Displacement Reactions. IX. The Reaction of Cyanide Ion with Cystine. An Example of Amino Group Participation as Detected with Nitrogen-15 during Cleavage of a Sulfur–Sulfur Bond[1]

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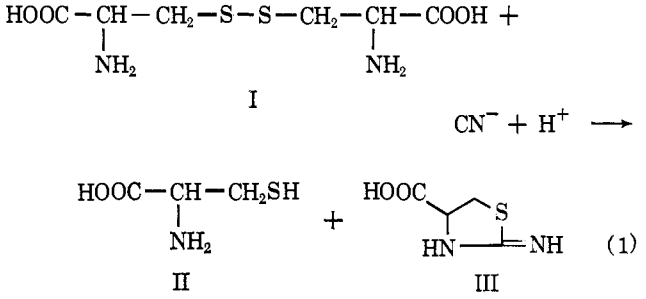
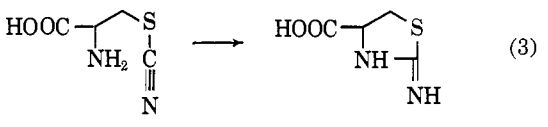
Abstract: In an earlier paper a relationship,[1] $E_a r^3$ = constant, was demonstated relating the activation energy, E_a, to the ground-state bond distance, r (in Å.), of a sulfur–sulfur bond cleaved in the rate-determining reaction. For cyanide ion in aqueous solution at 25° the value of the constant is 99 kcal. Å.^{3}/mole ($NC^- + RSSR \rightarrow RSCN + RS^-$). However, the reaction of cyanide with the amino acid disulfide, cystine, does not fit this relationship. The reaction has a higher activation energy, and a lower rate constant than predicted. Rather than disprove the empirical relationship, further work has demonstrated that cyanide ion is not the true nucleophile but that the amino nitrogen atom is moving in the rate-determining step. The k_{14}/k_{15} nitrogen isotope effect is small (0.94%) but the 95% confidence limit is ±0.05%. A mechanism is suggested which involves amino nitrogen participation.

Schöberl[4] has re-examined the well-known reaction of cyanide ion with the amino acid disulfide cystine (I), and has shown that the products are cysteine (II) and 2-imino-4-thiazolidinecarboxylic acid (III).



The reaction product[5] of cysteine and cyanogen chloride is the same cyclic compound III.

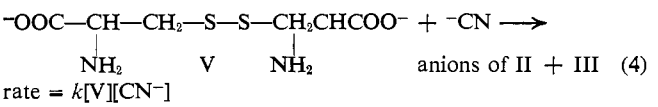
Thus any product of either reaction 1 or 2 having the structure IV immediately undergoes ring closure and



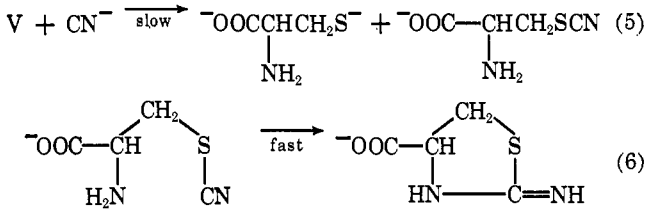
formation of the thiazolidine. Compound III is even detected in tissues following injection of cystine into the blood and then subcutaneous cyanide ion injection.[6]

The kinetics of the reaction 1 have been investigated by Hata and Matsushita who observed a first-order disappearance of cystine with excess cyanide ion.[7]

Gawron[8] has studied the kinetics of the reaction of the dianion of cystine and cyanide ion at pH 12.5 by removing aliquots of the reaction mixture and determining the ultraviolet absorption[8a] in aqueous solution.

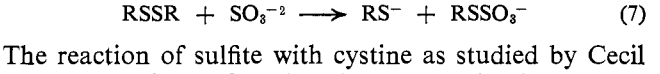


The reaction was second order, first order in cystine dianion and first order in cyanide ion. The energy of activation was reported to be 16.8 kcal./mole and ΔS^* was reported to be −7.4 gibbs. Gawron suggested a two-step mechanism at high pH: a slow rate-



determining cleavage of the sulfur–sulfur bond (5) followed by ring formation. In another study,[8b] the pH dependence of the reaction was studied at 28° over the pH range 7.5–12.5.

Reaction 5 was suggested in analogy with the cleavage of amino acid disulfides as cystine with sulfite ion to form a Bunte salt.



