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Chromatographic Determination of Cystine as Cysteic Acid

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The determination of the cystine content of proteins is frequently complicated by the instability of cystine and cysteine during protein hydrolysis, particularly in the presence of carbohydrate (cf. Martin & Synge, 1945; Block & Bolling, 1951). Oxidation of the cystine and cysteine residues in the intact protein can convert the amino acids into cysteic acid, which is stable under the conditions of acid hydrolysis. The present method is based upon this step, followed by the quantitative determination of cysteic acid by ion-exchange chromatography on columns of Dowex-2. The chromatographic identification of cysteic acid as the end product of the oxidation contributes to the specificity of the method. The procedure does not distinguish between cystine and cysteine residues in the protein.

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The conditions of Toennies & Homiller (1942) for the oxidation of the sulphur-containing amino acids by performic acid have been applied to proteins in several investigations. Toennies (1942) oxidized casein to convert methionine into the sulphone and observed the disappearance of part of the cystine and all of the tryptophan. Sanger (1949) used the reaction to split the —S—S— bridges of insulin as a first step in the investigation of the structure of the hormone. The reaction has been applied to oxytocin by Mueller, Pierce, Davoll & du Vigneaud (1951). The oxidation of wool by performic acid has been studied by Blackburn & Lowther (1951).

A quantitative study of the yield from the reaction requires an accurate method for the determination of cysteic acid in protein hydrolysates. Paper chromatograms have been used qualitatively to identify cysteic acid after its formation from

cystine (Dent, 1948). Consden, Gordon & Martin (1948), Consden & Gordon (1950), and Sanger & Tuppy (1951) have used a weakly basic ion-exchange resin (IR-4B) to separate cysteic acid and cysteic acid peptides. The quantitative method of ion-exchange chromatography developed for the present study is based upon the principles and techniques used in the determination of amino acids on sulphonated polystyrene resins (Moore & Stein, 1951), but uses a strongly basic resin under conditions similar to those employed with Dowex-1 and Dowex-2 in the chromatography of nucleotides by Cohn (1950) and of acids of the citric acid cycle by Busch, Hurlbert & Potter (1952).

Cysteic acid may also be determined on Dowex-50 columns, through which it passes without measurable retardation (Moore & Stein, 1951). Its determination under these conditions would be reliable only if one were certain that the hydrolysate contained no other strongly acidic, ninhydrin-positive constituents. The use of Dowex-2, with hydrochloric or chloroacetic acid as eluent, provides preferable resolving power in a system in which all of the common amino acids, including taurine and aspartic and glutamic acids, emerge first, with cysteic acid and its homologues having distribution coefficients strongly in favour of the resin phase. Mixtures of homocysteic and cysteic acids, for example, are resolved under the conditions of the present experiments, with the former emerging as a peak just preceding that of cysteic acid.

PROCEDURE

Oxidation. The performic acid reagent was prepared by the addition of 1 vol. of 30% (w/w) H_2O_2 to 9 vol. of 88% (w/w) formic acid. The solution was allowed to stand for 1 hr. at room temp. to permit the performic acid concentration to reach the maximal value (Toennies & Homiller, 1942). 25 ml. of the reagent, previously cooled to 0°, were added to the sample to be oxidized (preferably taken to contain 1–2 mg. of cystine). In the oxidations of cystine the amino acid was dissolved in formic acid (20 mg. in 10 ml.) and 1 ml. portions were added to the performic acid solution. With amino acids and soluble proteins the oxidation was allowed to proceed at 0° for 4 hr. With insoluble samples the ice-water bath was placed in a refrigerator overnight (16 hr.). At the end of the reaction time most of the reagent was removed under reduced pressure (water pump) at bath temp. 30–40° on a rotary evaporator of the type described by Craig, Gregory & Hausmann (1950). The concentration was stopped as soon as the bulk of the reagent was removed, yielding a syrupy ribbon on the wall of the rotating flask. This stage was reached in 10–15 min. Attempts to carry the evaporation to dryness under these conditions may in some instances lead to low recoveries. (If desired, the residue from the concentration on the water pump can be taken to dryness without loss of cysteic acid by placing the flask for 1–2 min. on a high-vacuum line (oil pump) equipped with a solid- CO_2 trap.)

