Higher Homolog and N-Ethyl Analog of Creatine as Synthetic Phosphagen Precursors in Brain, Heart, and Muscle, Repressors of Liver Amidinotransferase, and Substrates for Creatine Catabolic Enzymes*

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Tissues of chicks fed 5% N-methyl-3-guanidinopropionate (N-amidino-N-methyl-L-alanine) for 12 days accumulated the following amounts of free plus phosphorylated derivatives as μmol/g, wet weight: brain, 5.5; heart, 7.3; leg muscle, 21.0; and breast muscle, 24.4. Since total creatine levels remained nearly the same in brain, N-methyl-3-guanidinopropionate-P provided brain with a supplemental reservoir of high energy phosphate. Tissues of rats fed 2% N-ethylguanidinoacetate (N-amidino-N-ethylglycine) accumulated large amounts of N-ethylguanidinodiacetate-P, which has thermodynamic properties similar to creatine-P and is the kinetically most reactive synthetic phosphagen yet described. N-Ethylguanidinodiacetate derivatives replaced creatine derivatives mole-for-mole, and the fraction of synthetic to total phosphagen after 19 days was 60% in heart, 54% in slow oxidative muscle, 42% in fast glycolytic muscles, and 22% in brain. N-Ethylguanidinodiacetate served as a false end product co-repressor of liver arginine:glycine amidinotransferase in both chicks and chick embryos; N-methyl-3-guanidinopropionate and N-propylguanidinoacetate were relatively inactive. Creatinine amidohydrolase reversibly cyclized both N-ethylguanidinodiacetate and N-propylguanidinoacetate with even lower Kₘ values than for creatine derivatives, but it did not react significantly with N-methyl-3-guanidinopropionate, 3-guanidinopropionate, or 1-carboxymethyl-2-iminoimidazolidine (cyclocreatine). Creatine amidinohydrolase also hydrolyzed N-acetimidoylsarcosine, but was relatively unreactive toward N-ethylguanidinodiacetate, N-methyl-3-guanidinopropionate, 3-guanidinopropionate, and cyclocreatine. Amidinohydrolase can therefore be used to remove interfering creatine in assays of tissues for coexisting N-ethylguanidinodiacetate or N-methyl-3-guanidinopropionate. Assays are now available to follow changes during metabolic stresses of any combination or all of the following phosphagens accumulated by the same tissue: creatine-P, N-ethylguanidinodiacetate-P, cyclocreatine-P, N-methyl-3-guanidinopropionate-P, and homocyclocreatine-P.

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The principal functions of the creatine-P-creatine kinase system are to serve as a regulator of Pₐ levels (1, 2) and as a thermodynamic buffer (3) in near-equilibrium with the adenylate system in brain, heart, skeletal and smooth muscle, skin, lung, brown fat, macrophages, and certain other tissues. It has been suggested that as a consequence of this near-equilibrium relationship, intracellular diffusion of ATP and ADP is greatly facilitated, and up to 99% of the intracellular high energy phosphate flux in these tissues is transported by creatine-P as a surrogate for ATP, with creatine serving as a surrogate for ADP (4, 5). Creatine has also been implicated as a co-repressor of liver arginine:glycine amidinotransferase, the first of two enzymes involved in creatine biosynthesis (1, 6–8). Unregulated biosynthesis of creatine would cause wasteful diversion of 3 of the 20 amino acids involved in protein synthesis (1); even under normal conditions the daily loss of methyl groups in urinary creatinine and creatine approximates the total methyl groups provided by dietary methionine (9). Creatine is not catabolized by vertebrate tissues, but it can be cyclized to creatinine or hydrolyzed to sarcosine and urea by inducible enzymes of bacteria found in the soil (10–12) and apparently in the gut (13). All of the activities in which creatine is known to participate are depicted in Fig. 1. Each of these activities, plus membrane transport in intact animals (14), might be expected to have its own characteristic structural specificity toward creatine analogs, a number of which are shown in Fig. 2.

