[CONTRIBUTION FROM THE DEPARTMENT OF CHEMISTRY, OKLAHOMA STATE UNIVERSITY]

Reaction of Cystine with Sodium Sulfide in Sodium Hydroxide Solution

G. SATYANARAYANA RAO AND GEORGE GORIN

Received November 12, 1958

The reaction of cystine with sodium sulfide in 0.2*M* sodium hydroxide solution produces a product with an absorption maximum at 335 mμ; it is postulated that this absorption is due to ($^{-}SSCH_2CHNH_2COO^{-}$). In the presence of excess sulfide this species reacts further, and, overall, there are produced two cysteinate ions and disulfide or polysulfide ions. When the reaction mixture is made strongly acid, the absorption at 335 mμ disappears, sulfur precipitates, and cysteine is found; with 5–10 equivalents of sodium sulfide the reaction of cystine is more than 95% complete. The change of optical rotation with time in mixtures of cystine and sulfide has been measured, and the approximate speed of reaction determined.

Cystine is a substance of considerable importance in biochemistry, primarily because of its participation in the composition of many proteins, and of the unique role which it plays in establishing and maintaining protein structure. Owing to the interest which attaches to this substance, many of its physical and chemical properties have been intensively studied, but these studies have not extended, except in a cursory way, to the reaction with sodium sulfide.[1,2] This is rather surprising, because the solubilizing and softening action of sodium sulfide on wool and hair, a phenomenon of some practical importance, is largely due to reaction with the cystinyl residues in these protein materials.[3] Sodium sulfide also interacts in an interesting way with globular proteins, for instance with the enzyme papain.[4] The present investigation was undertaken to obtain fundamental information concerning this type of reaction.

Before entering upon a discussion of the experimental results, it is necessary to make reference to two problems which are pertinent to that discussion, namely, the acid-base properties of sulfide ion, and the nature and absorption of polysulfide ions.

The second ionization constant of hydrogen sulfide is so small that sulfide ion is almost completely hydrolyzed in aqueous solution; unfortunately, there is lack of agreement in the values reported for the ionization constant,[5,6] so that the relative concentrations of sulfide and hydrosulfide cannot be calculated accurately. In most of the experiments to be described, 0.2*M* sodium hydroxide was used as a medium. This would serve to reduce the hydrolysis, and maintain a nearly constant, although undetermined, ratio between the sulfide and hydrosulfide ions. Even in this alkaline medium, considerable hydrosulfide ion is present,[7] and it should be kept in mind that reactions and equations ascribed to the sulfide ion might involve hydrosulfide ion instead, or both ions.

With reference to the nature and properties of polysulfide ions, it should first be admitted that they are not well understood. However it is known that polysulfide ions form when sulfur is dissolved in sodium sulfide solution, and that they exist in mobile equilibrium with each other;[8] these facts are of importance in the work to be discussed. This usually involved comparatively small amounts of sulfur and an excess of sulfide, and disulfide was, accordingly, the predominant species, but higher polysulfide ions were probably present to an appreciable extent. This should be understood in the discussion to follow, even though disulfide will be the only species specifically named. Disulfide has a characteristic lemon-yellow color, and absorbs fairly strongly in the near ultraviolet. Attempts to determine its spectrum by dissolving known amounts of sulfur in excess sodium sulfide resulted in an absorption that changed gradually with time, probably because of the gradual establishment of the polysulfide equilibria previously alluded to, and of the occurrence of side reactions, such as interaction with oxygen. The solutions were protected from air as well as was conveniently possible, but oxidation could not be completely prevented. Qualitatively, the spectrum exhibits a broad band with a maximum about 270 mμ, and a gradually decreasing absorption toward higher wave lengths; for the purposes of comparisons to be made below, it should be noted that the molar absorption coefficient (calculated from the amount of sulfur) at 335 mμ is about 310, and the absorption at 310 mμ is four times greater.[9]

Figure 1 shows a series of curves that represent spectra obtained in the reaction of cystine and sodium sulfide. In all cases, development of the absorption was fairly rapid, although slow alterations

