formamide containing 1% acetic acid and subjected to gel filtration on a 0.95 × 59 cm column of Sephadex LH-20 in the same solvent mixture. Sixty 1-ml fractions were collected and examined at 275 mμ. A major peak was observed at 39% of column volume, and its component fractions were pooled and lyophilized. The resulting material (39 mg), which gave the expected amino acid and ammonia analyses for *N*-benzyloxycarbonyl-*S*-benzylcysteinyltyrosylisoleucylglutaminylasparaginyl-*S*-benzylcysteinamide, was treated with sodium in liquid ammonia, oxidized with potassium ferricyanide, and isolated in a manner analogous to the same steps in the synthesis of deaminotocinamide. The crude product was subjected to partition chromatography in the solvent system 1-butanol–ethanol–pyridine–acetic acid–water (4:1:1:0.4:6.4) and eluted with the upper phase with an $R_f$ of 0.26. The material solated from this peak was subjected to gel filtration on Sephadex G-25 in 0.2 *N* acetic acid and isolated by lyophilization. The hygroscopic material became light and fluffy after relyophilization from 2 ml of 0.2 *N* acetic acid: 7.5 mg; $[\alpha]^{23}D$ −4.9° (*c* 0.5, 1 *N* acetic acid).

*Anal.* Calcd for $C_{30}H_{44}N_8O_{12}S_2 \cdot C_2H_4O_2$: C, 48.0; H, 6.18; N, 15.8. Found: C, 47.7; H, 5.92; N, 16.2.

Amino acid analysis gave the following molar ratios: aspartic acid, 1.00; glutamic acid, 1.00; cystine, 0.92; isoleucine, 0.99; tyrosine, 0.94; and ammonia, 2.98.

**Acknowledgments.** The authors thank Miss Paula Glose and Mrs. Janet Huisjen for the bioassays done under the direction of Dr. Louis Nangeroni, New York State Veterinary College, Cornell University.

# On the Specificity of Creatine Kinase. New Glycocyamines and Glycocyamine Analogs Related to Creatine

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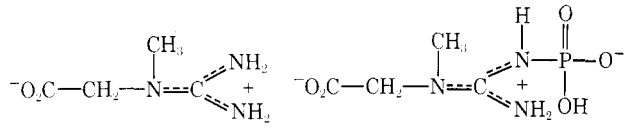
**Abstract:** The specificity of rabbit muscle creatine kinase (adenosine triphosphate–creatine phosphotransferase, EC 2.7.3.2) for a series of new synthetic analogs of creatine has been investigated. Two of these analogs, 1-carboxymethyl-2-iminoimidazolidine and *N*-methyl-*N*-amidinoaminomethylphosphinic acid, are more reactive (31 and 13% as reactive as creatine, respectively) as substrates in the creatine kinase reaction than any analogs of creatine reported to date. New synthetic routes to substituted glycocyamines have been developed as well as some improvements made on existing synthetic procedures. Earlier synthetic difficulties are discussed in terms of solubility properties of the glycocyamines and their ease of cyclization to glycocyamidines. In the presence of the enzyme, adenosine triphosphate has been shown to phosphorylate the highly reactive analog 1-carboxymethyl-2-iminoimidazolidine on the primary amino group to give 1-carboxymethyl-2-(phosphonoimino)imidazolidine. This result and the other specificity results are discussed in terms of bulk tolerance and geometrical requirements at the active site of the enzyme for optimal activity of the creatine analogs.

Creatine (*N*-methylglycocyamine (**1**)) is found in relatively large quantities in the muscles of vertebrates[2] as its phosphorylated derivative *N*-(phosphonoamidino)sarcosine (phosphocreatine **2**). Phosphocreatine is thought to be a storage form of energy made available for sustained muscular contraction[3,4] by its reversible reaction with adenosine diphosphate (ADP) to form adenosine triphosphate (ATP) and creatine, a reaction catalyzed by the enzyme creatine kinase (ATP–creatine phosphotransferase).[5] Creatine kinase constitutes an important fraction of the protein of vertebrate muscle.[6] The enzyme is currently of unusual medical interest since it has been noticed that creatine kinase levels in human serum rise dramatically following cellular damage, particularly after myocardial infarction.[7]



1



2

In the past only a few glycocyamines related to creatine have been prepared. For example, syntheses of creatine itself,[8-11] glycocyamine,[9-14] *N*-ethylglycocyamine,[15] DL-*N*-amidinoalanine[9,16] and DL-*N*-amidinoproline[16-18] have been reported. Of these, only glycocyamine[19] and *N*-ethylglycocyamine[20] have been reported to be active as substrates for creatine kinase.[2]

(1) (a) U. S. Public Health Service Special Fellow, National Institute of General Medical Sciences, 1968–present; (b) National Science Foundation undergraduate research participant, Summer 1968.

(2) S. A. Kuby and E. A. Noltmann, *Enzymes 1959–1963*, **6**, 515 (1962).

(3) D. F. Cain and R. E. Davies, *Biochem. Biophys. Res. Commun.*, **8**, 361 (1962).

(4) W. F. H. M. Mommaerts, K. Seraydarian, and A. Wallner, *Biochem. Biophys. Acta*, **63**, 75 (1962).

(5) M. Cohn, *Quart. Rev. Biophys.*, **3**, 61 (1970).

(6) S. A. Kuby, L. Noda, and H. A. Lardy, *J. Biol. Chem.*, **209**, 191 (1954).

(7) R. J. Duma and A. L. Siegel, *Arch. Int. Med.*, **115**, 443 (1965).

(8) A. Strecker, *Jahresber. Fortschr. Chem. Ver.*, 686 (1868).

(9) H. King, *J. Chem. Soc.*, 2374 (1930).

(10) E. Schütte, *Z. Physiol. Chem.*, **279**, 52 (1943).

(11) K. Odo and E. Ishikawa, *Nippon Kagaku Zasshi*, **77**, 1413 (1956).

(12) A. Strecker, *C. R. Acad. Sci., Paris*, **52**, 1212 (1861).

(13) H. L. Wheeler and H. F. Merriam, *Am. Chem. J.*, **29**, 478 (1903).

(14) E. Brand and F. C. Brand, "Organic Syntheses," Collect. Vol. III, E. C. Horning, Ed., Wiley, New York, N. Y., 1955, p 440.

(15) M. D. Armstrong, *J. Org. Chem.*, **21**, 503 (1956).

(16) M. Mourgue and R. Barat, *Bull. Soc. Chim. Fr.*, 1224 (1955).

(17) J. Kamphammer and H. Müller, *Z. Physiol. Chem.*, **225**, 1 (1934).

(18) H. Tuppy, *Monatsh. Chem.*, **84**, 342 (1953).

(19) M. L. Tanzer and C. Gilvarg, *J. Biol. Chem.*, **234**, 3201 (1959).

(20) A. H. Ennor, H. Rosenberg, and M. D. Armstrong, *Nature (London)*, **175**, 20 (1955).

No attempts have been made to prepare and examine a range of close structural analogs of creatine in enzymatic and other biological systems. As part of our research program to investigate the mechanism of action of creatine kinase from rabbit muscle, we have synthesized several new structural analogs of creatine and investigated their relative initial rates as substrates in the creatine kinase reaction. In an accompanying paper[21] we report on the chemistry of some closely related glycocyamidines.

## Results and Discussion

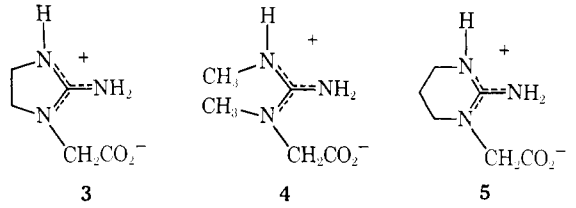
The initial rates[22,22a] afforded by several creatine analogs relative to creatine as substrates in the enzyme-catalyzed reaction are shown in Table I. In each case

**Table I.** Relative Initial Rates for Some Analogs of Creatine in the Creatine Kinase Reaction

| Substrate | Relative initial rate[a] (%, in order of decreasing reactivity) |
|---|---|
| Creatine (*N*-methyl-*N*-amidinoglycine (**1**)) | (100) |
| 1-Carboxymethyl-2-iminoimidazolidine (**3**) | 31 |
| *N*-Methyl-*N*-amidinoaminomethylphosphinic acid (**7c**) | 13 |
| *N*-Ethyl-*N*-amidinoglycine | 7.1 |
| DL-*N*-Methyl-*N*-amidinoalanine | 4.9 |
| Glycocyamine (*N*-amidinoglycine) | 1.0 |
| *N*-Propyl-*N*-amidinoglycine | 0.68 |
| D-*N*-Amidinoproline | 0.65 |
| *N*-Methyl-*N*-amidino-β-alanine | 0.19 |
| Methyl hydrogen *N*-methyl-*N*-amidinoaminomethylphosphonate (**7b**) | 0.092 |
| 1-Carboxymethyl-2-iminohexahydropyrimidine (**5**) | 0.078 |
| L-*N*-Amidinoproline | ~0.006 |
| *N*-Methyl-*N*-amidinoaminomethylphosphonic acid (**7a**) | ~0.003 |

[a] Relative initial rates (the average of at least two determinations) were measured on a recording pH-stat using the concentrations, procedures, and conditions described by Mahowald, Noltmann, and Kuby.[22a,29] Creatine and creatine analog concentrations were always the same, but in order to obtain conveniently measurable rates the enzyme concentration was varied from 0.16 μg/ml to 36 μg/ml. In all cases, however, the molar concentration of substrate was at least $10^4$ times greater than the molar concentration of enzyme. The values are corrected for spontaneous ATP hydrolysis. Under the conditions used there was no detectable reaction between ATP and creatine in the absence of the enzyme.

