

A PHOTOMETRIC METHOD FOR THE DETERMINATION OF PROLINE*

BY WALTER TROLL† AND JOHN LINDSLEY

(From the May Institute for Medical Research of the Jewish Hospital Association, Cincinnati, Ohio)

(Received for publication, October 21, 1954)

The characteristic red color produced when proline interacts with ninhydrin in acidic solutions has recently been proposed for use in the quantitative assay of this imino acid (1, 2). These methods could not, however, be applied directly to the estimation of proline in biological fluids, since other amino acids interfere to some extent and must be removed either by chromatography (1) or by destruction with nitrous acid (2). In the present method, which is a modification of the procedure of Chinard (1), the interfering basic amino acids lysine, hydroxylysine, and ornithine are removed by shaking the solutions with Permutit. Under these circumstances, the method becomes entirely specific for proline when applied to protein hydrolysates, urine, or plasma.

The reaction product of proline in this acidic ninhydrin reaction has been assumed to be the compound isolated by Grassmann and von Arnim (3, 4) from a neutral reaction mixture (1, 2). The distinctly different absorption spectra of the acid (1) and neutral ninhydrin condensation products (5) required elucidation. The acid condensation product has been isolated and appears to have properties of a tautomer of the compound formed in the neutral condensation (3, 4).

EXPERIMENTAL

Proline Method—The following procedure was developed for the determination of proline. The solutions (1 to 5×10^{-5} M proline) were shaken with approximately one-tenth their weight of Permutit¹ for 5 minutes. 5 ml. of this solution, 5 ml. of glacial acetic acid, and 5 ml. of ninhydrin reagent² are heated in a water bath for 1 hour in test-tubes with plastic screw caps. The solutions are cooled to room temperature and extracted with 5 ml. of benzene by shaking them vigorously for 5 minutes.

* This study was supported by a grant from the United States Public Health Service.

† Present address, Cancer Research Institute, New England Deaconess Hospital, Boston, Massachusetts.

¹ Permutit "according to Folin" obtained commercially.

² 125 mg. of ninhydrin per 3 ml. of glacial acetic acid and 2 ml. of 6 M phosphoric acid dissolved with heating to 70°. This reagent was stable for at least 24 hours.

The phases are allowed to separate, the benzene phase is transferred to a colorimeter tube, and the optical density determined at 515 m μ .³

The Permutit treatment removes lysine and ornithine quantitatively from solutions within the pH range of 1 to 7, while removing no proline (Table I). Cysteine is the only neutral amino acid shown to react in the Chinard method (1), but appears to be absent in the biological materials tested (2). Satisfactory recovery of proline was obtained when added to protein hydrolysates, urine, and plasma (Table I), and the absorption spectrum obtained when these biological materials were subjected to the procedure was identical with that obtained when proline alone was used. Urine was refluxed with 6 M phosphoric acid for 24 hours to liberate proline from conjugates or peptides. Without hydrolysis, urine gave one-third to one-half the proline values obtained after hydrolysis. The initial value,

TABLE I
Proline Recovery

Substance added	Proline added	Proline recovered	
	γ	γ	per cent
40 γ bovine serum albumin hydrolysate	2.30	2.25	97.8
	3.35	3.38	100.9
Hydrolyzed urine diluted 20-fold	2.30	2.18	94.8
	3.35	3.40	101.5
Deproteinized plasma diluted 1:14	3.25	3.25	100.0
	3.00	3.08	102.7

however, does not represent free proline, since some hydrolysis of the proline conjugates must take place in the 1 hour reaction period. Unsatisfactory recovery of proline was observed unless urine was diluted at least 20-fold and deproteinized plasma 10-fold with water before assay. The reason for this low recovery appears to be the binding of ninhydrin by other substances such as urea, thereby making it unavailable for reaction with proline. Satisfactory recovery was obtained in situations in which the proline concentration was too low to allow such a dilution by doubling the volume of the reaction system and extracting the color with one-sixth the volume of benzene. Protein hydrolysates were prepared by hydrolyzing 10 mg. of protein in 1 ml. of 6 N hydrochloric acid in a sealed tube at 110° for 24 hours. The protein hydrolysates tested were diluted to 250 ml. before analysis by the method described. The results are summarized in Table II. The standard deviation of the fifteen determinations of proline in protein hydrolysates was computed to be 0.10 per cent.

