SEPARATION OF CYSTEINE AND ITS OXIDATION PRODUCTS BY PAPER AND THIN-LAYER CHROMATOGRAPHY

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Many workers have attempted to identify free cysteine by paper chromatographic methods[1,2], but usually it is indistinguishable from the principal oxidation product, cystine. During a study of the products of reaction of tobacco smoke condensate with cysteine, it has been necessary to assess the amount of breakdown of cysteine itself during the procedure for chromatography. Whilst the chromatogram is running, cysteine does not oxidise appreciably, as shown by a well defined spot with little "tailing". The major breakdown of cysteine appears to occur whilst the material is standing at the origin of the chromatogram during equilibration prior to development, and also on a two-dimensional run in the period between removal of the first solvent and re-development with the second component. Oxidation is particularly noticeable in the latter case, when papers are allowed to stand for an appreciable time before the second development stage. In this connection it is advantageous to use the technique of thin-layer chromatography, where a full two-dimensional separation can be achieved in one working day.

The breakdown of cysteine after standing on the origin of a paper chromatogram was first noticed by TOENNIES AND KOLB[3], but they did not identify the oxidation products produced. Similar patterns of products are formed during the breakdown of methionine[4,5] and other amino acids[6,7], the multiplicity of spots produced on chromatography were readily shown by autoradiographic techniques, but again only a tentative identification of a few of the products was made.

In the present work we have attempted to elucidate the nature of the breakdown products obtained during the procedure for chromatography of mixtures containing cysteine labelled with sulphur-35. The oxidation of cysteine, when placed for long periods at the origin of chromatograms, has provided an accelerated test, and has also indicated a possible mechanism for the degradation process.

EXPERIMENTAL

Preparation of [^{35}S]-cysteine hydrochloride

A convenient preparation of microgram amounts of fresh [^{35}S]-cysteine hydrochloride, suitable for an autoradiographic investigation, was achieved by means of the disulphide exchange reaction[8]. Cysteine hydrochloride (0.33 μmole) was spotted at the origin of the chromatogram, followed immediately by [^{35}S]-cystine hydrochloride (1 μC, 0.033 μmole) (Radiochemical Centre, Amersham, Bucks. England). The mixture

was exposed to ammonia vapour for one minute to complete equilibration before acidifying with hydrogen chloride. Approximately 80 % of the radioactivity in the cystine is located in the cysteine after this equilibration process.

[^{35}S]-Cysteine hydrochloride was found to undergo appreciable radiolysis on storage, even in acid solution at temperatures near 0°, whereas [^{35}S]-cystine hydrochloride stored under nitrogen, was considerably more stable over long periods under the same conditions[9,10]. Reduction of [^{35}S]-cystine hydrochloride with tin and hydrochloride acid was found to be wasteful of material, particularly on a micro-scale, with no guarantee of complete freedom from metal and other impurities.

Chromatography and autoradiography

The technique of ascending chromatography was used throughout this work, except where streaking of material was necessary as a prelude to isolation, when a descending method was employed. Three main solvent systems were employed[11], as with slight modification they were also applicable to thin-layer plates.

Mixture 1: Butanol–acetic acid–water (120:30:50, v/v/v)

Mixture 2: Butanol–pyridine–water (1:1:1, v/v/v)

Mixture 3: Phenol–water (4:1, w/v).

The method of BRENNER AND NIEDERWIESER[12] was most satisfactory for thin-layer chromatography, using unactivated Kieselgel-G plates and the above solvents with lower water contents.

Radioactive substances on the chromatograms were located by autoradiography using "Kodirex X-ray film" (Kodak Limited, England), exposed for periods of up to 48 h. The film was protected from acidic materials on the papers and plates by thin polythene sheets. Parallel series of experiments were performed using labelled and unlabelled cysteine to detect any appreciable concurrent radiolytic breakdown of starting materials.

Oxidative breakdown of cysteine on chromatography paper

Figs. 1 and 2 illustrate the decomposition of cysteine hydrochloride on standing at the origin of a paper chromatogram for increasing periods of time. Even after 100 h, a small quantity of cysteine remained, but the number of degradation products and their concentration usually increased with time.

When acid-washed and hardened paper (Whatman No. 540) was used, either untreated or washed with a solution of oxalic acid, a slight decrease in the extent of breakdown of cysteine was observed. In contrast, a significant decrease in both oxidation and degradation was found when the procedure for chromatography was carried out with exclusion of light. The disappearance of cysteine was accelerated by both ultra-violet irradiation and the addition of metal ions to the cysteine solution. For example, the presence of 0.3 μmole Fe^{2+} catalysed the removal of all the cysteine in 48 h, compared with 81 h for 0.03 μmole Fe^{2+}. A little breakdown of cysteine occurred during the actual chromatographic development, as shown by a slight streaking superimposed on the discrete spots due to the degradation products. Variations in the moisture content of the paper did not appear to be a critical factor

