Interaction of *N*-Alkyl-*N*-nitrosourethanes with Thiols

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(Received 5 March 1965)

1. The interaction at room temperature of thiols (cysteine and certain of its derivatives, and glutathione) with *N*-alkyl-*N*-nitrosourethanes and with diazomethane gave complicated mixtures of products. 2. *S*-Methylcysteine and *S*-ethoxycarbonylcysteine, the main products of the reaction between cysteine and *N*-methyl-*N*-nitrosourethane, were isolated and unequivocally identified. Paper-chromatographic and other evidence was used for the identification of several other components of the mixtures of products obtained. 3. *S*-Methyl derivatives and their methyl esters were the common products formed from the thiol compounds with both *N*-methyl-*N*-nitrosourethane and with diazomethane. 4. The esters were unstable and hydrolysed readily. 5. *S*-Ethoxycarbonyl derivatives, the primary products of the reaction of the thiols with *N*-alkyl-*N*-nitrosourethanes, isomerized to the respective *N*-ethoxycarbonyl derivatives when the pH increased above 7.0; the migration of the ethoxycarbonyl group from S to N was particularly easy with cysteine, probably owing to the spatial proximity of the amino group. 6. It is suggested that the non-enzymic reactions *in vitro* of thiols with both *N*-methyl-*N*-nitrosourethane and diazomethane may represent models for events *in vivo* and might help in the studies of the carcinogenic process.

N-Alkyl-*N*-nitrosourethanes are very effective carcinogens, able to induce a variety of tumours by one or a few doses (Schoental, 1960, 1963*a,c*; Schoental & Magee, 1962), or by feeding (Druckrey, Preussmann, Schmähl & Müller, 1961).

Diazoalkanes may possibly be intermediates in the alkylation of cell constituents by alkyl nitroso compounds (Hultin, Arrhenius, Löw & Magee, 1960) and may be involved in the carcinogenic action of the parent compounds. Diazomethane itself proved to be carcinogenic (Schoental, 1960).

The development of oesophageal and stomach tumours after intragastric treatment with *N*-alkyl-*N*-nitrosourethane suggests that the compound interacts with tissues at the site of application. Indeed tissue homogenates have been found to interact with alkyl nitrosourethanes at or below body temperature with evolution of nitrogen (Schoental, 1961). The reaction was traced to the presence of ionizable thiol groups in tissue preparations, and does not require enzymes. Thiols react with alkyl nitrosourethanes at room temperature and at neutral pH, evolving nitrogen in the process. Hence, the course of the reaction can be followed manometrically in the apparatus of Warburg (Schoental, 1961).

N-Alkyl-*N*-nitrosourethanes are suitable compounds for the study of the mechanism of carcino-

genesis. Experiments *in vitro* may possibly give indications for the course of the reactions taking place *in vivo*. As a preliminary to the study of the metabolic fate of [*Me*-¹⁴C]-labelled *N*-methyl-*N*-nitrosourethane in animals, its interaction with thiols *in vitro* has been investigated. Cysteine and glutathione were chosen for this purpose, as these represent biologically important thiols, having in addition two other reactive groups: CO₂H and NH₂. Complicated mixtures of products were formed, and this paper describes their separation and the identification of some of the products. In experiments performed on gram-scale quantities it was possible to isolate and identify the main products; the structures of some of the other reaction products have been deduced from chromatographic and other evidence, and by comparison with the products formed by the action of diazomethane and of ethyl chloroformate on cysteine and on certain of its derivatives.

Some of the results obtained have been reported in a preliminary communication (Schoental & Rive, 1963).

MATERIALS

Cysteine, cystine, *S*-methylglutathione, *N*-methyl-*N*-nitrosourethane and *N*-ethyl-*N*-nitrosourethane were obtained from L. Light and Co. Ltd. (Colnbrook, Bucks.),

glutathione was from the British Drug Houses Ltd. (Poole, Dorset), *S*-methylcysteine was from California Corp. for Biochemical Research (Los Angeles, Calif., U.S.A.), the hydrochloride of cysteine methyl ester was a gift from Berks Pharmaceuticals Ltd. (London, W.1) and *S*-ethylcysteine was a gift from Dr J. J. Roberts and Dr G. P. Warwick. *N*-Methylcysteine was prepared from thiazolidinecarboxylic acid by the action of sodium in liquid NH_3 , as described by Greenstein & Winitz (1961). A crude preparation was used. Ethyl chloroformate was from Hopkin and Williams Ltd. (Chadwell Heath, Essex) and was redistilled.

METHODS

All the reactions involving alkylnitrosourethanes and diazomethane were performed in a fume cupboard under good exhaust. The danger of handling these very effective carcinogens should not be underestimated.

Ethereal solutions of diazomethane were prepared by the action of propanolic KOH on MNU* in ether and distillation (Bachmann, Cole & Wilds, 1940). After drying over KOH pellets the ethereal solutions were stored at -5° until used.

