A Micro-Method for the Estimation of Cystine in Protein Hydrolysates

Among the many methods of estimating cystine[1], one of the simplest is that described by SULLIVAN, HESS, and HOWARD[2] based on the reduction of cystine to cysteine and thiocyanate, the cysteine thus formed producing a red colour with sodium 1,2-naphthoquinone-4-sulphonate. A micromethod has been derived from this original method, which enables the estimation of cystine in protein hydrolysates in concentrations ranging from 20 to about 400 nmoles/ml.

Materials. All compounds used were of analytical grade, though from different commercial sources.

Spectrophotometric measurements were made with the Bausch and Lomb recording spectrophotometer, Spectronic 505.

Experimental. The original method of SULLIVAN, HESS, and HOWARD[2] was tested in the conditions of volume given by these authors: to 5 ml of saturated cystine solution in 0.1 *M* Tris buffer pH 7.2 were added 2 ml of 5% NaCN; after 10 min, 1 ml of 1% sodium 1,2-naphthoquinone-4-sulphonate was added and shaken for 10 sec; then 5 ml of 10% anhydrous Na_2SO_3 in 0.5 *N* NaOH were added, mixed, and allowed to stand 30 min; 1 ml of 5 *N* NaOH was added, followed by 1 ml of 2% $Na_2S_2O_4$ in 0.5 *N* NaOH. The maximum absorption was found at 500 nm.

(1) Comparison between NaCN and KCN as reducing agents. By means of this method, the 5% NaCN solution was compared with a 5% KCN solution. 7 min after the final addition of $Na_2S_2O_4$, the optical densities at 500 nm were respectively 0.180 with NaCN and 0.252 with KCN. Although the final colour obtained under these conditions was quite unstable, KCN was used in all further reactions instead of NaCN.

(2) Effect of changing the relative concentrations of the reagents. In order to increase the sensitivity of the method and to handle smaller volumes, the following solutions were tried: 1 ml of cystine solution (150 nmoles/ml); 0.1 ml of 20% KCN; 0.1 ml of 2% naphthoquinone-sulphonate; 1 ml of 10% Na_2SO_3 in 0.5 *N* NaOH; 0.2 ml of 5 *N* NaOH; and 0.1 ml of 4% $Na_2S_2O_4$ in 0.5 *N* NaOH. These volumes and concentrations correspond exactly to the original method. It was observed that the maximum absorption of the final colour was shifted to 466 nm, and that its value increased for the first 20 min then decreased during the next 40 min. Allowing the colour to develop in darkness did not change this result.

(3) Effect of the $Na_2S_2O_4$ concentration. Conditions were next investigated for producing a final colour that would remain stable for a reasonable time. Reagents were used in concentrations as in the original method, but the volumes were 5 times smaller. Only the concentration of $Na_2S_2O_4$ was increased from 2% to 10%. This gave a final colour whose optical density did not vary for at least 30 min.

A higher concentration of $Na_2S_2O_4$ was also tested. The use of 0.1 ml of a 20% aqueous $Na_2S_2O_4$ solution instead of 0.2 ml of the 10% solution in NaOH gave the same result. It was observed, however, that this highly concentrated aqueous solution loses its activity very rapidly; on the other hand, this concentration cannot be obtained in the alkaline solution because of the limited solubility of the reagent.

Thus, for the final method, the 10% solution in 0.5 *N* NaOH was used.

(4) Effect of the Na_2SO_3 concentration. If Na_2SO_3 is added 10 min after the naphthoquinone-sulphonate instead of immediately, the final maximum absorption is shifted from 500 to 470 nm, and the value is much decreased. This means that the complex forming reaction between cysteine and naphthoquinone-sulphonate should take place in a highly reducing medium.

Therefore, a higher concentration of Na_2SO_3 was tested: a 20% solution in 0.5 *N* NaOH was used instead of the 10% one. The result was a shift of the absorption maximum to 506 nm and a steady decrease of the optical density.