(1) J. C. Andrews, *J. Biol. Chem.*, **69**, 211 (1926); **80**, 196 (1928).
(2) W. Küster and W. Irion, *Hoppe-Seyler's Z. Physiol. Chem.*, **184**, 237 (1929).
(3) H. Ward and H. P. Lundgren, *Adv. Protein Chem.*, **9**, 259 (1954).
(4) J. R. Kimmel and E. L. Smith, *Adv. Enzymology*, **19**, 285 (1957).
(5) H. Kubli, *Helv. Chim. Acta*, **29**, 1962 (1946).
(6) P. Souchay and R. Schaal, *Bull. soc. chim. France*, 819 (1950).
(7) G. E. Martin, *Tappi*, **33**, 84 (1950).
(8) F. Fehér, *Angew. Chem.*, **67**, 337 (1955).
(9) The spectrum of Na_2S is reported by L. H. Noda, S. A. Kuby, and H. A. Lardy, *J. Am. Chem. Soc.*, **75**, 913 (1953).

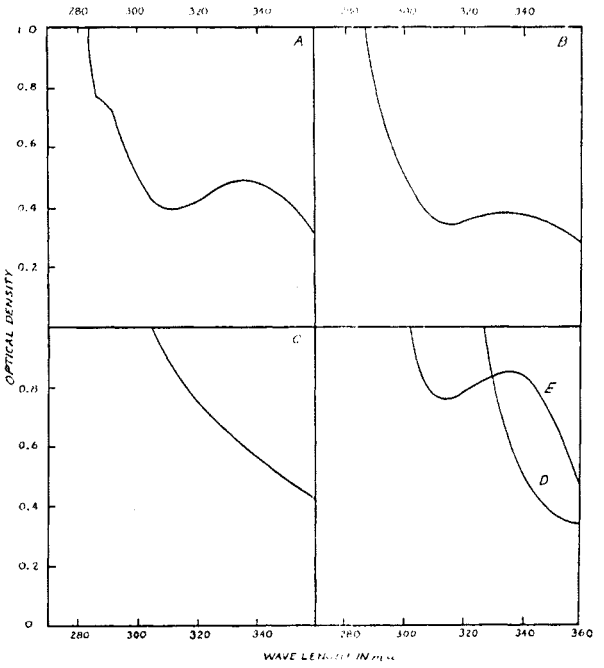
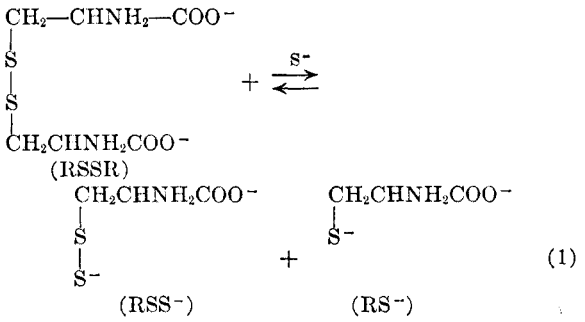


Fig. 1. A: 25 ml. 0.04*M* cystine + 1 ml. 0.052*M* Na_2S, 1 hr. after mixing, *vs.* 0.0385*M* cystine. B: 25 ml. 0.04*M* cystine + 4 ml. 0.52*M* Na_2S, 28 min. after mixing and diluted 25 times with 0.2*M* NaOH *vs.* 0.2*M* NaOH. C: 3 ml. 0.008*M* cystine + 2 ml. 0.516*M* Na_2S + 7 ml. 0.2*M* NaOH, 25 min. after mixing, *vs.* 0.086*M* Na_2S. D: 0.05*M* disulfide solution prepared by dissolving 0.054 g. sulfur in 30 ml. of 1.5*M* Na_2S, diluted 25 times with 0.2*M* NaOH *vs.* 0.06*M* Na_2S. E: 0.002*M* disulfide solution + 0.36 g. of cysteine *vs.* 0.06*M* Na_2S + 0.36 g. cysteine. (Cystine and Na_2S solutions prepared in 0.2*M* NaOH).

of the spectrum would generally be observed over a long period of time; the curves represent the spectra developed when the first rapid reaction had reached essential completion. Curve A represents the spectrum developed from a large excess of cystine and sodium sulfide, and a clearly defined absorption maximum is seen at 335 mμ. This maximum cannot be due to disulfde, or to cysteinate ion,[10] and it is postulated that it is due to the species RSS^-, formed according to Equation (1).