Observations on Reaction Product—The reaction product of the proline-

³ Coleman and Beckman spectrophotometers were used in this study.

ninhydrin interaction which occurs in a mixture of acetic and phosphoric acid was prepared as described by Chinard (1). The crude product was recrystallized from a 1:10 mixture of benzyl and ethyl alcohols. An identical product could be obtained by starting the preparation with the yellow intermediate of the neutral condensation product of proline and ninhydrin, mono-(diketohydrindylidene) pyrrole (prepared by adding 1 mole of ninhydrin to 1 mole of proline in ethyl alcohol (3)) and treating the solution

TABLE II
Determination of Proline

Proteins	Observed results*	Other methods
	<i>gm. per 100 gm. protein</i>	<i>gm. per 100 gm. protein</i>
Bovine serum albumin† (crystalline)	4.95 ± 0.03	5.07 (6)† 5.02 (7) 4.75 (8)
Casein§	11.2 ± 0.05	10.5 (9) 11.2 (9) 13.3 (10)
Bovine Achilles tendon	12.8 ± 0.0	
Horse Achilles tendon¶	12.9 ± 0.1	
Carp ichthyocol**	11.1 ± 0.1	
	<i>mg. per 100 ml.</i>	<i>mg. per 100 ml.</i>
Plasma	4.12 ± 0.03	1.5-5.7 (11)
	<i>mg. excreted per day</i>	<i>mg. excreted per day</i>
Hydrolyzed urine	69.9 ± 0.2	43-86 (12)

* The ± figures represent the standard deviation obtained on three determinations.

† Purchased from Armour and Company, Chicago.

‡ Bibliographic reference numbers.

§ Hammarsten casein purchased from the Nutritional Biochemicals Corporation, Cleveland.

|| Prepared by the method of Neuman (10).

¶ Obtained through the courtesy of the Lederle Laboratories Division, American Cyanamid Company.

** Prepared by the method of Schmitt *et al.* (13).

with ninhydrin in the acetic-phosphoric acid mixture. Analyses of this compound for nitrogen were identical with those obtained for the neutral condensation product and agreed with the formulation of a di-(diketohydrindylidene) pyrrole (3, 4). The spectra of the acid and the neutral condensation products are strikingly different. The spectrum of the acid condensation product has a maximum at 515 mμ (millimolar extinction 27.2) (Fig. 1), while that of the neutral condensation product has a maximum at 550 mμ (millimolar extinction 82.3) (5). Another difference between the two compounds is their behavior toward alkali in 50 per cent

methyl Cellosolve-water solutions. Only the acid condensation product turned blue when a drop of saturated sodium carbonate was added, the maximum of the spectrum moving to $630\text{ m}\mu$ (Fig. 1). The blue form of this compound, unlike the red form, is insoluble in benzene and soluble in polar solvents.

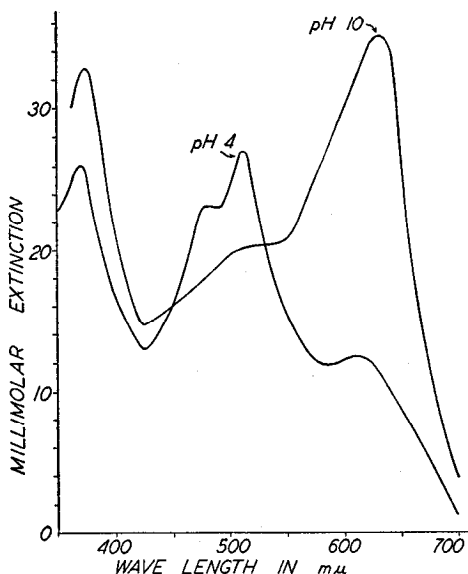


Fig. 1

Fig. 1. Absorption spectrum of the acid condensation product of proline and ninhydrin in 50 per cent methyl Cellosolve-water.