Reaction of thiols

With N-alkyl-N-nitrosourethanes. The thiol compound, about 0.5–1.0 m-mole, was dissolved in a small quantity of water, or Sørensen's 0.1 M-phosphate buffer (about 1 ml.), and treated with 10% (v/v) solutions of *N*-alkyl-*N*-nitrosourethanes in aq. ethanol (1:1, v/v) at room temperature, with frequent shaking. Various molecular proportions were tested, but it was found most convenient to use an excess of the *N*-alkyl-*N*-nitrosourethanes, about 2–3 moles/mole of thiol; this simplified the chromatographic separation of products, as only traces of unchanged thiol remained. The pH of the mixture (usually 7.0 ± 0.5) was maintained by the addition of 0.25 N-NaOH if necessary. Evolution of gas commenced soon after the mixing of the solutions; the tube was then shaken frequently until no further gas evolution was observed. The reaction products were usually resolved by paper chromatography. Reactions designed for isolation of products were performed in a similar way with 10 m-mole quantities of the thiol and 20 m-mole quantity of MNU; no buffer was then used and the desired pH (6.0, 7.2 or 8.0) was maintained by the addition of dilute NaOH or HCl as required.

With diazomethane. The compound (about 0.5 m-mole or less) was dissolved in 5 ml. of water (or phosphate buffer, pH 7.2), neutralized and treated, with shaking, at room temperature with ethereal diazomethane. The latter was added gradually from a burette, 1–2 ml. at a time. After each addition the solution was left to decolorize and spotted on paper for chromatography. This stepwise treatment with diazomethane and spotting was continued till more than 10 min. was required for the yellow colour of the diazomethane to disappear from the solution. These experiments were designed for the identification of the groups which are preferentially methylated by diazomethane. In other experiments, excess of ethereal diazomethane was added at once and the mixture after frequent shaking was left overnight in a fume cupboard at room temperature before spotting on paper.

* Abbreviation: MNU, *N*-methyl-*N*-nitrosourethane.

Paper chromatography

All paper chromatograms were run on Whatman no. 1 filter-paper sheets by the descending method. The solvents used for both the one- and two-dimensional chromatograms included: (A) butan-1-ol-acetic acid-water (12:3:5, by vol.); (B) butan-1-ol-water-aq. NH_3 (sp.gr. 0.88) (10:10:1, by vol.), alcohol layer; (C) propan-2-ol-water-aq. NH_3 (sp.gr. 0.88) (25:12:2, by vol.); (D) butan-1-ol saturated with water.

The chromatograms were run for about 16 hr., allowed to dry in a fume cupboard and sprayed with, or dipped in, location reagents. These comprised: (a) 0.25% ninhydrin in acetone; (b) the nitroprusside reagent of Toennies & Kolb (1951) containing KCN, made up in double strength to show up both SH and S·S groups; (c) the platonic iodide reagent of Toennies & Kolb (1951); (d) the indicator-permananganate reagent of Paskova & Munk (1960); (e) ethanolic 0.1% (w/v) bromocresol green adjusted to a blue-green colour with 1N-NaOH and diluted five times with acetone. Papers were sprayed with ninhydrin (a), allowed to dry and to develop. They were then sprayed with the platinum reagent (c). Positive reaction with platinum reagent could be demonstrated only with ninhydrin-negative compounds.

Isolation of *S*-methylcysteine

Cysteine (1 g.) was dissolved in deionized water (10 ml.) and the solution adjusted to pH 8.0 with 0.25 N-NaOH. An excess of MNU (2.5 ml.) dissolved in 25 ml. of aq. ethanol (1:1, v/v) was added dropwise. The pH was maintained between 7.5 and 8.0 by the addition of 0.25 N-NaOH. When gas evolution ceased and the pH remained unchanged, the excess of MNU was removed *in vacuo*, acetone was added (500 ml.) and the mixture left overnight in the cold at 4° . The white precipitate was filtered and crystallized repeatedly from methanol. It formed white needle-like crystals (240 mg.), m.p. $223\text{--}227^\circ$ (decomp.) not depressed by admixture of authentic *S*-methylcysteine $[\alpha]_D^{24} -26^\circ$ (c 1 in water) (Found: C, 35.6; H, 6.7; N, 10.0; S, 23.6. Calc. for $\text{C}_4\text{H}_9\text{NO}_2\text{S}$: C, 35.7; H, 6.7; N, 10.4; S, 23.7%). du Vigneaud, Loring & Craft (1934) recorded for *S*-methyl-L-cysteine $[\alpha]_D^{26-27} -31.2^\circ$ and -32.0° (c 1 in water). Oxidation with hydrogen peroxide according to Synge & Wood (1956) yielded *S*-methylcysteine *S*-oxide, which crystallized from aq. ethanol in white prisms, m.p. 164° (decomp.) (Found: C, 31.2; H, 6.3; N, 8.8; S, 21.6. Calc. for $\text{C}_4\text{H}_9\text{NO}_3\text{S}$: C, 31.8; H, 6.0; N, 9.3; S, 21.2%).

