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Paper Electrophoretic Quantitation and Measurement of the Radioactivities of Labelled Sulphur Amino Acids as Their Oxidation Derivatives

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Performic acid treatment at 0° for 16 h quantitatively converts both free and protein-bound cysteine/cystine and methionine to cysteic acid and methionine sulphone. These acidic derivatives, in contrast to original neutral amino acids, are stable and more suitable for separation and quantitation.

Most of performic acid is removed under reduced pressure at room temperature. Total hydrolysis is carried out in 6 N hydrochloric acid at 100° for 24 h and the acid removed after filtration under reduced pressure at 40°. The residue is dissolved in performic acid, evaporated to dryness *in vacuo* at room temperature and redissolved in 0.1 N hydrochloric acid. Cysteic acid and methionine sulphone are separated by paper electrophoresis at pH 3.6 and 2.65, respectively. Paper strips are stained with cadmium-ninhydrin reagent, and coloured fractions eluted and their optical densities measured at 505 nm. Duplicate strips stained with ninhydrin only are used for measurement of radioactivities direct on papers by liquid scintillation.

The quantitative determination of amino acids in biological materials is usually performed according to the classical method of Moore and Stein,[1] using ion exchange chromatography. This method, however, is elaborate and time-consuming, especially when there are only one or a few amino acids to be determined. Additional difficulties are encountered when the naturally occurring sulphur amino acids cysteine, cystine, and methionine are the objects of quantitation. Oxidation of cysteine [2,3] and methionine [4] during chromatography produces a bewildering number of derivatives. Several distant fractions must be collected and analysed to obtain reliable results. The major oxidation products are usually cystine, cysteic acid, methionine sulphoxide, and methionine sulphone. Their relative amounts are dependent on the preparation of samples, on chromatographic procedures, and on other factors difficult to standardize rigorously.

Quantitation of sulphur amino acids in animal tissues and measurement of their specific radioactivities was essential in studies concerning some aspects of sulphur metabolism going on in this laboratory. The present method based on the quantitative oxidation of sulphur amino acids and subsequent separation of the acidic derivatives by paper electrophoresis is fairly rapid, accurate and suitable for serial work.

EXPERIMENTAL

The oxidation of sulphur amino acids. The level of oxidation is not easily controlled when strong oxidizing agents, such as iodine, are used. Splitting of the carbon chain can occur, too. Somewhat milder oxidants, *e.g.* hydrogen peroxide, have been frequently used, especially for oxidation of cysteine. After hydrogen peroxide treatment of methionine variable amounts of methionine sulphoxide and methionine, in addition to methionine sulphone, were invariably found on chromatograms. For quantitative purposes oxidation with hydrogen peroxide proved to be inadequate, also when catalytic amounts of molybdate were present.

In appropriate conditions the performic acid oxidant of Schram *et al.*[5] produced cysteic acid and methionine sulphone as the only ninhydrin positive derivatives from the corresponding amino acids. It also had the advantage of being volatile and easily removed. Oxidation of free amino acids was completed in 4 h at 0°, for proteins an adequate reaction time was 16 h at 0°. After oxidation at room temperature lower recoveries of sulphur amino acids were obtained. 50 ml of performic acid reagent was used for approximately 1 mg of sulphur. After incubation the reagent was removed under reduced pressure at room temperature.

The following solvents and ascending paper chromatography were used for the identification of sulphur compounds on Whatman No. 1 paper:

Solvent A: t-butanol-methylethylketone-water 2:2:1 (v/v/v). 8 ml of diethylamine was added to 200 ml of the mixture.

Solvent B: butanol-acetic acid-water 12:3:5 (v/v/v).

Solvent C: phenol-water 4:1 (v/v).

The spots were visualized by spraying with ninhydrin and with cadmium-ninhydrin reagent (see later). As references, compounds purchased from the Sigma Chemical Company (St. Louis, Mo. U.S.A.), were used. The R_F-values obtained are presented in Table 1. The relative mobilities of methionine sulphoxide and sulphone are reversed in phenolic solvent mixture as compared with the values obtained in butanol-acetic acid or pyridine base solvent mixtures, an observation also made by Hais.[6] Erroneous R_F-data for these compounds are given in many handbooks.

Table 1. R_F-values × 100 of sulphur amino acids and some derivatives. Ascending paper chromatography, Whatman No. 1 paper. For solvents see text.

Compound	Solvent A	Solvent B	Solvent C
Cysteine	5	9	23
Cystine	7	7	14
Cysteic acid	20	4	7
Homocystine	11	20	20
Homocysteic acid	13	11	15
Methionine	41	50	90
Methionine sulphoxide	17	22	76
Methionine sulphone	31	28	58

Hydrolysis of proteins and peptides. Alkaline hydrolysis destroys most of the sulphur amino acids. Acidic hydrolysis with 6 N hydrochloric acid at 100° for 24 h is convenient to perform and high and reproducible recoveries of sulphur amino acids of proteins treated with performic acid were obtained in the experiments made. Approximately 5 ml of 6 N hydrochloric acid was used for 0.1 g of protein (dry weight). Lower recoveries were obtained when smaller amounts of acid were used. Performic acid treatment of tissue samples before total hydrolysis is essential. Carbohydrates and lipids of high molecular weight are partially hydrolysed in relatively mild conditions and formation of humin in subsequent total hydrolysis is diminished. Definitely lower recoveries of sulphur amino acids were found when total hydrolysis of tissue samples was carried out before performic acid treatment.