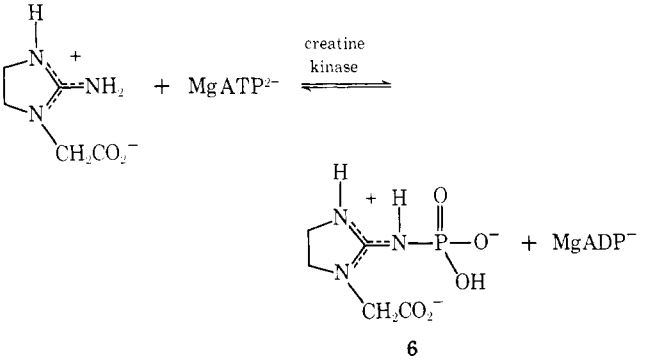
parallel product studies were performed by polyethylenimine cellulose ion exchange thin-layer chromatography,[23] and spots corresponding to ATP, ADP, and phosphorylated analogs of creatine could be detected in the proper ratios. The most reactive analog found in this study, and the most reactive creatine analog reported to date, was the nearly planar 1-carboxymethyl-2-iminoimidazolidine (**3**), which was 31% as reactive as creatine. On the other hand, no detectable rate of



**3** **4** **5**

reaction was observed for the acyclic analog of this compound, *N*-methylamidino-*N*-methylglycine (**4**). Our analytical methods were sensitive enough to allow the conservative estimate that **3** reacted at least 10,000 times faster than **4**. The corresponding puckered six-membered ring analog, 1-carboxymethyl-2-iminohexahydropyrimidine (**5**), reacted approximately 400 times slower than **3**. These results indicate a very limited tolerance by the active site of creatine kinase for substituents which project from the plane defined by the three guanido nitrogen atoms.[24]

In the enzymatic reactions of both **3** and of the corresponding six-membered ring analog **5** either the primary amino group or the secondary amino group could potentially be phosphorylated by the ATP. By isolation and characterization of the product of the enzymatic conversion of **3**, we have shown that the primary amino group is phosphorylated, *i.e.*




**6**

This enzymatic conversion was carried out using a low $Mg^{2+}$–ATP ratio in order to shift the equilibrium toward the side of formation of the phosphocreatine analog.[25] Undoubtedly this shift occurs because the stability constant for $Mg\text{–}ATP^{2-}$ is much greater than that for $Mg\text{–}ADP^{-}$.[26] A relatively high enzyme concentration of 40 μg/ml was used since the rate of attainment of equilibrium is diminished considerably by lowering the $Mg^{2+}$–ATP ratio.[27] We chose the volatile buffer triethylammonium acetate over the more volatile buffer triethylammonium bicarbonate because bicarbonate has been shown to be a moderately competitive inhibitor for the enzyme whereas acetate is only a weak inhibitor.[28] After completion of the reaction, the equilibrium was

(21) G. L. Kenyon and G. L. Rowley, *J. Amer. Chem. Soc.*, **93**, 5552 (1971).

(22) It was not feasible to make direct measurements of maximal velocities for creatine or its analogs without resorting to extensive kinetic studies of each since (1) binding constants are unfavorably high, even for creatine itself,[2] and (2) solubilities are generally rather low. Therefore, a uniform concentration, 40 m*M*, which is close to a saturated solution for most compounds, was chosen.

(22a) NOTE ADDED IN PROOF. More thorough kinetic studies using these analogs, including measurement of $V_{max}$ and $K_m$ values, have been carried out by Professor M. Cohn and Mr. A. McLaughlin, Johnson Research Foundation, University of Pennsylvania, and will be reported at later date.

(23) K. Randerath and E. Randerath, *J. Chromatogr.*, **16**, 111 (1964).

(24) For example, inspection of a molecular model of compound **4** indicates that in a conformation in which both methyl groups are on the same side of the molecule (thereby resembling compound **3**), the methyl groups sterically interfere with one another and both cannot lie in the plane defined by the three guanido nitrogen atoms.

(25) L. Noda, S. A. Kuby, and H. A. Lardy, *J. Biol. Chem.*, **210**, 83 (1954).

(26) J. F. Morrison and E. James, *Biochem. J.*, **97**, 37 (1965).

(27) S. A. Kuby, L. Noda, and H. A. Lardy, *J. Biol. Chem.*, **210**, 65 (1954).

(28) L. Noda, T. Nihei, and M. F. Morales, *ibid.*, **235**, 2830 (1960).

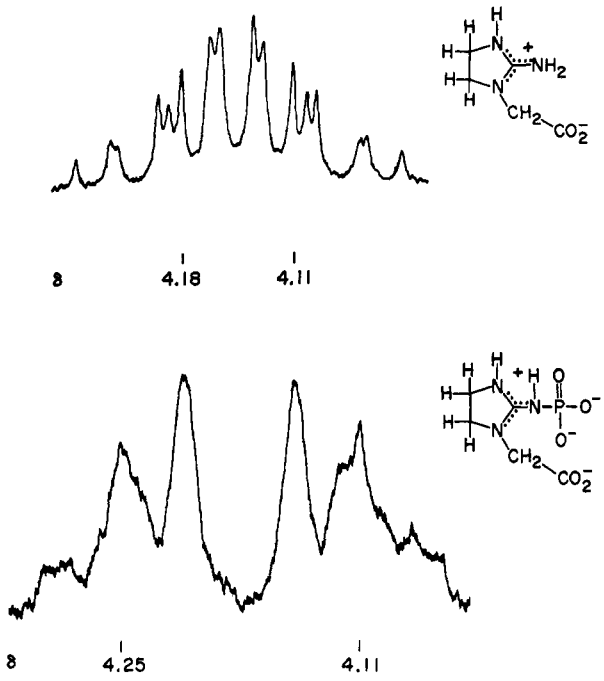
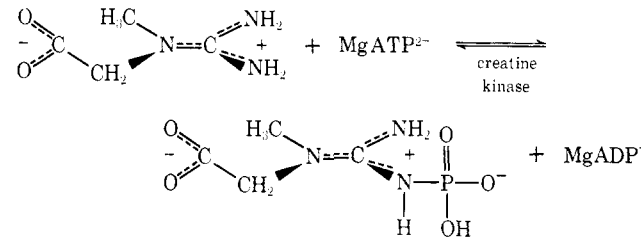



Figure 1. Proton nmr spectra taken at 220 MHz ($D_2O$) showing the ring methylene hydrogens of a 0.10 *M* solution of 1-carboxymethyl-2-iminoimidazolidine (above) and a 0.04 *M* solution of dilithium 1-carboxymethyl-2-(phosphonoimino)imidazolidine (below). Both spectra are interpretable as AA'BB' systems (see text). The lower spectrum was measured at a higher spectrum amplitude than the upper spectrum.

frozen with the rapidly acting, irreversible inhibitor of creatine kinase, 2,4-dinitrofluorobenzene.[29] After evaporation of solvent and buffer, the product was isolated by column chromatography following the method of Martonosi[30] with the exception that the volatile buffer triethylammonium bicarbonate[31] was used in place of potassium bicarbonate to elute the product from the column, a change which simplifies the purification process.

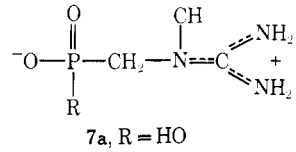
The nmr spectra at 220 MHz for the ring methylene hydrogens of both 1-carboxymethyl-2-iminoimidazolidine (**3**) and the dilithium salt of the N-phosphorylated product, **6**, are shown in Figure 1. Both spectra are interpretable as AA'BB' systems with only very small differences in chemical shift between the two methylene groups in the ring.[32] Significantly, the spectrum of product **6** shows at most only very slight spin–spin coupling to phosphorus (probably $<1$ Hz),[33] and more importantly, no readily detectable *differences* for the two methylene groups in coupling to phosphorus. If the phosphorus were on the secondary amino group, then PNCH coupling (usually having $J = 7$–12 Hz)[34] to the hydrogens of only one of the methylene groups in the ring would have undoubtedly been observed. We therefore conclude that the primary amino group was phosphorylated. This result, in context with the other kinetic specificity results shown in Table I, strongly implies that creatine itself is phosphorylated on the guanido nitrogen that is trans to the methyl group when it is bound to the enzyme's surface, *e.g.*




This *N*-methyl group, an important structural feature since creatine is 100 times more reactive than glycocyamine itself, has been implicated by James and Morrison[35] in causing a favorable conformational change to occur at the active site of the enzyme. Our results (Table I) show that hydrophobic substituents on one side (presumably the side of the molecule normally bearing the *N*-methyl group) of the creatine structure can be tolerated without completely abolishing the reactivity of the analog as a pseudosubstrate. The relative initial rates for the D- and L-amidinoprolines, for example, are striking. The L enantiomer was about 100 times slower than the D enantiomer.

