Creatine Biosynthesis during Embryonic Development. False Feedback Suppression of Liver Amidinotransferase by *N*-Acetimidoylsarcosine and 1-Carboxymethyl-2-iminoimidazolidine (Cyclocreatine)†

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ABSTRACT: The level of arginine:glycine amidinotransferase in liver of the developing chick embryo is partially suppressed following injection of arginine into the yolk, and the level can be completely suppressed following injection of guanidinoacetate or creatine (Walker, J. B. (1963), *Proc. Soc. Exp. Biol. Med. 112,* 245; Walker, J. B., and Wang, S.-H. (1964), *Biochim. Biophys. Acta 81,* 435). In this investigation structural requirements for the physiological suppressor were examined by testing certain analogues of creatine and its biosynthetic precursors for their ability to suppress liver amidinotransferase levels in developing chick embryos and growing chicks. The creatine analogues, *N*-acetimidoylsarcosine and 1-carboxymethyl-2-iminoimidazolidine (cyclocreatine), were found to suppress liver amidinotransferase levels of both developing embryos and growing chicks. Compounds ineffective as suppressors included: the arginine analogue, N^5-acetimidoylornithine; the guanidinoacetate analogue, *N*-acetimidoylglycine; and the creatine analogue, 1-carboxymethyl-2-iminohexahydropyrimidine. Our findings suggest that (i) arginine and guanidinoacetate must be converted to creatine before serving as a suppressor, and (ii) creatine, not phosphocreatine, is most closely related to the physiological suppressor of amidinotransferase.

The biosynthesis of creatine by developing chick embryos requires diversion of 3 of the 20 amino acids from the major pathway of tissue-specific protein synthesis (reactions 1 and 2); consequently creatine biosynthesis must be carefully regulated (Walker, 1963a, 1965a).

$$\text{L-arginine} + \text{glycine} \overset{\text{ATase}}{\rightleftharpoons} \text{L-ornithine} + \text{guanidinoacetate} \quad (1)$$

$$\text{guanidinoacetate} + S\text{-adenosylmethionine} \overset{\text{MTase}}{\longrightarrow} \text{creatine} + S\text{-adenosylhomocysteine} \quad (2)$$

Several factors are believed to participate in the control of creatine biosynthesis. By injecting amino acid precursors of creatine into eggs and subsequently assaying for creatine and for amidinotransferase in embryonic liver, we have shown that the availability of arginine is normally rate limiting for creatine biosynthesis during a major period of embryonic development (Walker and Walker, 1962; Walker, 1963b; Walker and Wang, 1964). The next rate-limiting precursor is glycine (Walker, 1963b); injection of methionine alone does not stimulate creatine biosynthesis. Formation of guanidinoacetate is the rate-limiting step and also the step which is most carefully regulated; when this step is bypassed by injecting guanidinoacetate into the egg, creatine biosynthesis is greatly increased (Walker and Wang, 1964) and methyl groups are correspondingly depleted. Of particular interest is the observation that the level of embryonic liver amidinotransferase is lower the higher the concentration of creatine in that tissue, regardless of whether the increased creatine concentration is of exogenous or endogenous origin (Walker and Wang, 1964; Walker, 1965b).

Studies on the specificity of negative feedback suppression of amidinotransferase with respect to the low molecular weight effector suggested that either creatine or phosphocreatine is the physiological suppressor (Walker and Wang, 1964); it was not possible at that time to distinguish between these two compounds under in vivo conditions. Recently, a number of novel analogues of creatine have been synthesized which differ in their ability to undergo N-phosphorylation by creatine kinase and ATP (Rowley et al., 1971; Wang, 1974). In this investigation the ability of certain of these analogues (Figure 1) to suppress liver amidinotransferase levels in vivo has been studied.

