A Method for Separating and Determining the Oxidation Products of Cysteamine

By G. G. JAYSON, TERENCE C. OWEN* AND A. C. WILBRAHAM
(*Department of Chemistry and Biology, Liverpool College of Technology, Liverpool, 3*)

A method is described for analysing and determining thiolsulphonate, hypotaurine and taurine in the presence of cysteamine or cystamine, or both, in aqueous solutions. The separation of all these compounds is achieved by paper chromatography. By treating the paper with 2,4-dinitrofluorobenzene the 2,4-dinitrophenyl derivatives can be formed. These derivatives are leached out of the paper with acetone or water. Their concentration can then be determined by measuring the ultraviolet absorption at 430 mμ.

It is often necessary to determine small amounts of oxidation products in the presence of relatively large amounts of the parent material. The study of the radiation chemistry of aqueous solutions of organic thiols and disulphides gives rise to a similar problem. For cysteamine and cystamine, trace amounts of thiolsulphonate, hypotaurine and taurine are formed. Earlier attempts to separate these substances chromatographically have been only partially successful[1]; further oxidation usually took place during the separation process (of the oxidation products) and this precluded a quantitative study of the radiation mechanism.

In this method, a detailed scheme has been worked out for separating these compounds and for their subsequent quantitative analysis. Before work on the analytical system itself could be commenced the compounds involved had to be synthesised.

Cysteamine (**I**), in the form of its hydrochloride, was prepared by hydrolysis of thiazolidinethione. Cystamine (**II**), thiolsulphonate (**III**) and taurine (**V**) were synthesised by adding the stoicheiometric amounts of hydrogen peroxide to cysteamine hydrochloride in the presence of a trace of iodine at a temperature of less than 20° C.[2]

TABLE I

PREPARATION OF OXIDATION PRODUCTS OF CYSTEAMINE

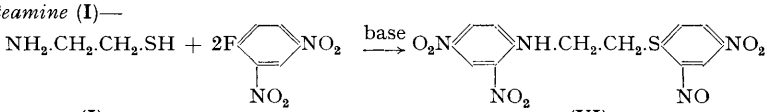
Reactants, moles			
Cysteamine	Hydrogen peroxide	Product	Yield, per cent. (recrystallised)
2	1	Cystamine	76·5
2	3	Thiolsulphonate	74·6
1	3	Taurine	79·6

For irradiation purposes, the sulphates of cysteamine and cystamine were preferred to the hydrochlorides. Cystamine sulphate was prepared by adding the stoicheiometric amount of sulphuric acid to a solution of the hydrochloride and removing the hydrogen chloride by rotary evaporation under reduced pressure.

Hypotaurine (**IV**) had to be prepared by a separate method, which involved the alkaline dismutation of the thiolsulphonate.[3,4]

All the prepared compounds contained amino, thiol or sulphinate groups. These are known to react with 2,4-dinitrofluorobenzene in the presence of alkalis (sodium hydrogen carbonate; pH about 9·5), as in the equations below—

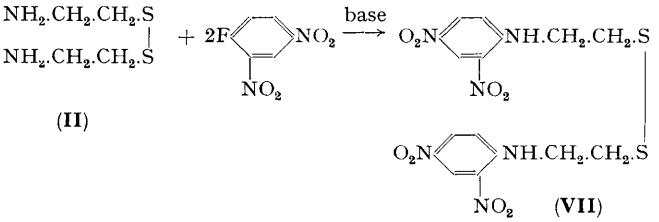
Cysteamine (**I**)—



(**I**) (**VI**)

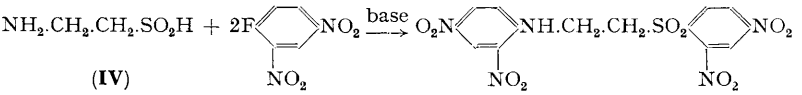
* Present address: University of South Florida, Tampa, Florida, U.S.A.

Cystamine (**II**)—



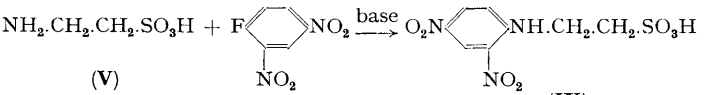
(**II**) (**VII**)

Hypotaurine (**IV**)—



(**IV**) (**VIII**)

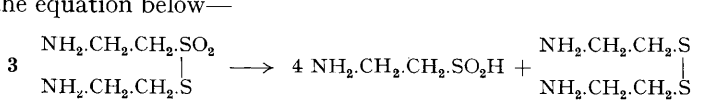
Taurine (**V**)—



(**V**) (**IX**)

All the 2,4-dinitrophenyl derivatives (**VI**, **VII**, **VIII** and **IX**) were prepared and found to have a yellow colour. Their composition was confirmed by elemental analysis. All were found to be soluble in acetone, but only the taurine derivative was soluble in water.