Isolation of *S*-ethoxycarbonylcysteine

For the isolation of *S*-ethoxycarbonylcysteine the reaction between L-cysteine (1.0 g.) and MNU (2.5 ml.) was performed under conditions similar to those used for the isolation of *S*-methylcysteine, but the pH was maintained at about 6.0. After cessation of gas evolution, addition of acetone resulted in a flocculent precipitate. This was collected and recrystallized repeatedly from methanol; it yielded 670 mg. of white prisms, m.p. $164\text{--}166^\circ$ (decomp.) (Found: C, 37.2; H, 5.7; N, 7.3; S, 17.1. Calc. for $\text{C}_6\text{H}_{11}\text{NO}_4\text{S}$: C, 37.4; H, 5.7; N, 7.3; S, 16.6%).

This product had the same chromatographic behaviour as, and was identical with, *S*-ethoxycarbonylcysteine, synthesized by the action of ethyl chloroformate on cysteine.

Preparation of S-ethoxycarbonylcysteine hydrochloride

Cysteine (1g.) was dissolved in 50 ml. of deionized water, the solution was adjusted to pH 7.0 with 0.1 N-Ba(OH)₂ and the flask surrounded by a freezing mixture. A slight excess of ethyl chloroformate (0.9 ml.) in 15 ml. of ice-cold ethanol was added slowly with mechanical stirring in the course of half an hour. The pH was maintained at 6.5–7.0 with the aid of a direct-reading pH-meter by the addition of 0.1 N-Ba(OH)₂.

When the reaction was completed, as indicated by the stability of pH, dilute H₂SO₄ was added till the precipitation of BaSO₄ was completed. The precipitate was removed by centrifugation followed by filtration of the supernatant. The filtrate was evaporated to dryness *in vacuo* and the residue was dissolved in conc. HCl and evaporated under reduced pressure. The remaining solid was recrystallized by the addition of acetone to its aqueous solution. *S-Ethoxycarbonylcysteine hydrochloride* formed fine needles, m.p. 184–185° (decomp.) (Found: C, 31.6; H, 5.4; Cl, 15.7; N, 6.2; S, 13.5. Calc. for C₆H₁₁NO₄SHCl: C, 31.4; H, 5.3; Cl, 15.4; N, 6.1; S, 14.0%).

Preparation of NS-diethoxycarbonylcysteine

Cysteine (1g.) was dissolved in deionized water (15 ml.) and 2.5 N-NaOH (2.8 ml.) was added to the cooled solution. Ethyl chloroformate (1.6 ml.) in ethanol (15 ml.) was added in the course of half an hour with continuous mechanical stirring, the temperature being maintained at –5°. After

completion of the reaction, acetone was added to the neutralized solution and the precipitated NaCl filtered off. The filtrate was concentrated under reduced pressure to a small volume and acidified with dilute HCl, whereupon an

Table 1. *Chromatographic behaviour of reference substances*

Solvents: A, butanol–acetic acid; B, butanol–ammonia; C, propanol–ammonia; D, butanol–water.

Substance	Mean R_F			
	Solvent A	Solvent B	Solvent C	Solvent D
Cystine	0.08	0.0	0.31	0.0
<i>S</i> -Methylcysteine	0.17	0.0	0.53	0.02
<i>S</i> -oxide				
Cysteine	0.30	0.0	0.31	0.0
<i>S</i> -Methylcysteine	0.37	0.10	0.59	0.13
<i>S</i> -Ethoxycarbonyl-cysteine	0.56	0.08	0.55	0.30
<i>NS</i> -Diethoxy-carbonylcysteine	0.93	0.45	0.84	0.51
Cysteine methyl ester	0.16	0.0		
<i>N</i> -Methylcysteine	0.12			
<i>S</i> -Ethylcysteine	0.54			
Glutathione	0.24		0.1	
<i>S</i> -Methylglutathione	0.28		0.49	

Table 2. *Behaviour of the products of interaction of N-methyl-N-nitrosourethane with cysteine on chromatography in various systems and towards locating reagents*

Solvents: A, butanol–acetic acid; B, butanol–ammonia; C, propanol–ammonia; D, butanol–water. a, Ninhydrin; b, nitroprusside; c, platinum reagent; d, indicator–permanganate reagent; e, bromocresol green.