The fact that 1-carboxymethyl-2-iminohexahydropyrimidine (**5**) reacted approximately 400 times slower than 1-carboxymethyl-2-iminoimidazolidine is interesting in light of the finding of Taylor, Leigh, and Cohn[36] that the five-membered ring spin label, *N*-(1-oxyl-2,2,5,5-tetramethyl-3-pyrrolidinyl)iodoacetamide, reacts readily with the cysteine sulfhydryl moiety at the active site of rabbit muscle creatine kinase whereas the corresponding six-membered ring analog, *N*-(1-oxyl-2,2,6,6-tetramethyl-4-piperidinyl)iodoacetamide, does not. These parallel findings that the enzyme greatly prefers to interact with five- rather than six-membered ring structures suggest that portions of the active site of the enzyme are only readily accessible to relatively flat or compact structures.

The enzyme must also have a very tight steric requirement in the region of the active site which is adjacent to the carboxylate moiety of bound creatine. Our results on the comparison of the initial rates of *N*-methyl-*N*-amidinoaminomethylphosphonic acid (**7a**), methyl hydrogen *N*-methyl-*N*-amidinoaminomethylphosphonate



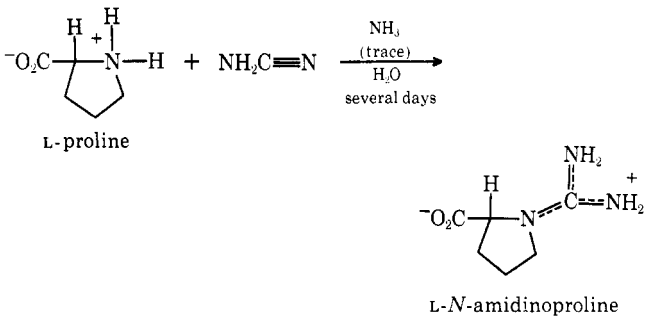
**7a**, R = HO
**7b**, R = $CH_3O$
**7c**, R = H

(**7b**), *N*-methyl-*N*-amidinoaminomethylphosphinic acid (**7c**), and creatine indicate that the steric bulk of the $-OCH_3$ group on the phosphorus in **7b** relative to the –H group on the phosphorus in **7c** has a large detrimental effect. This is not likely to be attributable to an

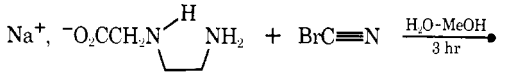
(29) T. A. Mahowald, E. A. Noltmann, and S. A. Kuby, *J. Biol. Chem.*, **237**, 1535 (1962).
(30) A. Martonosi, *Biochem. Biophys. Res. Commun.*, **2**, 12 (1960).
(31) J. Moffatt, *Can. J. Chem.*, **42**, 599 (1964).
(32) J. W. Emsley, J. Feeney, and L. H. Sutcliffe, "High Resolution Nuclear Magnetic Resonance Spectroscopy," Pergamon Press, Oxford, 1965, p 350.
(33) PNCNCH coupling of phosphorous to hydrogen in phosphocreatine is less than 0.5 Hz; δ 3.00 (3 H, singlet), 3.88 (2 H, singlet) (G. Rowley, unpublished results).
(34) F. Kerst, Ph.D. Thesis, Harvard University, 1967, p 118.
(35) E. James and J. F. Morrison, *Biochim. Biophys. Acta*, **128**, 327 (1966).
(36) J. S. Taylor, J. S. Leigh, Jr., and M. Cohn, *Proc. Nat. Acad. Sci. U. S.*, **64**, 219 (1969).

**Chart I**

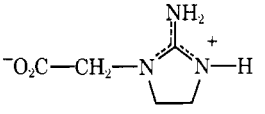
Method A (cyanamide method)




Method B (cyanogen bromide method)

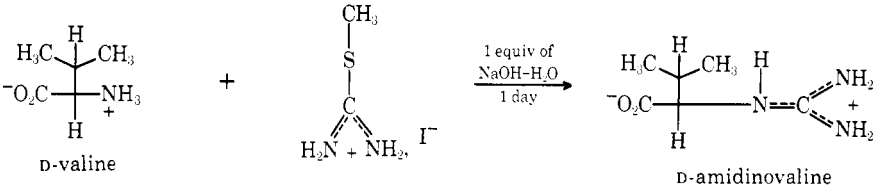



*N*-carboxymethyl-1, 2-diaminoethane, sodium salt

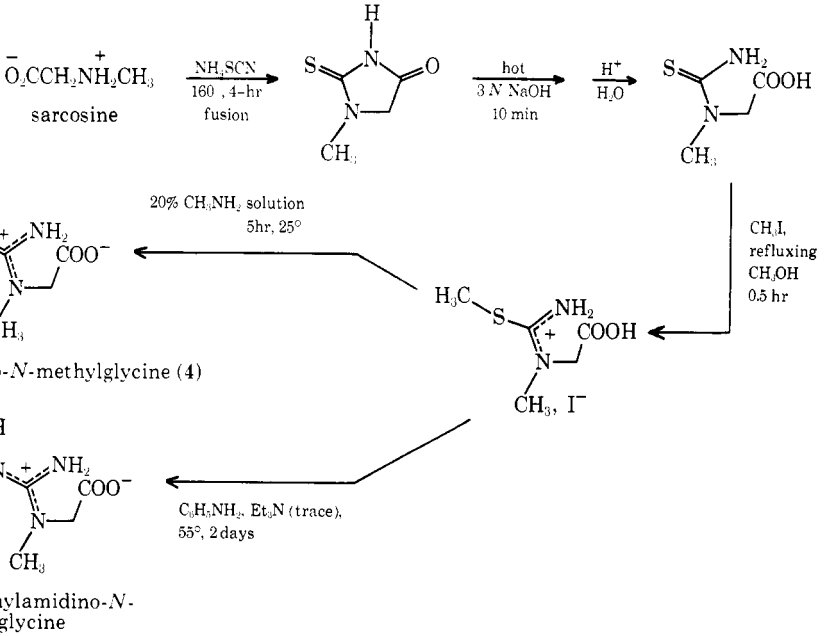


1-carboxymethyl-2-iminoimidazolidine (3)

Method C (*S*-alkylthiuronium halide method)




Method D (*N*-carboxymethyl-*N*-methyl-*S*-methylisothiuronium method)




Method E (glycocyamidine method)

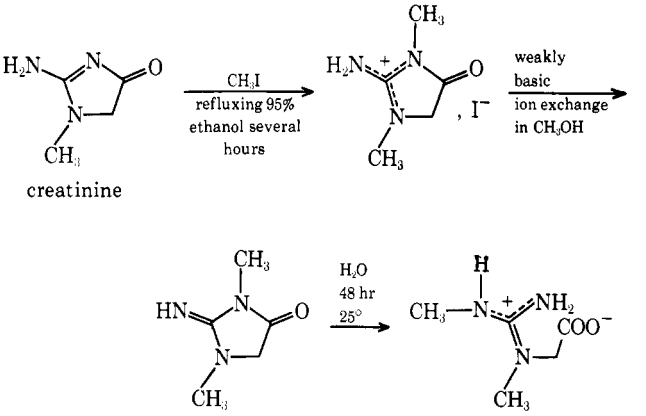
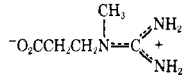
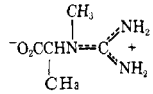
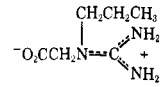
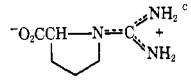
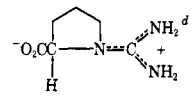
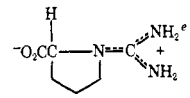
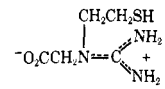
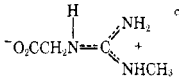
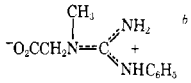
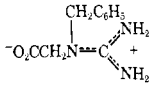
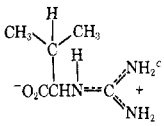
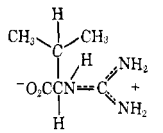


