Determination of Free Proline in Serum.* (28058)

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Chinard(1) proposed a method for analysis of proline which depends upon formation of a characteristic red color when the amino acid interacts with ninhydrin in acidic solution. Troll and Lindsley(2) later modified the method and reported satisfactory recovery of proline from hydrolysates, plasma, and urine. No information, however, was given on the type of precipitating agent used for deproteinizing plasma. In determining blood levels of proline after a proline load it was found that the protein precipitant used greatly influenced the results. This investigation was, therefore, undertaken to ascertain the recovery of proline from serum when various protein precipitating agents were employed and to study the absorption spectrum and rate of formation of the proline-ninhydrin acid condensation product with each precipitant.

Materials and methods. Protein-free filtrates (10 ml total volume) were prepared using 1 ml of serum to which 9 ml of one of the following precipitants was added—2.2% tungstic acid in 0.15 M sulfuric acid, 10% trichloracetic acid, and acidic 95% ethanol. The latter precipitant was prepared with 95 ml of absolute ethanol and 5 ml of 0.1 N HCl. Standards of proline in water were prepared in concentrations of 28, 14, and 7 μg/ml. One ml of each standard was mixed with 9 ml of each precipitant to make the solutions from which a standard curve was constructed. Proline was added to serum in concentrations ranging from 7.2 to 57.5 μg/ml for recovery studies. The standard solutions of proline and the deproteinized serum were shaken with approximately 1/10th their weight of Permutit (1 g) for 5 minutes to remove interfering basic amino acids, then centrifuged 30 minutes at 2,000 rpm. Five ml of the supernatant solution and 10 ml of color reagent (60 ml of 6 M phosphoric acid, 240 ml glacial acetic acid, and 3.75 g ninhydrin) were heated at 100°C in a water bath for one hour in test tubes with plastic screw caps. Solutions were then cooled to room temperature and extracted with 5 ml of redistilled benzene for 5 minutes. After the phases had separated, the benzene layer was transferred to a 10 mm × 20 mm cuvette and optical density determined at 515 mμ in a Bausch and Lomb Spectronic 20 colorimeter. Turbidity of the benzene solution occasionally developed but could be cleared by heating at 40°C for 3 minutes. Solutions were also scanned at 5 mμ increments from 475 to 615 mμ.

Results. Table I shows recovery data for proline in serum using the various protein precipitating agents. Recovery of proline was 98-101% when acidic ethanol was the protein precipitant. With trichloracetic acid and tungstic acid, recovery was 111-130%. For each concentration used in the recovery with trichloracetic acid and tungstic acid, the results were reproducible within ± 10% for 10 separate determinations. With acidic ethanol the reproducibility was within ± 3% for 10 separate determinations.

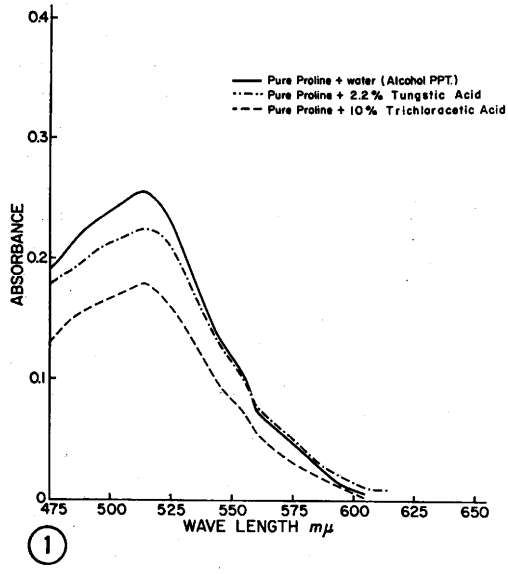
One ml of a 14 μg/ml proline standard was added to 9 ml of each protein precipitant to study absorbance characteristics. Fig. 1 shows the spectra of the pure proline-ninhydrin acid condensation product in benzene.

TABLE I. Proline Recovery from Serum.

Protein precipitating agent	Proline added,	Proline recovered,	
	μg/ml	μg/ml	%
10 % trichloracetic acid	28.7	33.4	116
	14.4	18.8	130
	7.2	8.8	122
2.2% tungstic acid + 0.15 M H_2SO_4	28.7	31.9	111
	14.4	17.9	124
	7.2	8.5	118
95 ml absolute ethyl alcohol + 5 ml 0.1 N HCl	57.5	57.5	100
	14.4	14.6	101
	7.2	7.1	98

* Aided by a grant from the Easter Seal Research Foundation, National Society for Crippled Children and Adults, Inc.

The absorption maximum for the product formed in acidic solution is 515 mμ. A suggestion of a secondary absorption peak was noted at 550 mμ, the absorption maximum for the neutral condensation product of proline and ninhydrin. There was lower maximal absorbance with trichloracetic acid and tungstic acid as compared to water in the 475 mμ to 550 mμ range.