The reaction of sulfite with cystine as studied by Cecil and McPhee[9] was found to have an activation energy

(1) Part VIII: *J. Am. Chem. Soc.*, **88**, 1 (1966); presented at the 148th National Meeting of the American Chemical Society, Chicago' Ill., 1964.
(2) Taken from the Ph.D. Thesis of E. S. W., 1964, in biochemistry.
(3) Alfred P. Sloan Fellow, 1962–1966.
(4) A. Schöberl, M. Kawohl, and R. Hamm, *Chem. Ber.*, **84**, 571 (1951).
(5) W. N. Aldrich, *Biochem. J.*, **48**, 271 (1951).
(6) C. Volgtlen, J. M. Johnson, and H. A. Dyer, *J. Pharmacol. Exptl. Therap.*, 27, 467 (1926).
(7) T. Hata and S. Matsushita, *Mem. Res. Inst. Food Sci., Kyoto Univ.*, [9] **19**, 39 (1955).
(8) (a) O. Gawron and J. Fernando, *J. Am. Chem. Soc.*, **83**, 2906 (1961); (b) O. Gawron, S. Mahbobb, and J. Fernando, *ibid.*, **86**, 2283 (1964); reviewed by O. Gawron, article for "Organic Sulfur Compounds," in press.
(9) R. Cecil and J. R. McPhee, *Biochem. J.*, **60**, 496 (1955); **59**, 234 (1955); *Advan. Protein Chem.*, **14**, 303 (1959).

of 12.8 kcal./mole for the second-order rate constant. Data on cystine with sulfite ion fits the r^{-3} relationship,[1] while the cyanide ion data *do not fit* the $E_a r^3 = 99$ equation.[1] Indeed the activation energy for reaction 4 ought to have an E_a of about 11 kcal./mole rather than the larger 16.8 kcal./mole reported.[8]

The anomoly could mean (1) that the reported E_a of 16.8 kcal./mole is in error, (2) that the r^{-3} relationship is not general, or (3) some new mechanism or another complication is present in the reaction of cystine and cyanide ion. Since data which do not correlate are generally of more interest than systems which fit an empirical relationship, the system was re-examined. Recalculation of the experimental data of Gawron[8] confirm the large E_a, but the value of ΔS^* is now -15.4 gibbs using the value of the rate constant in units of M^{-1} sec.$^{-1}$. An error of conversion of minutes to seconds is the cause of the change in ΔS^*.

Kinetics Study of Reaction 4. The reaction of cystine with cyanide ion was studied by noting the rate of change of the ultraviolet absorption spectrum in 1.000 ± 0.002 cm. quartz cells in a Beckman DU spectrophotometer equipped with an effective thermostated cell compartment.[10] In such a system the solution could be kept *deoxygenated* and thus the extremely facile air oxidation of cysteine anion was prevented. Dilution errors of the aliquot technique were also minimized. The data are presented in Tables I and II.

Table I. Kinetic Data on the Reaction of Cystine with Potassium Cyanide in Aqueous Solution

Cystine, $M \times 10^{4}$ [a]	KCN, $M \times 10^{2}$ [b]	pH[c]	Temp., °C.[d]	k_2, M^{-1} sec.$^{-1}$ [e]
1.00	5.00	12.5	25	6.01×10^{-3}
1.03	5.03	12.5	25	5.88×10^{-3}
1.02	5.00	12.5	25	6.04×10^{-3}
0.993	5.00	12.5	35	1.21×10^{-2}
0.993	5.00	12.5	35	1.25×10^{-2}
1.03	5.00	12.5	35	1.21×10^{-2}
1.95	5.00	11.3	35	1.05×10^{-2}
0.98	5.00	11.3	41	1.82×10^{-2}
0.99	5.00	11.4	41	1.74×10^{-2}
1.87	5.00	11.0	41	1.80×10^{-2}
1.00	5.00	11.0	41	1.84×10^{-2}
0.980	5.01	12.5	41	1.90×10^{-2}
0.989	5.00	12.5	41	1.86×10^{-2}
0.959	5.00	12.5	41	1.84×10^{-2}
2.04	0.50	11.3	47	3.07×10^{-2}
0.986	5.00	12.5	47	3.08×10^{-2}
1.01	5.00	12.5	47	3.16×10^{-2}
0.972	5.00	12.5	47	3.12×10^{-2}
1.05	5.01	12.5	53	4.30×10^{-2}
1.05	5.00	12.5	55	4.30×10^{-2}
1.00	5.00	12.5	53	4.38×10^{-2}

[a] Computed from the mass of cystine. [b] Computed from the silver nitrate titration of the freshly prepared stock solutions. [c] Value on a Beckman Model G pH meter making any cation corrections. [d] Temperature ±0.02° by NBS thermometers. [e] The average second-order rate constant computed from the separate rate constants.

The reaction order of reaction 5 is not in doubt since Gawron[8] varied the concentrations of both reactants. The main question to be settled is whether or not E_a is such that it fits the $E_a r^3 = 99$ relationship. The kinetic data at an ionic strength of $\mu = 0.15$ were obtained at five temperatures. The E_a value obtained from the data of Table I is 13.9 kcal./mole with a standard deviation, σ, of +0.4 kcal./mole and a ΔS^* value of -24 ± 2 gibbs. The application of the Debye–Hückel theory[11] allows a calculation of the effect of ionic strength on the activation energy.