Table II. Isolation Methods and Properties of Some Glycocyamines and Glycocyamine Analogs

| Structure | Name | Nmr in $D_2O$ | Ir (major peaks in $\mu$) | Synthetic method |
|---|---|---|---|---|
| **4** | *N*-Methylamidino-*N*-methylglycine | $\delta$ 2.80 (3 H, s), 2.92 (3 H, s), 3.81 (2 H, s) | 3.17, 3.30, 5.89, 6.26, 7.26, 7.73, 8.90, 9.27, 13.60 (br) | D, E |
|  | *N*-Methyl-*N*-amidino-$\beta$-alanine | $\delta$ 2.43 (2 H, t, $J$ = 6 Hz), 2.95 (3 H, s), 3.54 (2 H, t, $J$ = 6 Hz) | 3.04, 3.20, 5.86, 6.13, 6.26, 6.98, 7.07, 7.54, 8.63, 8.89, 9.07, 9.44, 13.03 (br) | A |
|  | DL-*N*-Methyl-*N*-amidinoalanine | $\delta$ 1.42 (3 H, d, $J$ = 7 Hz), 2.84 (3 H, s), 4.25 (1 H, q, $J$ = 7 Hz) | 3.06, 3.24, 5.91, 6.21, 7.00, 7.13, 7.35, 8.80, 9.11, 9.67, 14.50 (br) | A |
| **3**[a] | 1-Carboxymethyl-2-iminoimidazolidine | $\delta$ 3.64 (4 H, m), 3.83 (2 H, s) | 3.10, 3.30, 5.84, 6.03, 6.23, 7.28, 7.65, 7.97, 8.27, 8.64, 9.08, 10.45, 12.31 (br), 13.62, 14.02 | B |
| **5**[b] | 1-Carboxymethyl-2-iminohexahydropyrimidine | $\delta$ 2.75 (2 H, m), 3.88 (4 H, m), 4.27 (2 H, s) | 3.10, 3.31, 5.96, 6.13, 6.23, 7.26, 7.60, 7.72, 8.31, 8.71, 14.34 (br) | B |
|  | *N*-Amidino-*N*-propylglycine | $\delta$ 0.82 (3 H, t, $J$ = 7 Hz), 1.52 (2 H, m), 3.21 (2 H, t, $J$ = 7 Hz), 3.83 (2 H, s) | 2.99, 3.21, 6.00, 6.12, 6.28, 7.52, 8.90 (br), 10.26 | A |
|  | DL-*N*-Amidinoproline | $\delta$ 2.07 (4 H, m), 3.49 (2 H, t, $J$ = 6 Hz), 4.22 (1 H, m) | 3.03, 3.18, 5.86, 6.10, 6.24, 7.08, 7.25, 7.57, 7.65, 8.79 | C |
|  | D-*N*-Amidinoproline | $\delta$ 2.05 (4 H, m), 3.46 (2 H, t, $J$ = 6 Hz), 4.22 (1 H, m) | 3.00, 3.17, 6.12, 6.32, 7.20, 7.68, 7.73, 7.89, 9.16, 14.42 (br) | A |
|  | L-*N*-Amidinoproline | $\delta$ 2.05 (4 H, m), 3.46 (2 H, t, $J$ = 6 Hz), 4.22 (1 H, m) | 2.97, 3.17, 6.03, 6.16, 6.31, 7.20, 7.75, 7.89, 14.42 (br) | A |
|  | *N*-Amidino-*N*-(2-thioethyl)glycine | $\delta$ 2.72 (2 H, t, $J$ = 7 Hz), 3.50 (2 H, t, $J$ = 7 Hz), 3.90 (2 H, s) | 3.10, 3.32, 5.86, 5.99, 6.21, 7.24, 7.70, 8.53, 8.70, 14.18 (br) | A |
|  | *N*-Methylamidinoglycine | $\delta$ 2.82 (3 H, s), 3.76 (2 H, s), | 2.93, 3.15, 5.83, 6.16, 7.42, 7.76, 8.42, 8.77, 14.25 | C, E |
|  | *N*-Phenylamidino-*N*-methylglycine | $\delta$ 3.60 (3 H, s), 4.83 (2 H, s), 7.92 (5 H, m) | 3.03, 3.15, 6.02, 6.21, 6.56, 6.99, 7.13, 8.13, 9.03, 13.14, 13.29, 14.43 | D |
|  | *N*-Amidino-*N*-benzylglycine | $\delta$ 3.82 (2 H, s), 4.55 (2 H, s) 7.37 (5 H, m) | 3.02, 3.20, 6.03, 6.16, 6.49, 7.23, 7.73, 8.74, 10.23, 10.99, 13.29, 14.26 | A |
|  | DL-*N*-Amidinovaline | $\delta$ 0.84 (3 H, d, $J$ = 3 Hz), 0.95 (3 H, d, $J$ = 3 Hz), 2.14 (1 H, m), 3.72 (1 H, d, $J$ = 5 Hz) | 3.06, 3.20, 5.97, 6.11, 6.34, 7.14, 7.67, 8.37, 13.06 (br) | C |
|  | D-*N*-Amidinovaline | $\delta$ 0.84 (3 H, d, $J$ = 3 Hz), 0.95 (3 H, d, $J$ = 3 Hz), 2.12 (1 H, m), 3.68 (1 H, d, $J$ = 5 Hz) | 2.97, 3.15, 5.96, 6.09, 6.30, 7.13, 7.39, 7.65, 7.99, 8.38, 13.06 (br) | C |
| **7b** | Methyl hydrogen *N*-methyl-*N*-amidinoaminomethylphosphonate | $\delta$ 3.14 (3 H, s), 3.41 (2 H, d, $J_{PCH}$ = 10 Hz), 3.46 (3 H, d, $J_{POCH}$ = 10 Hz) | 2.99, 3.16, 5.93, 6.18, 6.48, 8.40, 9.16, 9.40, 9.56, 11.64, 12.39, 12.80, 14.07 | A |
| **7a** | *N*-Methyl-*N*-amidinoaminomethylphosphonic acid | $\delta$ 3.04 (3 H, s), 3.43 (2 H, d, $J_{PCH}$ = 10 Hz) | 3.05, 3.20, 5.93, 6.01, 6.13, 6.46, 6.94, 8.67, 9.05, 9.58, 9.72, 10.62, 10.90, 12.38, 12.86 | A |
| **7c** | *N*-Methyl-*N*-amidinoaminomethylphosphinic acid | $\delta$ 3.19 (3 H, s), 3.57 (2 H, d of d, $J_{PCH}$ = 8 Hz, $J_{HPCH}$ = 1.5 Hz), 7.22 (1 H, t of t, $J_{PH}$ = 533 Hz, $J_{HPCH}$ = 1.5 Hz) | 3.11, 3.29, 4.34, 6.00, 6.23, 6.48, 8.48, 8.91, 9.17, 9.45, 9.70, 10.00, 10.21, 12.29 | A |

[a] The nmr spectrum at 220 MHz showing the ring methylene hydrogens for this compound is shown in Figure 1. [b] The nmr spectra of these compounds were determined using warm solutions in order to dissolve enough of the glycocyamine, and therefore the chemical shifts are less reliable than the others. [c] These compounds have been reported previously (see text) but were incompletely characterized. [d] $[\alpha]^{25}D$

| Recryst solvent | % yield | Mp, °C | Empirical formula | Calcd, % C | H | N | Other | Found, % C | H | N | Other |
|---|---|---|---|---|---|---|---|---|---|---|---|
| 95% EtOH–acetone | 72 | 203–204 | $C_5H_{11}N_3O_2$ | 41.37 | 7.64 | 28.95 | | 41.29 | 7.49 | 28.80 | |
| $H_2O$ | 25 | 248–249 (softens 230) | $C_5H_{11}N_3O_2$ | 41.37 | 7.64 | 28.95 | | 41.51 | 7.51 | 28.88 | |
| $H_2O$–$NH_3$ | 23 | 272–273 | $C_5H_{11}N_3O_2$ | 41.37 | 7.64 | 28.95 | | 41.14 | 7.37 | 28.85 | |
| $H_2O$ | 62 | 341–342 | $C_5H_9N_3O_2$ | 41.95 | 6.34 | 29.35 | | 41.91 | 6.27 | 29.38 | |
| $H_2O$ | 16 | 253–254 (sinters) | $C_6H_{11}N_3O_2 \cdot H_2O$ | 41.13 | 7.48 | 23.99 | | 40.98 | 7.30 | 24.16 | |
| 95% EtOH | 45 | 252.5–253.5 (sinters) | $C_6H_{13}N_3O_2 \cdot H_2O$ | 40.66 | 8.53 | 23.71 | | 40.74 | 8.53 | 23.92 | |
| $H_2O$ | 89 | 305–305.5 | $C_6H_{11}N_3O_2$ | 45.85 | 7.05 | 26.74 | | 45.78 | 7.18 | 26.69 | |
| $H_2O$–$NH_3$ | 17 | 256–257 | $C_6H_{11}N_3O_2 \cdot H_2O$ | 41.14 | 7.48 | 23.98 | | 40.87 | 7.44 | 24.09 | |
| $H_2O$–$NH_3$ | 9 | 255–256 | $C_6H_{11}N_3O_2 \cdot H_2O$ | 41.14 | 7.48 | 23.98 | | 41.23 | 7.51 | 23.88 | |
| $H_2O$ | 82 | 187–189 | $C_5H_{11}N_3O_2S$ | 33.88 | 6.26 | 23.71 | S, 18.09 | 33.71 | 6.07 | 23.82 | S, 17.99 |
| $H_2O$ | 73 | 239–240 | $C_4H_9N_3O_2$ | 36.63 | 6.92 | 32.05 | | 36.58 | 6.84 | 32.14 | |
| Acetone | 18 | 207–208.5 | $C_{10}H_{13}N_3O_2$ | 57.96 | 6.32 | 20.28 | | 58.04 | 6.08 | 20.42 | |
| $H_2O$ | 49 | 268.5–269.5 | $C_{10}H_{13}N_3O_2$ | 57.96 | 6.32 | 20.28 | | 57.89 | 6.57 | 20.40 | |
| $H_2O$ | 75 | 266–268 | $C_6H_{13}N_3O_2$ | 45.27 | 8.23 | 26.40 | | 45.28 | 8.23 | 26.28 | |
| 95% EtOH–$H_2O$ | 63 | 251–253.5 | $C_6H_{13}N_3O_2$ | 45.27 | 8.23 | 26.40 | | 45.29 | 8.49 | 26.62 | |
| EtOH–$H_2O$ | 87 | 287–288 (sinters 75–80) | $C_4H_{12}N_3O_3P \cdot H_2O$ | 24.12 | 7.09 | 21.10 | P, 15.56 | 24.21 | 6.97 | 21.98 | P, 15.70 |
| $H_2O$ | 67 | 290–291 | $C_3H_{10}N_3O_3P$ | 21.56 | 6.03 | 25.15 | P, 18.54 | 21.58 | 5.95 | 25.16 | P, 18.49 |
| $H_2O$ | 53 | 294–294.5 | $C_3H_{10}N_3O_2P$ | 23.84 | 6.67 | 27.81 | P, 20.50 | 23.94 | 6.68 | 27.69 | P, 20.42 |

