A Spectrophotometric Method for the Direct Determination of Cysteine in the Presence of Other Naturally Occurring Amino Acids

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1. An acid ninhydrin reagent was found to react specifically in forming a pink product (E_{max} 560m μ) with cysteine. 2. The method was highly sensitive for the determination of cysteine (ϵ 28.0 $\times$ 10³). Homocysteine, glutathione, proline, ornithine and other naturally occurring amino acids tested did not give a similar reaction. 3. The reaction product was stable for at least 3–4 hr. at room temperature and the extinction was proportional to the concentration in the range 0.05–0.5 μ mole of cysteine. 4. The acid ninhydrin reagent also gave yellow products (E_{max} 370–404m μ) with tryptophan, 5-hydroxytryptophan, 5-hydroxytryptamine and indol-3-ylacetic acid. 5. The method was applied for the determination of cysteine in perchloric acid extracts of rat brain, liver and blood.

Ninhydrin reacts with α -amino acids and some other amino compounds to give a characteristic purple colour (E_{max} 570m μ), provided that the reaction is carried out at pH 5.0 or higher pH (Moore & Stein, 1948; 1954; Troll & Cannan, 1953; Yemm & Cocking, 1955). Under these conditions cystine gives 50–55% of the molar extinction expected for an amino acid (ϵ 21.6 $\times$ 10³); cysteine does not react and proline gives a yellow product (E_{max} 440m μ). At an acid pH the reaction of ninhydrin with proline and ornithine results in a red product and, as shown by Chinard (1952), this reaction may be employed at pH 0.3 for the quantitative determination of these two compounds (E_{max} 515m μ). Lysine, citrulline and cysteine also react with the acid ninhydrin reagent giving an orange to pink colour, but their molar extinction coefficients are considerably lower than those obtained from proline and ornithine. Chinard's acid ninhydrin reagent was modified by Work (1957) and by Gilvarg (1958) for the specific determination of diaminopimelate: it was also used by Work (1957) for the determination of several diamino acids such as lysine, cystine and cystathionine. In these methods, which employ an acid pH of 0.3–0.9 for the reaction, it was shown that several other naturally occurring amino acids did not give an appreciable colour.

In the course of my studies on sulphur amino acids the acid ninhydrin reagent as modified by Work (1957) was used for the ultraviolet spectrophotometric determination of cystine. The fractions

containing cystine and small amounts of cysteine, eluted with 0.5N-hydrochloric acid from ion-exchange columns, on reaction with this reagent gave an intense pink colour (E_{max} 560m μ). Under these conditions cystine does not give a pink product with extinction peak at 560m μ . However, Chinard (1952) has reported the formation of a pink product on treatment of cysteine with ninhydrin. On reference to the published spectrum (Chinard, 1952) the intense pink colour indicated the presence of large amounts of cysteine in the fraction. Further experiments showed (i) that the conditions used had increased the sensitivity of the reaction for cysteine, the molar extinction coefficient being several times that reported for cysteine by Chinard, and (ii) that in the presence of hydrochloric acid the reaction was specific for cysteine in that proline, ornithine, citrulline, cystine, homocyst(e)ine, glutathione (GSH and GSSG) and all other naturally occurring amino acids that were tested did not give a similar pink reaction product.

EXPERIMENTAL

Chemicals. Citrulline, glutathione (GSH and GSSG), homocysteine, homocystine (99% pure), L-cystathionine and DL-penicillamine were obtained from Koch-Light Laboratories Ltd., Colnbrook, Bucks. DL-5-Hydroxytryptophan was supplied by Shandon Scientific Co. Ltd., London, W. 10; 5-hydroxytryptamine creatinine sulphate by British Drug Houses Ltd., Poole, Dorset; indol-3-ylacetic acid by Sigma Chemical Co., St Louis, Mo., U.S.A.;

adrenaline (B.P.) by Boots Pure Drug Co. Ltd., Nottingham; noradrenaline bitartrate (Levophed) by The Bayer Products Co., Surbiton, Surrey. All other amino acids and dithiothreitol were supplied by California Corp. for Biochemical Research, Los Angeles, Calif., U.S.A. Ninhydrin, hydrochloric acid (sp.gr. 1.36) and acetic acid of analytical grade were obtained from British Drug Houses Ltd., and phosphoric acid (AnalaR) was from Hopkin and Williams Ltd., Chadwell Heath, Essex.

Acid ninhydrin reagent 1. This was prepared as described by Work (1957) by dissolving 250mg. of ninhydrin in a mixture of 6ml. of acetic acid and 4ml. of 0.6M-phosphoric acid. The reagent was stable for at least 2 weeks at 4°. Chinard (1952) used 6M-phosphoric acid in the ninhydrin reagent.

