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An Improved Colorimetric Method for the Determination of Proline in the Presence of other Ninhydrin-Positive Compounds

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1. The conditions required for sensitive and specific colorimetric determination of proline with acidified ninhydrin were investigated. 2. A method applicable to protein samples was developed. 3. The only compound found to interfere appreciably was a hydroxyproline.

The ninhydrin reagents that are commonly used for colorimetric determination of amino acids are relatively insensitive for proline (Light & Smith, 1963). Strongly acidified ninhydrin reagents are more specific because only transient coloration occurs when they react with many amino acids, whereas with imino acids and basic amino acids they form fairly stable red derivatives. Chinard (1952) based a method for colorimetric determination on this reaction. Through some elaborations, including removal of basic amino acids on Permutit cation exchanger, Troll & Lindsley (1955) claimed that they made the method 'entirely specific' for proline. Their procedure was subsequently quoted (Block & Weiss, 1956) and used widely. However, Finch & Hird (1960) reported that coloration was markedly stimulated by amino acids, and it was found (J. J. Wren & A. J. C. Smith, unpublished work) that Troll & Lindsley's (1955) procedure gave values that were 40% too high for proline in milk-protein hydrolysates. Later Messer (1961) reported interference by various amino acids, dipeptides and tripeptides, and partly overcame it by adding glycine in excess.

The present paper describes an improved method in which remaining problems of interference are overcome. It also includes some results that extend and slightly modify Messer's (1961) findings.

EXPERIMENTAL PROCEDURE

Materials

The acetic acid, benzene, formic acid, glycine, hydrochloric acid and orthophosphoric acid used were AnalaR reagents; 100-volume hydrogen peroxide was used. Ninhydrin was obtained from British Drug Houses Ltd. or Hopkin and Williams Ltd., and L-proline from British Drug Houses Ltd. Bovine serum albumin was obtained from Nutritional Biochemicals Corp., U.S.A., and dried at 80° *in vacuo*.

Permutit natural zeolite (British Drug Houses Ltd.) was washed with water, to remove fine particles, and left on the bench to dry before use.

Glycine solution contained 4 μmoles/ml. in acetic acid–6 M-phosphoric acid (3:2, v/v) mixture. The solution was stable at room temperature for several months.

Ninhydrin solution contained 40 mg./ml. in acetic acid and was prepared by warming (at a temperature not exceeding 70°). The solution was stable at room temperature for several hours only.

Method

Step 1: *performic acid oxidation.* Protein was dissolved in formic acid (not less than 0·1 ml./mg.) in a Pyrex test tube and hydrogen peroxide (0·002 ml./mg.) was added. After 1 hr. the mixture was evaporated to dryness in a stream of warm filtered air. (Residual formic acid may cause an explosion during step 2.)

Step 2: *hydrolysis.* 6 N-HCl (not less than 1 ml./mg.) was added, and the tube was sealed and heated at 110° for 20 hr. The hydrolysate was evaporated to dryness (*a*) in a stream of air if there were only a few tubes containing not more than 5 ml. each, or (*b*) over silica gel and KOH pellets in a vacuum desiccator, at 20–40° and approx. 1 mm. Hg, if the tubes were numerous, or (*c*) in a rotary evaporator if the volumes were greater than 5 ml. The residue was dissolved in a measured volume of water to give a proline concentration of 3–30 μg./ml.

Step 3: *removal of basic amino acids.* A 2 ml. sample of the solution was added to 200 $\pm$ 20 mg. of Permutit in a test tube and shaken intermittently. After 15 min. the tube was centrifuged briefly and 1 ml. of the cleared supernatant was transferred to a stoppered tube of 20 ml. capacity.

Step 4: *ninhydrin reaction.* A 2·5 ml. portion of glycine solution and 2·5 ml. of ninhydrin solution were added and the tube was shaken, placed in a water bath at 95 $\pm$ 0·5°, stoppered and left for 40 min. After cooling with tap water the solution was extracted with 5 ml. of benzene and centrifuged to clarify the benzene layer. The extinction of the benzene layer was measured in a 1 cm. cell at 515 mμ against a blank layer prepared from 1 ml. of water (steps 3 and 4). If the benzene layer was accidentally warmed in the

centrifuge it sometimes became turbid later, interfering with spectrophotometry.