Table II. Cyanide–Cystine at pH 12.5

Temp., °C.	$10^4 k_1$, sec.$^{-1}$ [a]	$10^2 k_2$, M^{-1} sec.$^{-1}$ [b]	E_a[c]	ΔH^*[d]	ΔS^*[e]
25.0	1.49 ± 0.02	0.597 ± 0.009	13.9	13.3	−24.1
35.0	3.05 ± 0.08	1.22 ± 0.03			
41.0	4.67 ± 0.08	1.87 ± 0.03			
47.0	7.87 ± 0.16	3.15 ± 0.07			
53.0	10.8 ± 0.2	4.33 ± 0.08			

[a] d(cysteine)/dt = k_1 (cystine). [b] $k_2 = k_1/[CN^-]$. [c] In kcal./mole; least-squares method used for calculation. [d] In kcal./mole, computed at 25°. [e] In cal./mole deg. (gibbs) computed at 25°.

$$\Delta E_a = \frac{\partial(\Delta\Delta E/T)}{\partial(1/T)} \tag{8}$$

$$E_a = \frac{-3Z_A Z_B e^2}{4D} K\left[1 + \frac{\partial \ln D}{\partial \ln T} + \frac{1}{3}\frac{\partial \ln V}{\partial \ln T}\right] \tag{9}$$

$$K = \sqrt{\frac{4e^2}{DkT}\Sigma n_i Z_i^2}$$

Assuming Debye–Hückel behavior, the reaction of a -2 anion with a -1 anion has an activation energy that ought to increase with increasing ionic strength. Computing from eq. 9, the change in E_a would be only 465 cal./mole. The E_a at $\mu = 0$ would be 13.4 kcal./mole. It must be noted that since the Debye–Hückel limiting slope of (9) overestimates the change in activation energy with ionic strength, the value of 13.4 represents the lowest possible value of E_a at infinite dilution. The experimental value is still too high to fit the inverse cubic relationship.

The reaction of cystine dianion with cyanide does not correlate with the r^3 relationship even though the reaction of sulfite ion with cystine dianion does fit this relationship.

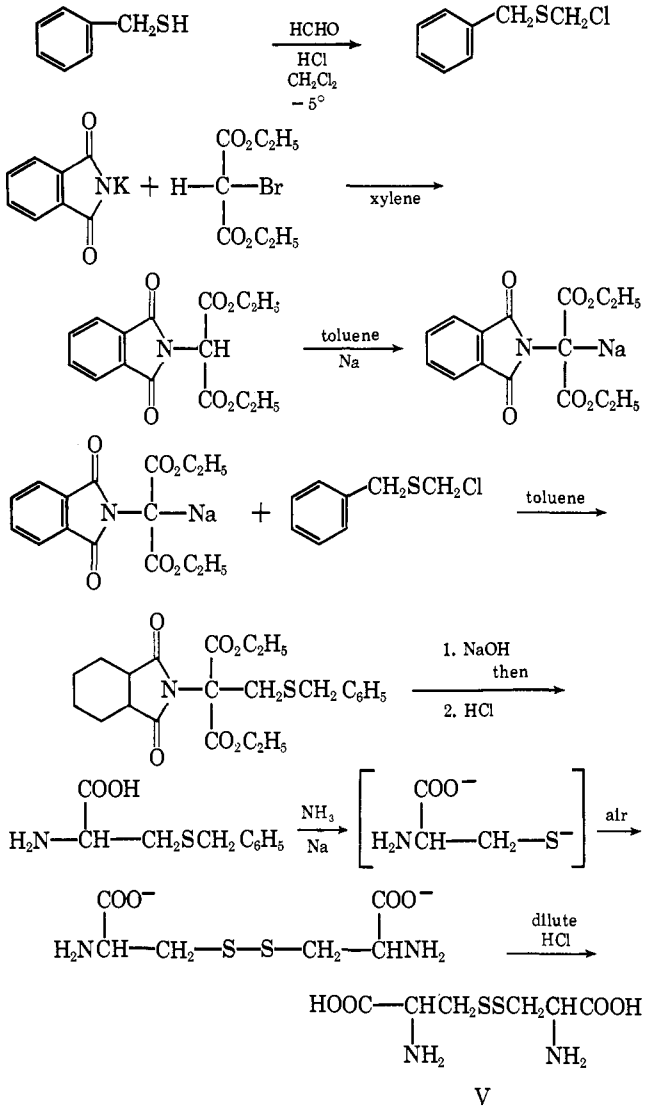
Measurement of the Amino Nitrogen Kinetic Isotope Effect. The larger activation implies that either the r^3 relationship is not general or that a new mechanism is operative.

Examination of molecular models shows that the two large sulfur atoms are very available for a colinear attack by a small nucleophile as cyanide. Moreover, as the negative cyanide ion would approach the cystine dianion, the negative carboxylate group would tend to remain as far away as possible. In such a configuration the amino nitrogen group is rotated up very close to the colinear line between the two sulfur atoms and the incoming cyanide ion. If the amino group is participating in the displacement reaction, then cyanide ion would no longer be the *true* nucleophile and the activation energy need not correlate with the other cyanide data reported.[1] Therefore the synthesis of N^{15}-labeled cystine was undertaken and is outlined in Chart I.

(10) P. D. Bartlett and R. E. Davis, *J. Am. Chem. Soc.*, 80, 2513 (1958).

(11) V. K. La Mer and M. E. Kammer, *ibid.*, 57, 2662 (1935).