Spot no.	Mean R_F				Spot detected with location reagents	Spot considered to represent:
	Solvent A	Solvent B	Solvent C	Solvent D		
1, 1', 1"	0.08*	0*	0.31*	0*	a b	Cystine
2	0.17*	0*	0.53*	0*	a	<i>S</i> -Methylcysteine <i>S</i> -oxide
3	0.30*				a b	Cysteine
4	0.37†	0.10†	0.59†	0.13†	a	<i>S</i> -Methylcysteine
5	0.45*				a	Decomposition product of <i>S</i> -ethoxycarbonylcysteine
6	0.56†		0.55	0.30†	a b	<i>S</i> -Ethoxycarbonylcysteine
7	0.73††	0.90*†		0.79††	a	<i>S</i> -Ethoxycarbonylcysteine methyl ester
8, 8', 8"	0.90†	0.08†	0.69†	0.07†	b c d e	<i>N</i> -Ethoxycarbonylcysteine
9	0.58*†	0.82*†	0.85*†	0.59*†	a	<i>S</i> -Methylcysteine methyl ester
10	0.90	0.32	0.80	0.34	c	<i>N</i> -Ethoxycarbonyl- <i>S</i> -methylcysteine
11	0.93	0.45	0.84	0.51	d e	<i>NS</i> -Diethoxycarbonylcysteine
12	0.93*†	0.95*†	0.92*†	0.96*†	b c	<i>N</i> -Ethoxycarbonylcysteine methyl ester

* Faint spot, not detected in all experiments.

† Main spot.

‡ Spot not detectable if mixture was chromatographed after standing at room temperature for a few days.

oil separated out. The oil was extracted with benzene and concentrated. It gave a single spot on paper chromatography from various solvents.

The oil (100 mg.) was dissolved in aq. ethanol (1:1, v/v), the pH adjusted to about 7.0, and treated with *p*-bromophenacyl bromide (100 mg.) in ethanol. The mixture was refluxed for 1 hr. on a water bath. The crystals which were deposited on cooling were recrystallized from ethanol. *NS-Diethoxycarbonylcysteine p-bromophenacyl ester* formed sharp needles m.p. 98° (Found: C, 44.1; H, 4.8; Br, 17.9; N, 2.9. Calc. for $C_{17}H_{20}BrNO_7S$: C, 44.2; H, 4.4; Br, 17.3; N, 3.0%).

The chromatographic behaviour of the various reference substances is summarized in Table 1.

RESULTS

Products of interaction of N-methyl-N-nitroso-urethane with cysteine. Interaction of MNU with cysteine in aq. ethanol solutions at room temperature and at the range pH 6.0–7.5 gave complicated mixtures of products. One-dimensional paper chromatography in butanol–acetic acid (solvent A) revealed eight spots. Of these, two were further resolved by two-dimensional chromatography with butanol–ammonia (solvent B) as the second solvent system and in addition spots appeared in this solvent as a result of transformation by the alkaline medium of some of the primary reaction products. A total of 20 spots were detected; those numbered 1–12 represent individual compounds (Fig. 1, Table 2). Spots indicated by dashed numbers represent compounds formed as a result of oxidation, isomerization or hydrolysis of the primary reaction products by the alkaline chromatographic solvent B.

The areas of the spots in Fig. 1 indicate the

Fig. 1. Two-dimensional paper chromatography of products formed from *N*-methyl-*N*-nitrosourethane and cysteine. Run first in the acidic system A, followed by a run in the basic system B.

approximate quantity of the respective products. Spots 4, 6 and 8 represent the main products of the reaction. Spots having dashed outlines were observed only in some of the experiments. The R_F values given in the Tables are mean values computed from several experiments.

Products represented by spots 1, 1', 1'', 2, 3, 4, 6, 12 were tentatively identified by direct comparison with authentic substances run on the same chromatograms. The identities of the compounds represented by the remaining spots were inferred by comparison of their chromatographic behaviour with that of products obtained from reference substances by the action of acids, alkali, hydrogen peroxide, diazomethane or ethyl chloroformate.

The two main products represented by spots 4 and 6 respectively (Fig. 1) were isolated in crystalline form and unequivocally identified by analysis and mixed m.p. with authentic *S*-methylcysteine and *S*-ethoxycarbonylcysteine respectively. Oxidation of the compound represented by spot 4 with hydrogen peroxide, according to Synge & Wood (1956), gave a product (spot 2) that was unequivocally identified as *S*-methylcysteine *S*-oxide by analysis and mixed m.p. with an authentic specimen, obtained by similar oxidation of commercial *S*-methylcysteine.

Product giving spot 6 was ninhydrin- and nitroprusside-positive like the product obtained from the action of ethyl chloroformate on cysteine. It did not react with *N*-ethylmaleimide or with MNU at neutral pH, hence no free thiol group was present, in spite of the fact that it gave a positive nitroprusside test. The latter is performed under alkaline conditions, and this compound was labile in alkali. It was possible to isolate it from reaction mixtures when MNU reacted with cysteine at slightly acidic pH (below 7.0) and to identify it with *S*-ethoxycarbonylcysteine (see the Methods section). When dissolved in phosphate buffer, pH 8.0, it readily underwent isomerization, even at room temperature, and formed a product which was no longer ninhydrin-positive, which reacted with both *N*-ethylmaleimide and MNU, indicating the presence of a free thiol group and of a substituted amino group. On paper chromatograms, the transformed product (spots 8, 8', 8'') could be detected by the platonic iodide reagent of Toennies & Kolb (1951), with which it gave a whitish spot on a pink background, or with bromocresol green, which showed its acidic reaction. These properties are compatible with those of *N*-ethoxycarbonylcysteine. This product was obtained as an oil by extraction with benzene from acidified solutions. When the reaction between MNU and cysteine was performed at pH 7.5–8.0 *N*-ethoxycarbonylcysteine was the main product and, being soluble in organic solvents (acetone), allowed the isolation in crystalline form

of product 4 and its identification as *S*-methylcysteine (see the Methods section).

