THE DISSOCIATION CONSTANTS OF RACEMIC PROLINE AND CERTAIN RELATED COMPOUNDS.*

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Recent developments (1) in the field of protein chemistry apparently indicate that a relationship exists between the ability of the protein molecule to combine with acids and with bases and its content of certain amino acids. Such correlations are at best only qualitative, since they are necessarily limited by the inaccuracies in the estimation of the amino acids and by the lack of physicochemical data concerning the components of the protein molecule. Although considerable progress (2) has in recent years been made in the estimation of the dissociation constants of the amino acids, such data are still lacking for proline, oxyproline, hydroxyglutamic acid, serine, and possibly for other components of the protein molecule.

The difficulty in obtaining pure proline has probably been the chief obstacle in the way of determining its dissociation constants. The syntheses of Fischer and Zemplén (3) and of Sörensen (4) are not wholly satisfactory. Our own attempts in this field have been negative. The method which is usually used for the preparation of proline was devised by Fischer and Boehner (5). The product as ordinarily obtained is highly impure and may contain as much as 30 to 40 per cent of its nitrogen in the form of amino nitrogen. Even after repeated crystallization of the copper salt of proline the product obtained by Van Slyke (6) contained 5 per cent of amino nitrogen. Gortner and Sandstrom (7) have commented upon the fact that even after a considerable number of steps had been taken by them in the attempt to obtain pure proline, their product still contained about 10 per cent of the total nitrogen in the form of amino nitrogen.

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nitrogen. They point out that possibly the pyrrolidine ring opens to some extent during the treatment with nitrous acid. Our success in obtaining a product which is practically free from amino nitrogen is due to recognition of the fact that the substance which usually contaminates proline is probably glycocholic, and it is this substance rather than proline which reacts with nitrous acid to give off nitrogen.

The following experiment was suggested to us by Dr. S. P. L. Sørensen. It would be expected that if the pyrrolidine ring opens on

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<th>Shaking time</th>
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<td>5</td>
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* Duplicate determinations were carried out in order to determine the experimental error.

treatment with nitrous acid, the reaction should be influenced by the time factor and there should be a gradual increase in the amount of nitrogen which is evolved. The results which are given in Table I do not indicate that this factor is one of any considerable magnitude. In the experiment 2 cc. portions of a solution which contained 0.29 per cent of proline nitrogen were shaken with nitrous acid in the usual manner for varying lengths of time and the nitrogen which was set free was determined. The increase in nitrogen at the end of the 2 hour period over that which was given off during the first 5 minutes
was less than 2 per cent of the proline nitrogen in solution. On the basis of the usual 5 minute period which is employed for the estimation of amino nitrogen, the nitrogen which is set free as the result of the breaking of the pyrrolidine ring during this time does not account for more than 0.1 per cent of the proline nitrogen. Estimation of the amino nitrogen was subsequently used as an index of the purity of our proline preparations.

The impure proline whose purification was attempted in the following experiments was prepared from gelatin in the usual manner by hydrolyzing with 50 per cent barium hydroxide and extracting the barium-free, dried amino acid mixture repeatedly with absolute alcohol. The alcohol was evaporated and the resulting residue was again extracted with absolute alcohol. The process was repeated until a product was obtained which readily dissolved in absolute alcohol. The attempts at further purification were carried out with this product.

1. In a recent study of the structure of the dipeptide glutathione, Quastel, Stewart, and Tunnicliffe (8) employed unsymmetrical trinitrotoluene to determine the position of the free amino group on the peptide. They note that unsymmetrical trinitrotoluene will react with amino groups but not with certain imino structures. Our experiments with trinitrotoluene were carried out with the hope that this agent might be used for the removal of the contaminating amino acid. The gamma isomer, 3, 4, 6-trinitrotoluene, was prepared by the method of Brady and Gibson (9). A specimen of proline which contained 37 per cent of amino nitrogen was dissolved in absolute alcohol, trinitrotoluene was added and the mixture was evaporated to dryness. The resulting brown, viscous mass was heated on the water bath with 20 per cent sulfuric acid and filtered hot. A brown precipitate resulted upon cooling. The ratio of amino nitrogen to the total nitrogen in the filtrate was approximately the same as at the beginning of the experiment. This method evidently is of no value as a means for the purification of proline.