Acid ninhydrin reagent 2. The reagent contained 250mg. of ninhydrin in a mixture of 6ml. of acetic acid and 4ml. of conc. HCl, which on mixing repeatedly at room temperature gave a solution in 20–30min. This reagent was prepared fresh before use.

Standard procedure for reaction with acid ninhydrin reagent. The reaction mixture, containing 0.5ml. of aq. cysteine hydrochloride solution (0.05–0.5 μ mole), 0.5ml. of acetic acid and 0.5ml. of acid ninhydrin reagent 1 or 2, was mixed thoroughly. As reported by Chinard (1952) the reaction may be performed with a sample volume of 1.0ml. or 1.5ml. provided that other reagents are increased in the same proportion. The tubes were covered with aluminium caps or glass marbles and heated in a boiling-water bath for 10min. They were then rapidly cooled in tap water; the contents of the tube were diluted to 5 or 10ml. with 95% ethanol and mixed. A reagent blank without cysteine was prepared under the same conditions. The spectral measurements of the reaction products were made against the reagent blank with a Unicam SP.800 recording spectrophotometer.

Reduction of cystine to cysteine. This was accomplished by treatment of 0.5ml. of cystine solution (0.25–0.5 μ mole) with 0.5ml. of aq. dithiothreitol solution (5 μ moles; Cleland, 1964); the reaction mixture was adjusted to pH 8.0–8.5 with 0.02–0.05ml. of N-NaOH, with phenolphthalein as indicator.

Perchloric acid extracts of rat tissues. Male rats weighing approx. 100g. were decapitated; brain and liver were quickly removed, blotted on filter paper and frozen in liquid nitrogen. Blood was collected in beakers coated with heparin and a known volume was pipetted into ice-cold 5% (0.5N-) perchloric acid. The frozen tissues were weighed and homogenized with Ultra-Turrax electric blender (Janke und Kunkel K.G., Staufen-in-Breisgau, Germany) in 5% ice-cold perchloric acid. Assuming 80% wet wt. as water in brain, liver and blood, the final volume of 3–4ml./g. or ml. of tissue was used. The suspension was centrifuged at 2800g for 1hr. at 5° and the supernatant solution filtered through Whatman no. 30 paper.

Determination of cysteine and cystine in rat tissues. A portion (1ml.) of perchloric acid filtrate was treated with acid ninhydrin reagent 2 according to the standard procedure described above. The extinction at 560m μ was measured and the amount of cysteine was calculated by reference to a calibration curve obtained, under similar conditions, for known amounts of cysteine. To another portion (0.5ml.) of perchloric acid filtrate was added first 10 μ l. of 10N-NaOH and then 0.1N-NaOH until just pink

to phenolphthalein, followed by 0.5ml. of dithiothreitol solution (5 μ moles) to reduce cystine to cysteine. After 30min. standing at room temperature total cysteine was determined by using acid ninhydrin reagent 2. The amount of cystine was obtained by difference between the two values.

RESULTS

Reaction of cysteine with acid ninhydrin reagents 1 and 2. Within 2–3min. of heating the reaction mixture a pink colour developed which showed a maximum extinction at 560m μ with a minor plateau region between 522 and 534m μ (Figs. 1 and 2). The colour development reached a maximum value on heating the tubes for 6–15min. at 100°. The pink colour faded gradually on standing at room temperature; it faded rapidly on diluting the reaction mixture with acetic acid, which was used as a diluent by Chinard (1952) and by Work (1957). The pink product was stabilized for at least 3–4hr. on dilution of the reaction mixture with methanol, ethanol or methylcellosolve; the loss in extinction was 20% in 20hr. In subsequent studies the reaction mixture was therefore heated for 10min., cooled rapidly and diluted immediately to 5 or 10ml. with 95% ethanol. As reported by Chinard, proline, ornithine and citrulline also gave coloured products under these conditions (Fig. 1). The molar extinction coefficients of cysteine, proline, ornithine, citrulline and lysine are given in Table 1. The reaction for cysteine with acid ninhydrin reagent 1 was about three times as sensitive as that first reported by Chinard (1952). The presence of 5% trichloroacetic acid or 6% perchloric acid did not appreciably affect the formation of the pink product from cysteine. The extinction was proportional to the concentration in the range 0.05–0.25 μ mole of cysteine.

The extinction for a given amount of cysteine increased with the concentration of hydrochloric acid in the sample (Table 2). The omission of acetic acid (0.5ml.) from the reaction mixture decreased the extinction at 560m μ and produced a second extinction peak at 610m μ .