Chart I. Synthesis of N^{15}-Cystine



V

The isotopic enrichment of N^{15} was determined by Kjeldahl digestion followed by conversion of the ammonia into nitrogen using sodium hypobromite. The ratio of $N^{14}N^{14}$ to $N^{14}N^{15}$ was then determined in an isotope ratio recording mass spectrometer.

Determination of the N^{15} Kinetic Isotope Effect. The reaction of cyanide ion with the slightly enriched cystine was studied. Defining the reacting system as

$$A + B \xrightarrow{k} \text{products}$$

$$A' + B \xrightarrow{k'} \text{products}$$

then

$$-dA/dt = kAB$$

$$-dA'/dt = k'A'B$$

where A and B are the concentrations of A and B, respectively. Solution of these two equations with the boundary conditions that $A = A_0$ at time $t = 0$ and $A' = A'_0$ yields an equation containing A, A_0, A', A'_0 and the ratios of the two rate constants. This equation can be solved using the experimental determined values of the isotopic ratio. Expressing this equation in terms of the fraction f of A reacted then

$$\frac{k}{k'} - 1 = \frac{\log R/R_0}{\log [(1-f)(1+R_0)/(1+R)]}$$

One can determine k/k' by measuring the isotopic ratio R_0 of the original substrate and R after fraction f of reaction. An error analysis shows that the minimum errors would occur in the k/k' ratio when f is about 0.80. Therefore analyses were made in the range of 0% reaction and then 50–80%. The isotope effect is reported in Table III.

Table III. Experimental Data on the N^{15}-Isotope Effect with 1-Cystine at 27.00°

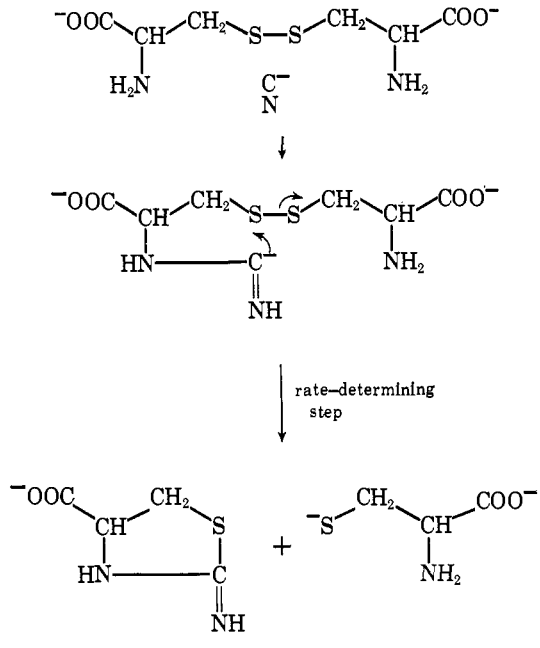
Measurement[a]	N_2 (28), v[b]	N_2 (29), mv.[c]	Ratio
	Initial Sample		
1	3.590	94.6	37.90
2	3.440	91.3	37.67
3	3.390	89.8	37.75
4	3.280	87.2	37.61
5	3.140	82.9	37.87
6	3.030	80.4	37.68
7	3.000	79.0	37.97
8	3.210	85.4	37.58
9	3.190	84.4	37.61
10	3.160	83.8	37.70
		Mean[d]	37.73
		S.D.[e]	0.14
		S.E.M.[f]	0.04
		95% C.L.[g]	37.73 ± 0.09
	Sample of 1-Cystine Recovered after 50% Reaction		
1	6.850	182.0	37.64
2	6.740	180.0	37.44
3	6.600	177.1	37.63
4	6.460	172.1	37.56
5	6.270	167.0	37.54
6	6.050	161.0	37.58
		Mean	37.56
		S.D	0.07
		S.E.M.	0.03
		95% C.L.	37.56 ±0.08
	Sample of 1-Cystine Recovered after 80% Reaction		
1	10.270	277.0	37.07
2	9.300	250.1	37.20
3	8.070	217.0	37.19
4	7.500	202.0	37.13
5	7.190	194.0	37.06
6	5.240	141.0	37.16
7	5.100	137.0	37.23
8	4.560	122.0	37.38
9	4.010	108.0	37.13
10	3.760	101.0	37.23
		Mean	37.18
		S.D.	0.09
		S.E.M.	0.03
		95% C.L.	37.18 ± 0.07
Isotope effect (randomization analysis)[h]			0.94 %
		S.D.	0.26 %
		S.E.M.	0.03 %
		95% C.L.	0.94 ± 0.05 %

[a] Three separate experiments were performed for each of the 0, 50, and the 80% reaction samples. The reproducibility of the trails were very good. The measurements cited are on one sample of N_2 gas from each of the three experiments. In all, about 270 separate estimates were made on the isotopic ratios in these three separate experiments. [b] This value is corrected for the background of N_2^+ (28) at the time of the measurements. Typically it is between 12 and 30 mv. [c] This value has been corrected for the *m/e* background at 29. Typically the correction is 0.400 to 1.000 mv. [d] Average. [e] Standard deviation. [f] Standard error of the mean. [g] Confidence limits. [h] W. J. Youden, "Statistical Methods for Chemists," John Wiley and Sons, Inc., New York, N. Y., 1951, p. 82.