The hydrolysate was filtered and evaporated to dryness at 40° *in vacuo*. 5 ml of performic acid was added for 0.1 g of original protein to ensure complete oxidation, the reagent removed under reduced pressure at room temperature and the dry residue stored at −20° until subjected to electrophoresis.

Paper electrophoresis. The following electrolytes and Whatman No. 1 or 3 MM paper were used:

Electrolyte A: pyridine-acetic acid-water 10:100:900 (v/v/v), pH 3.6.

Electrolyte B: pyridine-formic acid-acetic acid-water 10:40:100:1850 (v/v/v/v), pH 2.65.

Samples and references containing 0.02—0.2 μmole of cysteic acid and/or methionine sulphone in a volume of 5—40 μl were pipetted in a line at the centres of the strips (38 mm × 450 mm). A water cooled apparatus (AB Analysteknik, Vallentuna, Sweden), a constant potential gradient of 60 V/cm for 90 min and electrolyte A were used for separation of cysteic acid, which was very distinctly separated from other ninhydrin-positive bands. The nearest fraction was aspartic acid. In these conditions cysteic acid migrated 13.0 cm, aspartic acid 4.1 cm and glutamic acid 1.0 cm to anode and taurine 0.5 cm to cathode. Good separation was also obtained by means of a conventional non-cooled apparatus using a potential gradient of 10 V/cm for 180 min.

For separation of methionine sulphone electrolyte B and a potential gradient of 60 V/cm were applied for 180 min. The methionine sulphone band was located between the aspartic acid and glutamic acid + tyrosine fractions. Taurine migrated 0.7 cm, aspartic acid 4.0 cm, methionine sulphone 6.2 cm and the glutamic acid + tyrosine band 7.9 cm to the cathode. Satisfactory results were also obtained using as electrolyte 0.25 N acetic acid, pH 2.65, and a potential gradient 20 V/cm for 210 min in a non-cooled apparatus.

After electrophoresis the strips were oven-dried at 105° for 10 min.

Staining and photometric evaluation. A modification of the cadmium-ninhydrin staining method of Barrollier *et al.*[7,8] was found to be definitely superior, both in accuracy and sensitivity, to the staining methods involving the use of ninhydrin [9] or cupric ninhydrin.[10]

Staining solution (stock): cadmium acetate 1 g, water 100 ml, acetic acid 20 ml, and acetone to make 1 litre. The staining reagent was made up weekly by dissolving 1 g of ninhydrin in 100 ml of stock solution, stored at 4° in the dark.

Elution solvent: cadmium acetate 100 mg, acetic acid 10 ml, water 10 ml, ethanol 80 ml, prepared freshly when required. The presence of Cd ions is essential for colour stability.

The dried strips were drawn once through the staining reagent, most of the acetone was allowed to evaporate and the strips were placed on a stainless steel rack (LKB-Produkter AB, Stockholm, Sweden). The rack with strips was placed in a plastic container. A dish containing concentrated sulphuric acid to remove atmospheric ammonia was added and the closed container kept in an oven at 70° for 2 h for full colour development. The pink-coloured cysteic acid and methionine sulphone bands were localized by references, cut off and eluted in test tubes with 5 ml of the elution solvent for 2 h with frequent shaking. After elution the colour is stable for 24 h if kept at 4° in the dark. The absorbances were measured at 505 nm. The molar absorptivities ε ($cm^{-1} \times M^{-1}$) of cysteic acid and methionine sulphone were 35 000 and 32 000, respectively. In 1-cm light path cuvettes the absorbance was linear up to 0.7—0.8. With this method 0.01 μmole of amino acid can be measured accurately. The reference standards were run simultaneously with the unknown samples.

The measurement of radioactivity. On duplicate strips the cysteic acid and methionine sulphone bands were visualized by spraying with ninhydrin (0.5 % w/v in butanol) and

Table 2. Effect of cadmium-ninhydrin and ninhydrin staining on the counting of ^{35}S on paper strips by liquid scintillation. 4200 cpm applied as $Na_2{}^{35}SO_4$ in aqueous solution with increasing amounts of cysteic acid.

Cysteic acid μg	Radioactivity applied (cpm)	Radioactivity found (cpm) Cd-ninhydrin staining	Ninhydrin staining
0	4200	4225	4210
1	4200	3985	4222
3	4200	3624	4298
5	4200	3407	4201
10	4200	3074	4310
15	4200	2826	4277
20	4200	2500	4156

heating at 105° until the coloured fractions just appeared. The fractions were cut off and each strip placed as a cylinder in glass counting vials. 10 ml of the scintillator (toluene containing 0.4 % w/v 2,5-diphenyloxazole and 0.01 % w/v 1,4-bis-2-(4-methyl-5-phenyl-oxazolyl)-benzene) was added to each vial. The radioactivities were measured in a Packard Tri Carb Liquid Scintillation Spectrometer.