It was not possible to prepare the 2, 4-dinitrophenyl derivative of the thiolsulphonate (**III**) because this substance dismutes to cystamine and hypotaurine in alkaline solution, as in the equation below—



(**III**) (**IV**) (**II**)

Standard solutions of these 2,4-dinitrophenyl compounds were prepared and their absorption spectra between 300 and 450 mμ determined with a Unicam SP500 spectrophotometer.[5,6]

Hydrolysis of the excess of dinitrofluorobenzene produced 2,4-dinitrophenol, which in neutral aqueous solution exhibited an absorption maximum at about 350 mμ, *i.e.*, it overlapped with the absorption curves of all the other derivatives. This overlap was not complete, and from 410 mμ upwards, 2,4-dinitrophenol showed no absorption whereas all the other derivatives absorbed to an appreciable extent (see Fig. 1). All analytical optical-density measurements therefore were carried out at 430 mμ (see Table II).

TABLE II

2,4-DINITROPHENYL DERIVATIVES AND THEIR MOLAR EXTINCTION COEFFICIENTS (ϵ)

	2,4-Dinitrophenyl derivative	Wavelength of maximum absorbtion, mμ	Solvent	$\epsilon_{max.}$	$\epsilon_{430m\mu}$
(VI)	Bis-2,4-dinitrophenyl cysteamine	349	Acetone	29,330	4012
(VII)	Bis-2,4-dinitrophenyl cystamine	352	Acetone	35,706	7412
(VIII)	Bis-2,4-dinitrophenyl hypotaurine	346	Acetone	20,389	3398
(IX)	2,4-Dinitrophenyl taurine	359	Acetone	18,781	4372
(IX)	2,4-Dinitrophenyl taurine	359	Water	16,860	5233

VALIDITY OF THE METHOD—

In the analytical method, *e.g.*, on a chromatogram as shown below, the 2,4-dinitrophenyl derivatives initially appear in alkaline solution. Dinitrophenol is present as its sodium salt in which form it has an appreciable ultraviolet absorption at 430 mμ. It is essential therefore that the solution or chromatogram be made acidic before quantitative analysis.

Two series of samples were prepared; one containing known amounts of 2,4-dinitrophenyl taurine or bis-2,4-dinitrophenyl cystamine and the other containing the same amount of derivative *plus* known amounts of 2,4-dinitrophenol. The optical densities of the solutions containing the dinitrophenol were measured before and after the addition of 0·1 ml of concentrated hydrochloric acid per 100 ml of solution (see Table III).

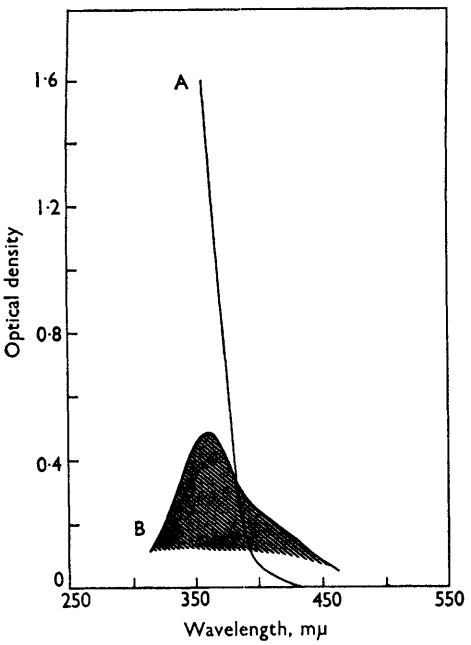


Fig. 1. Absorption curve for 2,4-dinitropheno in neutral solution (10 mg per 100 ml of water): curve A, showing how it interferes with other 2,4-dinitrophenyl derivatives (1 mg per 100 ml of solvent); curve B, at their $\lambda_{max.}$, but not at 430 mμ. The optical densities were read in 1-cm cells against water

Table III

Optical densities of 2,4-dinitrophenyl derivatives in the presence of 2,4-dinitrophenol

The optical densities were read at 430 mμ in 1-cm cells

Sample, mg per 100 ml	Optical density of— derivative	derivative *plus* 4 mg of 2,4-dinitrophenol per 100 ml	derivative *plus* 4 mg of 2,4-dinitrophenol and 0·1 ml of concentrated HCl per 100 ml
2,4-Dinitrophenyl taurine			
2	0·303	0·515	0·305
4	0·609	0·820	0·611
6	0·915	1·130	0·925
8	1·260	1·450	1·260
Bis-2,4-dinitrophenyl cystamine			
2	0·314	0·730	0·314
4	0·606	1·050	0·616
6	0·917	1·776	0·917
8	1·218	1·682	1·204

It is evident from Table III that there is no interference due to the presence of 2,4-dinitrophenol, providing the solution is made acidic before the optical density is measured.