The assumption is supported by the observation that the absorption maximum at 335 mμ was also formed when sulfur was dissolved in alkaline cysteinate solution.

(10) R. E. Benesch and R. Benesch, *J. Am. Chem. Soc.*, **77**, 5877 (1955).

As the proportion of sulfide was increased, the absorption at 310 mμ increased, both in absolute value and especially with respect to that at 335 mμ. In the experiment represented by Curve B, sodium sulfide and cystine were mixed in 2:1 molar ratio, and the maximum at 335 mμ is still visible, but the relative absorption at 310 mμ is a little higher than in Curve A. The concentrations of reaction product in experiments A and B are not known exactly, but are not the same, and therefore the intensities of absorption should not be compared directly. In the experiment represented by Curve C, sodium sulfide was used in large excess, and it is seen that the absorption at 310 mμ is now much higher than that at 335 mμ. It is postulated that, in excess sulfide, disulfide ion is formed, according to Equation (2), and that its absorption

$$RSS^- + S^- \rightleftharpoons RS^- + S_2^- \qquad (2)$$

obliterates the minimum at 310 mμ, observed in the other two cases. Unfortunately, the absorption of disulfide is not very distinctive, and its quantitative features are somewhat uncertain, as has already been stated; furthermore, the other product of the reaction, cysteinate ion, absorbs fairly strongly in the region of the disulfide maximum. For these reasons, a quantitative interpretation of the curves has not been attempted. However, it can be deduced from the appearance of Curve B that only a little disulfide could have been formed in that experiment, even though sufficient sulfide had been added to react with RSS^- completely according to Equation (2); it follows that the equilibrium constant of reaction (2) is small.

This conclusion is supported by Curves D and E. Curve D represents the spectrum of a disulfide solution prepared by dissolving 0.002*M* sulfur in excess sodium sulfide, and Curve E the spectrum developed when cysteine was added to this in a concentration comparable to that of sulfide, it is seen that the absorption typical of RSS^- is developed, by reversal of reaction (2).

When solutions showing the absorption maximum at 335 mμ were treated with strong hydrochloric acid, the maximum disappeared quickly, and sulfur precipitated. In the very dilute solutions used for spectrophotometric measurements, the precipitate was, of course, very light.

More concentrated solutions were next investigated. In this series of experiments, the reaction between cystine and sulfide was allowed to come to substantial completion, the reaction mixture was made strongly acid, excess hydrogen sulfide was expelled, and the remaining solution was titrated with iodine, in the conditions prescribed by Lavine[11] for the determination of cysteine. The results are shown in Table I. At sulfide-cystine ratios smaller than 2:1, the amount of cysteine formed was greater than the amount of

(11) T. F. Lavine, *J. Biol. Chem.*, **109**, 141 (1935).

sulfide; this shows that the reaction cannot be represented by an equation such as (3), according

$$RSSR + 2S^{=} \longrightarrow 2RS^{-} + S_2^{=} \quad (3)$$

to which the amount of cysteine and sulfide should be at most equivalent. The data can be explained, however, if it is further postulated that the species RSS^- is unstable in acid solution, and decomposes according to Equation (4). Such a postulate is

$$RSS^{-} + H^{+} \longrightarrow [RSSH] \longrightarrow RSH + S\downarrow \quad (4)$$

reasonable, in view of the similar behavior of inorganic disulfides toward acidification. The decomposition, however, is probably not instantaneous. In many cases, it was observed that the deposition of sulfur, although largely complete upon the first addition of acid, continued for some time afterwards. This may be due to the temporary existence of RSSH. In the experiments described, where acidification was followed by expulsion of hydrogen sulfide and long standing, the separation of sulfur appeared to be complete before other determinations were undertaken, and it has been assumed that no RSSH remained.