If up to 99% of the intracellular high energy phosphate flux is indeed transported by creatine-P in certain tissues when the creatine kinase reaction is in near-equilibrium (4), it should be of interest to determine the effects on energy metabolism of introducing into tissues analogs of creatine-P that (i) approach equilibrium with the adenylate system more slowly than creatine-P (15); or (ii) are thermodynamically poised to buffer the adenylate system and regulate Pₐ levels at lower cytosolic phosphorylation potentials and more acid pH, where the creatine-P system is ineffective (16, 17). The biochemical effects of introducing a given analog into tissues would also depend upon the extent of analog accumulation and the degree to which the introduced analog displaces or supplements the endogenous creatine-P pool. In brain, such experimental alterations of a regulatory system that helps buffer both the adenylate system and Pₐ levels should be of particular interest (16), since, in brain, even small changes in intracellular energy transactions, adenylate ratios, Pₐ levels, or ion distributions might result in subtle functional or behavioral changes.

Although studies employing intact animals have been initiated with a number of the creatine analogs depicted in Fig.
Membrane transport systems are not depicted. AdoMet, S-adenosylmethionine; AdoHcy, S-adenosylhomocysteine.

2 such as 3-guanidinopropionate (18-ZO), cyclocreatine (16,17,21), N-acetylimidoylsarcosine (8), and homocyclocreatine (E), comparable studies had not been reported for the two analogs that have been fed to animals. N-Methyl-3-guanidinopropionate, first synthesized by Rowley et al. (22), was synthesized by the following method devised for economical large-scale synthesis. 3-Chloropropionic acid, 290 g, was dissolved in 260 ml of water in a 1.5-liter beaker immersed in an ice bath and slowly neutralized with 158 g of 85% KOH in 160 ml of water. A solution of 440 ml of 40% methylamine in 600 ml of water was heated to 65 °C in a 2-liter Erlenmeyer flask, and to this the chloropropionate solution was added dropwise with stirring over a 1-1.5-h period. The rate of addition was adjusted to maintain a temperature of 65-75 °C. When the addition was complete, the temperature was maintained between 70 and 80 °C for 1 h, after which the reaction mixture was concentrated to a small volume in a 2-liter round-bottom flask, using a rotary evaporator at 70 °C. The concentrate was dissolved in 500 ml of methanol, chilled on ice, and filtered to remove precipitated KCl. The filtrate was concentrated to a small volume with a rotary evaporator and cooled to room temperature. The contents of a 100-g bottle of crystalline cyanamide were added to the round-bottom flask, and the bottle was rinsed with 20 ml of concentrated NH4OH, which was also added to the flask. After mixing, the reaction mixture was added to a 1-liter Erlenmeyer flask, which was then tightly stoppered and placed in an ice bath. After 12 h, the flask was removed from the bath, and after 48 h a solid cake of N-methyl-3-guanidinopropionate had formed. The cake was broken up, filtered, and washed with cold 95% ethanol. The filter cake was recrystallized twice from a minimum volume of boiling water. Yield: 80-90 g of N-methyl-3-guanidinopropionate·H2O; m.p. 248-250 °C (decomposition) (literature values 248-249 °C).

The 60-MHz proton NMR spectrum showed peaks at δ 2.57 (2H, triplets); 3.07 (1H, singlet), and 3.63 (2H, triplets). Chick embryos were injected and livers were harvested as described.
earlier (29, 30). Chicks were fed powdered Purina Startena mixed with analog and anesthetized with ether. Whole liver homogenates were assayed after freezing and thawing for amidonitratrelease activity by trapping the enzyme-active urea intermediate formed from arginine with NH$_4$OH to give N-hydroxyarginine, which was assayed at 480 nm after reaction with an aged 1% solution of N$_2$H$_4$(Fe(CN)$_3$)NH$_3$ at pH 7.0 (6, 31). Chick tissues containing creatine analogs were fast-frozen with aluminum tonsils chilled in liquid N$_2$, pulverized in a chilled steel percussion mortar, and homogenized in 0.4 M HCO$_3$ with a Tekmar Tissuemizer (21). N-Ethylguanidinoacetate and N-propylguanidinoacetate had molar extinction coefficients 80% that of creatine and N-methyl-3-guanidinoproprionate in the diacetyl assay (32). Guanidinoacetate and 3-guanidinopropionate were assayed by the Sakaguchi reaction (33). Cyclization of creatine analogs was followed by the increase in UV absorbance: creatinine, 6900 M$^{-1}$ cm$^{-1}$; N-ethylglycocyamidine, 6200 M$^{-1}$ cm$^{-1}$; N-propylguanidinoacetate, 6900 M$^{-1}$ cm$^{-1}$; N-propylglycocyanidine, 7900 M$^{-1}$ cm$^{-1}$; glycocyamidine, 7450 M$^{-1}$ cm$^{-1}$ (34). Hydrolysis was assayed by formation of diacetyl-reactive product.

