hearts were increased 10-fold. Additional work indicated that this peak level was followed by a decrease to 0.78 μmol/g after an additional 20 minutes. These elevated levels appear to indicate a blockage at some point of the glycolytic pathway, most probably at either the phosphofructokinase or glyceraldehyde-3-phosphate dehydrogenase reactions. Elevated NADH and lactate concentrations, lowered intracellular pH, and decreased levels of ATP could result in inhibition of one or the other of the two enzymes (Rovetto et al, 1975). Interestingly however, lactate levels measured in the same tissue samples continued increasing up to 100 minutes of ischemia, leveling off at that point. Perhaps only a partial inhibition of the pathway occurs.

Figure 33 illustrates the utilization of high-energy phosphate during ischemia. Much more high-energy phosphate was consumed by the cyclocreatinine-fed hearts than by control hearts due to the utilization of the increased phosphagen and glycogen stores present in cyclocreatinine-fed hearts. The shape of the utilization curves was quite similar to that of the lactate produced during ischemia (Figure 28) due to the dominant contribution of glycolysis to the regeneration of high-energy phosphate.
Figure 33. Time courses of high-energy phosphate (∼P) utilization in chick heart during ischemia. Chicks were previously fed a control diet (■) or 1% cyclocreatine (cCr) (●) for 11-13 days. Values were calculated using the equation: $P = 1.45 (Δ\text{lactate} - 2Δ\text{glucose}) + 2Δ\text{glucose} + 2Δ\text{ATP} + Δ\text{synthetic phosphagen}$. Each point represents mean ± SEM for 3 or 4 chicks.
In addition to the feeding of cyclocreatine or homocyclocreatine, another compound was fed to other batches of chicks. This was the naturally occurring compound creatine. It was found that the feeding of creatine as 5% of the diet resulted in a doubling of the total creatine, that is creatine plus creatine-P, contained in chick heart (Table 6). Upon subjecting these creatine-fed chick hearts to ischemia, no significant ATP-sparing effect was observed. ATP levels decreased to the same extent after 30 minutes of ischemia in creatine-fed hearts as in control hearts.

Normally, approximately 50% or more of the total creatine in the heart is phosphorylated under aerobic conditions. In these experiments creatine-P levels were found to be quite low at the time of the first tissue sampling, probably due to hypoxia and sympathetic discharge caused by ether anesthesia. Hearts of chicks fed 5% creatine contained 1.2 μmol/g of creatine-P at first sampling compared to the 0.5 μmol/g found in control hearts and 0.2 μmol/g found in analogue-fed hearts. Thus creatine-P levels were too low to buffer ATP/ADP during prolonged ischemia.

Total creatine levels were also affected in analogue-fed hearts. Total creatine content of hearts of chicks fed 1% cyclocreatine decreased to 1.8 μmol/g compared to the 5.9 μmol/g found in matched controls. These hearts, containing the lowest total creatine content yet observed in hearts from intact animals, nevertheless appeared to function quite adequately. Feeding of lower percentages of cyclocreatine
TABLE 6. EFFECT OF CREATINE FEEDING ON CREATINE CONTENT OF CHICK HEART AND ON ATP REMAINING AFTER 30 MINUTES OF ISCHEMIA

<table>
<thead>
<tr>
<th>DIET&lt;sup&gt;a&lt;/sup&gt;</th>
<th>TOTAL CREATINE&lt;sup&gt;b&lt;/sup&gt; (μmol/g)</th>
<th>REMAINING ATP (μmol/g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CONTROL (9)</td>
<td>6.6 ± 0.6</td>
<td>0.41 ± 0.18</td>
</tr>
<tr>
<td>5% CREATINE (12)</td>
<td>14.8 ± 2.1*</td>
<td>0.49 ± 0.15</td>
</tr>
</tbody>
</table>

<sup>a</sup>Five-day-old chicks were fed diets for 10-14 days. Following anaesthetization, hearts were excised and incubated at 37°C for 30 minutes, fast-frozen, and assayed for metabolites. Numbers in parentheses denote chicks for given diet. Means ± SD are given.

<sup>b</sup>Free plus phosphorylated derivatives of creatine contained in chick heart.

*<sup>p</sup> < .001 vs control-fed group.
resulted in a decrease in total creatine levels to intermediate values of 3.0 to 3.6 μmol/g. Total creatine levels in homocyclocreatine-fed animals were decreased to a lesser extent; hearts contained 4.2 μmol/g. However it was found that a large decrease in total creatine levels was not necessary for ATP protective effects. A group of chicks were fed a diet containing 1% cyclocreatine plus 1% creatine for 17 days. Subjection of the hearts to ischemia revealed that the hearts were indeed protected against depletion of ATP. After 30 minutes of ischemia ATP levels were found to be 1.2 ± 0.3 μmol/g. Cyclocreatine content was measured as 10.5 ± 1.5 μmol/g, while creatine levels were only slightly decreased to 5.3 ± 0.5 μmol/g. Thus it appears that depletion of creatine levels is not, of itself, the cause of the ATP-sparing effect observed in cyclocreatine-fed animals.

Utilization of adenosine triphosphate requires its hydrolysis, resulting in the formation of inorganic phosphate and adenosine diphosphate (ADP) after which the ADP can then react with adenylate kinase to form adenosine monophosphate (AMP). Figure 34 details the progressive depletion of ADP during ischemia. It is seen that ADP levels in control and cyclocreatine-fed chick hearts were initially the same. During ischemia, concentrations of ADP decreased more rapidly in control hearts, reaching a minimum after 30 minutes. ADP levels in cyclocreatine-fed hearts decreased for the first 15 minutes of ischemia, then plateaued for another 30 minutes. After a total of 30 minutes of ischemia ADP levels in
Figure 34. Time courses of changes in ADP levels in chick heart during ischemia. Chicks were previously fed a control diet (■) or 1% cyclocreatine (cCr) (●) for 10-14 days. Each point represents mean ± SEM for 3 to 5 chicks.
cyclocreatine-fed chick hearts were two-fold elevated over those in control hearts.

