SUMMARY

1. Some dithiocarbamates and dithiophosphonates potentiate the toxic action of mustard gas upon the pyruvate oxidase system; the effect of diethyl- (or diethanol-) dithiocarbamate has been closely examined.

2. Only compounds having a Cl atom which behaves kinetically as in mustard gas are potentiated; the effect is quickly established.

3. Potentiation is due to the formation of the half product of mustard gas with diethyldithiocarbamate (compound (I), 2-chloroethyl-2-(*N*-diethyldithiocarbamate)-ethyl sulphide), prepared crystalline under the conditions of the experiment. This is much more toxic than either of its components.

4. The toxicity of (I) is not due to the Cl group; the hypothesis is advanced that the $=N.C \begin{smallmatrix} S \\ \diagup \\ S- \end{smallmatrix}$ group is inherently toxic.

5. The bis-substitution product was only slightly toxic, which is explained by its inability to reach the active enzyme centre in the brain *brei*.

6. (I) is not toxic when injected into rats. This is interpreted as due to failure to reach the pyruvate system *in vivo*.

This work formed part of a programme of research carried out for the Ministry of Supply and reported in 1943; we are indebted to the Chief Scientific Officer, Ministry of Supply, for permission to publish. We are also grateful to other members of the team for help; and to Mr Philpot and Dr Stocken for thiophosphonates.

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Observations upon a Compound of Mustard Gas and Kerateine

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(Received 20 December 1946)

The fact that mustard gas will combine with some proteins is known from the work of Berenblum & Wormall (1939) on serum proteins, Berenblum (1940) on nucleoproteins, Pirie (1941) on collagen, and may be inferred also from the work of Fell & Allsopp (1940-1). Since enzymes are proteins, such combination is indicated from enzyme studies in Oxford (Peters & Wakelin, 1940) and Cambridge (see summary by Dixon & Needham, 1946), as also from kinetic considerations of the reactions of

mustard gas (Holiday, Ogston, Philpot & Stocken, 1940; Ogston, 1941).

Earlier work in this laboratory (Peters & Walker, 1924-5) had shown that mustard gas did not abolish the fixed SH reaction (Hopkins & Dixon, 1922) of extracted muscle tissue though dichlorodiethyl sulphone and phenyldichloroarsine readily did so. This is rather puzzling because mustard gas so readily combines with simple monothiols (Holiday *et al.* 1940; van Heyningen, 1941). The original object of

this work (Peters & Wakelin, 1941) was partly to determine whether mustard gas combines with the SH groups in protein products containing these and partly to provide material for experiment upon attempts to reverse any such combination.

EXPERIMENTAL

Kerateine, the soluble SH protein formed from keratin (Goddard & Michaelis, 1934) by reduction with alkaline cyanide, was used, owing to its relatively high content of SH and its successful use by Stocken & Thompson (1940, 1946). Upon the advice of Dr Thompson we used wool instead of human hair, which gave almost colourless products. The mixture containing the wool (100 g.) after digestion for 2–3 days in alkaline cyanide at 38° was acidified with acetic acid to pH 5.0 ± 0.3 . The ppt. was removed on a hardened filter paper, washed once with water and stirred into 1.7M-phosphate buffer (pH 7.4), alkali being added to bring the solution to pH 7.4 (total vol. c. 400 ml.). Glass-distilled water was used throughout to exclude the oxidizing effect of traces of metals. To reduce risk of oxidation of SH groups, the precipitation and solution at pH 7.4 were left until the last; 100 ml. were removed before proceeding further to act as control. The remaining 300 ml. were stirred in a flask under N_2 and treated with mustard gas dissolved in absolute ethanol (14% v/v or c. 20% w/w). This solution (25–30 ml. containing 5–6 g. mustard gas) was gradually stirred in over 0.75 hr. at 25°; slow stirring was continued for c. 1 hr. At the beginning, small samples gave a strong immediate nitroprusside reaction; later this became rather slight, and not much increased by cyanide addition, indicating a genuine reduction in SH-yielding groups. The final pH was about 7.0. The preparation was cooled and brought to pH 4.8 with acetic acid; the ppt. was filtered off, washed with 0.01N-acetate at pH 4.8 and then with successive portions of acetone; the first acetone washings usually contained detectable amounts of mustard gas. When there was no substance forming acid in presence of water in the acetone washings, the ppt. was transferred to a centrifuge tube, washed again with acetone and finally dried *in vacuo*. The final product was a fine, white, fluffy powder. In order to free it from the last traces of inorganic chloride under conditions least likely to affect the hydrolyzable organic chlorine, the product was purified by stirring up in minimal amounts of ice-cold water to which c. 2.5 ml. 20% NaOH were gradually added to bring to pH 7.4 (if moistened first with gradual addition of alkali, a better solution was obtained); the volume was finally adjusted with ice-cold water to 100–150 ml.; c. 1.8 ml. glacial acetic acid were then added to reprecipitate the 'mustard gas-kerateine' (pH 4.5–5.0). The ppt. was centrifuged in two 100 ml. tubes, washed first with dilute acetic acid (2 drops glacial acetic acid in 800 ml. water, once with acetone (100 ml.) and stirred with cool acetone for at least 2 hr., before drying after a further extraction with 100 ml. acetone.