The treatment of *S*-ethoxycarbonylcysteine with alkali (Table 4) usually gave an additional faint ninhydrin-positive spot corresponding to cystine (Fig. 1, spot 1"). This indicates that a small proportion of *S*-ethoxycarbonylcysteine undergoes hydrolysis of its ethoxycarbonyl group under the conditions used. An additional product giving a ninhydrin-positive spot (5) sometimes appearing on alkali-treatment of *S*-ethoxycarbonylcysteine was not identified.

Another product, which gave spot 11 on paper chromatograms, had the same properties as the one obtained by the action of ethyl chloroformate on cysteine, excess of the reagent (Table 4) being used (as described in the Methods section). Hence it is

probably *NS*-diethoxycarbonylcysteine. This compound is quite stable to 0.1N alkali at room temperature, but treatment with conc. ammonia (sp.gr. 0.88) for 30 min. at room temperature transformed it into *N*-ethoxycarbonylcysteine corresponding to spot 8". This provided additional evidence for the alkali-lability of the *S*-ethoxycarbonyl group, although in this compound it was less labile than in *S*-ethoxycarbonylcysteine.

Products of the action of diazomethane on cysteine, its N-, O- and S-monomethyl derivatives and S-ethoxycarbonylcysteine. The action of diazomethane on cysteine and its derivatives (Table 3) yielded mixtures of products of which only four have been identified. Two of the products showed the same behaviour on chromatography and towards locating reagents (R_F , positive to

Table 3. *Chromatographic behaviour of products formed by the action of ethereal diazomethane on cysteine and its monomethyl derivatives and on S-ethoxycarbonylcysteine*

Solvents: A, butanol-acetic acid; B, butanol-ammonia. a, Ninhydrin; b, nitroprusside; c, platinum reagent.

	Mean R_F					Spot detected with location reagents	Spot considered to represent:
	Solvent A				Solvent B		
	None	Small quantity	Excess	Excess (after standing 48 hr.)	Excess		
Diazomethane							
Cysteine	0.07 streak	0.07 0.16*	0.07 0.16*	0.07 0.16*	0 0.10	a b a b	Cystine Cysteine methyl ester
		0.26*	0.26	0.26	0	a	
	0.30 streak	0.30* 0.37 0.45	0.30 0.37 0.45 0.50 0.58	0.30 0.37 0.45 0.50 0.58	0 0.10 0.15 0.17 0.82	a b a c a	Cysteine <i>S</i> -Methylcysteine <i>S</i> -Methylcysteine methyl ester
Cysteine methyl ester	0.16	0.16 0.27	0.16* 0.27 0.48 0.58	0.08 0.16* 0.27 0.37 0.49 0.58*	0 0.10 0 0.17 0.82	a a b a a a	
<i>S</i> -Methylcysteine	0.16* 0.37	0.16* 0.37 0.43* 0.58	0.16* 0.37 0.43* 0.58	0.16 0.37 0.43* 0.58*	0.10 0.10 0.17 0.82	a a c a	<i>S</i> -Methylcysteine <i>S</i> -Methylcysteine <i>S</i> -Methylcysteine methyl ester
<i>N</i> -Methylcysteine	0.12	0.12* 0.26				b b	
<i>S</i> -Ethoxycarbonylcysteine	0.56	0.56 0.73	0.45 0.56* 0.73†	0.45 0.56* 0.73*	0.17 0.08 0.93	c a c a c	<i>S</i> -Ethoxycarbonylcysteine <i>S</i> -Ethoxycarbonylcysteine methyl ester

* Faint spot, not detected in all experiments.

† Main spot,

ninhydrin, negative to nitroprusside) as spots 4 and 9 (Fig. 1) from reaction mixtures of MNU and cysteine, and were *S*-methylcysteine and its methyl ester respectively. The latter was also present in mixtures obtained when diazomethane reacted with *S*-methylcysteine or with cysteine methyl ester (Table 3). It ran rather fast in solvent system B, in which it had a tendency to streak. When the reaction mixtures were left at room temperature for a few days, this product tended to disappear and gradually gave much fainter spots, or was no longer detectable by paper chromatography. On oxidation with hydrogen peroxide it gave a product represented by a spot corresponding to *S*-methylcysteine *S*-oxide. This behaviour indicates that spot 9 may represent *S*-methylcysteine methyl ester and that the ester grouping is labile and readily undergoes hydrolysis at room temperature under mild conditions.

