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The Splitting of some Thio-ether Linkages by Silver Salts under Mild Conditions

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In the attempt to decide (Peters & Wakelin, 1941, 1947) whether the compound of mustard gas and kerateine contained thio-ether linked (=C-S-C=) mustard gas, a new reaction was discovered; by suitable treatment at room temperature with Ag or Hg salts and adjusting to an alkaline reaction, some thio-ether linkages which are otherwise stable can be split. This splitting can be shown by a special application of the nitroprusside test.

EXPERIMENTAL AND RESULTS

Nitroprusside reactions

In order to throw light upon the finding that the nitroprusside reaction was reduced in the mustard

gas-kerateine compound as compared with kerateine itself, we studied the type of reactions given by nitroprusside with several sulphur compounds. There can be distinguished five types of nitroprusside reactions given by sulphur compounds upon addition of alkali with or without cyanide (0.05 ml. 20% KCN added to 0.5-1.0 ml. test solution) followed by 0.05 ml. of 5% sodium nitroprusside (NP). These are listed in Table 1.

To obtain convincing evidence of the splitting of a thio-ether linkage, it is necessary to find a compound which gives no (b), (c) or (d) reaction, but a powerful type (e) reaction. The colour must be strong since it is difficult to be certain that a rather weak type (d) reaction is not due to disulphide impurities.

Table 1. *Types of nitroprusside reaction*

	Reaction conditions	Colour development	Reagents
(a)	Cool	Immediate	Dilute ammonia + NP
(b)	Cool	Immediate	Cyanide + NP
(c)	Cool	Delayed	Cyanide + NP
(d)	Warm	Immediate	Cyanide + NP (see note 2)
(e)	Cool	Immediate	AgNO ₃ or Hg(OAc) ₂ + Cyanide + NP (see note 3)

Note 1. The use of cyanide was described by Walker (1925) for S—S groups, who stated that cystine might take 20 min. to reach maximum colour. If the cyanide is added and the solution allowed to stand for 15–45 min., immediate reaction occurs on addition of nitroprusside; sometimes reduction takes more than 30 min. In contrast, substances like dithiodiglycol react immediately (Type b).

Note 2. This is a variant of (c); for differentiation it is better to use 5–10% cyanide. The solution can be warmed to 90° and cooled, or placed in the water-bath at 50° for 10–15 min. (best stoppered).

Note 3. The test solution is treated with AgNO₃ or mercuric acetate, brought slowly to pH 7.5–8.0 by 0.1N-NaOH, and shaken for 3–5 min. Cyanide + NP are then added.

To the best of our knowledge (e) is a new reaction. It has not yet been thoroughly explored using metals other than Ag and Hg. It is given by cystine, due presumably to the intermediate formation of mercaptide.

Behaviour of some thio-ether compounds

The compound (I) has been found to satisfy the above conditions. It is 2,2-bis-(2'-amino-2'-carboxyethylthio)-diethyl sulphone, obtained by interaction of divinyl sulphone and cysteine, or from mustard gas sulphone (Ford-Moore, 1940). A specimen was kindly given to us by Mr A. H. Ford-Moore.

When an aqueous solution of (I) is treated with cyanide and nitroprusside, there is no magenta reaction; even after warming, this is very slight. When, however, the compound is treated with AgNO₃ followed by NaOH to pH 7.8 with shaking, an immediate strong reaction is given, equivalent in intensity to about 50% of its weight of cysteine. Controls showed that a concentration of (I) twenty times that of cysteine does not interfere with a cyanide + NP reaction for cysteine. The reaction occurs equally well *in vacuo*. It may be regarded as a hydrolysis as it involves the addition of water.

The cysteine formed from substance (I) can be isolated as cystine after a silver treatment. The AgNO₃ complex with (I) made at slightly acid reaction can be separated at pH 7.0 by centrifuging. After washing and suspending in water and addition of cyanide + nitroprusside, it gives no nitroprusside reaction. If, however, after suspension in

water, it is shaken with gradual addition of NaOH to c. pH 8.0, it then gives the usual nitroprusside reaction with cyanide; hence in the Ag complex the sulphur linkage is more sensitive to the reaction of OH ions.