In our experience, the final extraction by stirring in cold acetone is essential to free from the last traces of mustard gas, and Soxhlet extraction with ether is not so effective. Some preparations which had been well extracted with acetone, proved to be still irritant to the arm of a sensitive observer when 20 mg. powder was worn under cellophan for 2 hr. on the forearm. After thorough extraction by

stirring, for a fresh extracted preparation, this irritancy disappeared.

Eight preparations were made upon the above lines, the last two by the exact technique described above; the yields of mustard gas-kerateine have varied from 15 to 20 g./100 g. wool. The control preparations were precipitated in a similar way from solution containing an amount of NaCl equivalent to the total amount of hydrolyzable Cl available from the mustard gas added in the 'mustard gas-kerateine' samples, and then worked up in an exactly similar manner.

RESULTS

S and Cl content. In all cases, the S content of the treated protein did not increase beyond twice that of the control. While the Cl content of the control has been practically negligible, it has been found as high as 0.36% in the 'mustard-treated' kerateine. Analytical values by Carius's method are given in Table 1 for the last two experiments; Exp. K must be considered the more accurate.

Table 1. *Sulphur, chlorine and nitrogen content of kerateine and mustard gas-kerateine*

(Results as % of protein wt., 0.5 g. samples used for analysis.)

Pre- paration		Mustard gas- kera- teine	Control kera- teine	Differ- ence	Ratio (extra S/ extra Cl)
G	Cl	0.355*	0.03	+0.325	—
	S	2.23	1.38	+0.85	2.9
K	Cl	0.349	0.022*	+0.329	—
	S	2.29	1.21*	+1.08	3.6
	N	14.3	14.9	-0.6	—

* Average of duplicate estimations.

The S content of the final product seems to approach 2.3% and the Cl content 0.35% rather constantly under these conditions; about one-third of the extra S must be present in the form of the 'half product' (kerateine- $CH_2CH_2SCH_2CH_2Cl$).

Porphyridin titration of kerateine and mustard gas-kerateine. A more quantitative proof of the diminution of SH and S—S groups in mustard gas-kerateine is obtained by titrating with porphyridin (1 mg./ml., standardized with cysteine ester) after conversion of S—S to SH in the protein by reduction with monothioethylene glycol at pH 7.3.

After solution at pH 7.3, the protein was treated *in vacuo* in 0.25M-phosphate buffer for 2 hr. at 38° with monothioethylene glycol (20 mg./ml.); it was then rapidly reprecipitated at pH 4.5 by addition of N-HCl, and washed with dilute acetic acid until the centrifugate no longer gave the SH reaction (four washings necessary). NaCl (15%) at pH 4.5 (acetic acid) had to be used at this stage to keep the protein out of solution. When washing was complete, the protein was quickly redissolved in phosphate buffer (pH 7.2) and titrated with porphyridin until the nitroprusside reaction disappeared. All the solutions were made with glass-distilled water, boiled and cooled before use to remove

O₂. The porphyrindin and cysteine ester were as used by Stocken & Thompson (1940) and kindly supplied by Dr Stocken.

Table 2 gives the last of three experiments, which consistently showed that the mustard gas-kerateine contains definitely less S—S + SH groups than the control.