Separation of cysteamine and its oxidation products and their detection

Paper chromatography was used for separating the compounds without causing their interconversion.

Various solvent mixtures were investigated in conjunction with Whatman No. 4 chromatographic paper to find the most suitable chromatographic solvent for separating and detecting small amounts of oxidation products in the presence of large amounts of parent material.

Ethanol - water - sulphuric acid mixture (75 + 25 + 0·1, by volume) gave a good separation of the components, and this solvent was used in all subsequent paper-chromatographic work. Under these conditions the compounds gave the following R_F values—

Cysteamine = 0·65; hypotaurine = 0·59; taurine = 0·52; cystamine = 0·36; thiolsulphonate = 0·2.

Whatman No. 4 chromatographic paper was used in 2½-inch strips, as this gave a good solvent flow and at the same time kept the spots compact. Separation of synthetic mixtures of the above compounds could thus be achieved. When hypotaurine and taurine had to be separated, an inert atmosphere (nitrogen) was required with the acidic solvent to prevent hypotaurine being oxidised to taurine.

Prepared chromatograms were run for 8 hours. The strips were allowed to dry at 18° to 20° C for 15 minutes. In preliminary work, detection was carried out with ninhydrin-in-acetone reagent, *cf.* Grant, Mason and Link,[7] but this was used only in the initial experiments because the coloured derivatives were not stable and were unsuitable for quantitative work. The bright-yellow coloured 2,4-dinitrophenyl derivatives offered a more promising method of detection and subsequent quantitative analysis. The preparation of the derivatives on the paper after the parent material had been separated was carried out as described below. After a chromatogram had been run, the paper was dried and dipped into a solution of 2,4-dinitrofluorobenzene, 1 per cent. w/v in 2-propanol. The paper was partially dried and sprayed with 10 per cent. w/v aqueous sodium hydrogen carbonate solution. When the paper was warmed to 80° C a few isolated yellow spots appeared (owing to the rapid reaction of the reagent with cystamine and cysteamine), and eventually the entire paper became yellow (owing to sodium 2,4-dinitrophenate). The paper was again dried and sprayed with hydrochloric acid, 10 per cent. v/v in ethanol. The acid spray neutralised the excess of sodium hydrogen carbonate and converted the dinitrophenate to the colourless 2,4-dinitrophenol, but left the yellow areas due to the 2,4-dinitrophenyl derivatives of the cysteamine and its oxidation products. 2,4-Dinitrofluorobenzene thus was successful as a detecting agent; since the colours were stable, attempts were made to elute the derivatives from the paper so that the material could be determined quantitatively.

Areas containing the derivatives were cut out of the developed chromatogram and leached with various solvents. Water, ethanol, methanol, 2-propanol, glacial acetic acid, tetrahydrofurfuryl alcohol, chloroform, acetone and carbon tetrachloride were all tried as eluting agents, for 60 minutes at 18° C. Visual examination of the strips revealed that acetone was the most effective solvent, except for the taurine derivative, *i.e.*, 2,4-dinitrophenyl taurine, which was found to be much more soluble in water. None of the other derivatives were soluble in water. It was now possible to determine the amount of original material present from the ultraviolet absorption of their 2,4-dinitrophenyl derivatives at 430 mμ.

Once a suitable eluting agent had been found, experiments were undertaken to show that the formation of 2,4-dinitrophenyl derivatives on the chromatogram was quantitative. In particular, the effects of temperature and time on the reaction were studied.

Heat had to be used to form the coloured derivatives of thiolsulphonate, hypotaurine and taurine. Heating the strips directly was found to be unsatisfactory as the solvents were driven off and the paper charred. The paper strips were prevented from drying out during the heating process by sandwiching the still-moist strips between two thin sheets of glass. This assembly was tested for taurine (which gave the slowest reaction with 2,4-dinitrofluorobenzene) by placing it in an oven at 100° C for varying periods of time (see Table IV).