If the experiment was a control oxidation on cystine, the syrupy residue was taken up in water, transferred to a 10 ml. flask and a 1 ml. portion of the slightly acid solution was added to the Dowex-2 column.

Hydrolysis. In the analysis of proteins, the residue from the oxidation was immediately dissolved or suspended in 50 ml. of 6N-HCl and boiled under reflux for 20 hr. It is not necessary to remove the last traces of reagent before the addition of the HCl. If the initial sample contained more than 90% of carbohydrate, 100 ml. of HCl were used for hydrolysis. The oxidation, evaporation and hydrolysis were conveniently carried out in the same 250 ml. ground-joint flask to avoid transfers. The hydrolysate was filtered through sintered glass to remove humin. The filtrate was taken to dryness on a rotary evaporator. After the flask had been placed over NaOH in a vacuum desiccator for a few hours, the residue was taken up in water and transferred to a 10 ml. volumetric flask. Before making the sample to volume the pH was adjusted to about 5 by drops of 0.1N-NaOH, using methyl red as internal indicator.

Chromatography. From the treated hydrolysate, a 1 or 2 ml. portion (corresponding to 0.1–0.2 mg. cystine) was added to a 0.9 × 15 cm. column of the chloroacetate form of 200–400 mesh/in. Dowex-2-X 10 (Dow Chemical Co., Midland, Mich.). Cysteic acid was eluted with 0.1N chloroacetic acid (Fig. 1). A forerun of 100 ml. of 0.01N acid was used in the case of samples of very low cystine content (e.g. casein) or high carbohydrate content (Fig. 2). The effluent

Fig. 1. Chromatography of a hydrolysate of oxidized Bence-Jones protein. The sample on the column corresponded to 3.9 mg. of protein (79 μg . cystine). Column, Dowex-2 (0.9 × 15 cm.). Eluent, 0.1N chloroacetic acid.

Fig. 2. Chromatography of a hydrolysate of oxidized cow's milk. The sample on the column corresponded to 0.5 ml. of milk containing about 0.2 mg. cystine. Column, Dowex-2 (0.9 × 15 cm.). Eluent, aqueous chloroacetic acid.

was collected in 1 ml. fractions at 5 ml./hr. and analysed by the photometric ninhydrin method (Moore & Stein, 1948) as employed in this laboratory (Schram, Dustin, Moore & Bigwood, 1953), using 1 ml. of reagent for each fraction. All eluents contained 1% (v/v) of 33.3% (w/w) aqueous BRJ 35 (an ether of polyethylene glycol made by Atlas Powder Co., Wilmington, Del.) as detergent (as recommended by Moore & Stein, 1951). The 0.1 N chloroacetic acid fractions were neutralized with 0.1 ml. of 1.25 N-NaOH. The colour yield of cysteic acid (anhydrous) was 1.01 relative to leucine. Since the accuracy of the method is dependent upon the exact colour yield, the factor should be determined under the user's experimental conditions of sample size and neutralization. The yield in the oxidation of cystine should also be determined as a check on the procedure.

The Dowex-2 resin was washed between experiments with 50 ml. N chloroacetic acid, followed by equilibration with the strength of acid to be used for the chromatography. The resin was initially prepared for use by washing the hydrochloride form thoroughly with N-HCl and passing through N sodium chloroacetate until the effluent was free of chloride (cf. Groth, Mueller & LePage, 1952).

RESULTS AND DISCUSSION

The yield in the oxidation. The chromatographic technique permitted a detailed study of the yield of cysteic acid obtained from cystine in the performic acid oxidation. Under the prescribed conditions both cystine and cysteine gave $90 \pm 2\%$ of the theoretical yield of cysteic acid (Table 1). In the application of the method to proteins the quantities of cysteic acid measured in the hydrolysates have

routinely been divided by 0.90 to give the final percentage figures (Table 2). The justification for the use of the correction rests on the observation that the factor is applicable to cystine and cysteine, to N-substituted cystine derivatives (*NN'*-bis-chloroacetylcystine monohydrate, Table 1), and to proteins of established cystine content (e.g. bovine serum albumin, Table 2). A reaction which would give a 100% yield in the oxidation would be preferable, but the potential advantages of the cysteic acid approach indicate that with a 90% yield the method can serve the practical purposes for which it has been designed.