$+89.3°$; prepared from D-proline which had $[\alpha]^{24}D$ $+83.6°$. [e] $[\alpha]^{25}D$ $-93.5°$; prepared from L-proline which had $[\alpha]^{24}D$ $-84.7°$. [f] Specific rotation not determined; prepared from D-valine which had $[\alpha]^{25}D$ $-27.8°$.

inductive effect since phosphinic acids and phosphonic acid monoesters have similar $pK_a$ values, and both are much stronger acids than carboxylic acids. The fact that the phosphonic acid **7a** is only marginally reactive can be explained at least in part by the fact that under the conditions of the enzymatic assay (pH 9) this molecule would be largely in the form of its dianion.

A variety of synthetic approaches to the glycocyamine structures (Table II) were used. Examples of each approach are shown in Chart I.

Method A is probably the most versatile and most reliable procedure for generating glycocyamines which are unsubstituted on the amidino nitrogen positions. This is essentially the procedure first introduced by Strecker[12] for the synthesis of glycocyamine and later developed by Armstrong.[15] It is a rather slow reaction, usually requiring several days at room temperature for optimal yields. The amino acid to be amidinated should be very soluble in water since the reaction usually fails in dilute aqueous solutions. For example, we found this to be the case for the slightly water-soluble amino acid, *N*-benzylglycine. With a 1.0 *M* amino acid solution no reaction product was detected after 3 days of reaction. In more concentrated solutions of cyanamide, however, it was discovered that a 4.0 *M* solution of the *N*-benzylglycine could be obtained, and after 9 days a 49% yield of *N*-amidino-*N*-benzylglycine was isolated. We also noticed formation of very water-soluble complexes in the syntheses of both D- and L-amidinoproline by method A. These complexes were presumably the same type that Armstrong found in the synthesis of *N*-ethylglycocyamine. As recommended by Armstrong,[15] they were broken up and the glycocyamine crystallized from the solution on treatment with concentrated ammonia.

When DL-*N*-methyl-*N*-amidinoalanine was prepared by method A we noticed a pronounced tendency toward cyclization of the glycocyamine product to give the corresponding glycocyamidine. Duvillier[37] noted the same problem in reporting on unsuccessful attempts to synthesize a wider range of *C*-alkyl, *N*-alkyl-substituted glycocyamines. We found that by careful seeding of the reaction mixture and use of gentle isolation and recrystallization conditions, the DL-*N*-methyl-*N*-amidinoalanine could be isolated in quantity without contamination by the corresponding glycocyamidine.

Method B is patterned after the known reaction of ethylenediamine with cyanogen bromide to form 2-iminoimidazolidine[38] and is the only feasible method which we have found for the synthesis of the cyclic compounds **3** and **5**.

Method C has been employed widely in the past,[9,11,13,14] but often gives unsatisfactory yields with substituted amino acids or N-substituted *S*-alkylthiuronium salts. For example, in our hands several attempts to prepare *N*-methylamidino-*N*-methylglycine (**4**) by this procedure failed. Because of these failures, methods D and E were developed expressly for the purpose of synthesizing **4**. Of these two methods, method E is the most facile way to obtain **4**, and in an accompanying paper,[21] we discuss syntheses of several di- and tri-N-substituted glycocyamidines, the immediate precursors of even more highly N-substituted glycocyamines. Method D, however, is the only feasible route which we have devised for the synthesis of *N*-phenylamidino-*N*-methylglycine.

In the syntheses of *N*-ethylglycine, *N*-propylglycine, and several of the glycocyamines we made use of the well-known solubility of iodide salts (*e.g.*, sodium iodide) in acetone as a convenient way of separating these salts from the acetone-insoluble amino acids and glycocyamines. A search of the literature has revealed that this procedure, which we have found especially useful for isolation of these amino acids and glycocyamines in bulk quantities, has seldom been used previously. Usually, the more time-consuming procedure of ion-exchange chromatography has been employed to effect removal of the salts that are concomitantly formed in the preparation of these compounds.[39]

## Experimental Section

**Methods and Materials.** Infrared spectra were measured on a Perkin-Elmer Infracord spectrometer (Model 137). Unless otherwise specified, proton nmr spectra were determined on a Varian Model T-60 nmr spectrometer using tetramethylsilane as an external standard with 5–10% solutions in $D_2O$. Melting points are uncorrected. All decomposition melting points were determined by placing the sample into the heated bath at 3–5° below the decomposition point and heating at 1°/min. Microanalyses were performed by the Microanalytical Laboratory, Department of Chemistry, University of California, Berkeley, Calif.

Creatine kinase was either purchased from Worthington Biochemical Corp. (~20 Kuby units/mg of protein) or was isolated from rabbit skeletal muscle and recrystallized three times as described by Kuby, Noda, and Lardy[6] and Mahowald, Noltmann, and Kuby[29] (specific activity: 137 μequiv/mg of protein per min). The latter enzyme preparation, used exclusively in the initial rate studies, was free of detectable ATPase activity. Creatine kinase assays and initial rate studies were performed using the pH-stat method also described by Mahowald, Noltmann, and Kuby.[29] ATP was purchased from Calbiochem Corp. and was purified[40] to remove the bulk of the contaminating ADP. The resultant ATP contained less than 0.5% ADP as estimated by chromatography.[23]

Product studies for enzyme-catalyzed reactions were performed as follows. A sample of the glycocyamine or glycocyamine analog (24 mmol) was incubated at 30° with ATP (4 m*M*), $MgSO_4$ (4 m*M*), and creatine kinase (2.5 mg, ~20 Kuby units of activity) in a 5-ml solution buffered at pH 9.0 with NaOH–glycine. At intervals aliquots (1 ml) were withdrawn from this solution and pipetted into a 0.1-ml solution of the inhibitor 2,4-dinitrofluorobenzene (10 m*M*) in 2-propanol, and aliquots of the resulting solutions were spotted on polyethylenimine cellulose thin-layer plates[23] (microscope slides). Ascending chromatography was used with 1.2 *M* NaCl solution as the eluent. The spots were visualized using molybdate spray.[41] In each case spots corresponding to ATP, ADP, and the phosphorylated glycocyamine were detected. Further details will be published elsewhere.

**Amino Acids.** Sarcosine and *N*-(β-mercaptoethyl)glycine hydrochloride were purchased from Aldrich Chemical Co. DL-Proline, D-proline, L-proline, and D-valine were purchased from Calbiochem Corp. DL-valine was from Matheson Coleman and Bell. Glycine was the product of Eastman Organic Chemicals.

***N*-Methyl-β-alanine** was prepared by the method of Weinstein and Wyman.[42] From 40 g of acrylonitrile and aqueous methylamine, 44 g (70% yield) of β-methylaminopropionitrile, bp 70–71° (12 mm) (lit.[42] bp 37° (4 mm)), was obtained. The $^1H$ nmr spectrum (neat) showed δ 1.60 (1 H, singlet), 2.42 (3 H, singlet), 2.70 (4 H, multiplet, AA'BB' pattern). The nitrile was hydrolyzed in $Ba(OH)_2$ solution under reflux for several hours. After saturating the solution with $CO_2$ and filtering off the $BaCO_3$, the clear, colorless solution was evaporated to dryness. The product, isolated as a monohydrate, was crystallized from $CH_3CN$–ethanol and weighed

(37) M. E. Duvillier, *C. R. Acad. Sci. Paris*, **95**, 456 (1882); **96**, 1583 (1883); **97**, 1486 (1883); **100**, 916 (1885).
(38) P. Pierron, *Ann. Chim.*, **11**, 361 (1919).
(39) J. P. Greenstein and M. Winitz, "Chemistry of the Amino Acids," Vol. 2, Wiley, New York, N. Y., 1961, p 1393.
(40) L. Berger, *Biochim. Biophys. Acta*, **20**, 23 (1956).
(41) C. S. Hanes and F. A. Isherwood, *Nature (London)*, **164**, 1107 (1949).
(42) J. Weinstein and G. M. Wyman, *J. Org. Chem.*, **23**, 1618 (1958).