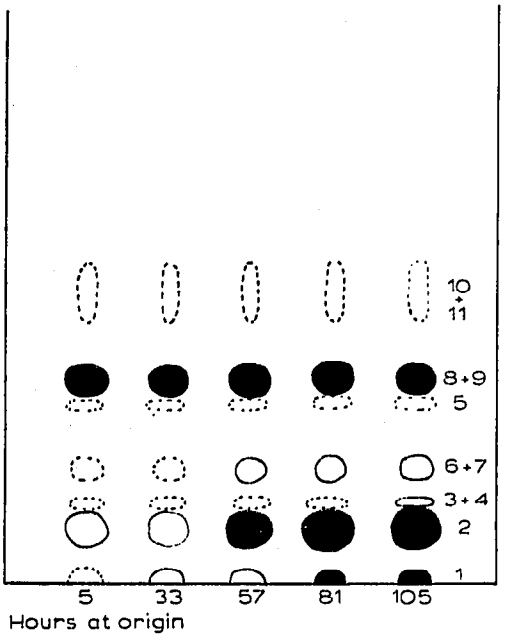

Fig. 1. Composite one-dimensional chromatogram and autoradiograph of cysteine hydrochloride and its degradation products. Solvent mixture 1. Whatman No. 1 paper. Identification of spots: 1 = cystine (unionised); 2 = cystine (ionised); 3 = cysteic acid, cystine disulphoxide and cystine monosulphoxide; 4 = β,β'-diamino-β-carboxydiethyl disulphide; 5 = cysteine sulphinic acid; 6 = cystamine and cysteine sulphoxylic acid; 7 = taurine; 8 = cysteine; 9 = unknown; 10 = β-aminoethylsulphinic acid; 11 = cysteamine. Approximate concentration in spot area: strong = solid circle; medium = continuous circle; weak = broken circle.

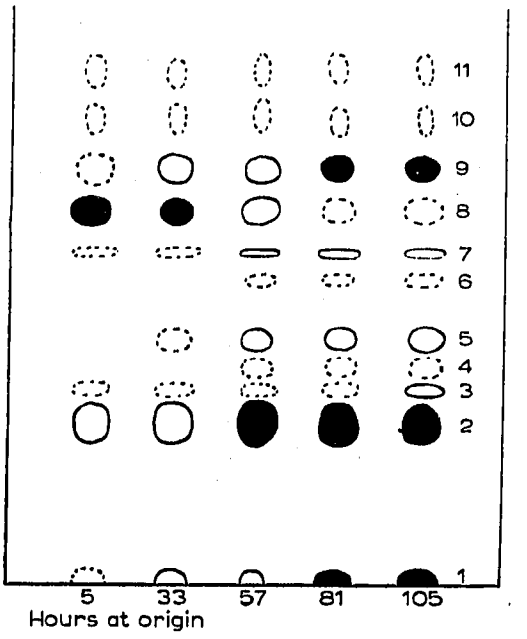

Fig. 2. Composite one-dimensional chromatogram and autoradiograph of cysteine hydrochloride and its degradation products. Solvent mixture 2. Whatman No. 1 paper. For key to spot numbers and symbols, see legend to Fig. 1.

in the oxidation process, as indicated by a comparison between papers hung in dry and water-saturated atmospheres.

In previous studies of the chromatography of cysteine and derivatives[1,2], low R_F values were quoted for cysteine itself, probably due to the rapid oxidation to cystine. In the present work, the R_F values for cysteine appear to be consistent with the structural relationships to the major oxidation products and other amino acids. Identification of the products from chromatography was made either from the R_F values, or by superposition of authentic ninhydrin-positive materials on radioactive cysteine at the origin, followed by comparison of the resulting chromatogram and autoradiograph. A sample of β,β'-diamino-β-carboxydiethyl disulphide was prepared by the exchange reaction between cystine and cystamine.

For two-dimensional chromatography, the preferred order of developing solvents was mixture 1 followed by mixture 2. Mixture 3 was not used extensively, as even redistilled phenol caused some breakdown of cysteine during the development[6,7]. The use of solvent mixtures 1 and 2 were also dictated by the requirements of a study of the reaction between cysteine and tobacco smoke condensate.

An example of an autoradiograph from a two-dimensional chromatogram is shown in Fig. 3. Here the [^{35}S]-cysteine hydrochloride was prepared on the origin of the chromatogram immediately prior to chromatography. Slightly differing results were obtained (Fig. 4) when oxalic acid-washed Whatman No. 540 paper was used.

The spots due to taurine (in Fig. 3) are now absent, even though the starting material had stood at the origin for 81 h before development. It is probable that the oxidations of cysteamine sulphinic acid to taurine, and cysteine sulphinic acid to cysteic acid, are catalysed by metal impurities in the paper. If untreated Whatman No. 1 paper is used, more extensive decomposition of the cysteine occurs.