TABLE IV

EFFECT OF TIME AND HEAT ON THE PRODUCTION OF 2,4-DINITROPHENYL TAURINE

Time at 100° C, minutes	2,4-Dinitrophenyltaurine: Amount on paper, μg	2,4-Dinitrophenyltaurine: Amount found, μg	Amount reacted to give the 2,4-dinitrophenyl derivative, per cent.
15	1·000	0·907	90·7
30	1·000	0·936	93·6
45	1·000	1·00	100·0
60	1·000	0·982	98·2

It can be seen from Table IV that the optimum conditions for quantitative conversion were obtained after the strips had been heated for 45 minutes at 100° C.

METHOD

APPARATUS—

Spectrophotometer—Unicam SP500 or similar instrument.
Whatman No. 4 chromatographic paper.

REAGENTS—

2,4-Dinitrofluorobenzene, 1 per cent. w/v in 2-propanol.
Hydrochloric acid, 10 per cent. v/v in ethanol.
Sodium hydrogen carbonate, 10 per cent. w/v in water.
Chromatographic solvent—A mixture of ethanol, water and sulphuric acid in the ratio 75 + 25 + 0·1, by volume.
Standard solutions of derivatives, 3×10^{-5} moles per ml.

PROCEDURE—

Load strips of Whatman No. 4 paper, $2\frac{1}{2} \times 16$ inches, with microgram amounts of material in aqueous solution. Insert the strips into the chromatographic tank and allow them to equilibrate for 10 minutes. Run ascending-solvent chromatograms for 8 hours with the ethanol - water - sulphuric acid mixture. Mark the solvent fronts and allow the strips to dry at 18° to 20° C. When the strips are dry, dip them into the 2,4-dinitrofluorobenzene solution. Partly dry the strips and then spray them evenly with the sodium hydrogen carbonate solution. Sandwich each paper, whilst still moist, between two thin pieces of glass and heat them at 100° C for 45 minutes. Allow the strips to dry and then spray them with the 10 per cent. hydrochloric acid solution and dry them. Cut out the individual spots and leach them with a mixture of 10 ml of acetone and 0·1 ml of hydrochloric acid. (Use water and hydrochloric acid for the taurine derivative.) Soak the paper for 60 minutes with slight agitation. Measure the optical densities of the resulting solutions in 1- or 4-cm silica cells. Carry out blank determinations alongside by using areas of developed chromatographic strip that are devoid of any derivatives. By using the molar extinction coefficients (see Table II) the concentration of each derivative, and hence, each oxidation product, can be determined from the optical density at 430 mμ.

NOTE—

No special treatment was required for thiolsulphonate. However, care had to be taken, because this compound was leached out from its position ($R_F = 0·2$) as hypotaurine and cystamine dinitrophenyl derivatives, and then determined via these dismutation products (see below under "Quantitative results," section *e*).

QUANTITATIVE RESULTS

(*a*) A solution containing 8·051 mg of cystamine sulphate per ml (*i.e.*, 3×10^{-5} moles per ml) and various amounts of taurine and thiolsulphonate as impurities was prepared. Aliquots of this solution were chromatographed and then developed with dinitrofluorobenzene reagent as described above. The spots were leached out and the concentration of the original substance determined from ultraviolet absorption at 430 mμ ($\epsilon = 7412$). The results are given in Table V.

(*b*) A solution containing 3·405 mg of cysteamine hydrochloride per ml (*i.e.*, 3×10^{-5} moles per ml) was prepared containing cystamine and taurine as impurities. Aliquots were

TABLE V

QUANTITATIVE RECOVERY OF CYSTAMINE FROM CHROMATOGRAMS (IN THE PRESENCE OF TAURINE AND THIOLSULPHONATE)

Cystamine sulphate			Also present in sample	
On chromatogram, μg	Recovered, μg	Recovered, per cent.	Taurine, μg	Thiolsulphonate, μg
8·05	7·78	96·7	0·72	0·69
16·10	15·23	95·7	1·44	1·38
24·15	22·45	91·8	2·16	2·07
32·20	30·40	94·5	2·88	2·76

chromatographed and after development the spots were leached out with acetone ($\epsilon = 4012$). The results are given in Table VI.

TABLE VI

QUANTITATIVE RECOVERY OF CYSTEAMINE FROM CHROMATOGRAMS (IN THE PRESENCE OF TAURINE AND CYSTAMINE)

Cysteamine hydrochloride			Also present in sample	
On chromatograph, μg	Recovered, μg	Recovered, per cent.	Taurine, μg	Cystamine, μg
3·405	3·25	95·5	0·72	8·05
6·81	6·57	96·5	1·44	16·10
10·215	10·04	98·2	2·16	24·15
13·62	13·14	96·5	2·88	32·20

(*c*) A solution containing 3·755 mg of taurine per ml (*i.e.*, 3×10^{-5} mole per ml) was prepared containing cystamine and cysteamine as impurities. Aliquots were chromatographed and after development the spots were leached out with water ($\epsilon = 5233$). The results are given in Table VII.