A lower concentration of Na_2SO_3 was next studied. Adding 0.5 ml of the 10% solution instead of 1 ml made no change in the result. This last condition was thus used in the following final method:

Final method. To 1 ml of cystine solution, add 0.2 ml of freshly prepared 10% aqueous KCN; mix and allow to stand 10 min; add 0.2 ml of 1% aqueous sodium 1,2-naphthoquinone-4-sulphonate and shake for 10 sec; add immediately 0.5 ml of 10% anhydrous Na_2SO_3 in 0.5 *N* NaOH, mix and allow to stand 30 min; add 0.2 ml of 5 *N* NaOH and 0.2 ml of 10% $Na_2S_2O_4$ in 0.5 *N* NaOH. Read at 504 nm against a blank similarly treated.

Figure 1 gives the absorption spectrum of the final colour thus obtained with a cystine solution of 200 nmoles/ml.

Figure 2 shows the standard curve obtained with cystine solutions prepared by dissolving crystalline cystine in *N* HCl and neutralizing with crystalline *tris*-(hydroxymethyl)aminomethane.

Results. The method described above was applied to the estimation of cystine in bovine serum albumin and in a preparation of total rat liver histone. The proteins were

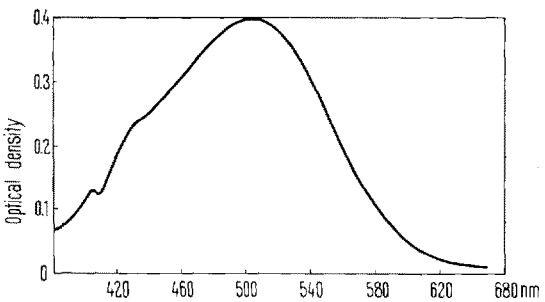

Fig. 1

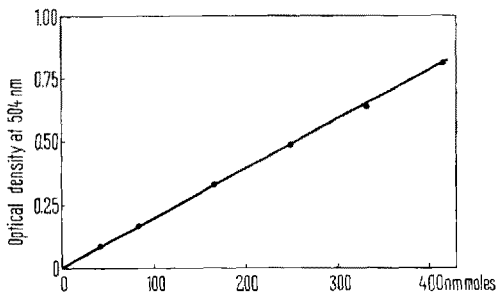

Fig. 2

[1] R. J. BLOCK and D. BOLLING, *The Amino Acid Composition of Proteins and Foods* (C. C. Thomas, Springfield, Ill. 1951).

[2] M. X. SULLIVAN, W. C. HESS, and H. W. HOWARD, J. biol. Chem. *145*, 621 (1942).

dried for 4 h at 105°C and hydrolysed in sealed tubes for 6 h at 120°C with 6*N* HCl. The hydrolysates were evaporated almost to dryness, taken up with 1 ml of distilled water, neutralized to pH 7.0 with crystalline *tris*(hydroxymethyl)aminomethane, and made up to 10 ml with 0.1*M* Tris buffer pH 7.0. The estimations were made on 1 ml aliquots.

Whereas the histone hydrolysate gave only a very faint colour, the maximum absorption of which was at 460 nm, the albumin hydrolysates gave valid results which are presented in the Table.

It may be concluded from these results that histone apparently does not contain any detectable cystine, and that the molar ratio of cystine in bovine serum albumin is 18; this last value agrees with that given by CATSIMPOOLAS and WOOD[3], whereas EDSALL[4] quotes that of 17.5.

Estimation of cystine in bovine serum albumin

Experiment no.	1		2		3
Dry weight of hydrolysed protein, mg	10.4		8.8		9.2
Hydrolysed protein in nmoles[a]	157.6		133.3		139.4
Total volume of neutralised hydrolysate, ml	10		10		10
Optical density at 504 nm	0.550	0.550	0.462	0.465	0.484
nmoles/ml	285.2	285.2	239.5	241.1	251.0
µg/ml	68.4	68.4	57.5	57.9	60.2
Molar ratio	18.1	18.1	18.0	18.1	18.0
Weight %	6.58	6.58	6.53	6.58	6.55

[a] The molecular weight used for the calculations was 66,000, given by EDSALL[4].