30 g (47% yield), mp 68–70° (lit.[43] mp of monohydrate, 66.5–67.5°). The $^1$H nmr spectrum showed $\delta$ 2.73 (3 H, singlet), 2.95 (4 H, multiplet, AA'BB' pattern).

DL-*N*-**Methylalanine** was prepared from 20 g of 2-bromopropionic acid and 100 ml of 40% aqueous methylamine by the procedure described by Fu and Birnbaum.[44] The product was recrystallized from ethanol–acetone to give 10.0 g (74% yield). The $^1$H nmr spectrum showed $\delta$ 1.46 (3 H, doublet, $J$ = 7 Hz), 2.71 (3 H, singlet), 3.60 (1 H, quartet, $J$ = 7 Hz).

*N*-**Propylglycine** was prepared from 14.8 g (80 mmol) of iodoacetic acid which was added slowly to an ice-cold rapidly stirred solution of 40 g (680 mmol) of *n*-propylamine in 40 ml of $H_2O$. After standing at room temperature for about 20 hr, the water was removed from the flask under reduced pressure. The residue was then dissolved in 40 ml of absolute ethanol, and this solution was added slowly to a mixture of 470 ml of acetone and 50 ml of absolute ethanol. A white precipitate formed which after filtering and drying weighed 5.8 g (62% yield), mp 191–193° dec (lit.[45] mp 196–198°). The $^1$H nmr spectrum showed $\delta$ 0.98 (3 H, triplet, $J$ = 7 Hz), 1.70 (2 H, multiplet), 3.00 (2 H, triplet, $J$ = 7 Hz), 3.57 (2 H, singlet).

*N*-**Benzylglycine** was prepared by modifying the general method of Quitt, Hellerbach, and Vogler.[46] Thus glycine (7.50 g, 0.10 mol) and 10 ml of 10 *N* NaOH were each added to 40 ml of $H_2O$. Benzaldehyde (10.1 ml, 0.1 mol) was added, and the mixture was stirred for 20 min. $NaBH_4$ (1.14 g, 0.03 mol) was then added in small portions over a period of 30 min while maintaining the temperature of the solution below 15° in an ice bath. The benzylation procedure was repeated twice more by the successive addition of 5.0 ml (0.05 mol) of benzaldehyde and 0.57 g of $NaBH_4$ (0.015 mol). The final solution was extracted twice with ether and then neutralized to pH 6.5. As the pH of the solution approached neutrality a white solid precipitated. After the mixture had stood for 3 hr, this solid was filtered off and dried to give 3.49 g of the by-product, *N*,*N*-dibenzylglycine, mp 192–194° (lit.[47] mp 200°). The $^1$H nmr spectrum of the $Na^+$ salt showed $\delta$ 2.83 (2 H, singlet), 3.38 (4 H, singlet), 7.00 (10 H, singlet). The filtrate was evaporated to dryness, 20 ml of $H_2O$ was added, and the solid cake was broken up. After the mixture had stood overnight, the product was filtered, washed with 5 ml of $H_2O$, and dried. The yield of *N*-benzylglycine was 8.49 g (52%), mp 196–198° dec (lit.[48] mp 198–200°). The $^1$H nmr spectrum showed $\delta$ 3.42 (2 H, singlet), 4.08 (2 H, singlet), 7.20 (5 H, singlet).

*N*-**(2-Aminoethyl)glycine, sodium salt,** was prepared by modifying the procedure of Bersworth.[49] Thus, NaCN (104 g, 2.12 mol) and ethylenediamine (100 ml, 1.50 mol) were dissolved in 800 ml of $H_2O$, and the solution was heated to 60°. Aqueous formaldehyde (112 ml, 37% solution, 1.38 mol) was dropped in slowly over about 2 hr with stirring. The mixture was then heated at 60° for 1 hr. The water was removed *in vacuo* and further drying was achieved by making a slurry of the salt in absolute methanol and removing the methanol under reduced pressure. Finally a slurry in dry hexane was prepared and then the hexane was removed to leave a dry cake. The product was taken up in absolute ethanol and the relatively insoluble excess NaCN was filtered off (filter aid). The alcohol was then removed *in vacuo* from the filtrate. Further purification was achieved by dissolving the product in a mixture of 50% absolute ethanol and 50% dry acetonitrile and filtering off the remaining undissolved solid (mostly NaCN). Finally the solvents were removed under reduced pressure and the hygroscopic solid product was dried and stored in a desiccator over $P_2O_5$. The yield of product was 50 g. The ir spectrum (Nujol) showed no cyanide absorption. The $^1$H nmr spectrum showed $\delta$ 3.24 (4 H, apparent triplet, $J$ = 3 Hz), 3.73 (2 H, singlet). Other minor peaks appeared in the nmr spectrum, but no attempts were made at further purification.

*N*-**(3-Aminopropyl)glycine, sodium salt,**[50] was prepared by a completely analogous series of reactions to those described above for the preparation of *N*-(2-aminoethyl)glycine, sodium salt. From 38.9 g (0.525 mol) of 1,3-diaminopropane, 25.75 g (0.525 mol) of NaCN, and 41.65 g of 37% aqueous formaldehyde (0.500 mol) the crude yield was 54.2 g, 75%. The product was hygroscopic and was stored in a desiccator over $P_2O_5$ until used. The $^1$H nmr spectrum showed $\delta$ 1.70 (2 H, multiplet), 2.62 (4 H, overlapping triplets, $J$ = 6 Hz), 3.18 (2 H, singlet). As in the case of the corresponding derivative from ethylenediamine, the nmr spectrum revealed other peaks, indicating the presence of minor amounts of impurities, but the product was used without further purification.

**Methyl hydrogen** *N*-**methylaminomethylphosphonate** was prepared in two steps. First, dimethyl iodomethylphosphonate was prepared by dropping 186 g (1.50 mol) of trimethyl phosphite (Aldrich Chemical Co.) into 134 g (0.50 mol) of methylene iodide (Matheson Coleman and Bell) over a period of 1 hr at 180°. It was necessary to pass a slow stream of $N_2$ through the system to sweep out the $CH_3I$ as it was formed. The product was fractionally distilled to give 33.2 g (27% yield), bp 78–83° (0.2 mm). Redistillation gave an analytical sample, bp 97–98.5° (1.5 mm). The nmr spectrum showed peaks at $\delta$ 3.31 (2 H, doublet, $J_{PCH}$ = 10 Hz), 3.85 (6 H, doublet, $J_{POCH}$ = 11 Hz). *Anal.* Calcd for $C_3H_8IO_3P$: C, 14.41; H, 3.23; I, 50.77; P, 12.39. Found: C, 14.59; H, 3.24; I, 50.62; P, 12.54.

This dimethyl ester (16.25 g, 65 mmol) was treated with 130 ml of 40% aqueous methylamine (1.68 mol) at 55° for 36 hr, bubbling a slow stream of methylamine into the solution throughout the reaction period, to yield crude methyl hydrogen *N*-methylaminomethylphosphonate which was isolated as a viscous syrup after removal of excess methylamine and $H_2O$ *in vacuo*. This syrup was dissolved in 25 ml of ethanol and slowly added with vigorous stirring to a mixture of 500 ml of acetone and 55 ml of ethanol. The crystals of product which formed were filtered and dried. The yield was 5.86 g (63%). After recrystallization from 95% ethanol–ethyl acetate, the product (5.17 g) had a mp 170.5–171.5°. The nmr spectrum showed peaks at $\delta$ 2.81 (3 H, singlet), 3.21 (2 H, doublet, $J_{PCH}$ = 12 Hz), 3.61 (3 H, doublet, $J_{POCH}$ = 10 Hz). *Anal.* Calcd for $C_3H_{10}NO_3P$: C, 25.90; H, 7.25; N, 10.07; P, 22.27. Found: C, 25.81; H, 7.37; N, 9.98; P, 22.29.

*N*-**Methylaminomethylphosphonic acid** was prepared from the monomethyl ester (2.78 g, 20 mmol) by reaction with 40 ml of 48% HBr (237 mmol) at reflux for 0.5 hr. After removal of the excess HBr solution, the product was obtained as the hydrobromide salt. This salt was dissolved in 4 ml of water and 30 ml of 2-propanol was added in small portions with rapid stirring to give 1.99 g of white needles of analytically pure, free *N*-methylaminomethylphosphonic acid (80% yield), mp 274.5–275.5° dec. The nmr spectrum showed peaks at $\delta$ 2.81 (3 H, singlet), 3.15 (2 H, doublet, $J_{PCH}$ = 13 Hz). *Anal.* Calcd for $C_2H_8NO_3P$: C, 19.20; H, 6.45; N, 11.20; P, 24.77. Found: C, 19.19; H, 6.54; N, 11.07; P, 24.86.