During ischemia ADP is acted upon by the enzyme myokinase, resulting in the formation of one molecule of AMP and one molecule of ATP from two molecules of ADP. The time course of AMP during ischemia is diagrammed in Figure 35. Beginning from the same initial concentration, it is seen that AMP initially decreased in cyclocreatine-fed hearts while increasing in control hearts. This is probably a result of the declining ATP concentrations in control heart (see Figure 23) while these levels remain elevated in cyclocreatine-fed hearts. While only the AMP levels for the 30 minute and 100 minute time points were significantly different (P<.02 and P<.005 respectively) from the AMP level at initial measurement for control hearts, it was found that both the 60 minute and 100 minute AMP levels differed significantly (P<.01 and P<.001 respectively) from the AMP level at the 30 minute time point in control hearts. Thus, it is seen that as ischemia progressed AMP levels began to decrease in controls after 30 minutes, while AMP levels in cyclocreatine-fed hearts were increasing at this time. Once again, referring to Figure 23, this is in accordance with the fluctuation of the ATP levels. ATP levels reached a minimum in 30 minutes in control hearts while levels were elevated and were still being depleted in cyclocreatine-fed hearts. AMP concentration continued to increase in cyclocreatine-fed animals, plateauing after 60 minutes of ischemia. 100
Figure 35. Time courses of changes in AMP levels in chick heart during ischemia. Chicks were previously fed a control diet (■) or 1% cyclocreatine (cCr) (●) for 10-14 days. Each point represents mean ± SEM for 3 to 5 chicks.
minutes after the initiation of ischemia AMP levels remained elevated nearly 3-fold higher in cyclocreatine-fed hearts than in control hearts. Further work indicated that AMP levels began to decline after 100 minutes. Certainly the protective effect against ATP depletion by cyclocreatine contributes to this elevation of AMP levels during ischemia; however, the plateau at 60 to 100 minutes after the essential exhaustion of ATP indicates that there may be an additional factor. It is possible that cyclocreatine or cyclocreatine-P acts directly or indirectly to cause the inhibition of AMP hydrolases, presumably 5'-nucleotidase, which bring about the breakdown of AMP to yield adenosine.

Figure 36 indicates the changes in the total adenylates, which consist of the summation of the ATP, ADP and AMP pools. It was observed that total adenylates remain greatly elevated in hearts of chicks fed cyclocreatine relative to those of controls; at 45 minutes adenylate pools were 2-fold higher in cyclocreatine-fed hearts than in controls. The early elevation was due to the increased ATP levels, while that in the later stages of ischemia was effected by the increased AMP levels. As was stated earlier, maintenance of adenylate pools is of prime importance in cardiac tissue. Loss of these pools has been found to correlate with irreversible damage. Furthermore, during reperfusion following ischemic insult, sufficient adenylate must remain to permit resynthesis of levels of ATP sufficient to permit normal cardiac function. As adenylate levels decline the resulting
Figure 36. Time courses of changes in total adenylate levels during ischemia. Chicks were previously fed a control diet (■) or 1% cyclocreatinine (cCr) (●) for 10-14 days. Total adenylates are equal to the sum ATP + ADP + AMP. Each point represents mean ± SEM for 3 to 5 chicks.
adenosine diffuses through the membrane, which, coupled with the relatively slow rate of de novo biosynthesis, results in inadequate reformation of ATP pools during reperfusion.

It was desired to determine the relationship of feeding of lower percentages of cyclocreatine with the amount of the ATP-sparing effect observed during ischemia. Figure 37 shows the accumulation of cyclocreatine in hearts of animals fed cyclocreatine as 0.25%, 0.5%, 0.7% or 1.0% of their diet. In Figure 38 the results of this accumulation upon ATP depletion after 30 minutes of ischemia are displayed. Feeding of the different levels resulted in differences in the accumulation of total cyclocreatine. As expected, an increase in the percentage fed resulted in an increase in accumulation. The concentration of ATP remaining after 30 minutes of ischemia correlated with the increased accumulation. With each increase in percentage of cyclocreatine fed in the diet, increased ATP-sparing was observed. While all levels of cyclocreatine feeding demonstrated the ATP-sparing effect in the work presented in Figure 38, it should be noted that the effect was not always observed in additional experiments with 0.25% cyclocreatine feeding.

In Figure 39 total cyclocreatine in hearts of chicks fed diets containing various concentrations of cyclocreatine diets is graphed against ATP remaining after 30 minutes of ischemia in these hearts. Most of these values are obtained from Figures 38 and 39 and represent the average of 3 chicks in most cases; additional data is included for control and 1%
Figure 37. Accumulation of total cyclocreatine (free + phosphorylated) by hearts of chicks fed diets containing various percentages of cyclocreatine. Diets included either 0.25% cyclocreatine (0.25% cCr) (△), 0.5% cyclocreatine (0.5% cCr) (△), 0.7% cyclocreatine (0.7% cCr) (○), or 1.0% cyclocreatine (1.0% cCr) (●). Each point represents mean ± SEM for 3 chicks, except for the 0.25% cCr, day 10 point which represents 2 chicks.
Figure 38. Development of ATP-sparing ability after 30 minutes in hearts of chicks fed diets containing various percentages of cyclocreatine, relative to control hearts. Diets included control (□), 0.25% cyclocreatine (0.25% cCr) (■), 0.5% cyclocreatine (0.5% cCr) (□□), 0.7% cyclocreatine (0.7% cCr) (■■), or 1.0% cyclocreatine (1.0% cCr) (■■■). Values obtained from chicks fed each cyclocreatine diet are compared with control values on different bar graphs. Each bar represents mean ± SEM for 3 chicks, except for the 0.25% cCr, day 10 value which represents 2 chicks.
Figure 39. ATP-sparing ability in chick heart after 30 minutes of ischemia correlated with total cyclocreatine (free + phosphorylated) present in the heart. Chicks were fed a control diet (■), 0.25% cyclocreatine (0.25% cCr) (▲), 0.5% cyclocreatine (0.5% cCr) (▲), 0.7% cyclocreatine (0.7% cCr) (○) or 1% cyclocreatine (1.0% cCr) (●) for 2-18 days. Each point represents the average of 3 chicks fed the same diet and killed on the same day, except for the highest value for 0.25% cCr which represents only 2 chicks.
cyclocreatinine chicks fed for several additional days. It is seen from this graph that a correlation exists between cyclocreatinine accumulation and protection against ATP depletion in chick heart. The linear correlation coefficient for the data was found to be 0.84 and was significant (P<.001). As total cyclocreatinine accumulation increases so also does protection of ATP during ischemia.