Table 2. *Total SH groups present in kerateine and mustard gas-kerateine as determined by porphyrindin titration*

(Results before and after treatment with mono-thioethylene glycol.)

	Sulphydryl-S (%)	
	Before treatment	After treatment
Kerateine	0.12	0.34
Mustard gas-kerateine	Nil	0.11

The conditions of reduction were more severe than those of Mirsky & Anson (1934) for egg albumin; they used thioglycolic acid at 30° for 45 min. The value obtained by Stocken & Thompson (1940, 1946) for SH initially present was c. 0.39 %, approaching our value of 0.34 %. Nitroprusside reactions in presence of cyanide, which therefore include S—S groups, were in agreement with the titration values. We interpret this to mean that about one-quarter of the mustard gas has combined with available SH groups; the remainder must be in combination with other groupings. Some of the kerateine sulphur will be unavailable initially owing to conversion to —SCN by the cyanide used in preparation. (Since these experiments were completed, there is evidence in the work of Northrop (1943–4), Bailey, Banks, Bournsnel, Francis, Webb, Wormall & Dixon (1945), and Banks, Bournsnel, Francis, Hopwood & Wormall (1946) using radioactive sulphur, that mustard gas combines with carboxyl groups in proteins.)

The presence of thio-ether-linked mustard gas was confirmed by the use of the new silver reaction (Peters & Wakelin, 1942, 1947); mustard gas-kerateine gave an intensification of the cyanide nitroprusside reaction after silver treatment, which was not given by kerateine. Furthermore, from a fraction obtained after enzymic digestion (Exp. I below) the silver reaction indicated the presence of thio-ether-linked sulphur.

Experiment 1. Mustard gas-kerateine was thoroughly digested with pepsin followed by trypsin, giving a soluble preparation not entirely free from biuret-yielding substances. The digest was first treated with cuprous chloride in acid solution to precipitate S—S and SH compounds (following Hopkins's method for isolating glutathione as applied in quantitative work by Roussouw & Wilkin-Jorden (1935)); after removal of excess Cu with H₂S, the filtrate was treated with mercuric acetate at pH 4.5 ac-

cording to the standard technique for precipitating methionine (Hill & Robson, 1934). After removal of Hg from the ppt. with H₂S, the substance present, which was not further isolated, resembled mustard gas-cysteine giving the faint cystine-like reaction with cyanide and a strong 'silver' reaction (type (e) reaction of Peters & Wakelin (1947)); the filtrate was inactive. No Sullivan reaction (for cysteine and cystine) was given by this fraction from the mercuric ppt. even after an acid hydrolysis, though glutathione when similarly hydrolyzed gave the reaction strongly. It was found that care had to be taken not to treat the thio-ether fractions with the metallic precipitant in neutral solution, otherwise it tended to give cystine-like reactions.

Reactive chlorine in mustard gas-kerateine. The presence of Cl in organic combination was shown by hydrolysis experiments.

Two 2.0 g. samples of mustard gas-kerateine were dissolved in 50 ml. ice-cold water at pH 7.4, and incubated at 38° for 1 and 3 hr. respectively. They were then cooled, reprecipitated and dried with acetone as before. Analytical values for Cl are shown in Fig. 1. These values confirmed and extended a previous experiment in which a similar treatment of 2 hr. had reduced the Cl content of the ppt. to 30 % of the original. It seems clear that the Cl present is hydrolyzable, but much more slowly than the Cl of mustard gas itself (Peters & Walker, 1923); all would not be expected to have disappeared at 38° under 6 hr.

From the above we feel justified in concluding that in addition to any kerateine-CH₂CH₂SCH₂CH₂OH there is an organic chloride present in mustard gas-treated kerateine, and that this is most likely to be a slowly hydrolyzing half product: kerateine-CH₂CH₂SCH₂CH₂Cl. From this it follows that fairly stable half products of mustard gas with proteins can be formed, and must be reckoned with in a final picture of the action of mustard gas on tissues, because they could link up over a period of hours with other protein tissue components.

Properties of mustard gas-kerateine. The powder almost completely dissolves at pH 7.4 to form an opalescent solution, provided that it is dissolved as recommended. The control kerateine is a similar white powder, more completely soluble at pH 7.4. Using as a measure the liberation of N not precipitated by trichloroacetic acid, both were digested by pepsin at equal rates, so that no linkage attacked by pepsin has been upset in the combination with mustard gas.