RESULTS

Accumulation of N-Methyl-3-guanidinopropionate Derivatives by Chick Tissues—Young chicks fed 1% 3-guanidinoproprionate stopped eating and often died within 1 week. They accumulated large amounts of 3-guanidinoproprionate and the free compound in breast muscle, as indicated in Fig. 3. This dietary regimen resulted in near-equal amounts of this synthetic phosphagen and creatine-P coexisting in breast muscle. The relative fractions phosphorylated indicated that N-methyl-3-guanidinopropionate-P has a Gibbs standard free energy of hydrolysis as high or higher than creatine-P and markedly higher than cyclonucleotide-P and homocyclocreatinine, both of which were at least 91% phosphorylated in chick breast muscle (15).

Large amounts of N-methyl-3-guandinopropionate derivatives were also accumulated from the diet by a number of other chick tissues, as indicated in Table I. At similar cytosolic phosphorylation potentials (35), all tissues containing creatine kinase would have fractions of N-methyl-3-guanidinopropionate in the phosphorylated form similar to that found in breast muscle; we have established that this general principle holds even for the extremely slow-reacting homocyclocreatine and its phosphate (15). Levels of coexisting creatine derivatives are also given in Table I, along with average values for chicks fed a control Startena diet. In the case of brain, this net increase in high energy phosphate reserves and thermodynamic buffering capacity occurred without a significant decrease in total creatine levels (Table I). This is the first report of a supplemental accumulation by brain of intact analogs of a synthetic phosphagen that is thermodynamically poised to buffer the adenylyl system at high phosphorylation potentials (cf. Ref. 16).

**Accumulation of N-Ethylguanidinoacetate Derivatives by Rat Tissues—N-Ethylguanidinoacetate-P is the most reactive analog and the free compound in tissues (15).**

**TABLE I**

| Tissue             | Total N-methyl-3-guanidinopropionate | Total creatine | Total phosphagen |
|--------------------|--------------------------------------|----------------|------------------|
| Breast muscle      | 24.4 ± 4.4                           | 20.0 ± 0.8     | 44.4 (37)        |
| Leg muscle         | 21.0 ± 2.3                           | 14.7 ± 2.3     | 36.7 (27)        |
| Brain              | 5.5 ± 1.5                            | 7.2 ± 0.3      | 12.7 (8)         |
| Heart              | 7.3 ± 1.0                            | 4.0 ± 1.0      | 11.3 (7)         |

The abbreviation used is: Heps, 4-(2-hydroxyethyl)-1-pipera- zineethanesulfonic acid.

**Fig. 3. Separation on a Dowex 1 (HCO$_3$) column of N-methyl-3-guanidinopropionate-P (PmGP) and creatine-P (PCr) from their nonphosphorylated derivatives coexisting in an extract of breast muscle from a chick fed 5% N-methyl-3-guanidinopropionate for 11 days. Phosphorylated derivatives were eluted with the KHCO$_3$ gradient as described previously for homocyclocreatine-P (15).**
kinetically of the synthetic phosphagens studied thus far, being even more reactive than cyclocreatine-P (15, 24). N-Ethylguanidinoacetate-P had earlier been detected in tissues of animals fed N-ethylguanidinocacetate, but no quantitative assay was then available that could distinguish between this analog and coexisting creatine derivatives (24). Use of creatine amidinohydrolase to remove interfering creatine has now resolved the analytical problem. Table II shows that feeding of 2% N-ethylguanidinoacetate to rats resulted in accumulation of large amounts of this analog plus its N-phosphorylated derivative in various muscles, heart, and brain. Moreover, the fraction of analog phosphorylated was the same as for creatine, which is consistent with our earlier experiments with the Ehrlich ascites tumor cell model system which demonstrated that both creatine-P and N-ethylguanidinoacetate-P had similar Gibbs standard free energies of hydrolysis (24). Unlike the case of N-methyl-3-guanidinopropionate (Table I) and other creatine analogs (15, 16, 18, 21, 28), net increases in total phosphagen content did not occur in any tissue of animals fed 2% N-ethylguanidinoacetate. Replacement of creatine derivatives by this analog was approximately mole-for-mole, as deduced from total color produced in the diacetyl assay. The greatest degree of replacement of creatine by N-ethylguanidinoacetate derivatives occurred in heart, followed by slow oxidative muscles (Table II). The characteristic variations between individual animals are illustrated here; exploratory experiments with mice and chicks gave similar results, with heart again showing the greatest accumulation of N-ethylguanidinoacetate derivatives, up to 90% of total phosphagens in chick hearts.