Summary

It has been found that in hearts of chicks fed cyclocreatinine and subjected to ischemia, ATP was sustained at high levels significantly longer than in hearts of chicks fed homocyclocreatinine or control feed.

Hearts of chicks fed one of the two synthetic compounds, cyclocreatinine or homocyclocreatinine, accumulated and phosphorylated high levels of these compounds. The accumulated cyclocreatinine-P can be utilized for regeneration of ATP during ischemia; the homocyclocreatinine-P, however, cannot.

Feeding of these two analogues caused a 3-fold increase of endogenous glycogen stores in cardiac tissues. Combined with the presence of increased phosphagen this resulted in greatly increased high-energy phosphate reserves available to the tissue. During ischemia glycolysis was increased in hearts from animals fed these analogues.

Feeding experiments demonstrated that the ATP-sparing effect during ischemia brought about by cyclocreatinine could be seen after only 2 days of feeding. Cyclocreatinine accumu-
lation was maximized after 6 days of feeding while ATP protection did not peak until after 10 days, indicating a possible adaptive effect in chicks fed the diet. Cyclocreatinine feeding also brought about depletion of creatine stores in the heart as did homocyclocreatinine feeding, which depleted total creatine to a lesser extent.

Further manipulation of creatine stores was accomplished by the feeding of a diet supplemented with 5% creatine. By so doing it was possible to more than double creatine stores in heart. However hearts from these animals demonstrated no significant elevation of ATP concentrations relative to controls following 30 minutes of ischemia.

Hearts from animals fed cyclocreatinine were better able to conserve total adenylate pools during ischemia than were control hearts. This was due to the early conservation of ATP during ischemia, accompanied by elevation of AMP later during ischemia. Because de novo synthesis of adenylates is relatively slow this conservation is a very important function performed by cyclocreatinine.

Hearts from chicks fed lower dosages of cyclocreatinine exhibited a decreased ATP-sparing protective effect. It was observed that a correlation exists between the amount of cyclocreatinine derivatives accumulated and the degree of protection against ATP depletion observed during ischemia.

This work has allowed the elimination of several possible mechanisms as being the sole cause of the protective effect of cyclocreatinine feeding. These include: 1)
lowering of myocardial creatine content, 2), the elevated levels of glycogen present in the cardiac tissue, 3), the increased rate and extent of glycolysis during ischemia, 4) an allosteric effect of the planar phosphorylated cycloguanidine ring. These are all possibilities which occurred in hearts of chicks fed homocyclocreatine. The finding that homocyclocreatine-fed hearts did not demonstrate a protective effect on ATP loss during ischemia resulted in the elimination of these possibilities as mechanisms solely responsible for the ATP-sparing effect observed in cyclocreatinine-fed chick hearts. It remains quite possible however, that increased glycolysis might be acting synergistically with cyclocreatinine-P utilization to help bring about the ATP-sparing effect observed during ischemia in hearts of chicks fed cyclocreatinine.

Although homocyclocreatine-P has thermodynamic properties similar to those of cyclocreatinine-P, it has been found that it reacts with creatine kinase 1000-fold slower than does cyclocreatinine-P, and 200,000-fold slower than does creatine-P. These observations have led to the current interpretation of the presented results. Homocyclocreatine-P is thermodynamically competent, but kinetically incompetent to effectively buffer ATP levels during prolonged ischemia. Creatine-P is kinetically competent but thermodynamically incompetent to buffer ATP levels during the later stages of ischemia as creatine-P is exhausted early in ischemia. The synthetic phosphagen cyclocreatinine-P alone possesses the
proper thermodynamic and kinetic properties to serve as a buffer of ATP levels in heart during prolonged ischemia.
Chapter V: Chick Skeletal Muscle

Voluntary striated muscle is often referred to as skeletal muscle. Through contraction these muscles cause movement of the body. In so doing skeletal muscle functions as a chemomechanical transducer; energy from chemical reactions is converted into mechanical work plus heat.

Muscle cells are much like the heart cells described earlier. They are covered by a sarcolemma, contain mitochondria and a Ca$^{2+}$-sequestering sarcoplasmic reticulum, and possess the communicating T-tubules. Contraction is theorized to occur by the sliding filament process, in which overlap between filaments of myosin and actin is increased. Several differences between the muscle types do exist, however. Notably skeletal muscles cells are multinucleated and do not contain intercalated discs as do cardiac cells. Skeletal cells are long fibers, 10-100 μm in diameter and ranging from a few mm to 100mm or greater in length.

Innervation is of greater importance to skeletal muscle than cardiac muscle. In the heart innervation by the autonomic nervous system functions mainly as a modulatory force; the sinoatrial node performs the pacemaker function in the heart. Skeletal muscle cells are innervated by peripheral neurons, motoneurones, which contact the cell at the motor endplate. Thus, contraction of skeletal tissue is elicited neurally by an endplate potential, under the control of the central nervous system. Skeletal tissue contraction
shows little dependence on extracellular Ca$^{2+}$, in contrast to cardiac muscle. Depolarization of the sarcolemma is followed by the conduction of an action potential through the T-tubules which in turn results in a release of Ca$^{2+}$ from larger sarcoplasmic reticulum stores. This Ca$^{2+}$ binds to troponin, releasing inhibition of contraction by tropomyosin and thus allowing increased interaction between actin and myosin, that is, contraction.

Upon examination of skeletal muscle fibers it becomes evident that there are at least two types of fibers, white and red. Red fibers appear to have a red color due to the high content of the heme protein, myoglobin. White fibers, pale in color, contain only small amounts of the protein. Other differences between the two are apparent. Red fibers are thin and contain large numbers of mitochondria and lipid droplets. White fibers are thicker and possess fewer fat droplets and mitochondria. As a result white muscle is stronger; with less room taken up by mitochondria a greater percentage of the muscle is composed of the contractile machinery. Red muscle has a greater oxidative capability and thus fatigues at a much slower rate than does white muscle.

White fibers (sometimes referred to as fast-twitch glycolytic) have high myosin ATPase activity enabling a rapid speed of contraction. Increased glycogen and creatine phosphate stores and high glycolytic capacity permit the muscle to function anaerobically, ideally suiting the tissue to rapid bursts of activity. Closer study of red fibers
reveals that there are actually two types of this fiber. These are commonly referred to as intermediate (slow twitch-oxidative) and red (fast twitch oxidative-glycolytic). Intermediate fibers are more resistant to fatigue, permitting them to function in such tasks as the maintenance of posture. Red fibers, with a higher rate of contraction and increased glycogen stores, are well suited for high intensity, prolonged work.