Among the reaction products of diazomethane with cysteine or with *S*-methylcysteine, paper chromatography revealed spots which were ninhydrin-negative, but reacted with the platinum iodide reagent of Toennies & Kolb (1951). These were evidently products of *N*-methylation. No comparable spots could be detected by chromatography among the products formed from MNU and cysteine.

The remaining products of the reaction between MNU and cysteine had no counterparts among the products formed from cysteine and diazomethane, or between cysteine and ethyl chloroformate as

judged by paper chromatography. The possibility that they may be products of both methylation and ethoxycarbonylation on the same molecule was therefore investigated.

When *S*-ethoxycarbonylcysteine was methylated with diazomethane (Table 5) the main ninhydrin-positive spot obtained on chromatography of the products in solvent system A had the same properties as spot 7 (Fig. 1, Table 2). A similar ninhydrin-positive spot represented the main product obtained when the methyl ester of cysteine reacted with ethyl chloroformate and also when the methyl ester of cysteine reacted with MNU (Table 5). Hence it is likely to be *S*-ethoxycarbonylcysteine methyl ester. In the solvent system B containing ammonia, this product was transformed into a ninhydrin-negative, nitroprusside-positive spot, probably *N*-ethoxycarbonylcysteine methyl ester, with characteristics similar to spot 12. This indicates that the *S*- to *N*- isomerization of the ethoxycarbonyl group under the influence of ammonia is more rapid than the hydrolysis of the ester group in this compound.

Reaction of ethyl chloroformate with S-methylcysteine and with the methyl ester of cysteine. When ethyl chloroformate reacted with *S*-methylcysteine, it gave one main ninhydrin-negative product (Table 4), which, on chromatography in solvent system A, gave a spot with the same properties as spot 11 (Fig. 1, Table 2), suggesting that this is probably *N*-ethoxycarbonyl-*S*-methylcysteine.

Mixtures of products obtained by the action of

Table 4. *Chromatographic behaviour of products of action of ethyl chloroformate on cysteine and on S-methylcysteine and on product 6 formed from cysteine + N-methyl-N-nitrosourethane*

Solvents: A, butanol-acetic acid; B, butanol-ammonia; C, propanol-ammonia. a, Ninhydrin; b, nitroprusside; c, platinum reagent; d, indicator-permanganate reagent; e, bromocresol green.

	Mean R_F			Spot detected with location reagents	Spot considered to represent:
	Solvent A	Solvent B	Solvent C		
Cysteine + ethyl chloroformate	0.08	0*	0.31	a b	Cystine
	0.56	—	0.55	a b	<i>S</i> -Carbethoxycarbonylcysteine
	0.90	0.08	0.69	b c d e	<i>N</i> -Ethoxycarbonylcysteine
	0.93	0.45	0.84	d e	<i>NS</i> -Diethoxycarbonylcysteine
Cysteine + excess of ethyl chloroformate (Cysteine + excess of ethyl chloroformate) + conc. ammonia	0.93	0.45	0.84	d e	<i>NS</i> -Diethoxycarbonylcysteine
	0.90	0.08	0.69	b c d e	<i>N</i> -Ethoxycarbonylcysteine
(Cysteine + MNU) Product 6	0.56	—	0.55	a b	<i>S</i> -Ethoxycarbonylcysteine
	—	0.08	0.69	b c d e	<i>N</i> -Ethoxycarbonylcysteine
(Cysteine + MNU) Product 6 + dilute alkali	0.08*	0*	0.31*	a b	Cystine
	0.56*	—	0.55	a b	<i>S</i> -Ethoxycarbonylcysteine
	0.90	0.08	0.69	b c d e	<i>N</i> -Ethoxycarbonylcysteine
<i>S</i> -Methylcysteine + ethyl chloroformate	0.90	0.32	0.78	c	<i>N</i> -Ethoxycarbonyl- <i>S</i> -methylcysteine

* Faint spot, not detected in all experiments.

ethyl chloroformate (and of MNU) on cysteine methyl ester (Table 5) gave on chromatography a main ninhydrin-negative, nitroprusside-positive spot, corresponding to 12 (Table 2). This probably represents *N*-ethoxycarbonylcysteine methyl ester.

This ester, like the methyl ester of *S*-methylcysteine described above, appeared to hydrolyse slowly in aqueous solution. It was present and was readily detected on paper chromatograms when spotted soon after preparation, but tended to be lost when the reaction products were kept at room temperature for 2 or 3 days, while the spot of its parent compound became more accentuated.

Products of interaction of MNU with glutathione. The interaction of MNU with GSH resulted in a mixture of products (Table 6), which revealed six spots on chromatography numbered 1–6. Spot 3 had the same R_F as authentic *S*-methylglutathione, and as a spot obtained from the action of diazomethane on GSH. On oxidation with hydrogen peroxide all three gave the same product, probably *S*-methylglutathione *S*-oxide.