Because the radiation energies of ^{35}S and ^{14}C are very similar, the same operating settings of spectrometer can be used for both isotopes. Losses of ^{14}C due to $^{14}CO_2$ formation in the amino acid-ninhydrin reaction must be corrected if the carboxyl-C of the amino acid is labelled. No loss of ^{35}S of sulphur amino acids takes place at staining. The counting efficiency for ^{35}S-labelled compounds on Whatman No. 1 paper was 65 %. A linear relation between the counts applied and yielded was obtained up to at least 20 000 cpm. Both cysteic acid and methionine sulphone are insoluble in toluene and the same scintillator can be used for several times. Filtration removed any radioactive paper lint from solution and normal backround values were again obtained.

The cadmium-ninhydrin complex has an effect on the counting efficiency (Table 2) and strips so stained are not suitable for measurement of radioactivity without corrections. In proper amounts of amino acids the ninhydrin colour has practically no effect on counting (Table 2) and no corrections are needed for different colour intensities of the strips.

The specificity of the method. An artificial mixture of 21 amino acids (10 mM solution of alanine, arginine, aspartic acid, asparagine, cysteine, cystine, glutamic acid, glutamine, glycine, histidine, leucine, lysine, methionine, phenylalanine, proline, hydroxyproline, serine, threonine, tryptophan, tyrosine, and valine) and appropriate amounts of crystalline ovalbulmin, rat liver, kidney, and pancreas were treated with performic acid and hydrolysed as described. Samples containing approximately 30—50 μg of cysteic acid and/or methionine sulphone were subjected to electrophoresis on strips 10 cm wide. The dried strips were cut longitudinally and the narrow guide strips stained with ninhydrin. The cysteic acid and methionine sulphone fractions on each unstained strip were localized by comparison with the stained guide and reference strips. Cysteic acid and methionine sulphone fractions were cut off and eluted with 5 ml of 0.1 N hydrochloric acid. Eluates of five fractions were pooled and evaporated to dryness *in vacuo*, redissolved in 5 ml of performic acid and evaporated to dryness under reduced pressure. The residue was dissolved in 1 ml of water and evaporated in a small conical tube *in vacuo*. The hydrochloric-acid-free residue was dissolved in 100 μl of water, and aliquots of 5, 10, and 30 μl, alone and mixed with authentic reference compounds, were subjected to ascending paper chromatography using solvent mixtures A, B, and C as described above. The spots were visualized by spraying the chromatograms with cadmium-ninhydrin reagent and heating at 105°. Only spots coincident with the authentic cysteic acid and methionine sulphone were found on the chromatograms.

$Na_2{}^{35}SO_4$, $Na_2{}^{35}SO_3$, and $Na_2{}^{35}S$ (The Radiochemical Centre, Amersham, England) added to boiled homogenates in amounts of 200 000 cpm/ml were not detected in the cysteic acid and methionine sulphone fractions after electrophoresis. The radioactivity

Table 3. Recoveries of cysteine and methionine added to rat liver homogenate. Values are means from three determinations.

Homogenate ml	Cysteine (μg) added	found	Recovery %	Methionine (μg) added	found	Recovery %
0.5	—	275		—	211	
1.0	—	540		—	415	
0.5	61	314	93	75	264	92
0.5	121	364	92	149	365	89
0.5	605	810	93	745	861	90
—	61	55	92	75	67	89
—	121	113	93	149	131	88
—	605	550	91	745	660	89

was almost quantitatively precipitated after performic acid treatment and acidic hydrolysis by adding $BaCl_2$.

Recoveries of sulphur amino acids. To discover the extent to which sulphur amino acids could be recovered from tissues by this method, known amounts of cysteine, cystine, cysteic acid, methionine, and methionine sulphone were added to homogenates of rat liver, kidney, and pancreas and to a solution of purified ovalbumin. These were treated as described and triplicate determinations were made from each. Results obtained from rat liver are presented in Table 3.

In rat kidney the recoveries for cysteine and methionine were 91 ± 2 % and 89 ± 3 %, respectively. The same mean recoveries were obtained from the pancreas. Slightly higher recoveries (94 ± 2 % and 91 ± 2 %) were obtained from ovalbumin.

The recoveries of added cysteic acid and methionine sulphone invariably were 4—6 % higher than recoveries of added cysteine/cystine and methionine treated in a similar way. This indicates that 4—6 % of cysteine and methionine are converted to products other than cysteic acid and methionine sulphone during performic acid oxidation. These unidentified compounds could not be detected on chromatograms with ninhydrin.

CONCLUSION

The present method allows a relatively rapid quantitation of cysteine/cystine and methionine in tissues. The sensitivity of the method is 0.01 μmole of amino acid, the precision ±5 %, the recovery approximately 90 % and the counting efficiency for ^{35}S- and ^{14}C-labelled amino acids 65 %. These results are not inferior to those obtainable by the more elaborate classical method using ion exchange column chromatography.

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