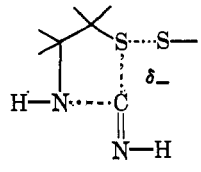
The isotope effect as determined in numerous experiments demonstrated that the amino group is participating in the reaction. An estimate of the maximum k_{14}/k_{15} isotope effect to be expected in cystine can be given by using the Bigeleisen–Mayer heavy-atom approximation[12] at 25°; the normal maximum would be expected to be about 1.030. Thus the value of 1.0094 represents a measurable effect, particularly when the 95% confidence limits is ±0.05%.

A Mechanism with Amino Group Participation. The magnitude of the kinetic isotope effect indicates that the amino nitrogen atom is moving in the rate-determining step and changing its bonding characteristics. Control experiments in progress suggest that there is no isotope effect on the reaction of sulfite with l-cystine. One might have argued that the isotope effect is due to hydrogen bonding or electrostatic attraction between the nitrogen and either the sulfite ion or the cyanide ion. Sulfite ion fits the empirical bond distance equation and has no isotope effect; cyanide ion does have an N^{15} isotope effect in the amino position and does not fit the empirical bond distance equation.

A postulated mechanism would involve the addition of the amino group to the cyanide to form a $-\bar{C}{=}NH$ ion which could then displace on the sulfur–sulfur bond and form the thiazolidine ring in one step.



Perhaps the movement of atoms and the change in bonding could be formulated as



The newly formed $-\bar{C}{=}NH$ ion ought to be a type of nucleophile different from the cyanide ion. Even if the equilibrium constant for formation of the $-\bar{C}{=}NH$ ion were low, the geometric and steric relationships would allow a facile ring closure and displacement of the cysteine dianion.

The reaction of sulfite ion with the amino acid disulfide must occur with only sulfur–sulfur bond cleavage. At present there is no evidence that the amino group can add to the sulfite ion to form the much more weakly nucleophile, –NH—S(=O)$\bar{O}$(OH). This thio ion could not displace on the S–S bond to form the Bunte salt.

Amino Group Participation. Nucleophilic participation of the amino group has been known in the ring closure of ω-aminoalkyl halides.[13,14] The amino group in aryl 4-(N,N-dimethylamino)butyrates causes a facile hydrolysis to a lactam cation which readily ring opens to give the butyrate anion.[15] The amino group can act as an internal general base for catalysis of the transesterification of cevadine orthoacetate.[16]

The dimethylammonium group is an effective intramolecular acid catalyst in the hydrolysis of 2-(N,N-dimethylamino)ethyl thioacetate.[16] A recent review article[17] discusses neighboring group participation in some detail. However, in these cases of participation the amino group *increases the rate of the reaction.* The novel aspect of the demonstration of amino group envolvement in the present study is that the amino group actually *reduces the nucleophilic power of the cyanide ion and raises the activation energy of the process by about 2 kcal./mole. The process using the* $-\bar{C}{=}NH$ *is only favored by the favorable entropy consideration.*

Experimental Section

Potassium Hydroxide. Potassium hydroxide (Baker A.R.) was used to prepare stock solutions. The solutions were stored in polyethylene containers and were protected from atmospheric carbon dioxide by a drying tube containing Ascarite.

Potassium Cyanide. Freshly opened potassium cyanide (Fisher A.R.) was used in the preparation of the cyanide solution. The cyanide solution was prepared directly in 0.040 *M* potassium hydroxide. The resulting solution was stored in a polyethylene container and was protected from atmospheric carbon dioxide by a drying tube containing Ascarite.

Cystine. Reagent grade cystine, purchased from Nutritional Biochemicals Corp., was used to prepare a solution just prior to each kinetic run.

Synthesis of *dl*-Cystine-N^{15}. Ethyl Bromomalonate.[18] A 1-l., three-necked, round-bottom flask was fitted with a stirrer, a reflux condenser with a tube leading to a flask of water for absorption of hydrogen bromide, and a pressure-equalizing dropping funnel equipped with a piece of capillary tubing which reached almost to the blades of the stirrer. In the flask was placed 160 g. (1.00 mole) of diethyl malonate (Matheson Coleman and Bell, b.p. 70–72°) and 150 ml. of carbon tetrachloride. In the pressure-equalizing dropping funnel was placed 165.8 g. (1.03 moles) of bromine (Baker reagent) which had been dried by shaking with an equal volume of concentrated sulfuric acid. The stirrer was started, and 2 ml. of bromine was run into the solution. A large electric bulb was held under the flask until the reaction started. Then the rest of the bromine was added at such a rate as to keep the liquid boiling gently. The solution then was refluxed until no more hydrogen bromide was evolved.

The mixture was cooled and washed five times with 50-ml. portions of 5% sodium carbonate solution. It then was distilled under

(12) J. Bigeleisen and M. G. Mayer, *J. Chem. Phys.*, **15**, 261 (1947); J. Bigeleisen, *ibid.*, **17**, 675 (1949).

(13) H. Freundlich and H. Kroepelin, *Z. physik. Chem.*, **122**, 39 (1926).

(14) C. A. Grob and F. A. Jenny, *Tetrahedron Letters*, **23**, 25 (1960).