Derivatives of N-propylguanidinoacetate were also found to be accumulated by tissues from the diet, but since accumulation was lower than for N-ethylguanidinoacetate or N-methyl-3-guanidinopropionate, further experiments were not performed. Differences in accumulation of these three analogs were most likely due to differences in membrane transport rather than activity with creatine kinase, since N-propylguanidinoacetate is more reactive than N-methyl-3-guanidinopropionate with creatine kinase (15, 22).

Repression of Liver Amidinotransferase by Creatine Analog—Evidence has previously been presented that creatine, and not guanidinoacetate, is the physiological repressor (or co-repressor) of chick liver amidinotransferase (1, 8, 30) and that the creatine analogs N-acetimidoylsarcosine and cyclocreatine could serve as false feedback repressors (8). N-Ethylguanidinoacetate was found to repress liver amidinotransferase levels in both growing chicks (Fig. 4A) and developing chick embryos (Fig. 4B). The degree of repression by N-Ethylguanidinoacetate was similar to that observed with the gratuitous co-repressor, N-acetimidoylsarcosine, acting on chick liver and chick embryonic liver amidinotransferases (8) and with creatine itself acting on amidinotransferase in rat kidney and pancreas (1, 36). N-Ethylguanidinoacetate, like creatine and N-acetimidoylsarcosine, is nontoxic even to the sensitive chick embryo system; embryos grew normally and hatched following injection of these compounds early in development. Mice continually fed 2% N-ethylguanidinoacetate for two generations likewise showed no toxic effects. Neither N-methyl-3-guanidinopropionate or N-propylguanidinoacetate at levels which did not inhibit growth of chicks was an effective repressor of liver amidinotransferase. The effects of these various analogs on derepression of previously repressed chick liver amidinotransferase (cf. Ref. 7) are shown in Table III. Chicks fed 1% 3-guanidinopropionate stopped eating and, as a consequence, had greatly decreased amidinotransferase levels; it will be recalled that chick liver amidinotransferase levels decrease during fasting (7). As can be seen from the

![Fig. 4. Repression of chick liver arginine:glycine amidinotransferase by N-ethylguanidinoacetate (eGA). Relative to control values (100%), in growing chicks (A) and developing chick embryos (B). Chicks were 7 days old at the start of experiment (n = 3). Eggs were injected with N-ethylguanidinoacetate on day 7 of incubation, and livers (15-30 per point) were harvested on day 12 or 13.](image-url)
CREATINE

The rate of decrease in amidotransferase levels, as per cent of controls, in livers of young chicks (n = 2) fed 2% creatine (Cr) and/or injected intraperitoneally at 0 and 24 h with the protein synthesis inhibitor, cycloheximide (CHX, 1 mg/kg).