When GSH was treated with ethyl chloroformate (Table 6) the product gave a spot with the same R_F as 4 and both were ninhydrin-positive and nitroprusside-negative. These represented probably *S*-ethoxycarbonylglutathione, which appeared to be labile to alkali though less so than the corresponding cysteine derivative. This was reflected in its not giving a positive nitroprusside reaction, which takes place in the presence of ammonia. However, when

treated with conc. ammonia for an hour, *S*-ethoxycarbonylglutathione appeared to lose the ethoxycarbonyl group and gave a main spot corresponding to GSH and a small spot which was ninhydrin-negative and nitroprusside-positive; the latter probably corresponds to *N*-ethoxycarbonylglutathione formed as a result of sulphur to nitrogen migration of the ethoxycarbonyl group. Spot 5 had the same R_F as a product formed by the action of diazomethane on *S*-methylglutathione or on GSH and was possibly the methyl ester of *S*-methylglutathione. Spot 6 was not identified.

Products of interaction of N-ethyl-N-nitrosourethane with cysteine. *N*-Ethyl-*N*-nitrosourethane interacted also with cysteine in aqueous ethanolic solutions at pH 7–7.5 though more sluggishly than MNU. Again, complicated mixtures of products were formed. Paper chromatography in solvent system A showed eight spots of a similar pattern to those described for MNU. The main spots represented *S*-ethylcysteine and *S*-ethoxycarbonylcysteine; faint spots representing cystine and cysteine were also present. The remaining faint spots were not examined in detail.

DISCUSSION

Thiol groups are very reactive at physiological pH. For the comparison of reactivities of various groups towards alkylating agents, their respective competition factors, introduced by Ogston (1948), are useful. These are related to the rate at which the groups are alkylated under standard conditions. In the Table reported by Ross (1958) the competition factor of terminal thiol groups is about 1000 times as great as that of the hydroxy or the amino groups. Pure compounds having ionizable free thiol groups have been found to interact with *N*-alkyl-*N*-nitrosourethanes at room temperature and at neutral pH, as did tissue homogenates (Schoental, 1961).

For the reaction of *N*-alkyl-*N*-nitrosourethanes with thiols aqueous ethanolic solutions were used. Ethanol was required as solvent for the sparingly water-soluble alkyl nitrosourethanes, so that the reaction could proceed in homogeneous solution, and water was needed as an ionizing medium for the thiol groups. In neutral aqueous ethanol, alkyl nitrosourethanes decompose only very slowly at room temperature, except when exposed to direct sunshine or ultraviolet light (Schoental, 1963b). In the presence of ionizable free thiol groups, however, alkyl nitrosourethanes decompose rapidly at neutral pH even in the dark, with evolution of nitrogen gas.

The reaction does not take place below pH 5.5, and its rate rises rapidly above pH 6.0. This reaction is quite distinct from the decomposition

Table 5. *Chromatographic behaviour of products formed from S-ethoxycarbonylcysteine with diazomethane and from cysteine methyl ester with N-ethyl-N-nitrosourethane and with ethyl chloroformate*

Solvents: A, butanol-acetic acid; D, butanol-water. a, Ninhydrin; b, nitroprusside; c, platinum reagent.

	Mean R_F		Spots detected with location reagents
	Solvent A	Solvent D	
<i>S</i> -Ethoxycarbonyl-cysteine + diazomethane	0.56*		a
	0.73†		a
Cysteine methyl ester + NMU	0.16*		a b
	0.27*	streak	a b
	0.73†	0.79†	a
	0.93†	0.95*	b c
Cysteine methyl ester + ethyl chloroformate	0.17*		a b
	0.26*	streak	a b
	0.73†	0.79†	a
	0.93*	0.95*	b c

* Faint spot, not detected in all experiments.

† Main spot.

Table 6. *Chromatographic behaviour of products of interaction of glutathione with N-methyl-N-nitrosourethane, diazomethane, ethyl chloroformate and of S-methylglutathione with diazomethane and effects of alkali and of hydrogen peroxide*

Solvents: A, butanol-acetic acid; C, propanol-ammonia. a, Ninhydrin; b, nitroprusside; c, platinum reagent.

	Mean R_F		Positive reaction with location reagents	Spot considered to represent:
	Solvent A	Solvent C		
Glutathione + NMU	1 0.08*	0.10	a b	GSSG
	2 0.24*		a b	GSH
	3 0.28†	0.49†	a	S-Methylglutathione
	4 0.44†	0.55†	a	S-Ethoxycarbonylglutathione
	5 0.58*	0.65*	a	S-Ethoxycarbonylglutathione
	6 0.66*	0.77*	a	methyl ester
(Glutathione + NMU) + dilute ammonia	0.06		a b	
	0.25†		a b	
	0.28†		a	
	0.44*		a	
	0.80		b c	
(Glutathione + NMU) + hydrogen peroxide	0.08		a	
	0.13†		a	
	0.44†		a	
Glutathione + ethyl chloroformate	0.10*	0.10*	a b	
	0.24*	0.10*	a b	
	0.44†	0.55†	a	
	0.80*		b c	
Glutathione + diazomethane	0.08		a b	
	0.25*		a b	
	0.28†		a	
	0.44*		a	
	0.58*		a	
S-Methylglutathione	0.28	0.49	a	
S-Methylglutathione + hydrogen peroxide	0.13		a	
S-Methylglutathione + diazomethane	0.08		a	
	0.17*		a	
	0.28*		a	
	0.44†		a	
	0.59†		a	

* Faint spot, not detected in all experiments.

