acid arises as a product of oxidation of the alkaloids. Guggenheim[21] has suggested that homarin, the betaine of α-picolinic acid, may be formed from pipecolic acid by dehydrogenation and methylation. Guggenheim's concept is that pipecolic acid should be derived from lysine by cyclization with the loss of ammonia. In fact the conversion of lysine to pipecolic acid in the developing fruit of the bean has now been established by Grobbelaar and Steward[22] and the same conversion has been proved in the rat by Rothstein and Miller.[23] It is evident, therefore, that "free" lysine is low in legumes where pipecolic acid is present. However, Stevens and Ellman[24] failed to replace lysine by pipecolic acid in the nutrition of the rat, in *Streptococcus faecalis* or *Leuconostoc mesenteroides*. With present knowledge the full role of pipecolic acid in metabolism remains obscure, though it has been suggested that it is a step en route from lysine to α-aminoadipic acid.

Acknowledgments.—The authors are indebted to the Beechnut Packing Company for the beans used in this work. The Grasselli Chemicals Division of E. I. du Pont de Nemours and Co. kindly supplied certain samples of piperidine carboxylic acids which were used. The authors are grateful to Dr. H. Posvic, then of the Chemistry Department of Cornell University, for assistance with the infrared absorption spectra.

(21) M. Guggenheim, "Die Biogenen Amine," J. Springer, Berlin, 1940.

(22) N. Grobbelaar and F. C. Steward, THIS JOURNAL, **75**, 4341 (1953).

(23) M. Rothstein and L. L. Miller, *ibid.*, **75**, 4371 (1953).

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ITHACA, NEW YORK

[CONTRIBUTION FROM THE DEPARTMENT OF BOTANY, CORNELL UNIVERSITY]

The Bulk Isolation of L(−)Pipecolic Acid from *Phaseolus vulgaris* and its Quantitative Determination[1]

BY N. GROBBELAAR,[2] R. M. ZACHARIUS AND F. C. STEWARD

RECEIVED JANUARY 11, 1954

Following the identification of L(−)pipecolic acid in the common bean (*Phaseolus vulgaris*) a procedure has been devised by which it may be isolated in high yield. This uses a method for the isolation of non-α-amino acids that was previously used for the isolation of γ-aminobutyric acid. After preliminary purification of the aqueous extract of beans, by absorbing all the soluble nitrogen compounds on a resin and then displacing them, the nitrogenous compounds were freed from other soluble products. The fractionation of the extract leading to the isolation of pure pipecolic acid was achieved by chromatographing the copper complexes of the nitrogen compounds on successive columns of mixed copper carbonate and alumina. Final recrystallization from pyridine–water yielded 13.4 g. of pure pipecolic acid, in the form of the free acid, from about 150 lb. of green beans. The preparation and characteristics of this material are described. The quantitative determination of pipecolic acid on paper chromatograms is described.

Pipecolic acid was first detected on paper chromatograms of extracts of *Phaseolus vulgaris*[3] as an unknown spot which was later identified.[4] It has also been isolated from extracts of *Trifolium repens*[5,6] and *Humulus lupulus* and identified.[7] The presence of pipecolic acid also has been observed in several legumes, in the edible mushroom, in potato tuber, green pepper, tulip, celery, asparagus,[8] Rhodesian teak,[9] barley[10] and coconut milk.[11] This compound is therefore of widespread occurrence in plants.

Thus far, pipecolic acid has been isolated in milligram quantities only by rather laborious methods.[4] The present paper describes a convenient method for the isolation of the compound in bulk and provides information on the quantitative determination of the compound by paper chromatography. As the metabolic reactions and nutritional role of this substance are investigated, access to stocks of the natural and optically active form of the compound become necessary, even though the racemic mixture may be readily synthesized by reduction of α-picolinic acid.[12] The isolation of the natural (−)pipecolic acid in bulk is therefore of importance for such studies.