(15) T. C. Bruice and S. J. Benkonic, *J. Am. Chem. Soc.*, **85**, 1 (1963).

(16) S. M. Kupchan, S. P. Eriksen, and Y.-T. Shen, *ibid.*, **85**, 350 (1963).

(17) B. Capon, *Quart. Rev.* (London), **18**, 45 (1964).

(18) C. S. Palmer and P. W. McWherter, "Organic Syntheses," Coll. Vol. I, John Wiley and Sons, Inc., New York, N. Y., 1961, p. 245.

reduced pressure, a fraction being collected at 124–130° (17 mm.). This fraction, the product, amounts to 187.7 g. (78.8%) of the theoretical amount.

Benzylthiolmethyl Chloride.[19] A 500-ml., three-necked, round-bottom flask was fitted with a stirrer, a thermometer, and a hydrogen chloride gas inlet tube. In the flask were placed 36.3 g. (0.293 mole) of benzyl mercaptan (Matheson Coleman and Bell), 8.7 g. (0.290 mole) of paraformaldehyde (Eastman Kodak), and 100 ml. of dichloromethane. This mixture then was placed in an ice–salt water bath. The stirrer was started and dry HCl gas was bubbled into the reaction flask at such a rate as not to raise the temperature of the reaction mixture above −5°. The reaction was allowed to proceed until the paraformaldehyde disappeared. The reaction mixture then was allowed to stand at room temperature for 24 hr. The reaction mixture was filtered and the filtrate was treated with an additional 20 g. of calcium chloride. After standing for an additional hour the mixture was filtered, and the filtrate was distilled. The product, benzylthiolmethyl chloride, was collected at 102° (2 mm.). The yield was 30.4 g. This represented 60.8% of the theoretical amount.

Ethyl Phthalimidomalonate.[20] A 1 l., three-necked, round-bottom flask was fitted with a stirrer, a thermometer, and reflux condenser. In the flask were placed 75.4 g. (0.315 mole) of freshly distilled ethyl bromomalonate, 58.2 g. (0.315 mole) of potassium phthalimide (Eastman Organic Chemicals), and 500 ml. of *m*-xylene (dried over $CaCl_2$.) The resulting mixture was stirred and heated at 110–120° for 4 hr. It was refluxed for an additional 2 hr. at 140°. The mixture was allowed to remain at 4° for 12 hr. and then was filtered. The residue was extracted with cold benzene. The washed residue was discarded and the filtrate and washings were combined and evaporated to dryness yielding a yellow oil. The yellow oil crystallized upon standing in an ice bath. The resulting crystals were washed with anhydrous ether until pure white. The product was recrystallized from ether by adding light petroleum ether (b.p. 60–70°). The yield of ethyl phthalimidomalonate melting at 73.5–74.5° was 41.9 g. (43.7% of the theoretical amount).

Ethyl N^{15}-Phthalimidomalonate.[20] A 200-ml., three-necked, round-bottom flask was fitted with a stirrer, a reflux condenser, and a thermometer. In the flask were placed 5.00 g. (0.027 mole) of potassium phthalimide-N^{15} (Volk), 7.15 g. (0.030 mole) of ethyl bromomalonate, and 50 ml. of dry xylene. The reaction mixture was heated with stirring at 110–120° for 2 hr. and further refluxed at 140° for 2 hr. The mixture then was stored at 4° for 12 hr. The solution was filtered and the residue was extracted with cold benzene. The washed residue was saved and the filtrate and washings were combined. The solution was evaporated to dryness yielding a yellow oil which crystallized on standing in an ice bath. After addition of 20.0 g. of nonisotopic ethyl phthalimidomalonate, the product was recrystallized from ether by adding petroleum ether (b.p. 60–70°). The amount of diluted ethyl phthalimidomalonate after recrystallization was 25.70 g. Therefore the yield of ethyl N^{15}-phthalimidomalonate was 5.70 g. (69.3% of the theoretical amount).

Sodium Phthalimidomalonic Ester.[21] A 500-ml., three-necked, round-bottom flask was fitted with a stirrer, a reflux condenser, and a glass stopper. In the flask were placed 90 ml. of toluene (dried over $CaCl_2$) and 25.70 g. (0.0785 mole) of ethyl N^{15}-phthalimidomalonate. After heating the mixture to dissolve the ester, metallic sodium was added piece by piece until no further reaction of the sodium was observed. The reaction mixture was refluxed for 12 hr. during this addition and then was allowed to cool. The light yellow product was filtered and washed with toluene. The yield of sodium phthalimidomalonic ester was 23.17 g. (84% of the theoretical amount).