Human muscle is typically composed of a mixture of these fibers. Examples of muscles which consist almost entirely of a single type of fiber do exist however. Cat soleus and guinea pig soleus, ankle extensors, are made up entirely of slow-twitch intermediate fibers. Lobster abdominal muscle and rabbit psoas muscle are composed almost totally of white fast-twitch fiber. More obvious in the usual course of life, and relevant to this work, is the muscle composition of the domestic fowl, the chicken. Examination of a chicken reveals that it is largely composed of two types of meat; white meat - the breast, utilized sporadically for flying, and dark meat - the leg, utilized for normal ambulation. The pectoral muscle is made up of virtually all white fast-twitch fiber. The leg contains, in addition, an increased proportion of red muscle.

Skeletal muscles are stimulated by the hormone epinephrine. Application of epinephrine brings about the well-known "fight or flee" response. Two types of receptors bind epinephrine, α-adrenoreceptors and β-adrenoreceptors. An
analogue of epinephrine, isoproterenol, binds primarily to the \( \beta \)-receptors. Injection of isoproterenol into the whole animal elicits a variety of responses, including vasodilation, lipolysis of adipose tissue, increased blood sugar and increased rate and force of the heartbeat. Most pertinent to this study is its action to cause increased glycogenolysis in skeletal muscle. Binding of isoproterenol to muscle \( \beta \)-receptors results in increased formation of cyclic AMP. Cyclic AMP activates protein kinase, which activates phosphorylase kinase, which in turn phosphorylates the inactive phosphorylase b to form the active enzyme phosphorylase a. Phosphorylase a then stimulates the breakdown of glycogen in conjunction with the debranching enzyme amylo-1,6-glucosidase. In the following studies performed by this author isoproterenol was utilized to bring about the depletion of glycogen, after which the muscle was subjected to ischemia.

Skeletal muscle is better able to withstand ischemia than is cardiac muscle. Injury is reversible in dog skeletal muscle subjected to ischemia for two hours. Irreversible injury occurs if the insult is extended to seven hours (Harris et al, 1986).

**Methods**

**Materials**

Cyclocreatine was synthesized as described in Chapter II. Isoproterenol bitartrate was obtained from Sigma
Chemical Company; enzymes and additional compounds were purchased as described in Chapters II and IV.

Protocol

White Leghorn eggs from Albers Hatchery in La Grange, Texas were hatched or one-day-old male Rhode Island Reds were obtained from Hendricks Grain Company in Houston, Texas and fed ground Purina chick chow. At five days of age some of the chicks were removed from the control diet and placed on diets containing, by weight, 0.25% cyclocreatine, 0.5% cyclocreatine, 0.6% cyclocreatine or 1% cyclocreatine. After up to 18 days of feeding, chicks were anesthetized with ether, killed by decapitation and the carcasses placed in a humid, 37°C incubator. (If ischemia was not to be carried out, muscle was removed either immediately prior to, or following death and the tissue was fast-frozen with aluminum tongs precooled with liquid nitrogen.) At the conclusion of ischemia the carcass was removed from the incubator and the appropriate muscle excised. Breast muscle was obtained by removing the skin from the chest and excising a portion of the pectoralis muscle on one side of the breast. Leg muscle was obtained by pulling the skin from the leg, cutting the tissue at the knee joint and at the tibio-metatarsal joint, and removing muscle from the back of the leg. Thus, a large portion of this muscle tissue was composed of the gastrocnemius muscle. The excised muscle was fast-frozen with aluminum tongs and immersed in liquid nitrogen. The
tissue was then powdered in a nitrogen-cooled steel percussion mortar and transferred to a preweighed 50 ml centrifuge tube. The tube and contents were then weighed, 6-20 ml of 0.4 M HClO₄ added and the mixture was homogenized on ice for one minute with a Tekmar Tissuemizer set at 20000 RPM. If glycogen was to be determined, a portion of the whole homogenate was removed, neutralized with 0.7 M KHCO₃ and put on ice to await later treatment with amyloglucosidase. The remainder of the acidic homogenate was centrifuged at 17000 x g for 8-10 minutes. A portion of the acidic supernatant was neutralized with 4 M KOH, the precipitated KClO₄ removed by centrifugation, and the sample later assayed for metabolites. Some samples were frozen and stored prior to assay. A second portion of the acidic supernatant was heated at 65°C for 15 minutes (for creatine-P) or 45-60 minutes (for cyclocreatinine-P), then neutralized and assayed for total creatine or total creatine analogue content.

In most of the studies chicks were injected with a solution of isoproterenol up to 4 hours prior to ischemia. A 5-10 mg/ml solution of isoproterenol was prepared by dissolving isoproterenol bitartrate in 0.9% saline solution. 5 mg/kg of isoproterenol was injected into chicks subcutaneously with a tuberculin (27 gauge) syringe; chicks were injected on the side, between the skin and the rib cage.

Assays

Adenosine triphosphate, cyclocreatinine, lactate and glycogen were assayed as specified in Chapters II and IV.
Results

Muscle glycogenolysis is stimulated by isoproterenol. In Figure 40, chicks were injected subcutaneously with isoproterenol (5 mg/kg), anaesthetized with ether and killed after a specified time period, and the breast assayed for the remaining glycogen. Although cyclocreatinine-fed chicks had higher initial glycogen levels, early glycogenolysis occurred at a greater rate in muscles of control chicks. Ninety minutes after the injection of isoproterenol, glycogen levels in control breast muscle had decreased to their lowest level. Cyclocreatinine-fed breast muscle still contained ample supplies of glycogen however, remaining 4-fold elevated over control glycogen levels. After this point, while glycogenolysis had ceased in control muscle, the continuation of this process decreased glycogen in cyclocreatinine-fed chick muscle until at 150 minutes the relative difference in glycogen content between the two levels had returned to the same as the preisoproterenol difference. This study was conducted in the White Leghorn variety of chicks. Further studies with Rhode Island Red chicks indicated that this latter strain contained increased muscle glycogen, with a greater relative initial level in cyclocreatinine-fed animals.