Materials and Methods

Materials. N^5-Acetimidoylornithine (Scannell et al., 1972), *N*-acetimidoylsarcosine, and *N*-acetimidoylglycine were prepared in this laboratory and kindly supplied by Dr. T. Wang (Wang, 1974). Cyclocreatine[1] and 1-carboxymethyl-2-iminohexahydropyrimidine were synthesized as described by Griffiths and Walker (1976). Trisodium pentacyanoammonioferrate $[Na_3(Fe(CN)_5NH_3)]$ was obtained from Fisher Scientific. Fertile White Leghorn chicken eggs, day-old male White Rock chicks, and Purina Chick Startena pelleted food were obtained from Hendricks Grain Co., Houston, Tex. Fertile New Hampshire Red eggs and chicks were obtained from a local farm.

Methods. Amidinotransferase assays were performed on whole homogenates, usually after freezing and thawing, as described previously (Walker, 1960). The use of whole homogenates is necessary for accurate results because amidino-

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[1] Trivial name and abbreviations employed: cyclocreatine, 1-carboxymethyl-2-iminoimidazolidine; ATase, arginine:glycine amidinotransferase; MTase, guanidinoacetate *N*-methyltransferase.

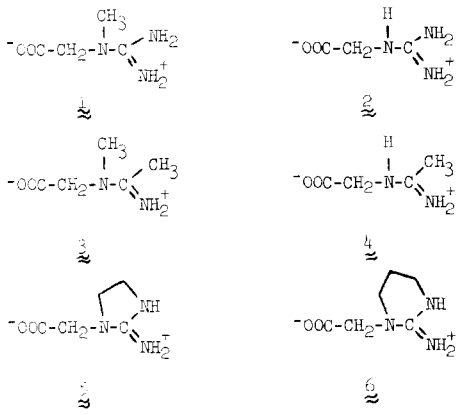

FIGURE 1: Structures of creatine (**1**) analogues utilized in this investigation. Guanidinoacetate (**2**); *N*-acetimidoylsarcosine (**3**); *N*-acetimidoylglycine (**4**); cyclocreatine (**5**); and 1-carboxymethyl-2-iminohexahydropyrimidine (**6**).

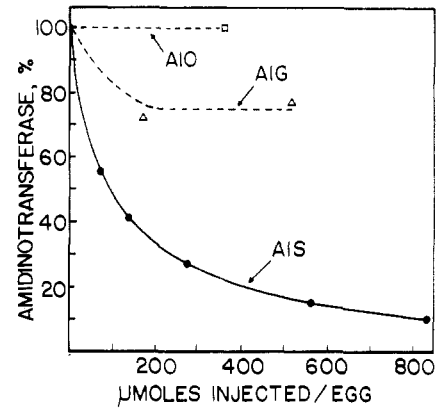

FIGURE 2: Suppression of embryonic liver amidinotransferase, assayed on 12th day of incubation, by various amounts of *N*-acetimidoylsarcosine (AIS) injected 5 days earlier, on the 7th day of incubation. *N*-Acetimidoylglycine (AIG) and N^5-acetimidoylornithine hydrochloride (AIO) are relatively inactive. The liver amidinotransferase levels of control embryos (100%) gave an absorbance of 0.485 in the standard assay for hydroxyguanidine produced. Each point on the curves represents assay of pooled livers from approximately 16 White Leghorn embryos. Similar results were obtained with New Hampshire Red embryos.

transferase is located in mitochondria of rat pancreas (Karelin, 1974), rat kidney (Magri et al., 1975), and chick liver (Richardson and Walker, unpublished experiments). Eggs were injected as described elsewhere (Walker and Walker, 1962; Walker and Wang, 1964), except that the volume injected was 0.4 ml per egg; all solutions, including controls, contained 5 mg of neomycin sulfate per ml to minimize bacterial contamination. *N*-Acetimidoylsarcosine, *N*-acetimidoylglycine, and N^5-acetimidoylornithine hydrochloride are quite soluble in water and were sterilized by autoclaving 15 min at 121 °C prior to injection; no differences in results were obtained when these compounds were not autoclaved prior to injection. Creatine, cyclocreatine, and 1-carboxymethyl-2-iminohexahydropyrimidine are less soluble in water and so must be injected as uniform aqueous suspensions of amorphous powder; crystals of these compounds, even if pulverized in a mortar, have a tendency to settle out and clog the needle, giving erratic results. Amorphous powders of the two cyclic creatine analogues were prepared by adding 20 g of an analogue to 500 ml of water in a 4-l. beaker, warming until dissolved, cooling slightly, and quickly adding with vigorous stirring 1500 ml of acetone. The precipitate was filtered with suction, washed with acetone, and dried in vacuo (yield: 19 g of each compound). Creatine powder was prepared in a similar manner (Walker, 1963b). Suspensions of amorphous powder cannot be autoclaved prior to injection because of the needle-clogging crystals which form on subsequent cooling.