The bulk isolation in high yield was from an extract from 175 lb. of green beans (*Phaseolus vulgaris*) made as described in the preceding paper.[13] The extract as made (sap + aqueous extract = 26 gallons) was concentrated to about 6 liters, which contained soluble compounds approximately equivalent to 20 g. of amino-N. Part of this extract was used in the first isolation and in the critical identification of a pure product.[13] After this had been done, the remainder was available for the attempt to isolate the bulk of the pipecolic acid in good yield. For this latter purpose the amount of extract available corresponded to about 147 lb. of beans, and consisted of 3.5 liters of the concentrated aqueous extract. The viscous dark-brown concentrate was found to contain about 16.5 g. of amino nitrogen as determined by the method of Moore and Stein.[14] The solution was centrifuged and the bulky pre-

(1) This work was supported by the Grasselli Grant to Cornell University for work being directed by one of us (F.C.S.).

(2) During the course of this work, N. Grobbelaar held a Fellowship awarded by the Rockefeller Foundation.

(3) F. C. Steward and J. F. Thompson, *Ann. Rev. Plant Physiol.*, **1**, 233 (1950).

(4) R. M. Zacharius, J. F. Thompson and F. C. Steward, THIS JOURNAL, **74**, 2949 (1952).

(5) R. I. Morrison, *Biochem. J.*, **50**, xiv (1952).

(6) R. I. Morrison, *ibid.*, **53**, 474 (1953).

(7) A. Harris and J. R. A. Pollock, *J. Inst. Brew.*, **59**, 28 (1953).

(8) R. M. Zacharius, Ph.D. Thesis, University of Rochester, New York, 1953.

(9) F. E. King, T. J. King and A. J. Warwick, *J. Chem. Soc.*, 3590 (1950).

(10) A. Harris and J. R. A. Pollock, *Chem. Ind.*, 931 (1952).

(11) E. Shantz, unpublished data obtained in an investigation at Cornell University.

(12) C. M. Stevens and P. B. Ellman, *J. Biol. Chem.*, **182**, 75 (1950).

(13) R. M. Zacharius, J. F. Thompson and F. C. Steward, THIS JOURNAL, **76**, 2908 (1954).

(14) S. Moore and W. H. Stein, *J. Biol. Chem.*, **176**, 367 (1948).

cipitate, probably denatured protein, was washed with water. The washings and original supernatant were combined and it was from this solution that the pipecolic acid was isolated in good yield by the method which is described below.

Bulk Isolation Procedure.—The basis of the first isolation of pipecolic acid from the extract of beans was the separation of the acid from the other soluble nitrogen compounds by chromatography on columns of synthetic resins. While this procedure yielded enough of a pure compound that sufficed for its identification, it was not adequate for the ready isolation of the compound in high yield.

The method adopted utilized a procedure applied by Thompson, Pollard and Steward[15] to the bulk isolation of γ-aminobutyric acid from the potato tuber. The essential step in this procedure was the separation of the non-α-amino acids from the α-amino acids by forming the copper complexes of these compounds and chromatographing these on alumina. These ends were achieved by the use of a mixed column consisting of alumina containing copper carbonate. Essentially the same device was adopted here and use was made of the different affinity of the copper complexes of pipecolic acid and the α-amino acids, respectively, for alumina. Although not a true α-amino acid, pipecolic acid forms a brilliant blue copper complex with a suspension of copper carbonate. This forms slowly at room temperature and very readily on warming. The color of this complex is distinct from that of the ordinary amino acid copper complexes. Unlike the copper complexes of the α-amino acids, that of pipecolic acid adsorbs only weakly on a mixture of copper carbonate and alumina. However, before applying the copper carbonate–alumina procedure the soluble nitrogen compounds in the crude extract were freed from other soluble compounds by the use of a synthetic resin. The bulk isolation was carried out as follows.