In succeeding experiments chicks were killed 2 hours after isoproterenol injection and subjected to ischemia. As is seen in Figure 41 breast muscle of chicks fed cyclocreatinine was protected against ATP depletion relative to controls. This effect is quite similar to that observed in
Figure 40. Time courses of glycogenolysis in breast muscle of chicks injected with isoproterenol. Chicks previously fed a control diet (■) or 1.0% cyclocreatinine (1.0% cCr) (●) for 13-17 days were injected with 5 mg/kg of isoproterenol bitartrate (s.c.). Each point represents the mean for 2 to 5 chicks. For points representing 3-5 chicks, ± SEM is presented.
Figure 41. Time courses of ATP depletion during ischemia in breast muscle of chicks injected with isoproterenol 2 hours prior to initiation of ischemia. Chicks previously fed a control diet (■), 0.5% cyclocreatinine (0.5% cCr) (▲), or 1.0% cyclocreatinine (1.0% cCr) (●) for 10-19 days were injected with 5 mg/kg of isoproterenol bitartrate (s.c.), killed 2 hours later, and subjected to ischemia. Each point represents the mean of 2 to 8 chicks. For points representing 3 to 8 chicks, ± SEM is presented.
chick heart. While ATP levels were somewhat lower in cyclocreatine-fed chicks initially, within an hour following the initiation of ischemia ATP levels in control muscle had decreased to less than 30% of the initial value while remaining almost unchanged in both 0.5% and 1.0% cyclocreatine-fed chicks. After 2 hours of ischemia ATP levels in breast muscle of chicks fed either cyclocreatine diet remained 6-fold elevated over control levels. An additional 2 hours of ischemia resulted in an almost complete disappearance of ATP from the breast muscle of chicks fed any of the diets. The time course of ATP depletion was quite similar in breast muscle of chicks fed either 0.5% or 1.0% cyclocreatine. The feeding of an increased amount of cyclocreatine appeared to cause a decrease in initial ATP levels. However ATP levels decreased at a slightly lower rate during ischemia in the 1.0% cyclocreatine-fed chicks relative to the 0.5% cyclocreatine-fed chicks. After 3 hours of ischemia ATP levels remained slightly higher in the 1.0% cyclocreatine-fed chick breasts than in 0.5% cyclocreatine-fed chick breasts. These studies were performed on chicks fed cyclocreatine for 10-19 days. Other experiments indicated that the ATP-sparing effect could be seen after 1 hour of ischemia after only 4 days of feeding cyclocreatine. The protective effect could be seen at 2 hours after only 4 to 6 days on the diet.

During ischemia previously accumulated cyclocreatine-P was utilized for regeneration of ATP (Figure 42). As
Figure 42. Time courses of the utilization of cyclocreatine-P during ischemia in breast muscle of chicks injected with isoproterenol 2 hours prior to initiation of ischemia. For details see Figure 41.
expected breast muscle of 1.0% cyclocreatine-fed chicks contained more total cyclocreatine than did muscle of 0.5% cyclocreatine-fed chicks. Total cyclocreatine in the 0.5% cyclocreatine-fed breast was 27.0 ± 2.8 μmol/g, while 1.0% cyclocreatine-fed breast contained 38.3 ± 3.0 μmol/g in these studies. A large amount (96-97%) of this accumulated cyclocreatine was phosphorylated. During ischemia, cyclocreatine-P decreased at approximately the same rate in chicks fed either diet. After 2 hours of ischemia, about half of the accumulated phosphagen had been consumed. An additional 2 hours of ischemia resulted in nearly total depletion of cyclocreatine-P stores.

As remaining glycogen stores were consumed during ischemia following isoproterenol injection, lactate was produced as an endproduct (Figure 43). Lactate production occurred at approximately the same rate during the initial hour of ischemia in breast muscle of chicks fed any of the three diets. After 2 hours of ischemia lactate levels were still quite similar in chicks of all three diets. At this point however lactate production ceased in control breast muscle, while continuing in cyclocreatine-fed breast muscle. After 4 hours of ischemia lactate was slightly elevated in muscle of chicks fed 1.0% cyclocreatine over that in chicks fed 0.5% cyclocreatine. Much more lactate was produced during ischemia following isoproterenol injection in breast muscle from chicks fed either of the cyclocreatine diets than in the controls; after 4 hours of ischemia lactate levels
Figure 43. Time courses of the accumulation of lactate during ischemia in breast muscle of chicks injected with isoproterenol 2 hours prior to initiation of ischemia. For details see Figure 41.
were elevated greater than two-fold in cyclocreatine-fed chicks relative to controls.

In an attempt to deplete glycogen levels to the same low level in breast muscle of cyclocreatine-fed animals as in controls, preliminary experiments were performed in which chicks were injected with isoproterenol and the amount of time before killing was doubled to 4 hours. As is seen in Figure 44 this procedure resulted in an ATP-sparing effect, much like that seen in Figure 41. Due to the decreased glycogen pools available for energy production, ATP depletion occurred at an increased rate, falling to practically zero after 2 hours of ischemia compared to the 4 hours necessary for total depletion when the time under isoproterenol was shortened to 2 hours. However, ATP remained greatly elevated during ischemia in the breast muscle of chicks fed cyclocreatine. After one hour of ischemia ATP levels in cyclocreatine-fed muscle were about 40-fold higher than in controls.

Lactate levels in chick breast muscle subjected to 4 hours of isoproterenol followed by ischemia (Table 7) were lower than in chicks subjected to only 2 hours of isoproterenol before ischemia (Figure 43). This indicated that glycogen levels were indeed depleted as anticipated, although lactate accumulation in breast muscle of chicks fed cyclocreatine diets was elevated relative to controls. Although this protocol held great promise, it was observed that results were not as consistent in chicks which were
Figure 44. Time courses of ATP depletion during ischemia in breast muscle of chicks injected with isoproterenol 4 hours prior to initiation of ischemia. Chicks previously fed a control diet (■) or 1.0% cyclocreatine (1.0% cCr) (●) for 19 days were injected with 5 mg/kg of isoproterenol bitartrate (s.c.), killed 4 hours later, and subjected to ischemia. Points at 1 hour represent mean ± SEM for 6 chicks. Other points in this preliminary experiment represent 1 chick.
<table>
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<tr>
<th>DIET&lt;sup&gt;a&lt;/sup&gt;</th>
<th>ATP REMAINING&lt;sup&gt;b&lt;/sup&gt; (µmol/g)</th>
<th>TOTAL CYCLO-β CREATINE (µmol/g)</th>
<th>LACTATE (µmol/g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CONTROL</td>
<td>0.07 ± 0.08 (6)</td>
<td>0 (6)</td>
<td>15.4 ± 1.9 (5)</td>
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<td>0.25% CYCLOCREATINE</td>
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<td>17.9 ± 8.6 (3)</td>
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<tr>
<td>0.6% CYCLOCREATINE</td>
<td>2.32 ± 1.94&lt;sup&gt;»&lt;/sup&gt; (4)</td>
<td>31.6 ± 3.9 (4)</td>
<td>22.6 ± 16.1 (3)</td>
</tr>
<tr>
<td>1.0% CYCLOCREATINE</td>
<td>2.71 ± 1.60&lt;sup&gt;»&lt;/sup&gt; (6)</td>
<td>41.8 ± 5.9 (6)</td>
<td>30.4 ± 5.4&lt;sup&gt;»&lt;/sup&gt; (5)</td>
</tr>
</tbody>
</table>