Comments on the method

Step 1. Performic acid oxidizes free and combined cysteine and cystine, which interfere in step 4, to cysteic acid (Harris & Ingram, 1960; Light & Smith, 1963), which does not. The quantity of hydrogen peroxide used is, very roughly, ten times the theoretical requirement for cysteine and cystine in a protein sample. Too great an excess causes a perceptible loss of proline, as Table 1 shows. Table 1 also shows how the colour yield from proline is depressed if cystine is not oxidized.

Step 2. A large acid/protein ratio ensures negligible loss of proline during hydrolysis when carbohydrates are present (Dustin, Czajkowska, Moore & Bigwood, 1953).

Step 3. Permutit (Troll & Lindsley, 1955) effectively removed basic amino acids including histidine (Block & Bolling, 1951). This zeolite was better than synthetic ion-exchange resins, which removed proline and neutral amino acids unless the pH was adjusted to a specific value.

Step 4. As in the work of Chinard (1952), the extinction was measured at 515 mμ ($\lambda_{max.}$ 516 mμ). Extraction with benzene, which was introduced by Troll & Lindsley (1955), is retained because it improves specificity. Thus the colour produced by tyrosine and much of that produced by histidine remain in the aqueous layer. Chinard (1952) chose 100° as the reaction temperature and Sarid, Berger & Katchalski (1959) chose 70°. For practical convenience and reproducibility we chose 95°.

Strong acid confers specificity for imino acids (Chinard, 1952; Piez, Irreverre & Wolff, 1956). The strength of phosphoric acid chosen is a compromise to meet the need for avoiding too rapid decomposition of ninhydrin and the needs for sensitivity and specificity. Silberstein, Adjarian & Thompson (1956) showed that the colour yield from pipecolic acid (a homologue of proline) and ninhydrin is much affected by phosphoric acid concentration. This is also true for proline. The colour yield is raised by diminishing the acid concentration if low concentrations of ninhydrin and glycine, and short reaction periods, are employed, but under some other conditions it may be raised or depressed. Diminishing the acid concentration incurs a major disadvantage in making the colour yield highly sensitive to changes in the concentrations of glycine and other amino acids: this is demonstrated in Fig. 1.

Table 1. *Effects of varying the addition of hydrogen peroxide on the proline content determined*

Experimental details are given in the text. The values in column *A* refer to bovine serum albumin, for which the reported proline content is 4·75 g./100 g., and the cystine/proline molar ratio 0·58 (Tristram, 1953). The values in column *B* refer to similar samples to which cystine had been added to raise the cystine/proline molar ratio to 2·05.

H_2O_2 (moles/mole of total cystine)	Proline found (g./100 g. of protein)	
	A	*B*
0	4·42, 4·22	3·36, 3·29
50		4·90, 4·80
100	4·80, 4·72	4·73, 4·51
250	4·28, 4·28	4·18, 4·25

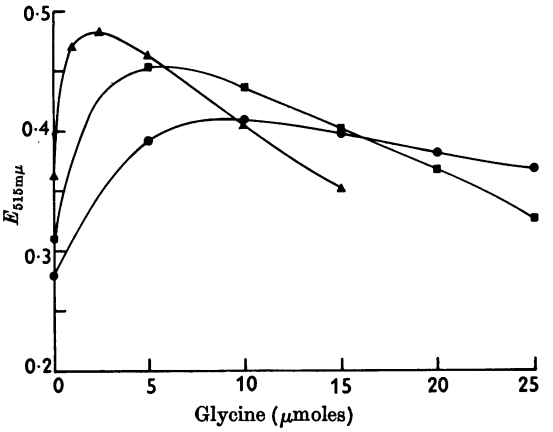

Fig. 1. Effects of changing the molarity of phosphoric acid (▲, 0·12 M; ■, 1·2 M; ●, 6 M) and the quantity of glycine on the colour yield from proline (0·114 μmole) in step 4 of the method described in the text. The reaction period was 60 min. in these experiments.