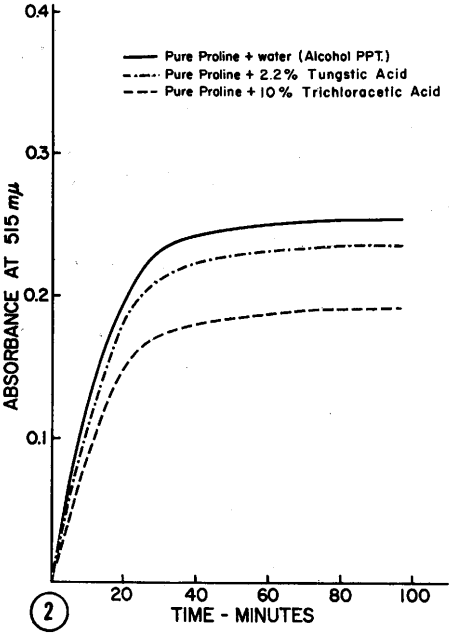


FIG. 1. Absorption spectra of proline-ninhydrin condensation product in benzene after dissolving proline in various protein precipitants and water.

FIG. 2. Rate of formation of proline-ninhydrin condensation product at 515 mμ and 100°C.

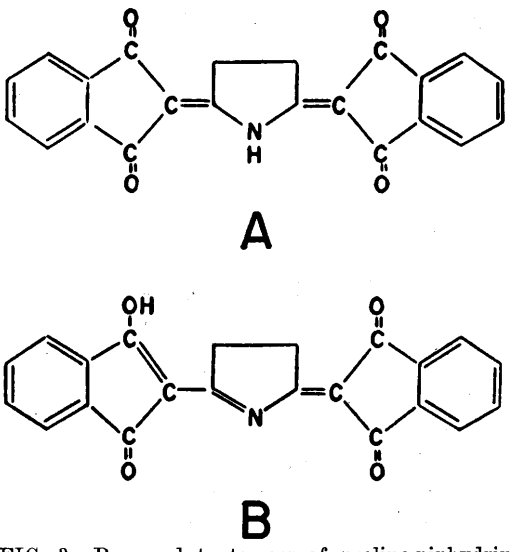


FIG. 3. Proposed tautomers of proline-ninhydrin condensation. A. Neutral product (keto form). B. Acid product (enol form).

Fig. 2 shows rate of formation of the proline-ninhydrin acid condensation product measured at 515 mμ. One ml of a 14 μg/ml proline standard was added to 9 ml of precipitant for this aspect of the study. The reaction follows the expectation for first-order kinetics and was complete in about 30 minutes at 100°C. There was little difference in rate of formation of the proline-ninhydrin condensation product from filtrates prepared by use of the various precipitating agents, but total absorbance differed as demonstrated in Fig. 1.

Discussion. Condensation products of proline and ninhydrin have been isolated from acidic and neutral solutions and their spectra have maxima at 515 mμ and 550 mμ respectively(3). These compounds are probably tautomeric and their postulated structures are shown in Fig. 3. The keto form (A) is the most likely structure for the neutral product. The acid condensation product would probably have properties of the enol form (B) since formation of a highly polar compound together with a striking color change would be expected from the ionization of a phenolic-type hydroxyl group. It is pre-

sumably the enol form which produces the red color noted in this method(4). No apparent interfering substances could be shown from the absorption spectra and very little of the keto form of the proline-ninhydrin condensation product appears to be present regardless of the precipitant.

The data suggest that the high recovery noted with strong acid precipitants may be related to lower maximal absorbance of standards at 515 mμ than that noted with acidic ethanol. The better solubility of pyrrolidines in ethanol than in strong acids may be an important factor in the excellent quantitative recovery of proline from serum after protein is precipitated by this solvent. Harrington(5) has reported that low water activiy represses ionization of the carboxyl group of proline and also dampens out the effect of the imino nitrogen charge. Optical rotation of proline in 95% ethanol is $I^D25 = -67°$. This is a substantial decrease from the rotation in water and must be attributed to dampening out of the charge effects on the proline molecule. Harrington has also reported that polyproline is insoluble in strong salt solutions, so that aggregation of proline molecules and solubility characteristics of the imino acid itself in the strong acid precipitating agents should be considered in interpreting the results. It is also possible that acid precipitants may alter solubility characteristics or reactivity of proline (or the proline-ninhydrin condensation product) to the extent that less is extracted with the relatively non-polar benzene solvent in the final step. The results indicate that acidic ethanol is a highly effective protein precipitating agent for determination of free proline in serum. Trichloracetic acid and tungstic acid are not recommended for deproteinizing serum in this method.

Summary. Various protein precipitating agents were used to obtain protein-free filtrates for determination of free proline in serum. Recovery of proline from serum was 98-101% after precipitation of protein with 95% acidic ethanol; with 10% trichloracetic acid and 2.2% tungstic acid in 0.15 M sulfuric acid, recovery was 111-130%. Accuracy of the method was ± 3% when acidic ethanol was used as a precipitant. Lower maximal absorption values for the proline-ninhydrin condensation product were obtained in aqueous standard solutions with trichloracetic acid and tungstic acid than with acidic ethanol, but rate of formation of the acid condensation product did not vary significantly in each system. Strong acid precipitants may alter physicochemical properties of proline itself or of the enol tautomer formed in the proline-ninhydrin condensation to produce the differences noted.

The authors express appreciation to Judson J. Van Wyk and D. W. Lamson for assistance in this work.

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Received November 21, 1962. P.S.E.B.M., 1963, v112.