<sup>a</sup>Five-day-old chicks were fed diets for 12-19 days. Chicks were injected with 5 mg/kg of isoproterenol (s.c.) and killed 4 hours later. Carcasses were incubated for 1 hour, breast muscle excised, fast-frozen and assayed for metabolites. Numbers in parentheses denote chicks for given diet fed and metabolite assayed.

<sup>b</sup>Free plus phosphorylated derivatives of cyclocreatine accumulated by breast muscle.

*P < .001 vs control-fed group.

<sup>»</sup>P < .005 vs control-fed group.

<sup>»</sup>P < .05 vs control-fed group.
subjected to 4 hours of isoproterenol prior to ischemia (note the large values of standard deviation for the ATP values listed in Table 7) as compared to chicks which were exposed to 2 hours of isoproterenol prior to ischemia. Thus, a greater amount of work was performed upon chicks subjected to 2 hours of isoproterenol.

It was decided to pursue preliminary experiments on the effect of isoproterenol on another tissue, leg muscle. Figure 45 illustrates glycogenolysis in chick leg muscle following the injection of isoproterenol. Cyclocreatine pre-feeding did not appear to result in slowed glycogenolysis in leg muscle as it did in breast muscle. Glycogen levels appeared to be initially higher in cyclocreatine-fed leg muscle but decreased rapidly until, after 1 hour, glycogen content appeared to be the same in both cyclocreatine-fed and control leg muscle. Glycogenolysis then continued until only about 2.5 μmol/g remained in muscle from chicks fed either diet after 4 hours. The data presented is for chicks which have been fed cyclocreatine for ten days. Results from chicks fed cyclocreatine for only five days indicated a smaller relative difference in initial glycogen levels. In these chicks also, glycogenolysis appeared to not be slowed in chicks fed cyclocreatine.

Figure 46 illustrates ATP depletion during ischemia in chick leg muscle. Initial ATP values are from non-injected chicks (isoproterenol has no effect on initial ATP levels), while the remaining values are for chicks injected with
Figure 45. Time courses of glycogenolysis in leg muscle of chicks injected with isoproterenol. Chicks previously fed a control diet (■) or 1% cyclocreatine (1% cCr) (●) for 10 days were injected with 5 mg/kg of isoproterenol bitartrate (s.c.). Points in this preliminary experiment represent 1 chick.
Figure 46. Time courses of ATP depletion during ischemia in leg muscle of chicks injected with isoproterenol 90 minutes prior to initiation of ischemia. Chicks previously fed a control diet (■), 0.5% cyclocreatine (0.5% cCr) (▲), or 1.0% cyclocreatine (1.0% cCr) (●) for 15 days were injected with 5 mg/kg isoproterenol bitartrate (s.c.), killed 90 minutes later, and subjected to ischemia. 0 hour animals were neither injected with isoproterenol nor subjected to ischemia. Points in this preliminary experiment represent 1 chick.
isoproterenol 90 minutes prior to death and then subjected to ischemia. Apparently, no ATP-sparing effect occurred with cyclocreatine prefeeding in chick leg muscle. ATP appeared to remain at higher levels in controls than in 1% cyclocreatine-fed chicks following initiation of ischemia. 0.5% cyclocreatine-fed leg muscle possessed intermediate levels of ATP. Within two hours almost all ATP has apparently been depleted in leg muscle of chicks fed any of the three diets.

Upon assay of leg muscle lactate levels in the experiment presented in Figure 46, it was found that lactate accumulation appeared to be approximately the same after 45 minutes of ischemia in chicks fed any of the three diets. Control and 1% cyclocreatine-fed leg muscle contained similar amounts of lactate after 2 hours of ischemia, while lactate levels appeared to be somewhat elevated in 0.5% cyclocreatine-fed leg muscle. Additional study appeared to indicate that lactate levels in 0.5% cyclocreatine-fed leg muscle treated in this manner were actually quite similar to those in leg muscle of chicks fed the other two diets. As was expected, cyclocreatine levels were increased when increased levels of cyclocreatine were added to the diet. Total cyclocreatine levels in leg muscle of chicks fed 0.5% cyclocreatine was 22.5 ± 3.0 μmol/g, while 1% cyclocreatine-fed muscle contained 29.2 ± 4.6 μmol/g. Accumulated cyclocreatine-P stores were consumed during ischemia in leg muscle from chicks fed either diet.
Returning to the chick breast muscle studies in which an ATP-sparing effect was observed in chicks injected with isoproterenol followed by ischemia, two additional graphs are presented (Figures 47 and 48). In these graphs data are depicted from experiments in which four different diets were fed: control, 0.25% cyclocreatine, 0.5% cyclocreatine, and 1% cyclocreatine. Each point on the graphs represents results for one chick injected with isoproterenol, killed 2 hours later and subjected to 2 hours of ischemia. In Figure 47 ATP content is plotted against total cyclocreatine. It is seen that as cyclocreatine content increases so does the ATP-sparing effect. Breast muscle from chicks fed 0.25% cyclocreatine demonstrated a partial conservation of ATP. Breast muscle which had accumulated greater than 20 μmol/g of total cyclocreatine (that from chicks fed 0.5% or 1.0% cyclocreatine) was maximally protected against ATP depletion. Thus it is seen that a correlation exists between accumulation of cyclocreatine derivatives and sparing of ATP levels during ischemia in chick breast muscle.