*N*-**Methylaminomethylphosphinic acid** was prepared from chloromethylphosphinic acid and methylamine by the method described previously[51] for the synthesis of aminomethylphosphinic acid. The product was recrystallized from 95% ethanol to give 2.72 g (41% yield), mp 214–216° dec. The nmr spectrum showed peaks at $\delta$ 2.76 (3 H, singlet), 3.03 (2 H, pair of doublets, $J_{PCH}$ = 10 Hz, $J_{HPCH}$ = 1.5 Hz), 7.18 (1 H, pair of triplets, $J_{PH}$ = 549 Hz, $J_{HPCH}$ = 1.5 Hz). *Anal.* Calcd for $C_2H_8NO_2P$: C, 22.02; H, 7.39; N, 12.84; P, 28.40. Found: C, 22.13; H, 7.26; N, 12.73; P, 28.28.

**Other Compounds. 1-Methyl-2-thiohydantoin** was prepared by the fusion of sarcosine (89.1 g, 1.0 mol) with $NH_4SCN$ (228 g, 3.0 mol) at 140° under a slow stream of $N_2$. After 12 hr of heating, the dark red solution was cooled. The solid cake which formed was broken up and washed with 400 ml of $H_2O$ onto a filter. The crystals were then washed successively with three 75-ml portions of $H_2O$, one 50-ml portion of 95% ethanol, and one 50-ml portion of hexane. After drying, 64.6 g (44%) of light tan crystals remained, mp 221–224° dec. A small amount of solid contaminant did not melt. The $^1$H nmr spectrum showed peaks at $\delta$ 3.23 (3 H, singlet), 4.27 (2 H, singlet). The ir spectrum (Nujol) showed major peaks at 3.18, 3.27, 5.80, 7.08, 7.49, 8.04, and 8.47 $\mu$. An analytical sample was prepared in quantitative yield by heating a highly purified sample of 3-methyl-4-thiohydantoic acid (see below) at 170° for 20 min, mp 227–229° dec (lit.[52] mp 224–226°). This sample had

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identical spectral properties with the contaminated product described above. *Anal.* Calcd for $C_4H_6N_2OS$: C, 36.91; H, 4.65; N, 21.52; S, 24.63. Found: C, 37.01; H, 4.56; N, 21.32; S, 24.52.

**3-Methyl-4-thiohydantoic acid** was prepared from a portion of the somewhat contaminated 1-methyl-2-thiohydantoin of which 41.9 g (0.322 mol) was dissolved in a solution prepared by adding 38.6 g (0.965 mol) of NaOH to 322 ml of $H_2O$. The resultant yellow-orange solution was heated with stirring to 100° (15 min was required to reach this temperature), held at this temperature for 5 min, and then quickly cooled. Concentrated HCl (100 ml) was added slowly with stirring to the solution cooled in an ice bath during which time a solid separated from the solution. The resultant strongly acidic mixture was stirred at 0° for 1.5 hr, after which the precipitated solid was filtered. After washing successively with $H_2O$, ethanol, and hexane, the pale tan solid was dried to yield 38.6 g (81% yield), which, according to the ir spectrum, contained some 1-methyl-2-thiohydantoin as an impurity. A portion of this crude acid (29.3 g, 0.198 mol) was then further purified by addition in small portions with stirring to an ice-cold solution of 16.6 g (0.198 mol) of $NaHCO_3$ in 190 ml of $H_2O$. The resulting mixture was stirred for 15 min after the final addition, and insoluble crystals were filtered off. The crystals were then washed with two 10-ml portions of water, and the combined filtrates were acidified with concentrated HCl (16.6 ml required) at 0°. The white solid which crystallized was filtered, washed successively with $H_2O$, ethanol, and hexane, and dried to give 20.3 g (69% recovery) of analytically pure 3-methyl-4-thiohydantoic acid, mp 230–232.5° dec. *Anal.* Calcd for $C_4H_8N_2O_2S$: C, 32.42; H, 5.44; N, 18.91; S, 21.64. Found: C, 32.32; H, 5.18; N, 18.81; S, 21.88. The $^1$H nmr spectrum showed $\delta$ 3.18 (3 H, singlet), 4.52 (2 H, singlet). The ir spectrum (Nujol) showed major peaks at 2.90, 3.00, 3.08, 3.5–4.5 (series of peaks), 5.80, 6.04, 6.52, 7.04, 7.26, 8.29, 9.19, 10.27, 12.06, and 13.33 $\mu$.

***N*-Carboxymethyl-*N*-methyl-S-methylisothiuronium iodide** was prepared by mixing 7.40 g (50 mmol) of 3-methyl-4-thiohydantoic acid with 7.10 g (50 mmol) of methyl iodide in 50 ml of absolute methanol and heating at reflux for 30 min. After removal of the methanol at room temperature *in vacuo*, the resulting yellow syrup weighed 14.87 g. The product crystallized after standing in the dark at 4° overnight and was subsequently triturated with two 3-ml portions of $CHCl_3$. After drying *in vacuo*, the product weighed 13.03 g (90% yield). An nmr spectrum of the product indicated that approximately 9% had cyclized to 1-methyl-2-methylthio-2-imidazolin-4-one hydriodide (see below). The analytical sample was prepared by recrystallizing in the dark from absolute ethanol–$CHCl_3$–hexane to give white needles, mp 130.5–132° dec. *Anal.* Calcd for $C_5H_{11}IN_2O_2S$: C, 20.70; H, 3.82; I, 43.74; N, 9.66; S, 11.03. Found: C, 20.74; H, 3.68; I, 43.80; N, 9.68; S, 10.90. The $^1$H nmr spectrum showed $\delta$ 2.68 (3 H, singlet), 3.27 (3 H, singlet), 4.47 (2 H, singlet).

**1-Methyl-2-methylthio-2-imidazolin-4-one hydriodide** was formed as a by-product of the methylation of 3-methyl-4-thiohydantoic acid as described above. It was found that longer reaction times and higher solvent evaporation temperatures enhanced the formation of this by-product. Thus, 3-methyl-4-thiohydantoic acid (11.83 g, 80 mmol) and iodomethane (12.47 g, 88 mmol) were heated at reflux in 80 ml of methanol for 1 hr. The solvent was removed at reduced pressure at 55° and 23.2 g of yellow syrup remained, which contained 22% of the cyclized by-product as indicated by nmr spectroscopy. A portion of this cyclized material was isolated by treating the sample with 80 ml of hot absolute ethanol and allowing the resulting slurry to cool with stirring in an ice bath. The solid was filtered and dried to give 1.82 g (38% recovery). An analytical sample was obtained by recrystallization from methanol, mp 226–228° dec (gas loss at 155–170°). *Anal.* Calcd for $C_5H_9IN_2OS$: C, 22.07; H, 3.34; I, 46.64; N, 10.30; S, 11.78. Found: C, 21.93; H, 3.21; I, 46.90; N, 10.31; S, 11.90. The nmr spectrum ($D_2O$) showed peaks at $\delta$ 2.79 (3 H, singlet), 3.29 (3 H, singlet).[53]

***S*-Ethyl-*N*-methylthiuronium bromide** was prepared by the method of Curd, Davey, Richardson, and Ashworth,[54] mp 74–76°.

***S*-Methylthiuronium iodide** was prepared by refluxing a solution of 78.1 g (1.00 mol) of thiourea in 1 l. of anhydrous methanol with 156 g (1.10 mol) of methyl iodide for 30 min. After removal of the methanol, the product was recrystallized from 300 ml of acetonitrile to give 190 g (81% yield) of colorless needles, mp 113.5–115°. *Anal.* Calcd for $C_2H_7IN_2S$: C, 11.01; H, 3.24; I, 58.20; N, 12.85; S, 14.70. Found: C, 11.00; H, 3.11; I, 58.18; N, 12.70; S, 14.54.

**2-Imino-3-carboxymethylthiazolidine** was prepared by treating *N*-($\beta$-mercaptoethyl)glycine hydrochloride (4.30 g, 25 mmol) with cyanogen bromide (2.65 g, 25 mmol) in the presence of 2 equiv of base (see method B below). The product, recrystallized from water, weighed 3.19 g (79%), mp 227–229.5° dec. *Anal.* Calcd for $C_5H_8N_2O_2S \cdot H_2O$: C, 33.70; H, 5.66; N, 15.72; S, 17.99. Found: C, 33.59; H, 5.36; N, 15.89; S, 18.25. The $^1$H nmr spectrum showed $\delta$ 3.78 (4 H, multiplet, AA'BB' pattern), 4.03 (2 H, singlet). The ir spectrum (Nujol) showed major peaks at 2.96, 3.12, 5.87, 6.03, 6.16, 6.30, 6.83, 6.97, 7.23, 7.30, 7.59, 7.73, 8.61, 13.58 (broad), and 14.09 $\mu$ (broad).