A column (65 cm. × 7.5 cm.) containing 2 liters of 60–80 mesh Zeo Rex resin (Permutit Co.) was used to adsorb the amino acids; 4% H_2SO_4 was used to activate the resin and 0.2 *N* NH_4OH for displacing the amino acids from the column. Because the extract contained enough material to saturate a Zeo Rex column of six times the capacity of the one used, the total extract, containing 16.5 g. of amino-N, was divided into six equal fractions. These fractions were consecutively adsorbed on the resin, extensively washed with water and then eluted. During the washing of the column, saturated with amino acids, the color of the eluate changed from a deep brown to a pale yellow. The only amino acids detected in these washings were the dicarboxylic acids, glutamic and aspartic acid. During the displacement of the amino acids with 0.2 *N* NH_4OH, the resin became slightly warm and the color of the eluate changed from pale yellow to brown probably due to some breakdown of the resin. The amino acids passed through the resin unselectively as the *p*H of the eluate became alkaline and the pipecolic acid accompanied them.

The combined fractions containing the soluble nitrogen compounds were concentrated *in vacuo* at 50° overnight and, during this operation the concentration of the NH_4OH was reduced to a negligible quantity. Further concentration of the solution was carried out on a water-bath under a stream of hot air until the volume was reduced to one liter. The dark brown solution could only be partially decolorized by treatment with Darco activated charcoal and so the volume of the solution was still further reduced to 200 ml. with heat.

Separation of the α-Amino Acids from Other Soluble Nitrogen Compounds.—As indicated above this was achieved by the method described by Thompson, Pollard and Steward.[15] Difficulty was, however, encountered when so concentrated an amino acid extract as that indicated above was added to dry-packed columns of copper carbonate and alumina. Evolution of gas and the generation of heat caused complications. These complications were, however, overcome by wet-packing of the mixed columns of copper carbonate and alumina. The mixed copper carbonate and alumina was added to the column in the form of a thick slurry. When necessary the materials of the column can be recovered by dissolving the copper from the alumina with 6 *N* HCl and reactivating the washed alumina by heating it to a dull red heat.

A concentrated extract containing mixed amino acids and pipecolic acid was added to the column, prepared as described above, either with or without the prior addition of copper carbonate to the solution. Water was used to wash the nitrogen compounds as their copper complexes down the column. (Adjusting the *p*H of the water to 9.0 with ammonia was not found to increase the efficiency of the column.) The eluate was collected in 20-ml. fractions using an automatic fraction collector. The first few of these contained pipecolic acid as the only ninhydrin reactive compound and these fractions were segregated. To test this, one drop from alternate fractions was spotted on a large sheet of paper, numbered and then the sheet was exposed to hydrogen sulfide prior to one directional chromatography with phenol–water. The fractions containing mixtures of amino acids were pooled and passed down two more, and similar, columns. In this way two isolates were obtained as follows. **Isolate A**, consisting of 1.7 liters of solution which contained no other ninhydrin reacting compounds than pipecolic acid, and **isolate B**, consisting of 2.5 liters of solution rich in pipecolic acid but also containing fair amounts of most of the original amino acids that were present in the bean extract.

Treatment of Isolate A.—The solution was treated with H_2S and the CuS removed by filtration. The brown filtrate was concentrated to 150 ml. with heat. Attempts to decolorize the solution with Darco charcoal, a mixture of the resins IR-45 and IRC-50 (Rohm and Haas Co.), and with various organic solvents, all failed.

Since pipecolic acid is very insoluble in pyridine, 700 ml. of pyridine was now added to the 150 ml. of solution and the mixture vacuum distilled. As the solution became progressively poorer in water (the distillate being an azeotropic mixture of 4:1 water:pyridine) pipecolic acid crystallized as colorless needles. However, a resinous black material also later precipitated from the dark brown solution. The distillation was continued until the volume was reduced to 300 ml. The cooled mixture was filtered and the pipecolic-acid-free filtrate discarded.

The extremely water-soluble bulky precipitate was redissolved in 10 ml. of water and passed down a column (80 cm. × 2 cm.) containing a mixture in equal amounts (100 cc.) of the resins IR-45 and IRC-50. Apparently the pyridine treatment had markedly altered the properties of the brown-black impurity of the extract for it now adsorbed completely on the resins while the pipecolic acid was collected in the colorless effluent. On concentrating the effluent to 20 ml. with heat, it became yellow. After the addition of 400 ml. of pyridine the pipecolic acid as the free acid immediately crystallized in the form of a bulky mass of colorless needles. The crystals were filtered off, washed with ether and dried at 80°; yield 6.4 g.