In Figure 48 ATP levels in breast muscle from chicks killed 2 hours after isoproterenol injection and then subjected to 2 hours of ischemia are plotted against lactate levels. It is observed that ATP content appears to increase with increasing lactate content in chicks not maximally protected against ATP depletion, that is, in controls and 0.25% cyclocreatine-fed chicks. However, upon examination of the curves drawn on this interpretational graph, an
Figure 47. Correlation of ATP remaining after 2 hours of ischemia with total cyclocreatinine content in breast muscle of chicks injected with isoproterenol 2 hours prior to ischemia. Chicks previously fed a control diet (■), 0.25% cyclocreatinine (0.25% cCr) (○), 0.5% cyclocreatinine (0.5% cCr) (▲), or 1% cyclocreatinine (1.0% cCr) (●) for 17-19 days were injected with 5 mg/kg isoproterenol bitartrate (s.c.), killed 2 hours later, and subjected to 2 hours of ischemia. Each point represents a single chick, except for the single control value which represents mean ± SEM for 7 chicks.
Figure 48. Relationship of ATP remaining after 2 hours of ischemia with accumulated lactate levels in breast muscle of chicks injected with isoproterenol 2 hours prior to ischemia. Chicks previously fed a control diet (■), 0.25% cyclocreatinine (0.25% cCr) (○), 0.5% cyclocreatinine (0.5% cCr) (▲), or 1% cyclocreatinine (1.0%cCr) (●) for 17-19 days were injected with 5 mg/kg isoproterenol bitartrate (s.c.), killed 2 hours later and subjected to 2 hours of ischemia. Each point represents a single chick.
interesting effect is observed. If a particular lactate concentration (that indicated by the asterisk) is chosen, it is seen that ATP levels are not the same in chicks fed the different diets. At identical lactate concentrations ATP levels in breast muscle from cyclocreatinine-fed chicks appear to remain much higher than in control chicks. In breast muscle from 0.5% and 1.0% cyclocreatinine-fed animals ATP levels were more than 3-fold greater than in control muscle. Thus it appears that although increased glycolysis is certainly helpful in retaining elevated ATP levels, this is not the sole reason for the ATP-sparing effect observed in cyclocreatinine-fed chick breast muscle during ischemia following isoproterenol injection. The cyclocreatinine-P accumulated by this tissue must be assisting to protect against ATP depletion.

Summary

Isoproterenol injection into chicks was performed for two reasons. First was to attempt to lower glycogen stores so events occurring during the following ischemia in breast muscle would be speeded up, permitting shorter ischemic periods and facilitating experimentation. Secondly it was hoped that glycogen content could be depleted to negligible amounts allowing the study of muscle forced to rely on other energy stores besides glycogen, primarily phosphagens, upon subjection to ischemia. In this manner it would be possible to assess glycogen contribution to effects noted during
ischemia in muscle from cyclocreatine-fed chicks. The second goal proved elusive since results from chicks treated in such a manner proved were too variable. Thus it was necessary to find other ways to assess the contribution of glycogen.

It was found that glycogenolysis following isoproterenol injection was initially slowed in breast muscle of chicks which had been fed cyclocreatine, relative to controls. Further work was performed in which chicks were subjected to ischemia 2 hours after the injection of isoproterenol. It was discovered that prior feeding of cyclocreatine elicited an ATP-sparing effect in breast muscle. In results quite similar to those previously found in heart, ATP levels remained 6-fold elevated after two hours of ischemia in chicks fed 0.5% or 1% cyclocreatine as compared to those fed a control diet. During ischemia accumulated cyclocreatine-P was utilized in addition to glycolysis to replenish ATP supplies. Glycolysis continued longer in breast muscle from chicks fed either cyclocreatine diet.

Interestingly, glycogenolysis following isoproterenol injection was not slowed in preliminary experiments with leg muscle of chicks fed cyclocreatine. Furthermore, no ATP-sparing effect was noted during ischemia following isoproterenol injection in leg muscle of chicks fed cyclocreatine, in contrast with the results described for breast muscles. The two muscles consist of differing amounts of individual fiber types. The breast muscle, which shows the ATP-sparing effect in cyclocreatine-fed animals, is
composed almost totally of white, fast-twitch fibers. The leg muscle, in which an ATP-sparing effect is not demonstrable in cyclocreatine-fed chicks, contains more red fibers than does breast muscle. Apparently, the differences in ATP-sparing ability in leg and breast muscle elicited by prior cyclocreatine feeding are related to this differential composition.

Finally, evidence was presented that if the contribution of energy from glycogen to the ischemic breast muscle is equalized, ATP levels in cyclocreatine-fed animals are still elevated compared to those in control muscle, indicating that glycogen is not the only contributing factor involved in the ATP-sparing effect. Furthermore, a correlation was demonstrated between the amount of cyclocreatine derivatives previously accumulated by breast muscle and the sparing of ATP during ischemia.
Chapter VI: Conclusions

This thesis dealt with the study of creatine and several of its analogues. Four separate areas of research were described: 1), studies utilizing various chick embryonic tissues as uptake systems, 2), the partial purification of chick liver amidinotransferase, 3), studies with chick heart, and 4), studies with chick skeletal muscle.

Several chick embryo tissues were used for the first time to study the uptake and utilization of creatine and its synthetic analogues, in addition to work performed with the previously studied chick embryo brain. It was found that chick embryo skin, heart, and muscle, with incubation in media containing cyclocreatinine, could take up and phosphorylate the compound, with skin and heart able to accumulate large amounts of cyclocreatinine-P. Optimization of various parameters of phosphagen accumulation in embryonic heart was attempted; 3-hydroxybutyrate was used as an energy source instead of glucose and heart was quartered prior to incubation, instead of minced, as was done with embryonic brain. Accumulated cyclocreatinine-P was utilized when embryonic heart or skin was subjected to nitrogen anoxia. Upon removing the nitrogen and placing the tissue back into incubation media, cyclocreatinine-P was resynthesized. In embryonic heart previously accumulated creatine-P or guanidinoacetate-P could be depleted by
anoxia, and resynthesized afterwards. It was furthermore noted that in heart tissue previously incubated with any of these compounds, ATP was depleted in response to anoxia and regenerated following anoxia. Several other compounds were applied to bring about depletion of accumulated phosphagen. In embryonic heart, this was accomplished by incubating the tissue with the cardiac glycoside, ouabain or with the respiratory inhibitor, cyanide. In embryonic brain, incubation with iodoacetate, an inhibitor of the glycolytic pathway, appeared to bring about a slight depletion of accumulated cyclocreatine-P. In addition, embryonic heart and brain were incubated with uptake media containing ATP. It was found that in contrast with brain, where no difference was observed, in embryonic heart accumulation of phosphagens was apparently increased when the tissue was incubated in the presence of ATP.