(*d*) A solution containing 2·183 mg of hypotaurine per ml (*i.e.*, 2×10^{-5} mole per ml) was prepared containing cystamine and cysteamine as impurities. Aliquots were taken and the chromatograms run in an inert atmosphere (nitrogen). After development the spots were leached out. Optical-density measurements at 430 mμ ($\epsilon = 3398$) gave the results shown in Table VII.

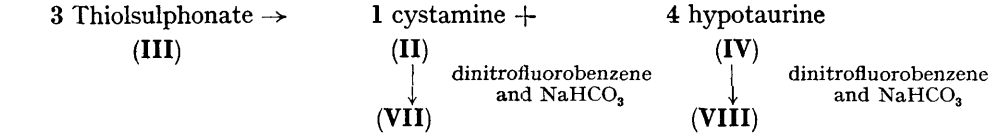
TABLE VII

QUANTITATIVE RECOVERY OF TAURINE AND HYPOTAURINE FROM CHROMATOGRAMS (IN THE PRESENCE OF CYSTAMINE AND CYSTEAMINE)

			Also present in sample	
On chromatogram, μg	Recovered, μg	Recovered, per cent.	Cystamine, μg	Cysteamine, μg
Taurine—				
3·755	3·647	97·25	8·05	3·405
7·510	7·24	96·40	16·10	6·81
11·265	10·77	95·7	24·15	10·215
15·02	14·48	96·4	32·20	13·62
Hypotaurine—				
10·92	9·57	87·66	40·25	17·02
21·83	18·33	83·98	80·51	34·05
32·75	29·33	89·50	120·76	51·07
43·66	39·78	91·13	161·02	68·10

(*e*) Thiolsulphonate is stable in acidic solutions, *i.e.*, the chromatographic solvent, and thus exhibits its own characteristic R_F value during the chromatography. When, however, the paper is treated with 2,4-dinitrofluorobenzene and subsequently with sodium hydrogen

carbonate, the thiolsulphonate dismutates, producing eventually bis-2,4-dinitrophenyl cystamine and bis-2,4-dinitrophenyl hypotaurine in the ratio of 1 + 4, *viz.*—



(A theoretical optical density can be calculated for a given weight of thiolsulphonate originally present in the solution.) The results in Table VIII were obtained by using aliquots of a solution containing 5·648 mg of thiolsulphonate sulphate per ml (*i.e.*, 2×10^{-5} moles per ml) in the presence of cystamine and taurine.

TABLE VIII

QUANTITATIVE RECOVERY OF THIOLSULPHONATE SULPHATE IN THE PRESENCE OF TAURINE AND CYSTAMINE

Thiolsulphonate sulphate			Also present in sample	
On chromatogram, μg	Recovered, μg	Recovered, per cent.	Taurine, μg	Cystamine, μg
28·24	21·38	74·85	3·5	40·25
56·48	44·33	78·48	7·0	80·51
84·72	64·05	75·60	10·5	120·76

It is apparent from the results that a chromatographic method is available for separating the oxidation products of cysteamine. By using a 2,4-dinitrofluorobenzene reagent with a subsequent hydrogen carbonate spray, it was possible to prepare the derivatives of the compounds on the chromatogram. Allowing for the different efficiency of derivative formation, it was possible to determine cysteamine, cystamine, thiolsulphonate, hypotaurine and taurine.

We thank the micro-analytical laboratory of the University of Liverpool for the analyses of all the compounds synthesised.

REFERENCES

1. Shapiro, B., and Eldjarn, L., *Radiation Research*, 1955, **3**, 255.
2. Field, L., Owen, T. C., Crenshaw, R. R., and Bryan, A. W., *J. Amer. Chem. Soc.*, 1961, **83**, 4414.
3. Owen, T. C., and Wilbraham, A. C., *J. Chem. Soc.*, in the press.
4. Cavallini, D., de Marco, C., and Mondovi, B., *Giorn. Biochim.*, 1953, **2**, 4–6, 338.
5. Lockhart, I. M., *Nature*, 1956, **177**, 393.
6. Block, R. J., and Weiss, K. W., "Amino Acid Handbook: Methods and Results of Protein Analysis," Charles C. Thomas, Publisher, Illinois, U.S.A., and Blackwell Scientific Publications, Oxford.
7. Grant, D. W., Mason, S. M., and Link, M. A., *Nature*, 1961, **192**, 352.

Received *June 15th*, 1964