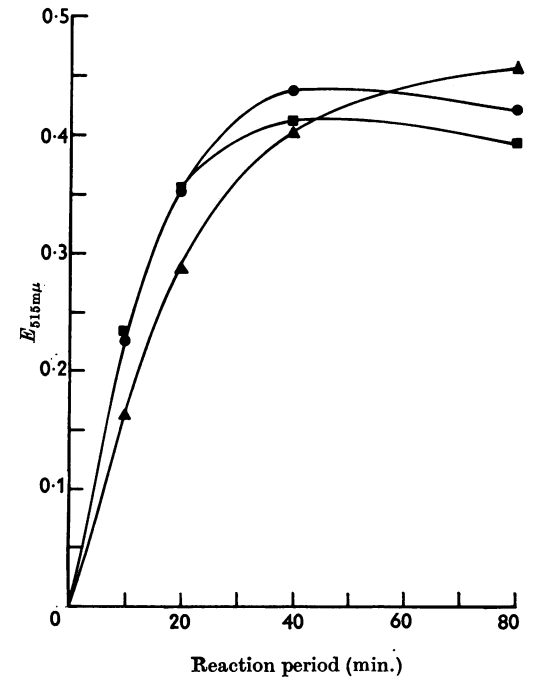

Fig. 2. Effects of changing the quantity of ninhydrin (▲, 50 mg.; ●, 100 mg.; ■, 125 mg.) and the reaction period on the colour yield from proline (0·113 μmole) in step 4 of the method described in the text.

The effects of variation of water and acetic acid concentrations were not studied directly.

An increase in the quantity of ninhydrin used may raise or depress the colour yield, depending on the reaction period. It appears to increase both the rate of coloration and the rate of disappearance of colour (Fig. 2). These effects also occur in the absence of glycine. However, increasing the quantity of ninhydrin diminishes sensitivity to changes in glycine concentration, no matter what reaction period is employed (Figs. 3 and 4); this is desirable, to minimize interference. The use of 100 mg. of ninhydrin is a compromise to achieve sensitivity to proline without undue sensitivity to glycine and other amino acids.

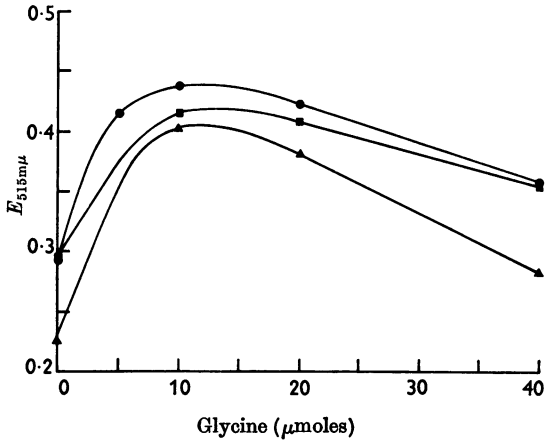

Fig. 3. Effects of changing the quantities of ninhydrin (▲, 50 mg.; ●, 100 mg.; ■, 125 mg.) and glycine on the colour yield from proline (0·113 μmole) in step 4 of the method described in the text.

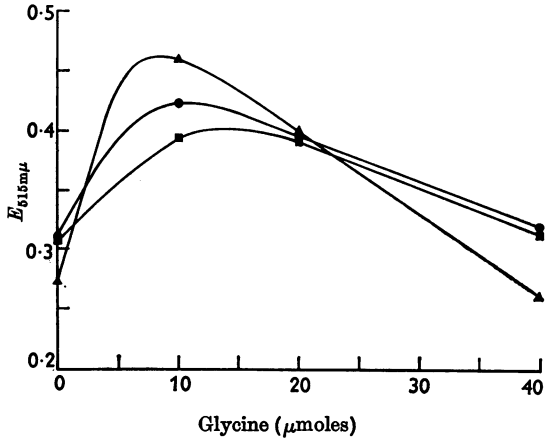

Fig. 4. Effects of changing the quantities of ninhydrin (▲, 50 mg.; ●, 100 mg.; ■, 125 mg.) and glycine on the colour yield from proline (0·113 μmole) in step 4 of the method described in the text, with the reaction period doubled (to 80 min.).