Résumé. Nous présentons une micro-méthode colorimétrique qui permet de doser la cystine dans des hydrolysats de protéines, dans un domaine de concentration compris entre 20 et environ 400 nmoles/ml.

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Service de Biochimie des Nucléoprotéides, Institut de Biologie physico-chimique, C.N.R.S., Paris (France), July 5, 1965.

[3] N. CATSIMPOOLAS and J. L. WOOD, J. biol. Chem. *239*, 4132 (1964).
[4] J. T. EDSALL, Exposés a. Biochim. méd. *18*, 86 (Masson et Cie., Paris 1956).
[5] Permanent address: Eisai and Co. Research Laboratories, Bunkyo, Tokyo (Japan).

A Differential Staining for Hypocotyl and Radicle

The radicle and the hypocotyl of seedlings may react very differently to environmental factors, such as temperature, illumination and growth regulators. In many developmental studies, it is essential to distinguish between these two organs.

Facing such a problem we were able to stain differentially the radicles and the hypocotyls of whole seedlings by *réactif genevois*. This reagent, which was developed by CHODAT, Genève (Switzerland), is a mixture of the dyes chrysoidin and Congo red (STRASBURGER[1], p. 813). CHODAT developed this reagent for differential staining of cell wall constituents. Cellulose is stained pink, lignin bright yellow and cutin and suberin yellowish brown. It was found that bathing the whole seedling in *réactif genevois* for 5 to 10 min followed by a rinse in water leaves only pink on the growing radicle. The hypocotyl may be stained somewhat yellowish-brown but the distinction between the two colours, and hence the two organs, is easily made (Figure 1). In all species tested the morphological boundary between the hypocotyl and the radicle, namely the place where the proximally (oldest) situated root hairs mark it, was identical with the boundary of the staining (Figure 2). This method was successfully tried in several species of different families of *Angiosperms*. There was no case in which this method did not work. The seedlings tested were of: *Lactuca sativa* (lettuce), *Pisum sativum* (pea), *Lycopersicum esculentum* (tomato), *Beta vulgaris* (beet), *Salsola inermis*, *S. volkensii* and *Atriplex dimorphostegia*.

It is suggested that the cuticular coating of the hypocotyl prevents the reaction of cellulose with *réactif genevois* from taking place. Since such a coating is absent from the epidermis of growing radicles, the cellulose of the epidermis reacts with the stain. Staining with $ZnCl_2$-KI-I

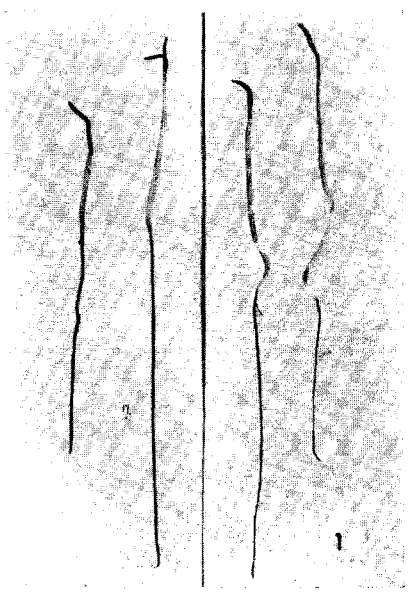

Fig. 1. Seedlings of *Atriplex dimorphostegia* stained with *réactif genevois* (2 on the right) and with carmine acetate (2 on the left). Note the sharp boundary between radicle (stained) and hypocotyl (unstained) with the former reagent.

[1] E. STRASBURGER, *Das botanische Praktikum*, 7th Ed. (Gustav Fischer, Jena 1923).