Isolate B.—The 2.5 liters which comprised this extract was treated with H_2S and the CuS removed by filtration. The pale brown filtrate was concentrated to 100 ml. with heat. The addition of two volumes of methanol precipitated a large amount of crystalline material which was found to consist mainly of asparagine and inorganic salts. Further attempts at fractional crystallization by the addition of organic solvents caused the formation of a heavy "oil," rich in amino acids.

The solution of isolate B was concentrated to 100 ml. by heating. After adding an excess of $CuCO_3$ to the solution, it was passed down $CuCO_3$–alumina columns in the same manner as before. At each such passage some fractions were obtained in which pipecolic acid was the only ninhydrin reactive substance and the remainder had a small concentration of miscellaneous amino acids together with a high concentration of the pipecolic acid. The latter fractions were combined and the procedure was repeated using three more columns, *i.e.*, in all six passages. In this way the final eluate contained a high concentration of pipecolic acid and the principal contaminants were small amounts of γ-aminobutyric acid and β-alanine.

As the fractions, especially rich in pipecolic acid, emerged from the column, the copper complex of pipecolic acid often crystallized in the form of deep blue prisms which grew to a considerable size in the tubes on the collector which received these fractions.

All the fractions from isolate B, which contained pipecolic acid as its copper complex, were pooled and treated with H_2S. The CuS was filtered off and the brown filtrate con-

(15) J. F. Thompson, J. K. Pollard and F. C. Steward, *Plant Physiol.*, **28**, 401 (1953).

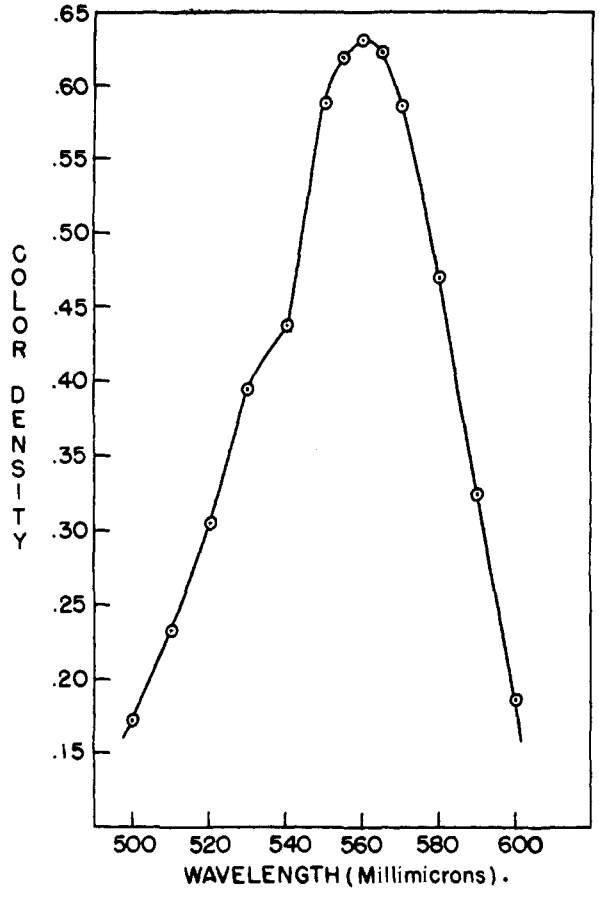


Fig. 1.—Absorption spectrum of the purple compound of ninhydrin with pipecolic acid.

centrated to 100 ml. by heating. The addition of an equal volume of methanol yielded a precipitate of inorganic salts, which was discarded. The filtrate was freed of methanol by concentration and passed down a column (80 cm. × 2 cm.) containing a mixture (200 cc.) of equal volumes of the resins IR-45 and IRC-50 in order to remove the last traces of salts. The resin now decolorized the solution completely (in contrast to previous experience).