Toxicity of mustard gas-kerateine

We have looked for any toxicity by (a) effects upon enzymes, (b) effects upon rats and (c) tests upon human skin.

(a) *Effects upon enzyme systems.* Two were tried, the succinoxidase and the pyruvate oxidase systems. In the former, only slight toxic non-specific effects were noticed, changes not exceeding 15 % for amounts of 5–10 mg./3 ml. (Table 3).

Table 3. *The effect of kerateine and mustard gas-kerateine upon the succinoxidase system* (heart muscle)*

Additions	O ₂ uptake	Change 50 min. (%)	Difference (%)
	Exp. 1		
Succinate only	309	—	—
+ kerateine (5 mg.)	279	-10	—
+ mustard gas-kerateine (5 mg.)	246	-20	-10
	Exp. 2		
Succinate only	277	—	—
+ kerateine (10 mg.)	248	-10	—
+ mustard gas-kerateine (10 mg.)	210	-24	-14

* We are indebted to Prof. Keilin for this preparation (Keilin & Hartree, 1940) and for cytochrome *c*. We added 0.1 ml. cytochrome *c* for these experiments and used 0.1 ml. succinic dehydrogenase preparation/3 ml. (pH 7.4).

For the pyruvate oxidase system pigeon brain dispersion was used (Peters & Wakelin, 1946) as it was unlikely that the mustard gas-protein product would penetrate a *brei*. The main effect of kerateine

it was purely an effect in presence of the pyruvate; there was no action on the residual respiration. There is a lower total O₂ uptake for mustard gas-kerateine as compared with kerateine, amounting to -10% in one experiment over a 50 min. period and -15% in another experiment (Fig. 2). This is a small effect, because the 10 mg. could not possibly contain more than 100 µg. mustard gas as a 'half product' which is practically without effect upon this enzyme; it is of a different order of toxicity from that observed for really toxic substances such as the sulphones.

Briefly, these enzyme tests did not reveal any toxicity of the mustard-treated product. The stabilization effect is likely to be due to the SH groups present in the kerateine.

(b) *Effects upon the rat.* The compound was fed to a growing rat (96 g.) by replacing 1 g. casein by 1 g. of the compound in each 10 g. of a diet containing 60% rice starch, 20% casein, 5% salt mixture, 2% agar agar, 10% dried yeast, 3% cod-liver oil. A control rat was fed with kerateine in a similar basal diet. Though the rat which was fed the compound did not eat this well and lost weight (13 g. during 5 days), at least 1.0 g. of the product was

Fig. 1.

Fig. 1. Hydrolysis of mustard gas-kerateine. 2.0 g. samples suspended in 50 ml. water pH 7.4 at 38°. Curve gives percentage Cl in the dried protein.

Fig. 2.

Fig. 2. Increase in O₂ uptake of pigeon brain 'dispersion' in presence of pyruvate (curve A) by addition of mustard gas-kerateine (curve B) or kerateine (curve C).

and mustard gas-kerateine was a remarkable stabilization of this rather labile enzyme system (see Fig. 2) leading to a total increase in O₂ uptake over the period studied. This is of interest, as it is the first time that we have observed such an action with brain dispersion. It was particularly significant that

consumed, equivalent to c. 50 mg. of mustard gas (calculated on the S content). The process of mixing the diet was carried out quickly and water reduced to a minimum to minimize possible hydrolysis of the 'half product'.

Dr Short (Prof. Cameron's Department, Experimental Station, Porton) reported (private communi-

cation) that there was no macroscopic or microscopic abnormality in the stomach. The fall in weight was due to low consumption of the diet as a result of the unappetizing character of kerateine, as the control rat also lost weight. Though only one rat was used, the result is regarded as valid evidence of absence of mustard gas toxicity in the dose consumed as even small amounts are detectable histologically. (Subsequent to this work, Kinsey & Grant (1946) fed mustard gas-casein to rats and observed no toxic effect, though the rats lost weight unless the diet was supplemented with methionine and threonine).

Dr Holiday, to whom we are grateful, injected 10 mg. of the compound in saline at pH 7.4 intraperitoneally into a rat without adverse results.