2. Under proper conditions proline probably reacts with nitrous acid in the manner characteristic of secondary amines.

\[ \text{HNO}_2 + R_1 \cdot \text{NH} \rightarrow R_1 \text{N} - \text{N} = \text{O} + \text{H}_2\text{O} \]
To remove the nitro group which attaches itself to the imino nitrogen requires boiling with acid. This drastic treatment was found to lead to extensive destruction of proline, probably due to the action of nitric acid which is formed in the reaction. Treatment of proline with amyl nitrite and hydrochloric acid in the cold, or hot, was found to be ineffective in reducing the content of amino nitrogen. Experiments in which proline was treated with sodium nitrite and sulfuric acid in excess showed that although the content of amino nitrogen could be reduced to about 5 per cent of the total nitrogen present there was invariably a loss of about 15 per cent of proline. Further attempts at diazotizing resulted in little reduction of the amino nitrogen and a heavy loss of proline.

3. The use of phosphotungstic acid suggested itself as a possible agent for the purification of proline. Sörensen (4) as early as 1905 showed that proline phosphotungstate is quite insoluble in 5 per cent sulfuric acid. Gortner and Sandstrom's attempts (7) at purification of proline included treatment of the impure product with phosphotungstic acid followed by several crystallizations of the copper salt. These procedures did not yield a pure product. Our attempts at purification were carried out in the reverse order to that followed by Gortner and Sandstrom, since it was suspected that the contaminating amino acid, when present in sufficient concentration, is likewise precipitated by phosphotungstic acid. Levene and Beatty (10), as well as Drummond (11), have shown that under proper conditions glycocoll and certain amino acids other than the hexone bases are precipitated from solution by phosphotungstic acid. A preparation of proline which contained 42 per cent of amino nitrogen was converted into the copper salt and recrystallized six times. This process resulted in a reduction of the amino nitrogen content to 1.5 per cent. Continued recrystallization is inadvisable, due to considerable loss of proline. Qualitative experiments indicated that only a slight precipitate of proline phosphotungstate was formed, after the solution had been allowed to stand for 24 hours at 8°C., when the concentration of proline was less than 0.6 per cent while heavy precipitates were obtained with 2.4 per cent solutions of proline. The final purification of proline was accomplished by precipitation of the proline containing 1.5 per cent of amino nitrogen by phosphotungstic acid, recrystallization of the
precipitate from hot water, and subsequent decomposition of the precipitate by means of barium hydroxide and removal of barium with sulfuric acid. 2 cc. of the resulting proline solution, containing 0.2 per cent of proline nitrogen, when treated with nitrous acid for 5 minutes, yielded within the limits of error no amino nitrogen. This specimen of racemic proline was used for the estimation of its dissociation constants.

In our attempts to synthesize proline a number of closely related compounds were prepared. This afforded the opportunity of determining the influence of structure upon dissociation constants. Pyrrolidone-α-carboxylic acid was prepared from glutamic acid according to the method of Haitinger (12) by heating for ½ hour at 150–160°C.

The amide of the α-carboxylic acid of pyrrol was prepared from ammonium mucate according to the method of Schwanert (13) and subsequently converted into the free acid by hydrolysis with barium hydroxide; the barium was removed with sulfuric acid, followed by ether extraction. Tetrahydropyromucic acid was prepared according to the method of Adams and Voorhees (14). Although this is the best available method it yields a product which is not absolutely pure, but is sufficiently so to indicate the magnitude of its dissociation constant.

For the estimation of dissociation constants the well known methods which have been described by Michaelis (15) and by Clark (16) were followed. In brief, the method consists in determining the hydrogen
ion concentration in solutions of the ampholyte to which varying quantities of standard acid or alkali have been added. Estimation of the pH was made in the usual manner with the aid of the Clark cell, n/10 KCl-calomel electrode, saturated KCl bridge, and a Leeds and Northrup hydrogen ion potentiometer. The system was carefully checked with the aid of potassium phthalate mixtures and by titration of glycocoll. The calculations were made with the assistance of the tables of Schmidt and Hoagland (17). The usual correction for the water blank was made (18).

The titration curves are graphically shown in Fig. 1 and the values for the dissociation constants and isoelectric points are given in Table II. The results indicate that proline functions as a weak ampholyte. Comparison of the dissociation constants of the various related compounds apparently indicates that the presence of oxygen as well as the unsaturated state of the ring both influence the magnitude of the acid dissociation constant. The titration curve of pyrrolidine-α-carboxylic acid gives no suggestion that the -COHN- group plays any rôle in the neutralization of either acid or base. This is in agreement with inferences which may be drawn from the work of Eckweiler, Noyes, and Falk (19).

**SUMMARY.**

1. It has been experimentally shown that breaking of the pyrrolidine ring of proline by nitrous acid is not a factor of sufficient magnitude to account for the amino nitrogen which is usually found in proline preparations.

2. A method for the preparation of racemic proline is described. The product was found to be free from amino nitrogen.
3. The dissociation constants of racemic proline and of certain structurally related compounds were determined.

BIBLIOGRAPHY.