The solution, now containing the free pipecolic acid, was concentrated to 50 ml. and 200 ml. of pyridine was added. After standing in the ice-box overnight a heavy yield of crystals of pipecolic acid, slightly contaminated with γ-aminobutyric acid, was obtained. By recrystallizing this crop of crystals 3 more times from water by the addition of pyridine a bulky crop of colorless needles of pure pipecolic acid was obtained. After the first crystallization, the pipecolic acid crystallized immediately from water on the addition of an excess of pyridine. The yield from isolate B was 7.0 g.

The combined yields of pure recrystallized free pipecolic acid from isolate A and isolate B therefore comprised 13.4 g. from about 150 lb. of fresh green beans. This represents the surprising yield of almost 1 g. of pipecolic acid to 10 lb. (4.54 kg.) of fresh green beans. This result emphasizes the fact that pipecolic acid is a major constituent of the soluble nitrogen in the fruit of *Phaseolus vulgaris*. The yield achieved by this procedure was estimated to be of the order of 60%.

Tests on the Crystalline Isolate

DL-Pipecolic acid (prepared from α-picolinic acid by catalytic hydrogenation[12]) and the isolate obtained as above, produced identical compounds with ninhydrin and isatin. With ninhydrin, the color developed is a brilliant violet while isatin produces a blue-green color. The isolated compound could not be separated from DL-pipecolic acid on paper chromatograms using phenol saturated with water, collidine–lutidine (3:1) saturated with water or butanol–

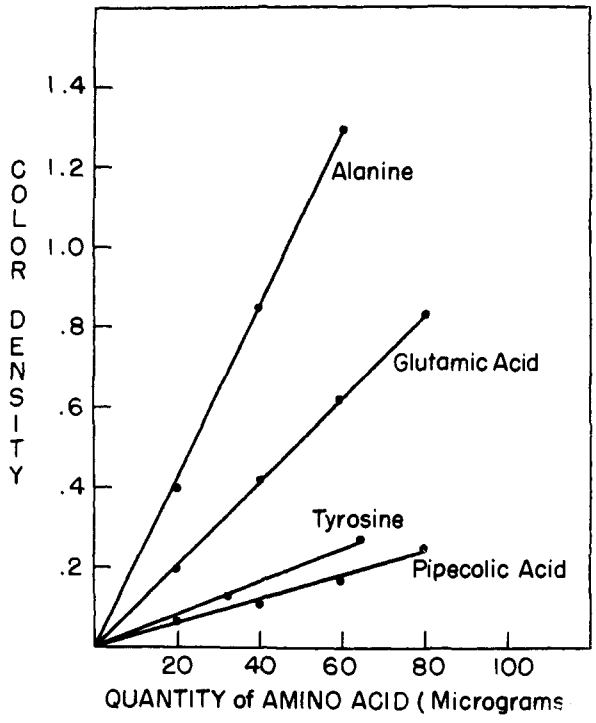


Fig. 2.—Standard curves of representative amino acids after chromatography on paper.

acetic acid (9:1) saturated with water. The isolate, however, gave a separate spot when mixed with either nipecotic acid or γ-pipecolic acid (its two closely related isomers) when chromatographed with collidine–lutidine or butanol–acetic acid. The isolate also could be distinguished readily from baikiain[9] on chromatograms by the yellow-brown ninhydrin color this latter compound produces with ninhydrin or isatin. Furthermore, after catalytic hydrogenation, the isolated compound was unchanged as shown by its behavior during chromatography and with ninhydrin or isatin, whereas baikiain gives pipecolic acid.[4]

An elementary analysis of the crystalline isolate was made.

Anal. Calcd. for pipecolic acid (free base): C, 55.79; H, 8.58; N, 10.84. Found for the isolate: C, 55.81; H, 8.48; N, 10.62.

The optical rotation of the isolated free base was determined on 1.087 g. dissolved in 25 ml. of water and this was found to be $[\alpha]^{23}D$ −24.6° which agrees very well with that found by earlier workers[7,9,6,16] for L(−)pipecolic acid, but it differs markedly from the value $[\alpha]^{25}D$ −34.9° found by Mende[17] for his synthetic preparation. As Harris and Pollock[18] have, however, recently pointed out, a trace of L(−)-baikiain as impurity will increase the optical rotation of L(−)pipecolic acid significantly and there is reason to believe that this compound did in fact contaminate Mende's[17] pipecolic acid preparation.