The acid ninhydrin reagent 1 was therefore modified and a new acid ninhydrin reagent 2 containing conc. hydrochloric acid was used: this considerably increased the molar extinction of the reaction product with cysteine (Table 1). The extinction was again directly proportional to the concentration in the range 0.05–0.5 μ mole of cysteine (Fig. 3). Solutions of cysteine hydrochloride prepared in water, 5% trichloroacetic acid or 6% perchloric acid gave approximately the same molar extinction (ϵ 25.0 $\times$ 10³; Table 1).

In view of the possibility of oxidation of cysteine to cystine in solutions of cysteine, the correct molar extinction of the reaction product of cysteine with

Fig. 1. Optical spectra of reaction products of 0.4 μ mole each of (1) cysteine, (2) ornithine, (3) proline and (4) citrulline with ninhydrin reagent 1. Reaction mixture (1.5 ml.) was heated in a boiling-water bath for 10 min., cooled and diluted to 5 ml. with 95% ethanol.

Fig. 2. Optical spectra of reaction products of 0.4 μ mole each of (1) cysteine, (2) ornithine, (3) proline and (4) citrulline with ninhydrin reagent 2. Reaction mixture (1.5 ml.) was heated in a boiling-water bath for 10 min., cooled and diluted to 10 ml. with 95% ethanol.

acid ninhydrin reagent 1 or 2 was obtained by reducing cystine to cysteine. This was accomplished by treatment of a solution of pure cystine with an aqueous solution of dithiothreitol (Cleland, 1964). Cysteine formed was treated with acid ninhydrin reagent 1 or 2; the extinction of the pink product when compared with that of cysteine hydrochloride showed that the reduction of cystine to cysteine by dithiothreitol was more than 95% in 5 min.

Pretreatment of freshly prepared solutions of cysteine with dithiothreitol gave a small increase in the extinction with both ninhydrin reagents

(Table 1); this was attributed to the presence of small amounts of cystine in these solutions. Known amounts of cysteine and of cysteine derived from cystine gave a similar extinction if both solutions were preincubated with dithiothreitol (Table 1 and Fig. 3).

Reaction of other amino acids with acid ninhydrin reagents 1 and 2. Proline, ornithine, citrulline and lysine gave red and orange products, similar to those described by Chinard (1952) with acid ninhydrin reagent 1. Chinard used 60 min. for the reaction with his acid ninhydrin reagent containing 6M-phosphoric acid, but under the conditions used

Table 1. *Molar extinction coefficients (ϵ) after reaction with acid ninhydrin reagents*

Proline and ornithine give maximum extinction at 510 m μ , lysine at 500 m μ and cysteine at 560 m μ with reagents 1 and 2. Citrulline gives maximum extinction at 510 m μ with reagent 1 and at 500 m μ with reagent 2. Values given in brackets were obtained after preincubation at pH 8.0 with dithiothreitol for 30 min. at room temperature.

Compound	$10^{-3} \epsilon$					
	Chinard (1952)		Present work			
			Reagent 1		Reagent 2	
	515 m μ	560 m μ	510 m μ	560 m μ	510 m μ	560 m μ
Proline	16.5	6.0	17.5	3.5	0.5	0.09
Ornithine	18.3	6.5	21.4	4.3	0.07	0.01
Citrulline			2.6	0.6	0.24*	0.04
Lysine	3.6*	1.0	1.2*†	0.4		0
	3.9†		1.6†‡			
Cysteine	2.0	3.3	4.6	10.4	10.3	26.0
Cysteine + dithiothreitol			[6.0]	[14.8]	[10.6]	[27.6]
Cysteine in 5% trichloroacetic acid			4.3	9.5	10.8	24.8
				[16.1]		
Cysteine in 6% perchloric acid			5.0	11.5	10.8	25.4
				[15.4]		
Cystine			0.13	0.06	1.4	0.9
			0.30§		1.54	
Cystine¶ + dithiothreitol			[5.9]	[12.4]	[11.2]	[28.0]

* At 500 m μ .

† At 440 m μ .

‡ These values were approx. doubled on heating the reaction mixture for 60 min.

§ At the extinction maximum of 450 m μ .

|| At the extinction maximum of 485 m μ .

¶ Molar extinction coefficient calculated for cysteine.

Table 2. *Effect of hydrochloric acid on the reaction of cysteine with acid ninhydrin reagent 1*

The reaction mixture contained 0.5 ml. of sample (0.5 μ mole of cysteine), 0.5 ml. of acetic acid and 0.5 ml. of reagent 1 and was heated for 10 min., cooled and diluted to 5 ml. or 10 ml. with 95% ethanol.