The variables in the process were systematically investigated. The yield of cysteic acid was not altered by increasing the concentration of hydrogen peroxide in the formic acid, by lengthening the time of reaction, by oxidation at -10° , or by removal of the reagent by freeze-drying. The choice of 0° was based upon the observation that the yield from cysteine was several per cent lower than the recovery from cystine when the oxidation took place at room temperature. The data in Table 1 demonstrate that the chromatographic procedure gives 100% recovery when cysteic acid is the starting material and that there is not more than 3% loss when cysteic acid is put through both the oxidation and hydrolysis steps of the procedure. Thus there appear to be side reactions in the oxidation leading to 10% of products other than cysteic acid.

Table 1. *Yields of cysteic acid in control experiments*

Starting material	Treatment before chromatography	Cysteic acid (% recovery or yield)
L-Cysteic acid	None	99
		99
		100
		99
	'Hydrolysis' in 6N-HCl	99
	'Oxidation' by performic acid	97.5
		96
L-Cysteine	'Oxidation' and 'hydrolysis'	97
		97
	'Oxidation' at $+20^\circ$	97
		97
	Oxidation and 'hydrolysis'	89
		90
		89
L-Cystine	Oxidation and 'hydrolysis'	89
		88
		92
<i>NN'</i> -bis-Chloroacetyl-L-cystine	Oxidation and hydrolysis	88
		90
L-Cystine	Oxidation at -10°	92
	Oxidation at $+20^\circ$	87
	Oxidation in 44% formic acid instead of 88%	84

Av. 90 ± 2

Table 2. *Application of the method to proteins*

Serum albumin and casein were dried (20 mm. Hg) at 100° over P_2O_5 . Hair was dried in air at 100° to constant wt., having been first de-fatted in ether (Soxhlet). Total nitrogen content is only given when actually determined in the same sample. This was not considered necessary in the case of Armour's 'Bovalbumin' and of human hair.

Material analysed	Treatment	Total N (%)	Cystine		Literature values	
			g./100 g.*	N as % of total N	g./100 g.	N as % of total N
50 mg. serum albumin, bovine (Armour)	Standard	—	6.28	—	—	—
		—	6.07	—	—	—
Same + 1 g. starch	Standard	—	6.43	Av. 6.34	—	—
50 mg. serum albumin	6N-HCl at 40° for 5 days before oxidation	—	6.50	—	6.52†	—
		—	6.42	—	—	—
50 mg. serum albumin	Oxidation at +20°	—	5.89	—	—	—
500 mg. casein‡	Standard (chromatogram started with 0.01N acid)	14.5	0.34	0.27	—	—
		—	0.33	0.27	—	—
375 mg. casein‡	Oxidation for 16 hr.	14.5	0.33	0.27	Approx. 0.3	—
20 mg. human hair (5.38% S)§	Oxidation for 16 hr.	—	18.0	—	—	—
		—	18.1	—	14–18	—
1 g. beef	Sample not ground (Oxid. 16 hr.)	3.50	0.28	0.93	—	Approx. 0.95
	Sample ground with sand (Oxid. 16 hr.)	—	0.27	0.90	—	—
1 g. veal	Sample not ground (Oxid. 16 hr.)	3.53	0.28	0.92	—	—
		—	0.28	0.92	—	—
1 g. barley¶	Ground whole meal	1.53	0.22	1.7	—	Approx. 0.6**
	(Oxid. 4 hr.)	—	0.24	1.8	—	—
		—	0.24	1.8	—	—
		—	0.23	1.8	—	—

* On basis of dry wt. for serum albumin, casein and hair; on basis of whole foods of specified N content for beef, veal and barley.

† Brand, Kassel & Saidel (1944) (cystine + cysteine).