In the second area of research, arginine:glycine amidinotransferase, the first enzyme of the two-step synthesis of creatine, was partially purified from liver for the first time. The enzyme has previously been purified from rat kidney and hog kidney. Critical steps of this purification from chick liver included ammonium sulfate fractionation resulting in a 4-fold purification and DEAE-cellulose chromatography with another 3 to 4 fold purification. Additional important steps included hydroxylapatite chromatography, resulting in a 4-fold purification and arginine-agarose chromatography, resulting
in a doubling of purity. Utilization of the outlined scheme resulted in a 160-fold purification of the enzyme arginine:glycine amidinotransferase.

A third area of study was that of chick heart and ischemia. Chicks were fed diets containing creatine analogues for a specified period of time, anaesthetized, and the heart excised and subjected to total ischemia in vitro. It was found that ATP levels remained as much as 6-fold higher during ischemia in hearts from chicks fed cyclo-creatine compared to those from controls. Cyclocreatine-P previously accumulated during feeding was utilized by the tissue during ischemia for the regeneration of ATP. Hearts of cyclocreatine-fed animals had greatly increased inorganic phosphate and lactate production during ischemia relative to controls; in addition these hearts contained greatly elevated glycogen stores prior to ischemia. In an attempt to differentiate some of these effects, additional chicks were fed another analogue of creatine, homocyclocreatine, a compound which has thermodynamic properties similar to cyclocreatine, but much slower kinetic properties. Hearts of homocyclocreatine-fed animals possessed elevated glycogen stores and also demonstrated increased lactate production during ischemia. However, accumulated homocyclocreatine-P was only slightly utilized during ischemia, and most importantly, ATP levels were not protected against depletion relative to similarly treated controls. Feeding of creatine resulted in a doubling of creatine levels in the heart but
no delay of ATP depletion during ischemia relative to controls. In hearts of chicks fed cyclocreatinine, in addition to the protection of ATP, total adenylate pools were also protected during ischemia, with AMP levels becoming elevated as ischemia progressed in cyclocreatinine-fed hearts relative to controls. Through the feeding of various levels of cyclocreatinine it was established that a correlation exists between elevation of ATP during ischemia and the amount of cyclocreatinine derivatives previously accumulated by the heart.

The final area of research dealt with the study of chick skeletal muscle and effects conferred upon it by the presence of cyclocreatinine-P. Chicks were fed cyclocreatinine for a period of time, then removed from the diet and injected with the compound isoproterenol, a synthetic analogue of epinephrine which yields only its β-adrenergic-agonistic effects, including increased muscle glyco- genolysis. At specific times after the injection the animals were killed and a portion of the breast muscle removed from each animal for analysis. It was found that in breast muscle of chicks which had been fed cyclocreatinine the breakdown of glycogen stores was slowed, so that glycogen levels remained 4-fold higher than in controls in animals killed 90 minutes after isoproterenol injection. Further experiments involved the subjection of the animal to ischemia two hours after the injection of isoproterenol. It was found that breast muscle of cyclocreatinine-fed chicks
exhibited prolonged glycolysis, utilization of cyclocreatine-P for resynthesis of ATP and a dramatic decrease in the rate of ATP depletion during ischemia relative to controls. In chicks killed two hours after the injection of isoproterenol and subjected to two hours of ischemia ATP levels remained 6-fold higher in breast muscle of chicks fed either 0.5% or 1.0% cyclocreatine as compared to controls. This ATP-sparing effect could be observed after one hour of ischemia after only four days of feeding of cyclocreatine. The protective effect could be seen at 2 hours of ischemia after only four to six days of feeding. In contrast, leg muscle from chicks fed cyclocreatine did not appear to demonstrate a protective effect against ATP depletion during ischemia following isoproterenol injection. In addition, glycogenolysis following the injection of isoproterenol was not slowed in cyclocreatine-fed chick leg muscle relative to controls. Apparently the difference between the two tissues is due to the difference in muscle composition. Chick breast muscle is composed of almost 100% white glycolytic fibers, while leg muscle also contains red fibers. As regards breast muscle, it was found that a correlation existed between ATP remaining after a period of ischemia following injection of isoproterenol and the amount of total cyclocreatine previously accumulated. In addition, by plotting ATP against lactate it was deduced that lactate, and therefore glycogen, was not the sole cause of the ATP-sparing effect.
In conclusion, several of the possible mechanisms of the ATP-sparing effect of cyclocreatine feeding on the heart have been eliminated by comparison of the metabolism during ischemia of hearts from homocyclocreatine-fed animals with those from cyclocreatine-fed animals. The mechanisms eliminated as being the sole cause of the ATP-sparing effect are: 1), an allostERIC effect of the planar phosphorylated cycloguanidine ring, 2), lowered myocardial creatine levels, 3), the increased glycogen stores in cardiac tissue, 4), the increased rate and extent of glycolysis during ischemia. However, a possible synergism between glycolysis and cyclocreatine-P utilization could function to aid in the sparing of ATP during ischemia in hearts of chicks fed cyclocreatine.

While homocyclocreatine-P is structurally quite similar to cyclocreatine and has quite similar thermodynamic properties, its kinetic properties are considerably slower. Homocyclocreatine-P reacts 1000-fold more slowly with creatine kinase than does cyclocreatine-P, and 200,000-fold more slowly with creatine kinase than does creatine-P. Thus, although homocyclocreatine-P is thermodynamically competent to buffer the ATP/ADP ratio during the low phosphorylation potential characteristic of the later stages of ischemia, it is kinetically incompetent to do so. Creatine-P is kinetically competent, but thermodynamically incompetent to buffer ATP during the later stages of ischemia. Cyclocreatine-P alone possesses the proper
thermodynamic and kinetic properties to serve as a buffer of ATP during prolonged ischemia.
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