S-Benzyl-*dl*-cysteine.[22] A 500-ml., three-necked flask was fitted with a stirrer, a thermometer, and a pressure-equalizing dropping funnel. In the flask were placed 160 ml. of a 1:1 mixture of 95% ethanol and water, 13 ml. of dioxane, and 16.8 g. (0.0381 mole) of benzylthiolmethyl phthalimidomalonic ester. One drop of phenolphthalein was added, and the mixture was heated to 50°. NaOH (2 *N*, 38 ml.) was added dropwise with stirring at a rate to maintain the temperature at 55–60°. When all the alkali had been added, the temperature was allowed to fall spontaneously. Then enough HCl was added to make the mixture acid to phenolphthalein. The solution was distilled to half its original volume *in vacuo*. Water was added to make the volume approximately 300 ml., and 30 ml. of concentrated HCl was added. Upon acidification, an evolution of carbon dioxide took place. The solution was heated for 1 hr. An additional 150 ml. of concentrated HCl was added, and the heating was continued another 1.5 hr. The solution was distilled *in vacuo* to dryness, the residue was taken up in water, and the solution was distilled again. It was taken up in 100 ml. of water and NH_4OH was added until the solution gave a reaction neutral to congo red. After the precipitate had been filtered and washed with water, it was suspended in boiling 95% ethanol, heated for a moment, and filtered while hot. The extraction was repeated twice to wash out the phthalic acid, leaving benzylcysteine as a crystalline residue. The product was recrystallized from hot, dilute HCl solution by the addition of NH_4OH. The yield of S-benzyl-*dl*-cysteine melting at 215–216° was 1.62 g. (20.2% of the theoretical amount). Analysis of the product in the mass spectrometer indicated that the compound contained 5.14% N^{15}.

S-Benzyl-cysteine-N^{15}.[22] Liquid ammonia (500 ml.) was collected in a 1-l., three-necked, round-bottom flask fitted with a mechanical stirrer, a $CaCl_2$ drying tube, and a glass stopper. Strips of metallic sodium were added to the liquid ammonia with stirring, and then cystine (Nutritional Biochemicals) was added in portions until the blue color of the dissolved sodium was dissipated. This procedure was repeated until 15 g. (0.062 mole) of l-cystine were reduced and a permanent blue color remained. The excess sodium was discharged with ammonium chloride, and 14.1 ml. (0.124 mole) of benzyl chloride (Baker Reagent) was slowly added with stirring. The ammonia then was allowed to evaporate and final traces were removed *in vacuo*. The residue was dissolved in 400 ml. of ice–water and the solution was filtered. Concentrated hydrochloric acid was added to the filtrate until precipitation began. Dilute hypochloric acid then was added until the thick mass gave a reaction acid to litmus. The precipitate was filtered and washed with water. The yield of S-benzyl-l-cysteine melting at 206–207° was 5.40 g. (20.7% of the theoretical amount).

***dl*-Cystine-N^{15}.**[22] Liquid ammonia (200 ml.) was collected in a 500-ml., three-necked, round-bottom flask fitted with a mechanical stirrer, a $CaCl_2$ drying tube, and a glass stopper. S-Benzyl-*dl*-cysteine (5.14% N^{15}) (1.62 g., 0.067 mole) and 3.54 g. (0.017 mole) of S-benzyl-l-cysteine were mixed and added portionwise to the liquid as fast as the metal reacted. When all the mixture had been added and the solution remained a permanent blue in color for 15 min., ammonia chloride was added until the excess sodium was destroyed. The ammonia was allowed to evaporate spontaneously and the residue was taken up in 100 ml. of ice–water. The solution was extracted with ether, and then concentrated hydrochloric acid was added to the highly alkaline solution until it was just alkaline of phenolphthalein. Two drops of 5% $FeCl_3$ solution was added and air was bubbled through the solution for 9 hr. The resulting solution was tested with nitroprusside solution and gave a negative test for the sulfhydryl group. The solution then was neutralized to litmus and allowed to stand for 2 hr. The solution was evaporated *in vacuo* to half its original volume and the resulting precipitate was filtered, washed with water, and dissolved in hot hydrochloric acid. To the resulting solution was added a small quantity of decolorizing charcoal and after boiling a few minutes the solution was filtered. The product was precipitated from the clear solution with ammonium hydroxide. The resulting white crystals were filtered and washed with water and alcohol. The yield was 1.33 g.; this represented 44.6% of the theoretical amount. The infrared spectrum of the compound was identical with that of the *dl*-cystine (Nutritional Biochemicals). Analysis in the mass spectrometer indicated that the compound contained 1.31% N^{15}.

Sodium Hypobromite. In a 500-ml., three-necked, round-bottom flask equipped with a mechanical stirrer, a dropping funnel, and a glass stopper were placed 133 g. (3.3 moles) of sodium hydroxide (Baker reagent) and 200 ml. of water. To half of this solution cooled in an ice bath was added with vigorous stirring about 40 ml. of bromine (Baker reagent) over a period of 10 min. The remaining sodium hydroxide solution then was added. The resulting hypobromite was stored in a refrigerator in a polyethylene bottle.

Kinetic Studies. All kinetic measurements were made in water on a Beckman Model DU spectrophotometer equipped with a cell compartment thermostated to ±0.01° using 1.00-cm. quartz cells. Stock solutions were thermostated in the same constant temperature bath which maintained the cell compartment temperature.

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Cystine solutions were prepared prior to each run by dissolving the necessary quantity of cystine (approximately 1.2 mg.) in 50 ml. of 0.0406 *M* potassium hydroxide and stored in a polyethylene container protected from atmospheric carbon dioxide by a tube containing Ascarite. The concentration of the cyanide solution was checked frequently with silver nitrate.