**Table IV**

| Substrate                  | $K_m$  | $V_{max}$ | $V_{max}/K_m$ | $K_m$       |
|----------------------------|--------|-----------|---------------|-------------|
| Cyclization                |        |           |               |             |
| Creatine                   | 79 ± 24| 100 ± 7*  | 100           | 0.72 ± 0.15 (0.86)* |
| N-Ethylguanidinoacetate    | 17 ± 2.4| 49 ± 2   | 228           | 1.48 ± 0.46 (2.04) |
| N-Propylguanidinoacetate   | 29     | 38        | 103           |              |
| Guanidinoacetate           | 125    |           | 100           | 0.14        |
| Hydrolysis                 |        |           |               |             |
| Creatinine                 | 43 ± 13| 100 ± 17* | 100           | 5.4 ± 1.6   |
| N-Ethylglycocyanidine      | 5.4 ± 1.6| 12 ± 1.8 | 105           |             |

* $V_{max}$ with creatine was 14.2 ± 1.0 S.D. μmol/min/mg of protein in the direction of cyclization (Reaction 1).
* First value is measured value of $K_m$ approached from both directions; value in parentheses is $K_m$ calculated from kinetic parameters with the Haldane relationship.
* $V_{max}$ with creatine was 9.0 ± 1.5 S.D. μmol/min/mg of protein in the direction of hydrolysis (Reaction 1).

Where $R$ is -CH$_3$ for creatine and creatinine. Its specificity for creatine analogs other than guanidinoacetate was determined here for the first time. We found that this enzyme did not react significantly with 3-guanidinopropionate, N-methyl-3-guanidinopropionate, cyclocreatin, or homocyclocreatin and reacted only extremely slowly with guanidinoacetate. However, it reacted readily with N-ethylguanidinoacetate ($R = -C_3H_7$) and N-propylguanidinoacetate ($R = -C_3H_7$) with even lower $K_m$ values than for creatine and creatinine, as shown in Table IV. In fact, the $V_{max}/K_m$ ratio for N-ethylguanidinoacetate was even higher than for the natural substrate, creatine.

The validity of the kinetic data was strengthened by the agreement of $K_m$ of Reaction 1 determined by direct measurement and calculated from the Haldane relationship (Table IV). The respective $K_m$ values are consistent with Lempert's suggestion (39) that bulky N-alkyl groups favor cyclization of glycocyanidines.

Specificity of Bacterial Creatine Amidohydrolase toward Creatine Analogs—Creatine amidohydrolase is an inducible enzyme (10, 12) that catalyzes Reaction 2,

\[
\text{Creatine} + H_2O \rightarrow \text{Creatinine} + \text{NH}_3 + \text{H}^+ (2)
\]

where $R$ is -NH$_2$ for creatine. The relative specificities of this enzyme for creatine and its analogs other than guanidinoacetate were determined here for the first time, with the results shown in Fig. 6. It can be seen that creatine is the preferred substrate and that neither N-methyl-3-guanidinopropionate nor N-ethylguanidinoacetate exhibited significant activity. This specificity can serve as the basis for assay of creatine analogs coexisting in tissues with creatine (Table II), in which interfering creatine is removed by incubation of neutralized extracts with amidohydrolase. The enzyme does not react with creatine-P. Preliminary experiments indicated that amidohydrolase readily hydrolyzed N-acetimidoylsarcosine (Reaction 2; $R = -CH_3$), a creatine analog that contains the

![Fig. 5.](image_url) **Fig. 5.** Rate of decrease in amidotransferase levels, as per cent of controls, in livers of young chicks (n = 2) fed 2% creatine (Cr) and/or injected intraperitoneally at 0 and 24 h with the protein synthesis inhibitor, cycloheximide (CHX, 1 mg/kg).

![Fig. 6.](image_url) **Fig. 6.** Relative reactivity of creatine (Cr) and various analogs (see Fig. 2 for abbreviations) with bacterial creatine amidohydrolase. Activities were measured as disappearance of substrate; the final enzyme concentration was 0.2 mg/ml in Hepes buffer, pH 7.5.
sarcosine moiety, presumably to sarcosine and acetamide. Further studies on this reaction are planned.