The isolate melted sharply with decomposition at 274° and could be sublimed successfully at 220° and 3 mm. The sublimate melted with decomposition at 256°.

Quantitative Determination of Pipecolic Acid by Paper Chromatography.—Pipecolic acid is best detected and was first discovered on two directional paper chromatograms. Thomson and Steward[19,20] have perfected a procedure, which enables quantitative determinations to be made of the common amino acids and amides that can be separated on two directional paper chromatograms. This method has been described at length but in the present context its principal feature is as follows. The paper after chromatog-

(16) R. Willstätter, *Ber.*, **34**, 3168 (1901).

(17) F. Mende, *ibid.*, **29**, 2889 (1896).

(18) A. Harris and J. R. A. Pollock, *Chem. Ind.*, 462 (1953).

(19) J. F. Thompson, R. M. Zacharius and F. C. Steward, *Plant Physiol.*, **26**, 375 (1951).

(20) J. F. Thompson and F. C. Steward, *ibid.*, **26**, 421 (1951).

raphy (phenol:collidine–lutidine) is dried, sprayed with ninhydrin and the color is developed in an anaerobic atmosphere (to CO_2), saturated with alcohol at 60°. Thereafter the colored spots are cut out, the ninhydrin color is extracted with 1:1 ethanol–water and the density is determined at 570 mμ for comparison with a standard curve.

Under these conditions pipecolic acid does not react as strongly as the typical α-amino acids, etc. Pipecolic acid produces a distinctive brilliant purple spot on paper when treated with a solution of ninhydrin in ethanol and when the paper is heated in air. The spot is stable for several days and produces a crimson fluorescence in ultraviolet light. Traces of collidine or lutidine in the paper during the development of the color, however, cause the color to fade rapidly to a yellow-brown. When the pipecolic acid–ninhydrin color is prepared on paper under the anaerobic conditions of Thompson, Zacharius and Steward,[19] which are preferred for most of the amino acids, a reddish rather poorly colored spot results. Some improvement in color production may be obtained by developing it at a higher temperature. Even so, both the aerobically and anaerobically produced spots dissolve only sparingly in 50% ethanol and the undissolved ninhydrin compound immediately turns brown on the paper due to the water content of the solvent. Therefore, the quantitative determination of pipecolic acid by procedures that are suitable for a wide range of amino acids still presents further problems.

Further information on the reaction between pipecolic acid and ninhydrin was obtained from two experiments. When a sample of pure pipecolic acid reacted with ninhydrin according to the method of Van Slyke,[21] it was found that only 0.58 mole of CO_2 was liberated per mole of pipecolic acid added. This is in striking contrast to the usual 1 mole of CO_2 liberated per mole from most α-amino acids and proline. If the method of Moore and Stein[14] was used to determine pipecolic acid, *i.e.*, treating it as a compound analogous to an α-amino acid, it behaved as if only 21% as much color is given by pipecolic acid as by α-amino acids. Under the procedure of Moore and Stein, the reaction mixture had the same reddish color that is produced anaerobically on paper under the conditions described.[19]

(21) D. D. Van Slyke, D. A. MacFayden and P. Hamilton, *J. Biol. Chem.*, **141**, 671 (1941).

Pipecolic acid produces a blue-green compound when treated on paper with a 4% solution of isatin in glacial acetic acid–*n*-butyl alcohol (4:96). Although the isatin compound proved to be extractable in pyridine, collidine, lutidine or glacial acetic acid, it rapidly faded and could not be used as a basis for a quantitative method. Therefore, the most hopeful method of quantitative determination is still the development of the ninhydrin color and its quantitative determination, even though this is not as sensitive a reaction as in the case of α-amino acids.