That cysteine is reconstituted in this reaction and that the cyanide takes no part in the hydrolysis we have proved as follows: 50–100 mg. of compound (I) are treated in 5 ml. of water with 5 ml. of 10% AgNO₃. At first, the addition causes solution; with continued addition a silver complex separates, which is washed after centrifuging. The silver content was 32.5%, slightly over 2 atoms/molecule of compound I. To obtain more complete splitting, the pH must be made more alkaline than that at which the nitroprusside reaction is detectable. The Ag complex is therefore treated with NaOH to pH 8.5, thoroughly shaken and allowed to stand about 5 min. The pH is then adjusted to pH 3.5 with N-HCl and the ppt. is washed with dilute HCl at this pH. The ppt., containing silver and cysteine, is suspended in 3–4 ml. water (pH 3.0) to which are added 50–100 mg. NaI, pH 3.0. Successive treatments with NaI in 3–4 ml. water will eventually liberate cysteine in solution; 0.05–0.1 ml. of each extract is tested, after centrifuging, for cysteine with addition of cyanide + nitroprusside. The first and second extracts may contain no cysteine and may be discarded. (That an actual SH rather than S—S compound is present is proved by tests with nitroprusside and ammonia alone.) The extracts are concentrated to dryness *in vacuo* (35–40°), treated with ammonia and left to oxidize in air. Ammonia is removed *in vacuo*, and the ppt. washed with ice-cold water; after centrifuging, the ppt. is then redissolved in ammonia and the cystine formed by oxidation allowed to crystallize gradually. Hexagonal plates of cystine are obtained by dissolving in acid and reprecipitating by addition of NaOH to pH 3.0; the mother liquor gives the typical slow reaction of Walker (1925) for cystine upon addition of cyanide + NP, and also the Sullivan (1929) reaction. No reaction for H₂S was obtained at any stage with lead acetate paper. After purification the yield of cystine is approx. 10% of the original weight of the compound.

We have similarly isolated cystine from (2-amino-2-carboxyethylthio)-ethylphenyl sulphone

prepared by Mr Ford-Moore at Porton; the procedure was the same except that we were unable to prepare a satisfactory silver complex (so having to use an excess of AgNO₃), and that a more alkaline pH was necessary. We treated 30, 100 and 500 mg. batches with excess of AgNO₃ at pH 4.5, followed by addition of 0.1N-NaOH to c. pH 10.0. The losses were considerable in purification, which was carried out with some modifications of the procedure described above. Some of the cysteine is lost, presumably at the silver complex stage. In this con-

nexion, it may be noted that Vickery & Leavenworth (1930) state that cystine, when precipitated as the Ag salt and brought to pH 6.0, consists largely of a Ag-cysteine compound giving the colour reaction for cysteine, but in addition, reduction is accompanied by oxidation giving, among other products, ammonia and cysteic acid. Further work upon this reaction is described elsewhere (Ford-Moore, Peters & Wakelin, 1942).

Some applications of the silver reaction for thio-ethers

As previously mentioned it can be shown by the use of this reaction that some thio-ether linked sulphur is present in mustard gas-kerateine (Peters & Wakelin, 1947). Some product formed upon treatment of cysteine with mustard gas also gives the type (e) nitroprusside reaction; this has not been isolated, but we presume it to be the half product;* it is left in solution after precipitation of the crystalline dicysteinyl mustard gas product 2:2-bis-(2'-amino-2'-carboxyethylthio)-diethylsulphide. The latter gives a faint type (e) reaction; this was confirmed upon a pure specimen of the bis-compound kindly sent to us by Dr Hellerman.

Preparation of mustard gas-cysteine product. Using Ogston's (1941) modification of the technique of Peters & Walker (1923), excess of mustard gas dissolved in ethanol was added gradually at room temperature to cysteine-HCl (50 mg.) dissolved in 40 ml. water containing a trace of bromothymol blue as indicator; precautions were taken to avoid oxidation by bubbling N₂ through the reaction vessel. NaOH solution (0.2N) was added at intervals to maintain the pH at c. 7.4. On completion of the reaction SH had disappeared (as shown by the absence of type (a) reaction). The type (e) reaction with Ag salts was strong, giving a colour intensity about 50% of that given by an equal weight of cysteine. Care must be taken to avoid excessive alkalinity as this thio-ether linkage is evidently more easily hydrolyzed by alkali alone than in the case of the sulphone compounds.

Reactions of other thio-ethers. Qualitative reactions with silver salts have been found with other substances containing the thio-ether linkage, though there is much quantitative difference in the splitting. With methionine, rather special treatment is needed. To 0.5 mg. or more of methionine in 0.2 ml. water, 0.05 ml. 10% AgNO₃ is added and 0.1N-NaOH drop by drop with constant shaking as the precipitate separates; cyanide and nitroprusside are then added.

Reactions have been obtained with *S*-carboxymethyl cysteine kindly given by Prof. F. Dickens. The reaction with diisopropyl sulphide was slight

* After completion of the above work, and after submission of this paper for publication, we had the opportunity of testing the half product mentioned, viz.

kindly given by Drs Kinsey and Grant; we can confirm that this compound gives the silver reaction.

and very transient. Pure specimens of thiodiglycol give no splitting with silver; impure specimens usually give a type (c) nitroprusside reaction because they contain dithiodiglycol as an impurity, as was shown by Peters & Walker (1925); 2:2'-dichlorodiethyl sulphone gives no reaction.

Of several other sulphur compounds tested, for which we are indebted to Dr du Vigneaud, using 0.5 mg. for each test, *dl*-cystathionine and *d*-lanthionine gave faint silver reactions; no reaction was given by *l*-djenkolic acid, *d*-*S*-benzyl homocysteine or *l*-*S*-benzylcysteine.