TABLE I

CONVERSION OF CYSTINE TO CYSTEINE

10^{-4} Moles in 20-Ml. Samples		
Cystine	Na_2S	Cysteine
5.0	2.5	3.8
5.0	5.0	7.0
5.0	7.5	8.8
5.0	10.0	9.0
5.0	15.0	9.4
5.0	50.0	9.6
3.8	3.8	5.4
7.6	3.8	6.2
26.6	3.8	7.2

The results of Table I indicate that the reactions do not go to completion. However, there is a gradual shift toward completion as the excess of either reagent is increased, in accordance with the Law of Mass Action. It cannot be deduced from the data to what extent reactions (1) and (2) contribute to the total reaction; indeed, the results might be explained in terms of reaction (1) and (4) alone. However, the occurrence of reaction (2) and formation of disulfide is clearly indicated by the spectrophotometric experiments, and by the strong yellow coloration observed in the more concentrated solutions containing excess disulfide.

Further confirmation of these conclusions was obtained by polarimetric measurements. In a typical experiment, an 0.02*M* solution of cystine in 0.2*M* sodium hydroxide, having a rotation of −0.415° (1-dm. tube; $[\alpha]_D$ −87°) was treated with varying amounts of approximately 1*M* sodium sulfide; in one case, 5.00 ml. was added, giving a concentration of 0.0166*M* cystine and 0.188*M* sulfide. The levo-rotation decreased rapidly, and finally leveled off at a value of −0.033°; this value varied little in the interval between 2000 and 6000 seconds. The final value of the rotation is significant only as to order of magnitude, because it was found that the final rotation given by samples of similar initial composition was not closely reproducible. It is believed that exposure to oxygen was a major cause of the variation, and care was taken to minimize such exposure, but it did not prove practicable to eliminate it. Fully significant values of the final rotation were consequently not obtained.

The reaction mixture was allowed to stand about 90 minutes after attaining constant rotation, and then was made strongly acid. After removal of precipitated sulfur and hydrogen sulfide, the optical rotation of the solution, then approximately 1*M* in hydrochloric acid, was found to be −0.016°; this corresponds to less than 2% of the original amount of optically active cystine. An aliquot portion of this solution was made 1*M* in potassium iodide, then treated with iodine solution drop by drop until a small excess had been added, and finally decolorized with a drop or two of sodium thiosulfate. Measurement of the optical activity then gave a value of −0.296°, which corresponded to regeneration of 95% of the original cystine activity (the solution having been diluted 3.3 times, but the activity now being measured in 1*M* hydrochloric, in which $[\alpha]_D$−215°). These results confirm that cysteine was a product of the reaction and that the reaction was better than 90% complete in those conditions.

Table II summarizes some data obtained with varying concentrations of sulfide. The values called "half-life" actually measure the times required for the initial rotation to decrease to half the value, and do not truly correspond to half-lives of reaction, because the final rotations were not zero. However, these values were small enough to make little difference. The values decrease as the concentration of sulfide increases, as might be expected, and serve to indicate the approximate speed of the reaction, which is fairly great. The final values of the rotation are of qualitative significance only, as has already been explained. Although not exactly reproducible, the values obtained in any one experiment were constant for long periods of time after the initial rapid decrease. The gradual decrease in the final levo-rotation with increasing sulfide concentration is consistent with the existence of an equilibrium which is gradually shifted toward completion of the reaction. The specific rotation of cysteine is so small[12] it can be neglected in the interpretation of the observed rotation in these experiments.

To evaluate from these results the possible utility of sodium sulfide for reducing disulfide bonds in proteins, one must keep in mind that the

(12) J. P. Greenstein, *Adv. Protein Chem.*, 9, 184 (1954).