(c) *Behaviour in human skin.* The behaviour of these products was peculiar. Tests were done upon two human subjects both reacting with E⁺ (raised erythematous patches 3 × 2.5 cm.) to 1/140 ml. of 1/1000 mustard gas in benzene; in every case 20 mg. of the powder was worn under a patch of cellophan 3 cm. diam., for periods of 2.5 hr. unless otherwise stated.

Kerateine itself was quite non-irritant, applied both in the dry state, even for 24 hr., and as a moist patch. Mustard gas-kerateine was never irritant after recent extraction by stirring for at least 2 hr. at room temperature with acetone. On at least three occasions, however, preparations which had been stood in a desiccator for from 4 to 6 weeks proved irritant (E⁺) upon a retest. In the first instance, this was considered to be due to improper extraction and a re-extraction gave a non-irritant powder which did not subsequently become irritant. On a second occasion, a preparation had acquired irritancy (removed by acetone extraction); this was believed at the time to be due to standing (though in a corked tube) in a desiccator, in which were other preparations containing small amounts of mustard gas. On a third occasion, the same preparation (known to have been freed from irritancy (2.5 hr. test) by second extraction with acetone) upon a retest 6 weeks later in which the patch was accidentally worn for 12 hr., gave an E⁺ patch, 3.8 × 4 cm. and again, for 2.5 hr., E, 3 × 3 cm. Hence we believe that the effect is genuine. Its extent must not be exaggerated; this recovery of irritancy does not correspond to more than 5 µg. as mustard gas in the 20 mg., i.e. 1/4000; but the repetition of the phenomenon is interesting and, if occurring *in vivo*, it would be important.

DISCUSSION

Though some mustard gas combined with the SH, the behaviour of the SH groups in kerateine to mustard gas is different from that found by Stocken & Thompson (1940) for the arsenicals, which attacked

the SH groups alone, 75% of which formed rings with As. We think the work provides some explanation of the reason why mustard gas does not appear to abolish the fixed SH reaction in muscle; there is not enough diminution to be noticeable.

It is curious that the mustard gas 'adsorbed' upon kerateine can only be removed from this very fine powder by prolonged extraction with an organic solvent. One might have expected that the step involving solution in ice-cold water would have been adequate to deal with this. Long ago, Dixon & Gooding (1924) drew attention to the permanence of mustard gas adsorbed on silk, etc. and the possibility of adsorbed mustard gas has been considered by Fell & Allsopp (1940-1). The properties of 'adsorbed' mustard gas as distinct from that dissolved in fats needs more study as the substance seems in some way to be stabilized. This might occur through van der Waals's attachment at O atoms, forming a 'pseudo-sulphoxide' structure in which Cl atoms are stable. Whatever is its explanation, it would be a factor in the retention of mustard gas in tissue, available for delayed effects.

This paper embodies the main features of a Report to the Ministry of Supply, no. 39, by Peters in 1941. For an account of the work as a whole, see Peters (1947).

SUMMARY

1. The soluble SH protein product kerateine has been treated with mustard gas at pH 7.4 and room temperature for about 1 hr.

2. The mustard gas combined with the kerateine as shown by the increase in sulphur content. By porphyrindin titration, it was estimated that about one-quarter of the combining mustard gas was in combination with SH groups originally present. This was confirmed by the use of the new silver reaction for thio-ethers.

3. About one-third of the mustard gas had combined to form a half product containing chlorine more slowly hydrolyzable than that of mustard gas.

4. The fine white powder, mustard gas-kerateine, even after thorough extraction with acetone, may show slight irritant effects to human skin on standing.

5. Mustard gas-kerateine was not toxic to the rat; it is not toxic to the succinoxidase system, and stabilizes O₂ uptake of the pyruvate oxidase system, a property also shown by kerateine itself.

This work was carried out as part of a larger programme of research directed by Prof. R. A. Peters for the Ministry of Supply. We are indebted to the Director General of Scientific Research (Defence) for permission to publish it. We are also indebted to Drs Holiday, Ogston, Stocken and Thompson for useful suggestions and to the Physiological Department, Porton, for histological aid. We are also grateful to Prof. Keilin for the succinic dehydrogenase preparation and for the cytochrome c.

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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 ($\text{C}=\text{S}-\text{C}=\text{C}$) 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.