Experiments with growing chicks were performed as described previously (Walker, 1960).

Results

Effects of N-Acetimidoylsarcosine and N-Acetimidoylglycine on Embryonic Liver Amidinotransferase Levels. The observations that N^5-acetimidoylornithine can serve as an inhibitory analogue of arginine and as a substrate for arginase (Scannell et al., 1972) suggested to us that acetimidoyl analogues of creatine and its biosynthetic precursors (Figure 1) might also have biological activity (Wang, 1974). Injection of N^5-acetimidoylornithine into eggs did not suppress liver amidinotransferase levels, nor was this arginine analogue toxic at a concentration of 380 μmol per egg (Figure 2). The corresponding guanidinoacetate analogue, *N*-acetimidoylglycine, also was relatively inactive as a suppressor (Figure 2). However, the corresponding creatine analogue, *N*-acetimidoylsarcosine, did suppress embryonic liver amidinotransferase levels (Figure 2); the curve obtained was quite reproducible with different batches of eggs. Neither of the latter two compounds is toxic during embryonic development; eggs injected on the 7th day of incubation with 540 μmol of *N*-acetimidoylsarcosine subsequently hatched, and these chicks grew normally when fed 1% of this compound in their diet following hatching. Wang (1974) has reported that neither *N*-acetimidoylglycine nor *N*-acetimidoylsarcosine is an active substrate of creatine kinase or inhibitor of creatine phosphorylation. The effect appears to be a suppression of enzyme levels and not an inhibition of enzyme activity, since *N*-acetimidoylsarcosine does not inhibit amidinotransferase activity in vitro, and mixing of control and suppressed homogenates gives additive results. Furthermore, activity is not increased following overnight dialysis of homogenates. Similar controls had previously been performed when the creatine suppression effect was discovered (Walker, 1960).

Effects of Cyclocreatine and 1-Carboxymethyl-2-iminohexahydropyrimidine on Embryonic Amidinotransferase Levels. Kenyon and coworkers have shown that cyclocreatine is an excellent substrate for creatine kinase in vitro, whereas the closely related cyclic analogue, 1-carboxymethyl-2-iminohexahydropyrimidine (Figure 1), is not a substrate (Rowley et al., 1971; McLaughlin et al., 1972). Griffiths and Walker (1976) have recently found that dietary cyclocreatine accumulates in high concentration as P-cyclocreatine in chick breast muscle. Both cyclic creatine analogues can play a regulatory role, like creatine itself, in stimulating the synthesis of actin and the heavy chain of myosin in cultured muscle cells from chick embryos (Ingwall et al., 1974). We found that a low concentration of cyclocreatine suppressed embryonic liver amidinotransferase levels as effectively as a similar concentration of creatine (Figure 3), but cyclocreatine, unlike creatine, is toxic to developing chick embryos in concentrations which would be needed to suppress amidinotransferase levels more completely. Many experiments were performed in an attempt to reverse this toxicity by injecting other compounds, such as creatine, along with cyclocreatine, but all results were negative. 1-Carboxymethyl-2-iminohexahydropyrimidine, on the other hand, does not suppress embryonic liver amidinotransferase levels to a significant extent (Figure 3) and is not toxic to developing chick embryos. As in the case of *N*-aceti-

TABLE I: Suppression by Creatine Analogues of Liver Amidinotransferase in White Rock Chicks.[a]

