CHROMATOGRAPHIC DETERMINATION OF CYSTEIC ACID*

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The instability of cystine in protein materials during acid hydrolysis has led to the development of various methods of hydrolysis[1]. The procedure of SCHRAM, MOORE AND BIGWOOD[2] in which cystine is oxidized to cysteic acid prior to acid hydrolysis eliminates this objection since cysteic acid is stable during this hydrolysis.

Much difficulty was experienced in this laboratory, however, in the determinations of cysteic acid with ninhydrin reagent[3] following its chromatographic separation on a resin column. This study was undertaken to investigate this difficulty and improve the conditions.

EXPERIMENTAL AND RESULTS

All the materials to be analyzed for cystine were oxidized with performic acid, according to the procedure of SCHRAM, MOORE AND BIGWOOD[2]. After the removal of excess oxidizing reagents by heating and evaporation on the steam bath, the residue from the oxidation was hydrolyzed by autoclaving with 50 ml of 20 % hydrochloric acid for 6 h at 15 lb. pressure. The hydrochloric acid was removed by evaporation on the steam bath. Water added twice, and the sample evaporated almost to dryness after each addition. The autoclaved sample was washed into a 25 ml volumetric flask and made to volume with water. The sample of protein taken for oxidation and hydrolysis was of such a size that a 1- or 2-ml aliquot from the 25 ml-hydrolysate contained approximately 100 or 200 μg of cystine.

The 1- or 2-ml aliquot was added to a 0.9 × 15 cm column of Dowex-2 X 10 resin in the chloroacetate form. Cysteic acid was eluted with a solution of monochloroacetic acid containing 15 g/l at the rate of 4 ml/h, and a 1-ml fraction was collected in each tube.

Since the fraction collector used was a Time-Flow Technicon machine, tubes on the rack did not always contain exactly the same volume, as would be the case with a Drop-Counter mechanism. Since the eluant was not a buffer, the addition of the same amounts of standard sodium hydroxide did not always produce the pH 5.0 required for the maximum color development with ninhydrin[3]. The values obtained under these conditions were very erratic; some duplicates varied as much as 50 %, while others gave good agreement.

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Since a strong sodium hydroxide solution was required to give the desired pH with the approximately 0.16 *N* monochloroacetic acid used as the eluant, it was decided that this alkali solution should have a buffer salt dissolved in it. After several trials, sodium citrate was selected as the most satisfactory. A solution of 1 *N* sodium hydroxide saturated with sodium citrate gave the required pH to the fractions collected in the tubes, but 4 drops were required for each ml eluate collected. The alkali was then changed to 2 *N* sodium hydroxide, saturated with sodium citrate, and 2 drops per tube used. With this procedure, excellent color development was

TABLE I

RECOVERY OF CYSTEIC ACID

Added γ	*Recovered* γ	*Adjusting solution**
29.5	29.1	4 drops *N* NaOH
29.5	29.8	2 drops 2 *N* NaOH
34.7	34.7	4 drops *N* NaOH
35.1	34.9	4 drops *N* NaOH
35.1	35.0	2 drops 2 *N* NaOH
325.0	322.0	2 drops 2 *N* NaOH
325.0	324.0	2 drops 2 *N* NaOH
362.0	370.0	2 drops 2 *N* NaOH

* Standard sodium hydroxide solution saturated with sodium citrate to give pH 5.0 in collection tubes, containing 1 ml eluate.

obtained, and the pH after dilution was always very close to 5.0. Recoveries were excellent. Table I shows the values obtained with cysteic acid added to the chromatographic column, eluted with a solution of monochloroacetic acid (15 g/l) and the pH of the tubes adjusted by addition of sodium hydroxide saturated with sodium citrate.

All the tubes used to collect the fractions from the column had been calibrated for 10 ml. After the adjustment to pH 5.0 with the buffered alkali, 2 ml of freshly-prepared ninhydrin solution[3] were added and the tubes heated for 20 min in the steam bath to develop the color. After cooling each tube was diluted to this mark with a solution of 1:1 isopropyl alcohol–water. The color was read in a Beckman Model B Spectrophotometer at 570 mμ using 1-cm cuvettes. A standard curve had been made using a definite weight of cysteic acid per tube (μg/ml), and reading at 570 mμ. From this curve, the micrograms of cysteic acid per unit of optical density can be calculated. Using these procedures, the concentration of cysteic acid can be determined directly from the sum of the optical densities of all the tubes containing cysteic acid. It is, therefore, not necessary to correct for color yields relative to leucine nor for evaporation during color development[3].

The addition of 2 drops of 2 *N* sodium hydroxide to each fraction containing approximately 1 ml eluate, gave pH 5.0, which is essential for maximum color development. The use of an unbuffered sodium hydroxide solution with the tubes from a Time-Flow fraction-collector frequently gave less than optimum color development, and, consequently, poor checks for duplicate samples.

As soon as the desired volume of the hydrolysate had been washed onto the column and the funnel containing the eluant had been connected, the column was moved onto the fraction-collector. With the set-up used in this laboratory and a collection rate of 4 ml/h, all the cysteic acid was collected in about 16 tubes, usually between tubes numbered 55–70.

With the materials used, grains, seeds, animal and vegetable proteins, test runs showed there was no added advantage in using a forerun with 0.01 *N* monochloroacetic acid[2]. The use of a solution of monochloroacetic acid containing 15 g/l for the elution of cysteic acid from the column, rather than the 0.1 *N* solution recommended[2], concentrated the cysteic acid in fewer tubes and made the separation sharper.

Blanks for each determination were chosen from the tubes showing very slight color immediately before and after the tubes containing cysteic acid.

The cystine was corrected for the 90% yield on oxidation, according to SCHRAM, MOORE AND BIGWOOD[2].

SUMMARY

Difficulty was encountered in the determination of cysteic acid with ninhydrin after elution from a resin column, using a Time-Flow fraction-collector. Since the trouble arose from the variable pH obtained on the addition of sodium hydroxide to the eluate, a buffered solution of 2 *N* sodium hydroxide saturated with sodium citrate was used to produce the pH 5.0 required for maximum color development. The calibration for 10 ml of the tubes collecting the eluate, and the use of a standard cysteic acid curve facilitated the calculation of the cysteic acid concentration in the sample.

REFERENCES

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