**DISCUSSION**

With the addition of the two synthetic phosphagens described in this paper, a total of five different synthetic phosphagens are now known to be accumulated by tissues of intact animals fed the corresponding creatine analogs. These phosphagens, in descending order of their relative kinetic reactivities with rabbit muscle creatine kinase (in parentheses), are: creatine-P (1), N-ethylguanidinoacetate-P (30°), cyclocreatine-P (150°), N-methyl-3-guanidinopropionate-P (500°), 3-guanidinopropionate-P (1,500°), and homocyclocreatine-P (200,000°). The respective kinetic reactivities might vary somewhat with different creatine kinase isozymes and the animal species involved. Cyclocreatine-P and homocyclocreatine-P are further distinguished (i) by possessing a planar pharmacologically active guanidinopropionate ring (Fig. 2) similar to that found in certain pharmacologically active drugs; and (ii) by being thermodynamically poised to buffer the adenylate system and Pi levels at cytosolic phosphorylation potentials 2 kcal/mol below the effective thermodynamic buffering range of the creatine-P system (15, 40, 41), although homocyclocreatine-P is kinetically limited in its ability to buffer the ATP/ADP ratio (15).

It is still not possible to predict either the extent to which derivatives of a novel creatine analog will be accumulated from the diet by a given animal tissue, or the degree to which it will decrease endogenous creatine levels (15). The results obtained must depend in part not only on the specificity of the creatine transport system (14), but also on the occurrence of alternative transport systems for that analog. Consequently, the requisite feeding experiments must actually be performed with intact animals of various species and suitable assay methods developed, as have been described here for N-methyl-3-guanidinopropionate (Table I) and N-ethylguanidinoacetate (Table II). With the development of the two new assays described here, assays are now available to follow the respective changes in response to imposed metabolic stresses of each of the following phosphagens coexisting with all the others in a single tissue: creatine-P, N-ethylguanidinoacetate-P, N-methyl-3-guanidinopropionate-P, cyclocreatine-P, and homocyclocreatine-P. Such assays should prove useful in conjunction with increased use of *in vivo* 31P NMR studies (20).

Even at this early stage in the investigation of N-methyl-3-guanidinopropionate-P and N-ethylguanidinoacetate-P as synthetic phosphagens, certain interesting features have emerged. Feeding of N-methyl-3-guanidinopropionate to chicks resulted in massive accumulation of its high energy phosphate derivative by muscle (Fig. 3) and increased the total phosphagen levels in heart and brain by approximately 60% (Table I). Accumulation by brain was unexpected because derivatives of the parent 3-guanidinopropionate were not accumulated by rat brain, even though accumulation occurred in both muscle (18) and heart (43). Accumulation of N-methyl-3-guanidinopropionate derivatives by brain represented a net increase in high energy phosphate reserves and an enhanced thermodynamic buffering capacity for this important organ, without a significant decrease in total creatine levels. This is of interest because normal energy reserves in brain will support a state of consciousness for less than 10 s after the blood supply has been cut off. The extent to which accumulation of this foreign dianion by brain displaces pools of neuroexcitatory amino acids (cf. Ref. 16) or alters neuronal polarization remains to be determined. Feeding experiments with this analog have not yet been undertaken with rats or mice. Since more prolonged feeding of cyclocreatine resulted in gradually increased accumulation of cyclocreatine-P by mouse brain (44), it is possible that different regimens would result in even greater accumulation of N-methyl-3-guanidinopropionate derivatives by brain. It will be interesting (i) to determine the extent to which the same brain can be loaded with derivatives of N-methyl-3-guanidinopropionate (Table I), N-ethylguanidinoacetate (Table II), cyclocreatine (16, 44), and homocyclocreatine (15), representing four different kinetic response ranges and two different thermodynamic buffering ranges; and (ii) to observe effects of such multiphosphagen loading on adenylate metabolism, neuronal polarization, P, regulation, and displacement of endogenous ions, including neuroexcitatory amino acids.