TABLE II

POLARIMETRIC STUDY OF CYSTINE-SULFIDE MIXTURES

Concn. Cystine *M*	Concn. Sulfide *M*	"Half-Life" Sec.	Final Rotation 1-dm. Tube
0.0185	0.0689	760	−0.068°
0.0180	0.103	503	−0.043°
0.0167	0.155	250	−0.030°
0.0166	0.188		−0.017°
0.0143	0.310		−0.012°

free energy of disulfide bonds in proteins is not the same as that of the bond in cystine; accordingly the values of the equilibrium constants may be different. To the extent that protein disulfide bonds would show the same reactivity as those in cystine, it can be inferred that sodium sulfide in moderate excess would reduce disulfide bonds almost completely to give —S^- and —SS^- residues, and that, in a large excess of sulfide, reduction to two —S^- residues would be expected. Acidification of the solution would convert both types of residue to —SH groups in any case; some time may be required for reaction. During and after acidification, excess reagent, now in the form of hydrogen sulfide, can be removed easily.

EXPERIMENTAL

Materials. All reagents were of analytical reagent grade, except as otherwise specified. L-Cystine, "cfp" grade, and L-cysteine, purified grade, were obtained from the California Foundation for Biochemical Research, Los Angeles; the former compound was estimated to be 98% pure, but no correction for the 2% impurity was made in the calculation of yields. The water used in the preparation of all solutions was distilled, deionized by passage through Amberlite MB-1 resin, deaerated by boiling and cooling with a stream of nitrogen bubbling through it, and stored in a siphon out of contact with the air. Sodium sulfide solutions were prepared by dissolving crystals of $Na_2S.9H_2O$ which had been washed clean of yellowish spots of sulfur and polysulfide; the titer was determined iodimetrically.[13] Cystine solutions were also prepared in sodium hydroxide, 0.2 or 0.6*M*, freshly before use, since the disulfide bond suffers slow hydrolytic fission in strong alkali; in the period of time involved in the experiments described, this reaction would occur only to a very small extent.

Nitrogen was of commercial grade, except in one case as noted below; the commercial gas was purified by passing it through a solution of vanadous ion.[14]

Reaction of cystine with sulfide and titration of cysteine. For the first set of data reported in Table I, 0.123 g. of cystine was weighed in a 50-ml. round-bottom flask, and sufficient 0.2*M* sodium hydroxide added so the final volume, after addition of sulfide, would be 20.0 ml. The solution was stirred with a magnetic stirrer until the cystine had dissolved, sodium sulfide in 0.2*M* sodium hydroxide solution was added, the air above the solution was displaced with nitrogen, the flask was closed, and the solution stirred for the length of time required to give constant cysteine titer (1 hr. for the smallest concentration of sulfide to 15 min. for the largest). The temperature was maintained at 30°. Then approximately 5 ml. of ice-cold 6*N* HCl was added, and the hydrogen sulfide was expelled: in one set of measurements, this was accomplished by stirring and bubbling nitrogen through the solution for 1.5 hr., and in another set by boiling the solution under vacuum with gentle heating and vigorous stirring for 10–15 min., both procedures giving the same results. The solution freed from hydrogen sulfide was added to about 25 ml. of standard iodine solution, prepared from standard potassium iodate and sufficient potassium iodide and hydrochloric acid to give a final concentration of approximately 1*M* in iodide and hydrogen ions; after standing a few minutes, the excess iodine was titrated with standard sodium thiosulfate. Blank experiments were run, first on solutions containing a representative amount of sodium sulfide, by which it was established that the procedure employed expelled all but a small amount of hydrogen sulfide; and secondly, on mixtures of cysteine and sodium sulfide, in which about 97% of the cysteine originally taken could be recovered; the two corrections, *i.e.* for residual hydrogen sulfide and for loss of cysteine during manipulation nearly cancelled one another, so no correction was applied to the results obtained by titration on the cystine-sulfide samples. The accuracy of the determination is believed to be about ±3%.