Glycine is added to improve specificity and sensitivity, as advocated by Messer (1961). Like him, we found that it increases both the rate of coloration and the rate of disappearance of colour, and it raises the maximum colour yield without giving any measurable colour itself (up to 40 μmoles tested). However, the quantity used by Messer (1961), 1·8 μmoles, is insufficient for swamping, and we prefer to use 10 μmoles. With this quantity the factor by which the colour yield from proline is raised is independent of the glycine/proline ratio. The factor is not simply related to the glycine/ninhydrin ratio (Figs. 3 and 4). When 6 M-phosphoric acid is used, and reaction periods of at least 20 min., the maximum increase in colour yield is always produced by about 10 μmoles of glycine (as used in the method).

The optimum reaction period depends on the concentrations of phosphoric acid, glycine and ninhydrin. In the method described the colour yield is fairly stable to changes in the reaction period between 40 and 80 min. (Fig. 2), but interference by amino acids rises slightly with increase in the reaction period.

Compounds tested for interference in step 4

Dissolving proline in 0·1 N-HCl instead of water only slightly depressed the colour yield. [The effect of HCl varies widely in other procedures (Schweet, 1954; Silberstein *et al.* 1956; Work, 1957; Gilvarg, 1958).]

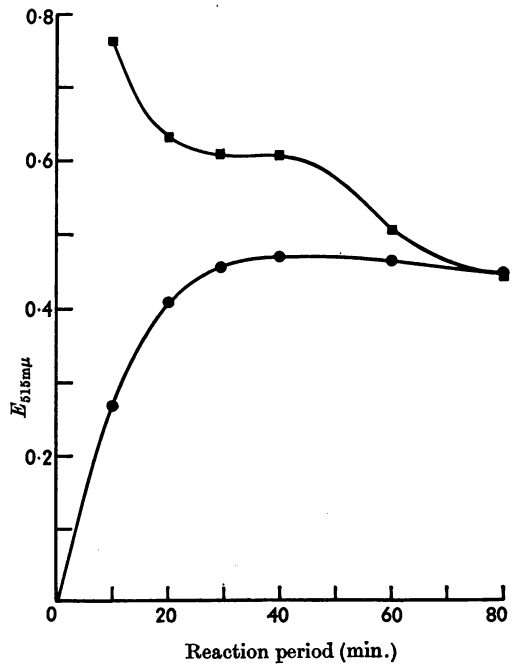

Fig. 5. Effects of adding cysteine (1·00 μmole) and varying the reaction period on the colour yield from proline (0·124 μmole) in step 4 of the method described in the text. ●, Proline alone; ■, proline + cysteine.

Up to 5 μmoles of acetamide, ammonium acetate, ammonium chloride, aspartic acid, cysteic acid, glutamic acid, glycine, methionine, methionine sulphone, phenylalanine, 2-pyrrolidone-5-carboxylic acid, serine or threonine did not interfere measurably, although aspartic acid and methionine gave weak brown colours in the absence of proline. Histidine and lysine gave strong brown colours. Hydroxyproline gave a faint colour ($\epsilon_{515\,m\mu}$ approx. 2% of that for proline). The sample tested probably contained both the 3- and 4-isomers; only the former gives a colour by Troll & Lindsley's (1955) procedure (Ogle, Arlinghaus & Logan, 1962).

Cysteine gave a violet colour ($\epsilon_{515\,m\mu}$ 4·4% of that for proline) that disappeared if the reaction was prolonged (Fig. 5). Cystine gave a brown colour ($\epsilon_{515\,m\mu}$ 1·7% of that for proline) that increased in time. However, cystine interfered more significantly by counteracting the increase in proline coloration caused by glycine. Tyrosine produced a similar effect, which was directly related to the tyrosine/glycine ratio but not to the tyrosine/proline ratio.