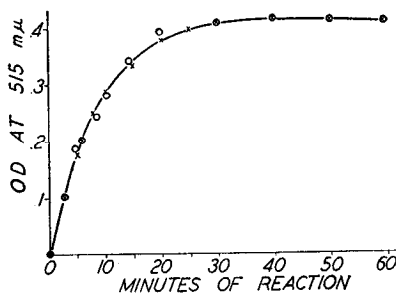
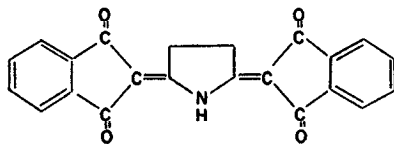


Fig. 2

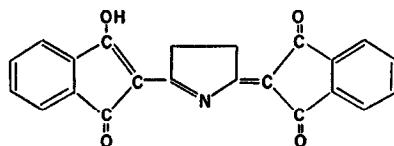
Fig. 2. Rate of formation of the acid condensation product of L-proline and ninhydrin as measured by the optical density at $515\text{ m}\mu$ in a Coleman junior spectrophotometer. O, results obtained when $5 \times 10^{-5}\text{ M}$ proline was used in the modified Chinard reaction described in the text; X, results obtained when $5 \times 10^{-5}\text{ M}$ mono-(diketohydrindylidene) pyrrole was used as the starting product in the same system.

With the modified Chinard reaction, as described above, the formation of this compound occurs with apparent first order kinetics, and the reaction is complete in about 30 minutes at 100° . It is of interest that the rate of formation of the red condensation product was identical when either proline or the mono-(diketohydrindylidene) pyrrole intermediate was used as the starting product (Fig. 2). The yellow mono compound is apparently an intermediate in the reaction, but the condensation of the 2nd ninhydrin molecule is the rate-controlling step. Yellow color formation has been observed before the red color formation in the Chinard procedure (1).

Two tautomers have been proposed as possible structures of the neutral proline-ninhydrin condensation product (3). The keto form (I) has been shown to be the most likely structure for the neutral condensation product (4). The acid condensation product appears to have the properties of the enol form (II) in that the formation of a polar compound together with a



I



II

striking color change (Fig. 1) would be expected from the ionization of a phenolic type hydroxyl group. No simple explanation is apparent for the complete lack of color formation from hydroxyproline in this system. We have tested the survival of hydroxyproline in the acid ninhydrin system by analyzing for hydroxyproline by a method specific for this imino acid (5) at several intervals during the reaction and have observed that it was rapidly destroyed. This suggests that hydroxyproline reacts with ninhydrin in this system, but that the colored compounds corresponding in structure to proline are too unstable to survive.

SUMMARY

1. A specific photometric method for proline and its application to protein hydrolysates, urine, and plasma has been described.

2. The colored reaction product of proline and ninhydrin formed in acidic solution is not identical with the neutral condensation product which is known to be a di-(diketohydrindylidene) pyrrole. Evidence is presented in support of the view that the acid condensation product is an enol tautomer of this compound.

BIBLIOGRAPHY

1. Chinard, F. P., *J. Biol. Chem.*, **199**, 91 (1952).
2. Schweet, R. S., *J. Biol. Chem.*, **208**, 603 (1954).
3. Grassmann, W., and von Arnim, K., *Ann. Chem.*, **509**, 288 (1934).
4. Grassmann, W., and von Arnim, K., *Ann. Chem.*, **519**, 192 (1935).

5. Troll, W., and Cannan, R. K., *J. Biol. Chem.*, **200**, 803 (1953).
6. Keston, A. S., and Udenfriend, S., *Cold Spring Harbor Symposia Quant. Biol.*, **14**, 92 (1949).
7. Troll, W., *J. Biol. Chem.*, **202**, 479 (1953).
8. Stein, W. H., and Moore, S., *J. Biol. Chem.*, **178**, 79 (1949).
9. Brand, E., *Ann. New York Acad. Sc.*, **47**, 213 (1946).
10. Neuman, R. E., *Arch. Biochem.*, **24**, 289 (1949).
11. Harper, H. A., Hutchin, M. E., and Kimmel, J. R., *Proc. Soc. Exp. Biol. and Med.*, **80**, 768 (1952).
12. Stein, W. H., *J. Biol. Chem.*, **201**, 45 (1953).
13. Schmitt, F. O., Gross, J., and Highberger, J. H., *Proc. Nat. Acad. Sc.*, **39**, 459 (1953).