Our experiments have made it clear that N-ethylguanidinoacetate and its high energy phosphorylated derivative are the synthetic analogs with properties most similar to creatine and creatine-P. Indeed, administration to animals of ethionine with or without added guanidinoacetate (7, 30) should result in endogenous synthesis of N-ethylguanidinoacetate (cf. Ref. 15). N-Ethylguanidinoacetate, like creatine, was tolerated at very high levels in the diet of animals and embryos with no observable adverse effects, and it replaced creatine derivatives in tissues mole-for-mole without affecting normal tissue levels of ATP, glucose-6-P, or glycogen (24). Feeding of high levels of creatine is known not to significantly increase total creatine levels in brain (46); the total high energy phosphate reserves of brain likewise were not increased by feeding N-ethylguanidinoacetate. Our experiments have indicated that N-ethylguanidinoacetate-P has the same thermodynamic properties as creatine-P, based upon their similar degrees of phosphorylation in rat leg muscle (Table II) and comparisons with the same secondary phosphagen standard in dual-phosphagen Ehrlich ascites tumor cells (24). Moreover, N-ethylguanidinoacetate, like creatine, has now been found to repress liver amidinotransferase levels in both chicks and chick embryos (Fig. 4) and to react readily with bacterial creatinine amidohydrolase (Table IV). The main differences appear to be that (i) N-ethylguanidinoacetate (14, 15, 22, 47) and its phosphate (15, 24) react 15-50-fold more slowly than the corresponding creatine derivatives with creatine kinase; (ii) N-ethylguanidinoacetate appears not to be taken up by cells as rapidly as creatine at similar concentrations (14, 24); and (iii) N-ethylguanidinoacetate reacts very much slower with bacterial creatine amidohydrolase than does creatine (Fig. 6). The ability of N-ethylguanidinoacetate to be tolerated by chick embryos and to repress embryonic amidinotransferase levels suggests that it might be possible to obtain chick embryos and young chicks for physiological studies that contain only N-ethylguanidinoacetate derivatives, and not creatine, in their tissues. This would be accomplished by injecting N-ethylguanidinoacetate into eggs, together with 3-hydroxy-4-aminobutyrate (1) and low levels of ethionine (1, 30), to help further reduce endogenous biosynthesis of creatine. In most animals, displacing the last remnants of creatine derivatives from even such favorable tissues as heart might require dietary regimens that include one or more other creatine analogs; coexistence of N-ethylguanidinoacetate-P and cyclocreatine-P has already been demonstrated in the Ehrlich tumor cell model system (24). Because of its complete lack of toxicity, N-ethylguanidinoacetate could be administered to all animals, including man, for sophisticated 31P NMR analyses (cf. Ref. 20), physiological experiments on muscle, heart, and neural tissues, or study of its possible sparing action on nutritional requirements for biosynthetic precursors of creatine (Fig. 1) by virtue of its repression of amidinotransferase levels (Fig. 4 and Table III) (1).
A clearer picture has now emerged concerning the structural requirements for co-repressor activity. Among the creatine analogs shown in Fig. 2, only N-acetylomylsarcosine (8), cyclocreatine (8), and N-ethylguanidinoacetate (Fig. 4) have been found to serve as false feedback co-repressors. With such structural information, it should be possible eventually to construct even more effective repressors of this important enzyme with the ultimate goal of completely shutting down creatine biosynthesis, as has been demonstrated to occur with creatine in the chick embryo system (42). Putative co-repressor candidates preferably should exert no toxic effects on the chick test system, since liver amidinotransferase levels also decrease markedly in response to a number of deleterious agents (1) (cf. Fig. 5).