When glycine was omitted the effects reported by Messer (1961) were confirmed for all amino acids tested except phenylalanine (which behaved qualitatively like glycine) and aspartic acid (which behaved like methionine).

Application of the method

The calibration graph for 0·03–0·3 μmole of proline in step 4 was a straight line of slope 3·89 ($E_{515\,m\mu}/\mu$mole) $\pm$ 0·09 (S.D.).

The method was tested on bovine serum albumin with cysteine, cystine, hydroxyproline, lysine, methionine, tyrosine and sucrose added in various proportions. These proportions were chosen to test for interference that might be caused by extremes of composition (Tristram, 1953) in different proteins. The results are given in Table 2. The effects of omitting step 1 or steps 1 and 3 can be seen: most of them are readily predictable from our findings about step 4.

In the method as described it appears that all interference is suppressed except that by hydroxyproline and tyrosine. From the results of our tests of step 4 we can predict a value about 5·0 in Table 2 for proline in the presence of hydroxyproline. The predicted value is nearer the value obtained when step 1 was omitted than that obtained by the complete method, suggesting that an oxidation product was chiefly responsible for interference. Interference by tyrosine is only marginally significant.

The mean value of all determinations by the complete method, except those in which hydroxyproline and tyrosine were added, is 4·78 $\pm$ 0·08 (S.D.). According to Tristram (1953) bovine serum albumin contains 4·75 g. of proline/100 g. [Spahr & Edsall (1964) reported that well-purified bovine serum mercaptalbumin contains 5·22 g. of proline/100 g.]

DISCUSSION

The method appears suitable for the determination of proline in most protein samples with an accuracy of about $\pm$5%. This margin seems reasonable for a procedure including oxidation, hydrolysis and adsorption. The final step is sensitive to less than 2 μg. of proline and gives highly precise values. Depending on the nature of a sample to be analysed, one or more of steps 1–3 may be omitted from the method. Thus step 4 alone can be used to determine free proline in the absence of certain interfering amino acids, notably cysteine, cystine, lysine and tyrosine; it should therefore be useful for eluates from ion-exchange columns, to which Chinard's (1952) and Troll & Lindsley's (1955)

Table 2. *Determination of proline in bovine serum albumin, with additives tested for interference*

Experimental details are given in the text.

Additive	Additive* (moles/mole of proline)	Proline found (g./100 g. of protein) Step 1 omitted	Steps 1 and 3 omitted	Method as described
None		4·5, 4·5	4·7, 4·6	4·81, 4·80, 4·72
Cysteine	0·28	4·0	4·5, 4·5	
Cystine	2·4	3·3, 3·4	3·7, 3·7	4·80, 4·78, 4·90, 4·69
Hydroxyproline	2·5	5·1		5·58
Lysine	6·2	4·2	5·5, 5·4	
Methionine	0·52	4·0	4·7, 4·6	
Methionine	2·6	4·5		4·71
Tyrosine	2·4	5·1, 4·5	4·5, 4·5	5·19, 5·01
Cystine	2·4			
Hydroxyproline	2·5	4·1		5·73
Tyrosine	2·4			
Cystine	2·4			
Methionine	2·6	3·6		5·08
Tyrosine	2·4			
Sucrose	9·8			
Sucrose	9·8	4·5		4·82

* Including that combined in the protein.

methods have previously been applied (Harfenist, 1953; Light & Smith, 1963).

Proteins in which hydroxyproline occurs will give high values for proline, but these can presumably be corrected. Although tyrosine interferes only slightly, its halogen derivatives (Thompson, 1954; Kirby, 1962; Sanger & Thompson, 1963) seem to interfere more, and so high concentrations of halide ions should be avoided in protein samples.

These investigations revealed a more complicated pattern of interference than was previously recognized. All the experimental variables in step 4 seem to be interdependent and the reactions occurring are certainly too complex to be explained from present knowledge of the chemistry of ninhydrin (McCaldin, 1960). Free radicals may be involved (cf. Lagercrantz & Yhland, 1963).

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