The second set of data was determined in a similar way, except that in all the experiments the sodium sulfide concentration was kept constant at about 0.019*M* and the concentration of cystine varied; 0.6*M* sodium hydroxide was used to dissolve the sodium sulfide and cystine.

Spectrophotometric measurements. Spectra were scanned with a Beckman DK-1 Spectrophotometer; some optical density measurements at a fixed wave length were made with a Beckman DU Spectrophotometer. Sodium sulfide solutions were prepared shortly before measurement. Both cystine and sulfide solutions were made up in deaerated 0.2*M* sodium hydroxide and stored under nitrogen. They were mixed and transferred to the spectrophotometer cell, taking care to minimize exposure to air. The cell had a ground glass stopper and was filled completely with liquid; *i.e.*, no air space was left above the solution.

Polarimetric measurements. Measurements were made with a Rudolph Model 80 High Precision Polarimeter, modified for photoelectric recording by Mr. Donald Sproul (Department of Biochemistry, University of California, Berkeley). The accuracy and precision of the instrument were checked with samples of cystine. Solutions of cystine in 0.2*M* sodium hydroxide solution were prepared and the temperature was allowed to rise to 25.0°, sodium sulfide was then quickly added with a pipet, and the time of mixing was noted. A sample was transferred to a jacketed polarimeter tube, and measurements of the optical rotation were made at 25° as soon as possible after mixing and at appropriate intervals thereafter. The bulk of the solution was kept in a flask under nitrogen; when the sample in the polarimeter tube had attained constant rotation, a fresh sample from the bulk of the solution was also measured. The two measurements usually disagreed by some appreciable amount; in very unfavorable cases, the discrepancy was as large as 0.03°, an amount which is not great when compared to the initial rotation, but of the same order of magnitude as the final rotation itself.

Polarimetric study of the reaction products. The solution, which typically would contain 0.016*M* cystine, 0.17*M* sodium hydroxide, and 0.18*M* sodium sulfide, was allowed to stand about 90 min. A 10-ml. aliquot was transferred to a 50-ml. centrifuge tube and 3.00 ml. of concentrated hydrochloric acid gradually added; the tip of a thin capillary was then introduced below the surface of the reaction mixture and *high-purity* (>99.8%) nitrogen was bubbled through for 30 min. The mouth of the tube was covered with foil and allowed to stand 2 hr. It was then centrifuged in a Servall Superspeed Centrifuge at about 8000 r.p.m. To an aliquot of the clear centrifugate was added sufficient solid potas-

(13) N. H. Furman, *Scott's Standard Methods of Chemical Analysis*, Vol. II, Van Nostrand Co., New York 1939, p. 2182.

(14) L. Meites, *Polarographic Techniques*, Interscience Publishers, New York, 1955.

sium iodide to make the solution approximately 1*M*, and 0.1*M* iodine was added drop by drop until a permanent iodine color remained. After the solution had stood for 10 min., 0.1*M* sodium thiosulfate was added drop by drop until the iodine color was just discharged.

Acknowledgment. The polarimetric measurements described in this paper were conducted largely in the Department of Biochemistry of the University of California at Berkeley; the kind hospitality afforded by that institution is gratefully acknowledged. Professor Joseph B. Neilands offered valuable advice and helpful discussion; Mr. Donald Sproul assembled and maintained the photoelectric polarimeter which was used. That portion of the work was performed under Contract No. AF 18(603)-135, with the United States Air Force, Office of Scientific Research and Development, Aeromedical Division. The remainder of this work was supported by Grant G-4669, United States Public Health Service.

STILLWATER, OKLA.