DISCUSSION

Some thio-ether linked compounds are known to be degraded in varying degree after drastic treatment with alkali (Schönberg & Iskander, 1942); compounds of thiol acids with aldehydes which readily dissociate in alkaline solution have long been known (Schubert, 1935, 1936). The compounds studied here are much more stable. Apart from the chemical interest and possible applications of our silver reaction, we feel that it presents an instance in which the thio-ether linkages can be split at comparatively low temperature near the biological pH. The mechanism of the reactions appears to be the formation of a silver complex, which is more susceptible to splitting by alkali.

This paper embodies the gist of two reports nos. 39 and 53 sent to the Ministry of Supply by Peters in 1941 and 1943.

SUMMARY

1. The compound of divinyl sulphone and cysteine, viz. 2:2-bis-(2'-amino-2'-carboxyethylthio)-diethyl sulphone (made by Ford-Moore) gives no nitroprusside reaction by treatment with cyanide; if, however, it is treated at room temperature with a silver salt at acid pH and the resultant mixture made slightly alkaline, a nitroprusside reaction is given in presence of cyanide. In this new reaction of silver salts it has been proved that cysteine is regenerated from the thio-ether linkage.

2. The reaction has been used as supporting evidence for the presence of some thio-ether linked mustard gas in mustard gas-kerateine.

3. The reactions of some other sulphur compounds have been investigated.

This work was carried out as part of a programme of research for the Ministry of Supply directed by Prof. R. A. Peters. We are grateful to Mr A. H. Ford-Moore, Experimental Station, Porton, and to others mentioned in the text for the provision of compounds. We are grateful also for valuable criticism to members of the team, and to Dr Whittaker for the specimen of monothioethylene glycol. We are indebted to the Director General of Scientific Research (Defence), Ministry of Supply for permission to publish this work.

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1:2:4-Fluorodinitrobenzene

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During the war we carried out extensive investigations on the synthesis and physiological properties of organic compounds containing fluorine (McCombie & Saunders, 1941, 1946 *a, b, c*). In connexion with this work we had occasion to prepare 1:2:4-fluorodinitrobenzene, and as several workers have recently used this compound for other purposes (e.g. Sanger, 1945), its preparation is now given in detail.

1:2:4-Fluorodinitrobenzene was first prepared by Holleman & Beekmann (1904) by nitrating *p*-fluoronitrobenzene which in turn was prepared from *p*-nitroaniline. Later, Gottlieb (1936) obtained it by the action of potassium fluoride on 1:2:4-chlorodinitrobenzene. Neither method is entirely satisfactory. The second method is obviously more direct, but as described by Gottlieb is laborious. It consists in heating 1:2:4-chlorodinitrobenzene dissolved in nitrobenzene with potassium fluoride (2/3 equiv.), cooling, adding more potassium fluoride, reheating, cooling, filtering, and then repeating the whole procedure twice more, thus using 4 equiv. of potassium fluoride. Gottlieb maintains that this procedure is essential, otherwise the potassium fluoride becomes coated with potassium chloride and the reaction stops. We have found, however, that the method can be greatly improved as described in the experimental part. Only 2 equiv. of potassium fluoride are required, and in particular the intermittent additions and filtrations are unnecessary.

Pure 1:2:4-fluorodinitrobenzene is a colourless liquid which darkens slowly on standing, and solidifies when cooled in ice water. In aliphatic compounds containing the C-F link, the fluorine atom

is usually less reactive than the chlorine atom in the corresponding chloro compound. In 1:2:4-fluorodinitrobenzene, however, the fluorine atom is more reactive than the chlorine atom in 1:2:4-chlorodinitrobenzene towards many reagents.

Whereas methyl fluoroacetate is a powerful convulsant poison (McCombie & Saunders, 1946*c*), 1:2:4-fluorodinitrobenzene is found to be relatively non-toxic, and does not exhibit any fluoroacetate-like activity. 1:2:4-Fluorodinitrobenzene, however, shows slight vesicant action on human skin, and in this respect it resembles 1:2:4-chlorodinitrobenzene. This vesicant action, though slight, persists with some individuals for a considerable time, therefore the compound should be handled with care.

EXPERIMENTAL

Preparation of 1:2:4-Fluorodinitrobenzene

1:2:4-Chlorodinitrobenzene (101 g., 0.5 mol.) was dissolved in dry nitrobenzene (101 g.) and to this solution was added dry, finely powdered KF (60 g., 1 mol.) and the mixture heated to 190–195° for 5 hr., under reflux, with vigorous stirring. The product was then cooled and filtered from inorganic salts, which were washed with hot toluene. The combined toluene filtrates were dried overnight over anhyd. Na_2SO_4 . After filtering off the Na_2SO_4 , the toluene was distilled off on the water pump and the residue fractionally distilled. The fraction of b.p. 136–138°/2 mm. was collected (yield 66 g., 71%). This sample was sufficiently pure for most purposes. A second fractionation gave a product which was entirely chlorine free and solidified readily on being cooled in ice water.

The fluorine was estimated by the method of Chapman, Heap & Saunders (1947). Found: F, 10.03; 10.38. Calc. for $\text{C}_6\text{H}_3\text{O}_4\text{N}_2\text{F}$: F, 10.2%.