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REFERENCES

1. Walker, J. B. (1979) Adv. Enzymol. Rel. Areas. Mol. Biol. 50, 177–242
2. Davuluri, S. P., Hird, F. J. R., and McLean, R. M. (1981) Comp. Biochem. Physiol. B Comp. Biochem. 69, 329–336
3. Stucki, J. W. (1980) Eur. J. Biochem. 109, 257–267
4. Meyer, R. A., Sweeney, H. L., and Kushnerer, M. J. (1984) Am. J. Physiol. 246, C365–C377
5. Mainwood, G. W., and Rakusan, K. (1982) Can. J. Physiol. Pharmacol. 60, 98–102
6. Walker, J. B. (1960) J. Biol. Chem. 235, 2357–2361
7. Walker, J. B. (1961) J. Biol. Chem. 236, 493–498
8. Walker, J. B., and Hannan, J. K. (1978) Biochemistry 15, 2519–2522
9. Mudd, S. H., and Poole, J. R. (1975) Metab. Clin. Exp. 24, 721–735
10. Tsuru, D., Oka, I., and Yoshimoto, T. (1976) Agric. Biol. Chem. 40, 1011–1018
11. Rikitake, K., Oka, I., Ando, M., Yoshimoto, T., and Tsuru, D. (1970) J. Biochem. (Tokyo) 86, 1109–1117
12. Yoshimoto, T., Oka, I., and Tsuru, D. (1976) Arch. Biochem. Biophys. 177, 508–515
13. Twort, F. W., and Mellonby, E. (1912) J. Physiol. (Lond.) 44, 43–49
14. Fitch, C. D., and Chevli, R. (1980) Molab. Clin. Exp. 29, 686–690
15. Roberts, J. J., and Walker, J. B. (1983) Arch. Biochem. Biophys. 220, 563–571
16. Woznicki, D. T., and Walker, J. B. (1980) J. Neurochem. 34, 1247–1253
17. Roberts, J. J., and Walker, J. B. (1982) Am. J. Physiol. 243, H911–H916
18. Fitch, C. D., Jellinek, M., and Mueller, E. J. (1974) J. Biol. Chem. 249, 1060–1063
19. Mainwood, G. W., Alward, M., and Eiselt, B. (1982) Can. J. Physiol. Pharmacol. 60, 120–127
20. Shoubridge, E. A., and Radda, G. K. (1984) Biochim. Biophys. Acta 805, 79–88
21. Annesley, T. M., and Walker, J. B. (1980) J. Biol. Chem. 255, 3924–3930
22. Rowley, G. L., Greenleaf, A. L., and Kenyon, G. L. (1971) J. Am. Chem. Soc. 93, 5542–5551
23. Armstrong, M. D. (1933) Fed. Proc. 12, 170–171
24. Roberts, J. J., and Walker, J. B. (1982) Arch. Biochem. Biophys. 215, 564–570
25. Walker, J. B., and Walker, M. S. (1959) J. Biol. Chem. 234, 1481–1484
26. Armstrong, M. D. (1966) J. Org. Chem. 21, 503–505
27. Wang, T. (1974) J. Org. Chem. 39, 3591–3594
28. Griffiths, G. R., and Walker, J. B. (1976) J. Biol. Chem. 251, 2049–2054
29. Woznicki, D. T., and Walker, J. B. (1979) J. Biol. Chem. 254, 12049–12054
30. Walker, M. S., and Walker, J. B. (1962) J. Biol. Chem. 237, 473–476
31. Walker, J. B., and Wang, S.-H. (1964) Biochim. Biophys. Acta 81, 435–441
32. Dubnoff, J. W. (1957) Methods Enzymol. 3, 635–639
33. Van Pilsum, J. F., and Carlson, M. (1970) Anal. Biochem. 35, 424–434
34. Borsook, H. (1935) J. Biol. Chem. 110, 481–493
35. Veech, R. L., Lawson, J. W. R., Cornell, N. W., and Krebs, H. A. (1979) J. Biol. Chem. 254, 6538–6547
36. Walker, J. B. (1965) Nature 206, 1043
37. Donaldson, W. E. (1975) Comp. Biochem. Physiol. B Comp. Biochem. 50, 391–394
38. McGuire, D. M., Gross, M. M., Van Pilsum, J. F., and Towle, H. C. (1984) J. Biol. Chem. 259, 12034–12038
39. Lempert, C. (1959) Chem. Rev. 59, 667–736
40. Annesley, T. M., and Walker, J. B. (1977) Biochem. Biophys. Res. Commun. 74, 185–190
41. Turner, D. M., and Walker, J. B. (1985) Arch. Biochem. Biophys. 238, 642–651
42. Walker, J. B. (1963) Proc. Soc. Exp. Biol. Med. 112, 245–247
43. Fitch, C. D., and Chevli, R. (1975) Anal. Biochem. 68, 196–201
44. Woznicki, D. T., and Walker, J. B. (1979) J. Neurochem. 33, 75–80
45. Stokol, J. A., Weiss, S., and Somerville, C. (1963) Arch. Biochem. Biophys. 100, 86–99
46. Chamutin, A. (1927) J. Biol. Chem. 75, 549–557
47. Ennor, A. H., Rosenberg, H., and Armstrong, M. D. (1955) Nature 175, 120