Normality of HCl in the sample	$E_{560 \text{ m}\mu}$ in 5 ml. of solution
0.001*	1.00
0.1	0.92
0.5	1.17
1.0	1.43
2.0	1.75
4.0	2.26
6.0	2.36
8.0	2.54
10.0	2.36
12.0	2.16

* Calculated from cysteine hydrochloride.

in this work proline and ornithine gave somewhat increased sensitivity on heating with acid ninhydrin reagent 1 for 10 min. (Table 1). Although the extinc-

tion peaks (E_{max} , 510 m μ) for proline and ornithine were well separated from that for cysteine (Fig. 1), the presence of proline and ornithine in solutions containing cysteine introduced a small error in the determination of cysteine with acid ninhydrin reagent 1. However, when acid ninhydrin reagent 2 was used proline, ornithine and citrulline did not produce significant amounts of red colour and only cysteine gave the pink reaction product with an extinction peak at 560 m μ (Fig. 2). The acid ninhydrin reagents 1 and 2 did not give significant amounts of reaction products, measurable at 560 m μ , with the following compounds: homocysteine, glutathione (GSH and GSSG), penicillamine, dithiothreitol, ascorbic acid, *S*-methylcysteine, cystine, homocystine, α -alanine, glycine, serine, threonine, leucine, valine, methionine, cystathionine, taurine, histidine, histamine, arginine, lysine, hydroxylysine, hydroxyproline, ethanolamine, aspartate, glutamate, glutamine, tryptophan, 5-hydroxytryptophan, 5-hydroxytryptamine, adrenaline, nor-adrenaline, tyrosine, phenylalanine, 2,4-dihydroxyphenylalanine, 3-hydroxytyramine, γ -aminobutyrate and indol-3-ylacetic acid. Cystine, however, gave small amounts of an orange reaction product

Fig. 3. Extinctions (at 560m μ) of reaction products of known amounts of cysteine on treatment with ninhydrin reagent 2: ▲, cysteine hydrochloride; ○, cysteine hydrochloride + 5 μ moles of dithiothreitol; ●, cysteine + 5 μ moles of dithiothreitol.

with extinction peaks at 450 and 485m μ on treatment with ninhydrin reagents 1 and 2 respectively (Table 1). Glutathione (GSH) produced negligible amounts of the pink product on heating with reagent 2; this was attributed to some hydrolysis of GSH to cysteine during the heating of the reaction mixture. Pipelicolic acid gave a significant amount (ϵ 2.9 $\times$ 10³) of pink product with an extinction peak at 560m μ on treatment with reagent 1 and negligible amounts (ϵ 0.19 $\times$ 10³) with reagent 2.

Additional reaction products of amino acids with acid ninhydrin reagents 1 and 2. The acid ninhydrin reagent 1 used in this work was that described by Work (1957) for the determination of diamino acids. The conditions used, however, were different in that the time of heating the reaction mixture was 10 min. and the reaction mixture was diluted with 95% ethanol instead of acetic acid. All of the diamino acids tested under these conditions also gave similar products (Table 3). Glutamine gave a product with $E_{\max}$ 365m μ on treatment with ninhydrin reagent 1 but not with ninhydrin reagent 2; likewise citrulline, ornithine, proline and lysine did not react with ninhydrin reagent 2. On treatment with ninhydrin reagent 1, cysteine and cystine produced

a reaction product with an extinction peak in the ultraviolet region ($E_{\max}$ 340–345m μ). The extinction of the product from cysteine was doubled when ninhydrin reagent 2 was employed (Table 3). Cystine or cysteine cannot be determined in the presence of each other with either ninhydrin reagents 1 or 2 in the ultraviolet range.

Tryptophan, 5-hydroxytryptophan and 5-hydroxytryptamine gave intense yellow products with both acid ninhydrin reagents: the reagent 1 gave somewhat higher extinction than reagent 2 (Table 3). Indol-3-ylacetic acid gave a yellow reaction product with a high molar extinction on treatment with acid reagent 2; it gave no significant yellow colour with acid reagent 1 (Table 3). Adrenaline and noradrenaline also gave yellow products with acid ninhydrin reagents 1 and 2.

Application of the method to rat tissues. On treatment with the acid ninhydrin reagent 2, the perchloric acid extracts of rat brain, liver and blood gave extinction maxima at 560, 470 and 380–390m μ . The recoveries of 0.05 μ mole of cysteine added to the perchloric acid extracts of the tissues were: brain, 98%; liver, 98%; blood, 92%. Brain and liver contained 55.9 and 94.9 μ moles of cysteine/g. fresh wt. of tissue respectively and little or no cystine; blood contained 30.8 μ moles of cysteine and 18.8 μ moles of cystine/ml. (Table 4).