**Glycocyamines and Glycocyamine Analogs.** Creatine (Matheson Coleman and Bell) and glycocyamine (J. T. Baker) were both recrystallized from water before use. *N*-Ethylglycocyamine was prepared by the method of Armstrong, mp 270.5–272° dec (lit.[15] 264–270° dec). The rest of the glycocyamines and glycocyamine analogs were prepared by the following general methods. The compounds synthesized, the synthetic methods employed, and properties of these compounds are listed in Table II.

**General Methods for the Preparation of Glycocyamines and Glycocyamine Analogs from Amino Acids. Method A (Cyanamide Method).** A sample of the amino acid (25 mmol) was dissolved in the minimum amount of $H_2O$ (usually 2.5 ml) and 1.26 g of cyanamide (a 20% molar excess) was added. A few drops of concentrated ammonia were then added to catalyze the reaction. After a few days at room temperature, the product usually crystallized out. In some cases a soluble complex formed[15] which could be broken up by saturating the solution with ammonia and keeping the solution overnight at 4°.

**Method B (Cyanogen Bromide Method).** The sodium salt of the amino acid (50 mmol) was dissolved in 10 ml of $H_2O$. Cyanogen bromide (5.3 g, 50 mmol), dissolved in 7.5 ml of $CH_3OH$, was added with stirring over a period of 3 hr to the salt solution which was kept in a water bath at room temperature. After addition was complete, the mixture was left to stand for about 20 min, during which time some darkening had occurred. By this time the product had crystallized from the solution. The crystals were filtered, washed with a small portion of cold water, and dried.

**Method C (*S*-Alkylthiuronium Halide Method).** A solution of the amino acid (15 mmol) in 3.75 ml of 4 *N* NaOH (15 mmol) solution was stirred in a water bath at room temperature (use hood!) while an equivalent molar quantity of the appropriate *S*-alkylthiuronium halide, dissolved in the minimum amount of $H_2O$ (approximately 2 ml), was dropped in slowly over a period of 5–10 hr, and then the solution was left in the hood overnight. In the case of relatively insoluble glycocyamines the crystals which formed were filtered off and washed with a small portion of cold water. Higher recoveries of relatively water-soluble glycocyamines were obtained when halide ion was iodide. Thus, the product, dissolved in the minimum amount of water or ethanol, was precipitated by addition to a large excess of vigorously stirred acetone containing 10% (v/v) of 95% ethanol, a solvent mixture in which the contaminating NaI is soluble.

**Method D (*N*-Carboxymethyl-*N*-methyl-S-methylisothiuronium Iodide Method).** The *N*-carboxymethyl-*N*-methyl-*S*-methylisothiuronium iodide (usually only about 90% pure as determined by nmr spectroscopy) was treated with 4 equiv (100% excess) of the appropriate amine in aqueous ethanol at room temperature for about 5 hr (aromatic amines required higher reaction temperatures, *ca.* 55°, longer reaction times, and catalysis by a molar equivalent of triethylamine). After removal of the water *in vacuo*, the product and alkylammonium iodide were isolated as an oil. The nonaromatic products were purified by dissolving in a minimum quantity of 95% ethanol and the resulting solution was added slowly with vigorous stirring to a large excess of acetone, a solvent in which all the contaminants were soluble, and in which the product was insoluble. In the case of aromatic creatine analogs the oil was taken up in a minimum quantity of water at which point crystallization occurred.

**Method E (Glycocyamidine Method).** The appropriate glycocyamidine was heated with a slight excess of an alkyl halide in 95% ethanol for several hours at reflux. After removal of the ethanol, the hydrohalide salt of the product was dissolved in the minimal amount of anhydrous methanol, and the resulting solution was passed through a column of a weakly basic ion exchange resin

(53) The methylene protons were probably masked by the HOD peak at $\delta$ 4.58.

(54) F. H. S. Curd, D. G. Davey, D. N. Richardson, and R. Ashworth, *J. Chem. Soc.*, 1742 (1949).

(*e.g.*, BioRad AG 3-X4 amino form) which had been washed thoroughly with anhydrous methanol just prior to use. After removal of the methanol at 25° *in vacuo*, the crude monoalkylated glycocyamidine was purified by vacuum sublimation. Upon spontaneous hydrolysis of an aqueous solution of the glycocyamidine at room temperature for 24–48 hr, the substituted glycocyamine was formed in high yield.

**Initial Rate Studies.** The initial rates afforded by selected creatine analogs relative to creatine as substrates in the creatine kinase reaction are shown in Table I. The relative rates (average of at least two determinations) were measured on a Sargent Model S-30240 recording pH–stat using a Corning 12 pH meter at 30.00 ± 0.05°. The concentrations, procedures, and conditions used in these studies were identical with those described by Mahowald, Noltmann, and Kuby[29] except that volumes were 50% as large. Creatine and creatine analog concentrations were always the same but in order to obtain conveniently measurable rates the enzyme concentration was varied from 0.16 to 36 μg/ml. In all cases, however, the molar concentration of substrate was at least $10^4$ times greater than the molar concentration of enzyme. The values were corrected for spontaneous ATP hydrolysis. Under the conditions used there was no detectable reaction between ATP and creatine in the absence of the enzyme. In each case parallel product studies were performed using PEI cellulose thin-layer chromatography, and spots corresponding to ATP, ADP, and phosphorylated analogs of creatine could be detected in the proper ratios. Only in the case of the slowest reacting analogs measured, *N*-methyl-*N*-amidinoaminomethylphosphonic acid and D-amidinovaline, could large amounts of inorganic phosphate be detected relative to the amounts of the phosphorylated creatine analog.

The other creatine analogs synthesized were estimated by PEI cellulose thin-layer chromatography to be either unreactive or only marginally reactive in the creatine kinase reaction.

**Phosphorylation of 1-Carboxymethyl-2-iminoimidazolidine Catalyzed by Creatine Kinase.** To 50 ml of 0.4 *N* triethylammonium acetate buffer (pH 9) were added disodium ATP (250 mg, 0.40 mmol), 1-carboxymethyl-2-iminoimidazolidine (57.1 mg, 0.40 mmol), magnesium acetate (0.8 ml of 0.05 *M* solution, 0.04 mmol), bovine serum albumin (0.2 ml of 1% solution), NaOH (1.2 ml of 1.0 *M* solution, 1.20 mmol), and creatine kinase (2 mg, specific activity: 100 μmol $mg^{-1}$ $min^{-1}$). The solution was allowed to stand for 12 hr at room temperature. Using PEI–cellulose thin-layer chromatography, an analysis indicated that the reaction had proceeded approximately 80% toward formation of the phosphocreatine analog. 2,4-Dinitrofluorobenzene (1 ml of 0.01 *N* solution in 2-propanol) was then added to the solution to inactivate the enzyme, and after 30 min at room temperature, the solvent and buffer were removed in a high vacuum at <35° to leave 0.465 g of pale yellow solid. This solid was dissolved in 15 ml of $H_2O$, and 1.8 ml of 1 *N* NaOH was added to adjust the pH to 7. This solution was applied to a BioRad AG 1-X8 column (43 × 1.5 cm, 200–400 mesh, $HCO_3^-$ form), and the column was washed with 150 ml of $H_2O$ to remove $NaHCO_3$, $MgCO_3$, and unreacted 1-carboxymethyl-2-iminoimidazolidine. The phosphocreatine analog was then eluted with 0.10 *N* triethylammonium bicarbonate, pH 8. A total of 72 10-ml fractions were collected. The analog was located in fractions 37–52 by spotting small aliquots of each fraction on a piece of filter paper (Whatman No. 42, low ash) and developing with molybdate spray reagent.[41] These fractions were pooled, the solvent and the volatile buffer were removed *in vacuo* to leave 103.5 mg (61% yield) of bis(triethylammonium)-1-carboxymethyl-2-(phosphonoimino)imidazolidine. A 55.2-mg portion of this product was converted to the dilithium salt by the addition of 0.083 ml of 3.70 *N* LiOH and evaporation of the resulting solution to dryness *in vacuo*. The resulting solid was treated with 4 ml of $H_2O$–methanol (30:70), and the solution was centrifuged to remove a small amount of insoluble $Li_3PO_4$, which was resuspended in a small portion of $H_2O$–methanol (30:70) and recentrifuged. The supernatant and wash were combined and taken to dryness to leave 37.3 mg of solid product. A 35.9-mg portion of this material was dissolved in 1 ml of $H_2O$, and ethanol was added to turbidity (4.5 ml). After standing for 12 hr, small needles had formed. These were collected by filtration and dried to give 25 mg of dilithium 1-carboxymethyl-2-(phosphonoimino)imidazolidine dihydrate. *Anal.* Calcd for $C_5H_8N_3O_5PLi_2 \cdot 2H_2O$: C, 22.16; H, 4.46; N, 15.50; P, 11.43. Found: C, 22.77; H, 4.36; N, 15.46; P, 11.56. The ir spectrum (Nujol) showed major bands at 3.06, 6.00, 6.17, 7.18, 7.28, 7.65, 8.48, 8.97, and 9.82 μ. The nmr spectrum at 220 MHz ($D_2O$) showed peaks at δ 4.18 (4 H, multiplet, AA′BB′ pattern) and 4.34 (2 H, singlet). The multiplet centered at δ 4.18 is shown in Figure 1.

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