Dietary Additive	Amidinotransferase Act.[b] (%)
None	100
N-Acetimidoylglycine	94
N-Acetimidoylsarcosine	24
Cyclocreatine	18
1-Carboxymethyl-2-iminohexahydropyrimidine	84
Creatine	6

[a] Male chicks, 14 days old at harvest, had been fed 8 days on powdered Startena and then were fed 5 days on powdered Startena plus indicated additives at a concentration of 1%. [b] Activity of 100% gave an absorbance of 0.795 at 480 nm in the standard assay for hydroxyguanidine produced.

TABLE II: Effect of Creatine Analogues on Recovery of Chick Liver Amidinotransferase from Suppression by Creatine.[a]

Dietary Additive	Amidinotransferase Act. A_{480}	Amidinotransferase Act. %
None	0.633	100
N-Acetimidoylglycine	0.645	102
N-Acetimidoylsarcosine	0.176	28
Cyclocreatine	0.152	24
1-Carboxymethyl-2-iminohexahydropyrimidine	0.373	59
Creatine	0.080	13
Guanidinoacetate	0.106	17

[a] Male White Rock chicks were fed from hatching for 7 days on powdered Startena plus 1% creatine and then fasted 1 day to give a suppressed liver amidinotransferase activity of $A_{480} = 0.015$ in the standard assay for hydroxyguanidine produced. Recovery of amidinotransferase from creatine suppression was measured after feeding for 2 days of diets containing 1% of the indicated additives.

midoylsarcosine, the effect of cyclocreatine was shown not to be due to an inhibition of enzyme activity.

Effects of Analogues on Liver Amidinotransferase Levels of Growing Chicks. Regulation of creatine biosynthesis must occur not only during embryonic development in the closed system of the hen's egg, but also throughout post-hatch life to conserve dietary essential amino acids and methyl groups (Walker, 1963a, 1965a). It was therefore of interest to determine whether those creatine analogues effective as suppressors when injected into the closed system of bacteria-free eggs are also effective when added to the food of growing chicks and subjected to the action of gut flora prior to absorption. The data of Table I show that liver amidinotransferase levels of 9-day old White Rock male chicks were lowered by ingestion of food containing *N*-acetimidoylsarcosine, cyclocreatine, or creatine, but amidinotransferase levels were relatively unaffected by food containing *N*-acetimidoylglycine or 1-carboxymethyl-2-iminohexahydropyrimidine. Next, the effects of various compounds on recovery of amidinotransferase from complete suppression by creatine were examined. Liver amidinotransferase levels of newly hatched White Rock male chicks were almost completely suppressed by feeding a creatine-supplemented diet for 7 days, followed by 1 day with no food (cf. Walker, 1960, 1961); various compounds were added to the diet during a 2-day recovery from suppression, with the results shown in Table II.

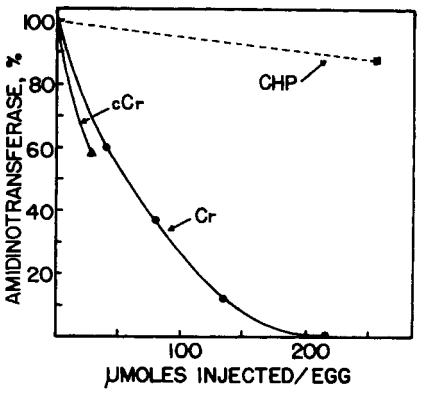

FIGURE 3: Suppression of embryonic liver amidinotransferase, assayed on 12th day of incubation, by cyclocreatine (cCr) and creatine (Cr) injected 5 days earlier, on the 7th day of incubation. 1-Carboxymethyl-2-iminohexahydropyrimidine (CHP) is relatively inactive. Embryos are killed by concentrations of cyclocreatine above 50 μmol per egg. Each point on the curves represents assay of pooled livers from approximately 18 White Leghorn embryos.