‡ Corrected for moisture (8.25%) but not for ash.

§ The cystine found accounts for 89% of the total sulphur.

|| Cf. Block & Bolling (1951).

¶ Dustin, Schram, Moore & Bigwood (1953).

** Ågren (1949).

Application to proteins. The rate of oxidation of cystine residues in proteins appears to be similar to that of the free amino acid. Bovine serum albumin gave the same yield of cysteic acid at 2, 4, 8 and 20 hr. of oxidation. Neither heat denaturation nor prior partial hydrolysis of the protein altered the yield (Table 2). The recovery was not affected by the presence of carbohydrate to the extent of 20 times the weight of the serum albumin. The reaction was effective on insoluble proteins; human hair or cubes of beef were fully oxidized after standing in the reagent overnight (Table 2). Stirring of the reaction mixture was not necessary. When samples of milk were oxidized, 5 ml. portions were concentrated to about 2 ml. in the flask before the addition of the 25 ml. of chilled reagent.

The need for the use of the 0.01N chloroacetic acid forerun is determined by whether or not the effluent curve with 0.1N acid falls to the base-line before the emergence of the cysteic acid peak. In the analysis of foods of high carbohydrate content, part of the possible interference in the early portion

of the effluent curve comes from the reddish colour yielded by the reaction of ninhydrin with carbohydrate decomposition products (Dustin, Czajkowska, Moore & Bigwood, 1953). In the developmental studies on the present method 0.010N- and 0.015N-HCl were used as the eluents with the chloride form of Dowex-2, under which conditions the rate of travel of cysteic acid is about the same as that obtained with the higher molarities of chloroacetic acid. In preparative chromatography the use of 0.015N-HCl would be preferable for the isolation of cysteic acid and cysteic acid peptides. In general, 0.1N reagents have the advantage of rendering the column relatively insensitive to any buffering action of the sample under analysis, and therefore give more reproducible results.

In addition to the applications referred to in Table 2, the method has been employed for the determination of cystine levels in a number of foods including human milk (Soupert, Moore & Bigwood, 1953) and cassava flour (Close, Adriaens, Moore & Bigwood, 1953).

SUMMARY

1. A method for the determination of the cystine plus cysteine content of proteins has been based upon oxidation of the protein by performic acid, hydrolysis by hydrochloric acid and chromatographic determination of the resulting cysteic acid on columns of a basic ion-exchange resin (Dowex-2).

2. The method can be used directly on products in which a high carbohydrate content may render other methods inapplicable, or in cases where the uncertainties which apply to the stability of cysteine and cystine even under favourable hydrolytic conditions may render an alternative method desirable. The procedure has been applied to the analysis of foods and purified proteins.

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Active Transport of Ions by Sub-Cellular Particles

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The biochemical aspects of active transport in a variety of cells have been studied intensively over the last few years (see reviews by Ussing, 1949, 1952; Davies, 1951), and it has now become clear that most secretory processes require supplies of energy from aerobic metabolism. The metabolic behaviour of a variety of sub-cellular particles has also been investigated, and this work has shown that the mitochondria are the site of nearly all the respiratory activity and oxidative phosphorylation of the cells that have been investigated (see reviews by Green, 1951; Schneider, 1953). Since experiments with 2:4-dinitrophenol indicate that oxidative phosphorylation is closely linked with active transport (Mudge, 1951; Krebs, Eggleston & Terner, 1951; Davies, 1951; Hodgkin & Keynes, 1953; Whittam & Davies, 1953), it seemed reason-

able to suppose that mitochondria are closely associated energetically with secretory activity in cells. The mitochondria may also be the special structures responsible for the appearance of secretions at one particular wall of a cell. It therefore seemed worth while to investigate the possibility of a direct association between mitochondria and active transport.

Harman (1950) failed to find a concentration gradient of potassium between mitochondria and the surrounding medium. However, the particles he had isolated had been kept for more than 20 min. at 0° and this may have so reduced their metabolism that effects attributable to active transport could no longer be observed. This paper describes experiments in which preparations of 'cyclophorase' and mitochondria from sheep kidney cortex were