[CONTRIBUTION FROM THE DEPARTMENT OF CHEMISTRY, CARNEGIE INSTITUTE OF TECHNOLOGY]

Synthesis of a Chloro Derivative of DL-Vasicine[1]

PHILIP L. SOUTHWICK AND SHELDON E. CREMER

Received December 3, 1958

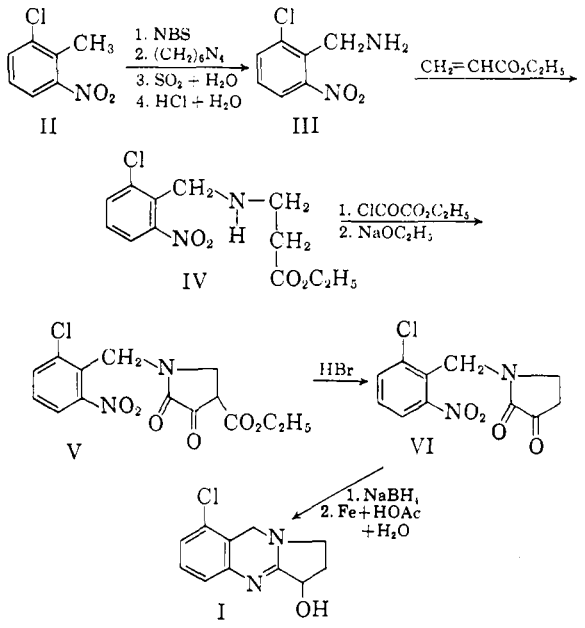
DL-7-Chlorovasicine (7-chloro-3-hydroxypeg-9-ene) has been prepared from 2-chloro-6-nitrotoluene. The compound displays a moderate activity against histamine-induced bronchospasm in guinea pigs, an activity which is potentiated by simultaneous administration of atropine. DL-Vasicine is less active, and DL-6-methoxyvasicine (6-methoxy-3-hydroxypeg-9-ene) showed no activity of this kind.

In a recent publication[2] from this laboratory there was described a new scheme of synthesis for the alkaloid vasicine which was successfully applied to the synthesis of an analog carrying a methoxyl group in the 6- position of the pegene ring system. The method has now been extended to the synthesis of a chloro derivative (I), which could be described as DL-7-chloro-3-hydroxypeg-9-ene according to the system of nomenclature introduced by Späth,[3] and which, for convenience, we have designated DL-7-chlorovasicine.

The general synthetic scheme was discussed in some detail in the previous paper.[2] In the present instance the starting point for the synthesis was the commercially available 2-chloro-6-nitrotoluene (II). The methyl group of II was brominated by use of *N*-bromosuccinimide (NBS),[4] and the crude bromide so obtained was converted into 2-chloro-6-nitrobenzylamine (III) in an over-all yield of 24.8% from II by first forming the hexaminium salt with hexamethylenetetramine and then hydrolyzing this product *via* an intermediate methylol sulfite.[2,5]

The 2-chloro-6-nitrobenzylamine (III) was treated with ethyl acrylate to produce a 93% yield of ethyl β-(2-chloro-6-nitrobenzylamino)-propionate, (IV), which was characterized in the form of the hydrochloride. Compound IV reacted with ethoxalyl chloride to from the *N*-ethoxalyl derivative, which, upon treatment with sodium ethoxide, underwent a cyclization of the Dieckmann type to yield 1-(2-chloro-6-nitrobenzyl)-4-carbethoxy-2,3-dioxopyrrolidine (V). The over-all yield of V from III was 44.6%.

Completion of the synthesis of DL-7-chlorovasicine (I) involved hydrolysis and decarboxylation of V to yield 1-(2-chloro-6-nitrobenzyl)-2,3-dioxopyrrolidine (VI) (74.5% yield), followed by two reduction steps. Reduction of crude VI with



(1) This investigation was supported by a research grant (RG-4371) from the Division of Research Grants, National Institutes of Health, Public Health Service.

(2) P. L. Southwick and J. Casanova, Jr., *J. Am. Chem. Soc.*, **80**, 1168 (1958).

(3) E. Späth, *Monatsh.*, **72**, 115 (1938).

(4) *Cf.* N. Kornblum and D. C. Iffland, *J. Am. Chem. Soc.*, **71**, 2137 (1949).

(5) *Cf.* B. Reichert and W. Dornis, *Arch. Pharm.*, **282**, 100 (1944); *Chem. Abstr.*, **45**, 1969 (1951).