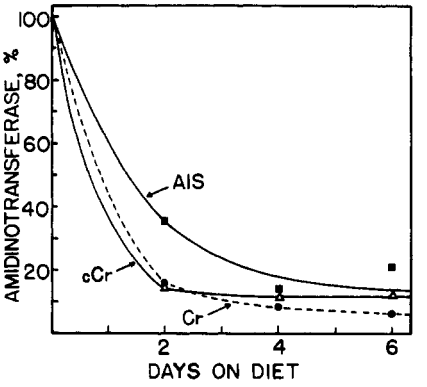

FIGURE 4: Suppression of liver amidinotransferase of New Hampshire Red chicks fed cyclocreatine (cCr), *N*-acetimidoylsarcosine (AIS), or creatine (Cr). Male and female chicks were 7 days old at start of experimental feeding. Each compound was present at a concentration of 0.5% in powdered Startena; each point on the curves represents the average value from livers of two chicks. Amidinotransferase levels are expressed as percentage of the value for control chicks at harvest.

Figure 4 shows the time course of suppression of liver amidinotransferase levels of New Hampshire Red chicks fed, starting at 7 days of age, on diets containing *N*-acetimidoylsarcosine, cyclocreatine, or creatine. It is apparent that *N*-acetimidoylsarcosine and cyclocreatine, when included in the diet, can each be absorbed by the gut and accumulate in liver in high enough concentration to suppress liver amidinotransferase levels in two different strains of growing chicks. As in the case of chick embryos, suitable control experiments established that the observed suppressions did not involve inhibition of enzyme activity.

Discussion

Compounds now known to be effective in suppressing the level of arginine:glycine amidinotransferase in liver of chick embryos and growing chicks include guanidinoacetate and other biosynthetic precursors of creatine (Walker, 1961, 1963b; Walker and Wang, 1964), creatine (Walker, 1960; Walker and Walker, 1962), and, as reported in this paper, two

creatine analogues, *N*-acetimidoylsarcosine and cyclocreatine. N^5-Acetimidoylornithine, *N*-acetimidoylglycine, and 1-carboxymethyl-2-iminohexahydropyrimidine are ineffective as suppressors.

We suggest that biosynthetic precursors of creatine must be enzymically converted to creatine before serving as suppressors. This suggestion is supported by the following observations. (i) Arginine (or citrulline) plus glycine cannot suppress embryonic liver amidinotransferase below approximately 30% of normal levels, whereas guanidinoacetate and creatine can completely suppress amidinotransferase levels (Walker and Wang, 1964). Complete suppression by arginine plus glycine presumably cannot occur because amidinotransferase is required for synthesis of the physiological suppressor from arginine and glycine. (ii) Although guanidinoacetate synthesis from arginine plus glycine is reversible, creatine synthesis from guanidinoacetate is irreversible; therefore the suppressing action of creatine does not result from its conversion to guanidinoacetate. (iii) Five days after injection of various quantities of arginine plus glycine, guanidinoacetate, or creatine into eggs, a plot of embryonic liver amidinotransferase vs. liver creatine concentration gave the same curve, regardless of whether the increments in creatine concentration were derived from increased endogenous synthesis or from exogenously supplied creatine (Walker and Wang, 1964). It is unlikely that any other parameter could exhibit such a correlation. (iv) Suppression of embryonic liver amidinotransferase levels by biosynthetic precursors of creatine was significantly reversed by the simultaneous injection of ethionine, which apparently inhibited conversion of guanidinoacetate to creatine; this ethionine effect was counteracted by methionine. On the other hand, suppression by exogenous creatine was not reversed by ethionine (Walker and Wang, 1964). (v) The arginine analogue, N^5-acetimidoylornithine (Scannell et al., 1972), and the guanidinoacetate analogue, *N*-acetimidoylglycine, are ineffective as suppressors, whereas the corresponding creatine analogue, *N*-acetimidoylsarcosine, is an effective suppressor (Figure 2).