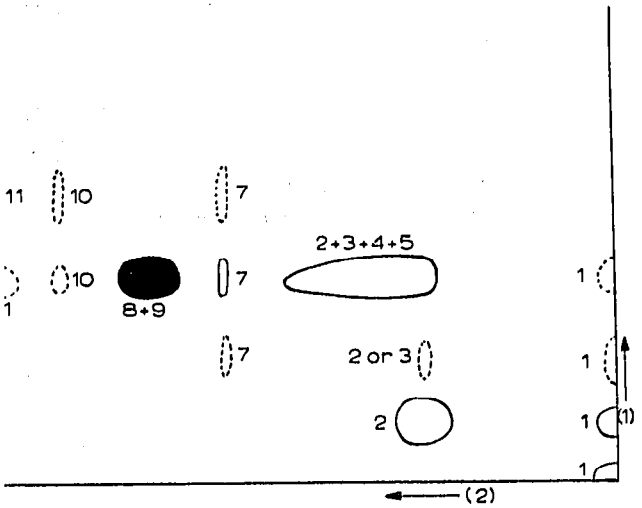

Fig. 3. Composite two-dimensional chromatogram and autoradiograph of cysteine hydrochloride after application to origin immediately prior to development. Whatman No. 540 paper. For key to spot numbers and symbols, see legend to Fig. 1.

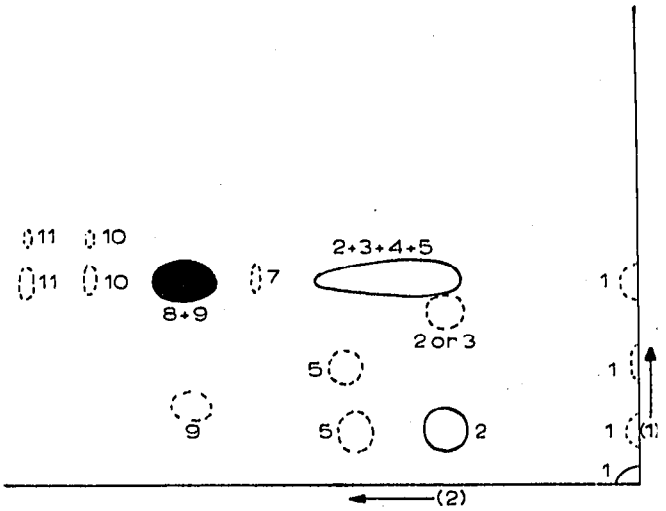

Fig. 4. Composite two-dimensional chromatogram and autoradiograph of cysteine hydrochloride after being allowed to stand at origin for 81 h before development. Whatman No. 540 paper washed with *M* oxalic acid. For key to spot numbers and symbols, see legends to Fig. 1.

When [^{35}S]-cysteine hydrochloride is spotted at the origin, treated with ammonia and allowed to stand for a long period of time, a different pattern of oxidation and degradation products is formed containing a considerable amount of fast-moving material. This result is due to the enhanced radiolytic decomposition of cysteine in alkaline solution, and details of the products will be reported in a subsequent communication.

Thin-layer chromatography of cysteine and its degradation products offers the advantages of speed of operation and high capacity of the stationary phase. A two-dimensional separation can be achieved over the period of one working day, thus lessening the inherent decomposition of the cysteine mixture. The separation of products is comparable to that obtained from paper chromatography over an equivalent solvent run.

Mechanism of oxidation of cysteine during the procedure for chromatography

The general mechanism of the oxidation of cysteine to cystine has been discussed in detail by TARBELL[13], the reaction probably being free radical in character and catalysed by metal ion impurities. In the present study this reaction occurs together with another oxidation and decarboxylation sequence, the first stage of which is the conversion of cysteine to cysteamine. Subsequently the cysteamine is oxidised to cysteamine sulphinic acid and then to taurine. It has been suggested that in biological systems, cysteine sulphinic acid is produced initially from cysteine followed by

decarboxylation and further oxidation to taurine[14]. This reaction sequence may also occur in the oxidation of cysteine on chromatography paper. The present results exclude the formation of taurine by decarboxylation of cysteic acid or cystine disulphoxide. No evidence was obtained for a progressive decomposition of cysteic acid or cystine disulphoxide when spotted onto chromatography paper and allowed to stand for long periods of time before development. The trace of cystamine observed is formed by the oxidation of cysteamine, whilst β,β'-diamino-β-carboxydiethyl disulphide is formed by the disulphide exchange reaction between cysteamine or cystamine and cysteine or cystine.

The metal-catalysed oxidations of cysteine on chromatography paper are decreased by using Whatman No. 540 paper washed with complexing agents. The overall oxidation is minimised by decreasing the time required for the process of chromatography, the latter preferably carried out with the exclusion of light.

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SUMMARY

The nature of the decomposition products formed from cysteine during the procedure for chromatography has been investigated. Concurrent oxidation and decarboxylation processes have been shown to occur when cysteine, either free or as the hydrochloride, is finely dispersed on chromatography paper and allowed to stand for extended periods of time. The total amount of decomposition is reduced by purification of chemicals and chromatography paper, and by exclusion of light.

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