† Main spot.

of *N*-alkyl-*N*-nitrosourethanes by alkali, which leads to the evolution of diazoalkanes (Pechmann, 1895), and which does not take place below pH 8.8.

The course of the reaction and the products formed appeared to be the same with phosphate buffer used to provide pH stability as in its absence.

The products formed with both cysteine and glutathione comprised *S*-ethoxycarbonyl and *S*-alkyl derivatives and their alkyl esters. The actual species of the alkylating entity was not established, nor is it known whether diazoalkane was an intermediate. Until the actual species of the alkylating entity formed is established, it will be referred to as 'active alkylene'. It alkylates readily both intra- and inter-molecularly and leads to formation of a complex mixture of products.

The relative quantities of the various products, formed when cysteine interacted with alkylnitrosourethanes, depended on conditions such as the pH, ratio of the reactants and also on the time which elapsed between the reaction and the detection or the isolation of the products.

Below pH 7.0 the *S*-ethoxycarbonyl derivatives predominated; with the increase in pH, the ethoxycarbonyl group migrated from the sulphur to the nitrogen atom of the amino group. This isomerization took place very readily with cysteine, probably because of spatial proximity of the thiol and amino groups. It was observed also with glutathione.

Similar isomerizations under alkaline conditions of the very readily formed sulphur derivatives to

the more stable nitrogen derivatives have been observed by Wieland (1954) and more recently have been reported for *S*-(2,4-dinitrophenyl)-cysteine (Burchfield, 1958) and for *S*-benzyloxycarbonylcysteine (Sokolovsky, Wilchek & Patchornik, 1964).

When the reaction between cysteine and MNU was performed above pH 7.0 the ethoxycarbonyl group primarily attached to the sulphur tended to migrate to the nitrogen and the restored free thiol was either alkylated by the 'active alkylene' to form *S*-alkyl-*N*-ethoxycarbonylcysteine and its ester, or interacted with another molecule of MNU to form *NS*-diethoxycarbonylcysteine.

The alkyl esters of the substituted cysteines were rather unstable and hydrolysed on standing at room temperature, even at neutral pH. This increased rate of hydrolysis is similar to the greatly accelerated rate of hydrolysis of esters having amino, carbonyl or hydroxyl as neighbouring groups (Bernhard *et al.* 1962; Shalitin & Bernhard, 1964*a,b*).

The action of diazomethane on cysteine gave also mixtures of products. This is not surprising. Cysteine contains four hydrogen atoms capable of substitution, and could form 11 methylated products: three isomers monosubstituted; four disubstituted; three trisubstituted; one tetrasubstituted. In addition, other possibilities such as the formation of methylene bridges between molecules of cysteine (as in djenkolic acid) cannot be excluded. In experiments in which diazomethane was added gradually to a solution of cysteine, a few drops at a time (Table 3), and the reaction mixtures were tested by paper chromatography after each addition, evidence was obtained that the compounds first formed were *S*-methylecysteine and cysteine methyl ester. The additional spots appearing later have not been identified. However, even after the addition of excess of diazomethane a mixture of products was present, giving a number of spots on paper chromatography, instead of a single spot expected from a fully methylated product. This might have been due to the tendency to hydrolysis of the esters of substituted cysteines.

Because of the complexity of the reaction mixtures and the instability of some of the products formed, the reaction of diazomethane with cysteine and its derivatives could not be used for preparative purposes. Nevertheless, the reaction helped in the identification of several of the products formed by the action of MNU on cysteine. The same was true in the reaction of diazomethane with glutathione.

Since thiols react non-enzymically with nitroso compounds and with diazomethane the results *in vitro* may represent models for the events *in vivo*. *S*-Methylcysteine and its methyl ester and *S*-methylglutathione and its methyl ester were the common products formed from the parent thiols with both MNU and diazomethane. Both these carcinogenic agents gave complicated mixtures of products when they acted on pure cysteine *in vitro*. It could be expected that when interacting with tissues *in vivo* they would produce much more complicated mixtures of products, which might present great difficulties to disentangle.

Our thanks are due to Dr J. J. Roberts and Dr G. P. Warwick for the gift of *S*-ethylcysteine and to Berks Pharmaceuticals Ltd. for the hydrochloride of cysteine methyl ester. Microanalyses were by Mrs G. Ostler.

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