Grassman and von Arnim[22] have reported that the ninhydrin–pipecolic acid compound is insoluble in water but soluble in glacial acetic acid, pyridine and dioxane. None of these solvents was found to dissolve the purple ninhydrin color produced aerobically on paper to any significant extent. Instead 1:1 ethanol–glacial acetic acid, 1:1:1 ethanol–glacial acetic acid–water, 1:1 methanol–glacial acetic acid or 9:1 glacial acetic acid–water (all by volume) will dissolve both the aerobically and anaerobically produced (red-purple) compound completely from paper. Even so, the pipecolic acid–ninhydrin compound dissolves much more slowly in ethanol–acetic acid than the compounds of the common amino acids do in 50% ethanol. The resulting reddish solution is stable for at least 24 hours at room temperature and has an absorption curve with a pronounced maximum at 560 mμ as shown in Fig. 1.

Quantities of 20, 40, 60 and 80 μg. of pipecolic acid were chromatographed two-directionally on washed and buffered Whatman No. 1 filter paper with phenol–water and collidine–lutidine–water, and the chromatograms further treated as described by Thompson and Steward.[19,20] The resulting ninhydrin complex was extracted from the paper with 1:1 ethanol–glacial acetic acid and the density of the color measured at 560 mμ on a Beckman spectrophotometer. The results are represented graphically in Fig. 2. There is a linear relationship between the concentration of pipecolic acid and the resulting color, and therefore an analytical method can conveniently be based on the calibration curve. However, as can be seen from Fig. 2, the method is less sensitive even than for tyrosine.

(22) W. Grassman and K. von Arnim, *Ann.*, **509**, 288 (1934).

ITHACA, NEW YORK

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

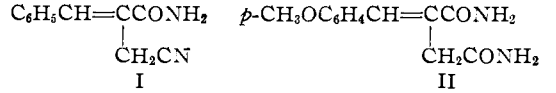
Reactions of Succinonitrile. I. Condensation with Cyclohexanone in the Presence of Sodamide[1]

BY PAUL E. FANTA AND SHELBERT SMITH

RECEIVED DECEMBER 21, 1953

The condensation of succinonitrile with cyclohexanone in the presence of sodamide yields a mixture of 4-cyclohexylidene 5-imino-2-pyrrolidone and 4-(1-cyclohexenyl)-5-imino-2-pyrrolidone. Evidence for these structures was obtained by degradation and measurement of the infrared and ultraviolet absorption spectra. The mechanism of the reaction is believed to be analogous to the well-known Stobbe condensation of succinic esters.

The aldol-type condensation of succinic esters with aldehydes and ketones was discovered by Stobbe in 1893 and recently has been investigated in great detail by Johnson and his co-workers.[2] Although a large number of similar reactions have also been reported for succinic acid and succinic anhydride,[3] only one report of such a condensation involving succinonitrile has been found in the literature.[4] The aldol-type condensations of succinonitrile with benzaldehyde or anisaldehyde in the presence of sodium ethoxide were reported in 1894,[5] but no unambiguous evidence was offered for the structures claimed for the products, I and II.



I II

A complication in the use of aldehydes or unsymmetrical ketones in this reaction is the possibility

(1) (a) Presented in part at the 118th Meeting of the American Chemical Society, Chicago, Illinois, September, 1950. (b) Based on a thesis by Shelbert Smith presented to Illinois Institute of Technology in partial fulfillment of requirements for the M.S. degree, June, 1954.

(2) W. S. Johnson and G. H. Daub in R. Adams, "Organic Reactions," Vol. VI, John Wiley and Sons, Inc., New York, N. Y., 1951, p. 1.

(3) D. Billet, *Bull. soc. chim. France*, [5] **16**, 297 (1949).

(4) "Cyanamid New Product Bulletin," Coll. Vol. I, American Cyanamid Co., New York, 1949, pp. 90–100. When the recent work was nearly completed, J. Stanek and V. Jarolim, *Chem. Listy*, **46**, 384 (1952), reported the preparation of cyclohexylidenesuccinic acid in 55% yield by the condensation of cyclohexanone with succinonitrile in the presence of potassium amylate in amyl alcohol followed by hydrolysis. They did not isolate intermediates or propose a mechanism for the reaction.

(5) C. Bechert, *J. prakt. Chem.*, [2] **50**, 1 (1894).