Both cystine and cyanide solutions were deoxygenated prior to use by passing purified nitrogen through the solutions for 15 min. To minimize access of air to reaction mixtures during the run, the initial reaction mixture of 1.5 ml. of cyanide solution and 1.5 ml. of cystine solution, as measured in a 3.0-ml. syringe, was placed in a stoppered quartz 1.00-cm. Beckman cell. Optical density measurements then were recorded as a function of time. The spectrophotometric determination of the appearance of the product cysteine at 235 mμ was actually followed. The relationship of a measured physical quantity such as optical density to concentration is well known.

Since the concentration of cyanide was greater by a factor of 500, pseudo-first-order rate constants could be obtained. The infinity point was determined in virtually all runs. Therefore one obtained, upon plotting the logarithm of the difference of the optical density at infinity and the optical density at the time in question against time, a straight line which was directly proportional to the pseudo-first-order rate constant for the reaction. The bimolecular rate constant for the reaction was obtained by dividing the pseudo-first-order rate constant by one-half the concentration of cyanide solution. An attempt was made to duplicate the experimental technique used in each run.

Preparation of a Sample for Analysis in the Mass Spectrometer. The determination of the N^{15} concentration of the nitrogen of an organic molecule, such as cystine, involves the conversion of the organic molecule to ammonia followed by the oxidation of the ammonia to elementary nitrogen.[23] The organic molecules used in this work were converted to ammonia by the Kjeldahl procedure.[24] This procedure consists of digesting sufficient organic compound to yield 1 to 3 mg. of nitrogen in 2 ml. of boiling concentrated sulfuric acid containing approximately 150 mg. of mercuric sulfate. Kjeldahl flasks (100 ml.) were used and the digestion was carried out for 12 to 18 hr. Rittenberg[25] found that this extended digestion was necessary in order to oxidize all organic material.

If a shorter digestion time was employed the nitrogen subsequently prepared from the ammonia could contain an impurity which would give rise to ions in the mass spectrometer at mass 45, 31, and 29. The most abundant ion would be at mass 31.

After digestion, the Kjeldahl mixture was diluted with 25 ml. of water and was made alkaline with 50% NaOH. The resulting ammonia was distilled off in a stream of air bubbled through sulfuric acid into an apparatus comprised of a 100-ml. Kjeldahl flask connected *via* glass tubing to a condenser whose outlet tube was drawn to a fine capillary.[25] All joints were of the ground-glass type. The ammonia was absorbed in 7 ml. of 0.05 *N* HCl. About 20 ml. of water was distilled over. The ammonia sample contained in HCl solution was tested with congo red paper to make certain of the presence of excess acid.

The oxidation of the resulting ammonia to elementary nitrogen was accomplished by first boiling the ammonium chloride solution down to a volume of approximately 3 ml. and then transferring it to a reaction vessel containing a side arm which is easily swiveled. This reaction vessel was attached to a vacuum line. In the side arm of the reaction vessel was placed 5 ml. of dilute hypobromite solution. Both reaction vessel and side arm were carefully chilled in liquid nitrogen followed by evacuation of the system. After evacuation the reaction vessel and side arm were allowed to warm to room temperature. This removed the dissolved gases in the solutions. The freezing and thawing were repeated and the solutions were mixed by tilting the side arm of the reaction vessel. When the resulting reaction subsided, the reaction vessel and side arm again were chilled in liquid nitrogen. Ammonia is quantitatively oxidized to nitrogen by hypobromite.

In the present study three basic conversions of cystine amino groups to elementary nitrogen were accomplished. They consisted of digestion of pure 1-cystine (1.31% N^{15}) and 1-cystine-N^{15} recovered from the reaction mixture after 50 and 80% reaction with cyanide had occurred. The separation of the unreacted 1-cystine-N^{15} from the reaction mixture consisted of acidifying the reaction mixture with glacial acetic acid to pH 5–6. The reaction mixture then was evaporated to one-fourth its original volume *in vacuo* after which the precipitated, unreacted cystine was separated by filtration. The product was washed first with water, followed by alcohol. The infrared spectrum of the cystine collected in this way is identical with that of the commercial sample (Nutritional Biochemicals).

The resulting gas was handled and manipulated by means of a high-vacuum line. Since the gas evolved from the oxidation of ammonia is nitrogen, analysis by means of a mass spectrometer was the most accurate method of determining its isotopic composition.

In the use of the mass spectrometer to measure the amount of isotopic nitrogen, it is assumed that the peak height intensity represents the concentration of the corresponding component and that the use of isotopes does not change the percentage of molecules ionized. A Consolidated-Nier 21-201 equipped with a Varian Vac-Ion pump and a Cary Vibrating Reed MS 31 electrometer was used to obtain the isotopic ratios.

Acknowledgment. The authors wish to thank the Research Corporation for a Cottrell grant, the Sloan Foundation, the Walter Reed Army Institute of Research for Grant WRAMC-MD-2107, the National Science Foundation for Grant NSF-GI5750, and the National Institutes of Health for Grants GM-07655 and RH-00279 over the past 5 years which have supported these investigations.

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