The next question which arises is whether creatine must be phosphorylated by creatine kinase to form phosphocreatine before serving as the physiological suppressor. The fact that cyclocreatine can serve as a suppressor is of little help in this regard since cyclocreatine can readily be phosphorylated by creatine kinase in vitro (Rowley et al., 1971; McLaughlin et al., 1972) and in vivo (Griffiths and Walker, 1976). However, the observation that *N*-acetimidoylsarcosine can suppress amidinotransferase levels suggests that creatine, not phosphocreatine, is the physiological suppressor, inasmuch as Wang (1974) has reported that *N*-acetimidoylsarcosine is not a significant substrate or inhibitor of creatine kinase in vitro.

Our earlier studies on suppression by creatine of liver amidinotransferase in developing chick embryos suggested that liver creatine concentrations above 6 mM completely suppressed amidinotransferase, with half-maximal suppression occurring at 1.5 mM creatine (Walker and Wang, 1964). Extrapolation of the experimental curve of liver amidinotransferase levels vs. liver creatine concentration to zero creatine concentration indicated that liver amidinotransferase of the 12-day chick embryo normally is suppressed approximately 33% by endogenous creatine (Walker and Wang, 1964). Additional unpublished experiments in this laboratory are consistent with this estimate. In studies of the mechanism of suppression, binding of radioactive creatine to any potential regulatory macromolecule might prove difficult to detect if the dissociation constant is as high as the 1.5 mM value estimated for half-maximal suppression of amidinotransferase by creatine. A relatively high dissociation constant with respect to creatine is presumably required because the regulatory system must permit a substantial rate of creatine biosynthesis during rapid growth of vertebrates on a creatine-free diet consisting of plants or lower animals. Hopefully, one of the creatine analogues will be found to have a much lower dissociation constant and thus aid in characterization of the postulated regulatory macromolecule.

It is anticipated that the wide variety of creatine analogues now available (Fitch et al., 1968; Rowley et al., 1971; Wang, 1974; Griffiths and Walker, 1976) will continue to prove useful in studies on the regulation, transport, compartmentation, and various functions of creatine in vertebrate muscle, heart, and brain tissues under both physiological and pathological conditions.

References

Fitch, C. D., Shields, R. P., Payne, W. F., and Dacus, J. M. (1968), *J. Biol. Chem., 243,* 2024.

Griffiths, G. R., and Walker, J. B. (1976), *J. Biol. Chem. 251,* 2049.

Ingwall, J. S., Weiner, C. D., Morales, M. F., Davis, E., and Stockdale, F. E. (1974), *J. Cell Biol. 63,* 145.

Karelin, A. A. (1974), *Vopr. Med. Khim. 20,* 406.

Magri, E., Balboni, G., and Grazi, E. (1975), *FEBS Lett. 55,* 91.

McLaughlin, A. C., Cohn, M., and Kenyon, G. L. (1972), *J. Biol. Chem. 247,* 4382.

Rowley, G. L., Greenleaf, A. L., and Kenyon, G. L. (1971), *J. Am. Chem. Soc. 93,* 5542.

Scannell, J. P., Ax, H. A., Pruess, D. L., Williams, T., Demny, T. C., and Stemple, A. (1972), *J. Antibiot. 25,* 179.

Walker, J. B. (1960), *J. Biol. Chem. 235,* 2357.

Walker, J. B. (1961), *J. Biol. Chem. 236,* 493.

Walker, J. B. (1963a), *Adv. Enzyme Regul. 1,* 151.

Walker, J. B. (1963b), *Proc. Soc. Exp. Biol. Med. 112,* 245.

Walker, J. B. (1965a), in Developmental and Metabolic Control Mechanisms and Neoplasia, Baltimore, Md., Williams & Wilkins, p 317.

Walker, J. B. (1965b), *Nature (London) 206,* 1043.

Walker, J. B., and Wang, S.-H. (1964), *Biochim. Biophys. Acta 81,* 435.

Walker, M. S., and Walker, J. B. (1962), *J. Biol. Chem. 237,* 473.

Wang, T. (1974), *J. Org. Chem. 39,* 3591.