DISCUSSION

The formation of a pink product, first reported by Chinard (1952), on heating a solution of cysteine with an acid ninhydrin reagent containing acetic acid and phosphoric acid, has been made the basis of a quantitative method for cysteine. In the preliminary studies the pink product formed was stabilized by adding organic solvents such as ethanol, methanol or methylCellosolve as diluent since acetic acid used as diluent by Chinard (1952) and by Work (1957) rapidly destroyed the pink product. The pink product gave an extinction peak at 560m μ . Under the same conditions proline, ornithine and citrulline also gave red products as reported by Chinard (1952).

The intense pink colour formed when acid ninhydrin reagent 1 was heated with small amounts of cysteine eluted with hydrochloric acid from ion-exchange columns was shown to be due to increased sensitivity of the reaction in the presence of hydrochloric acid. By replacing phosphoric acid with conc. hydrochloric acid in the ninhydrin reagent it was shown (i) that the interference from proline, ornithine and citrulline was abolished, (ii) that several naturally occurring amino acids, including homocysteine and glutathione, did not form any reaction products measurable in the visible band and (iii) that the molar extinction

Table 3. *Molar extinction coefficients of additional products of some amino acids on treatment with acid ninhydrin reagents 1 and 2*

Values given in brackets were obtained when acetic acid was used as diluent.

	$E_{\max.}$ (m μ)	$10^{-3} \epsilon$	
		Reagent 1	Reagent 2
Cystine	340-345	7.3	2.8
Cysteine	340-345	4.0	7.0
Penicillamine	370	0.24	0.24
Homocystine	340-345	0.4	0.3
Homocysteine	340-345	0.4	0.6
Cystathionine	340-345	6.2	2.2
Glutamine	365	2.0	0
Citrulline	320-330	1.5	0
Ornithine	335-340	6.0	0
Lysine	340	5.2	0
Hydroxylysine	340	14.0	0
	440	5.5	0
Proline	330-335	5.5	0
Hydroxyproline	325	2.0	5.2
Tyrosine	385	0.6 [0.4]	1.1 [0.9]
3-Hydroxytyramine (Dopamine)	—	0	0
Phenylalanine	—	0	0
2,4-Dihydroxyphenylalanine	—	0	0
Tryptophan	380	4.0 [2.9]	3.9 [3.5]
5-Hydroxytryptophan	370	—	3.70
	400	7.75	—
5-Hydroxytryptamine	398	—	9.50
	404	9.95	—
Indol-3-ylacetic acid	380-382	0.40	11.5
Adrenaline	384	—	2.70
	425	—	1.85
	460	0.40	—
Noradrenaline	375	—	6.40
	425	2.19	3.71

Table 4. *Cysteine and cystine content of rat tissues*

Values given under (a) were obtained by treatment of ninhydrin reagent 2 with 5% perchloric acid extracts and those given under column (b) were obtained from perchloric acid extracts preincubated with dithiothreitol at pH 8.0. All values are expressed as m μ moles of cysteine $\pm$ s.d.

	Cysteine (a)	Cysteine + cystine (b)	Cystine (b) - (a)
Brain	55.9 $\pm$ 10.7	62.5 $\pm$ 8.3	6.6 $\pm$ 5.8*
Liver	94.9 $\pm$ 12.7	107.5 $\pm$ 16.6	12.5 $\pm$ 8.1†
Blood	30.8 $\pm$ 7.3	68.3 $\pm$ 10.7	37.5 $\pm$ 8.8

* Range 0-14.2 m μ moles.† Range 0-22.0 m μ moles.

coefficient for cysteine, ϵ 28.0×10^3 , was several times that reported previously by Chinard (1952). The method described here for cysteine was more sensitive than the ninhydrin method normally used for α -amino acids at pH 5.0 (Moore & Stein, 1948, 1954; Troll & Cannan, 1953; Yemm & Cocking, 1955). Cysteine may be determined in the presence

of 5% trichloroacetic acid and 6% perchloric acid. By employing dithiothreitol (Cleland, 1964) it may also be used for the determination of cystine in protein hydrolysates and of mixed disulphides of cysteine.

The results (Table 1 and Figs. 1 and 2) suggested that the acid ninhydrin reagent 1 may be used for

the specific determination of proline, provided that the reaction product is diluted with acetic acid as recommended by Chinard (1952).

The acid ninhydrin reagent 2 was used in the present work for the specific determination of cysteine directly in perchloric acid extracts of rat tissues.

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