compounds in the blood after the injection of H^*, HO_2^* and HO^* might help to throw light on some of the problems discussed in this paper.

SUMMARY

1. The fate in rabbits of intravenously injected HO ($\beta\beta'$-dichlorodiethyl sulphoxide) and HO_2 ($\beta\beta'$-dichlorodiethyl sulphone), two oxidation products of mustard gas (H), has been studied with the aid of the radioactive S^{35} as a tracer element. (An asterisk indicates the presence of S^{35} in the substance.)

2. The injected HO^* or HO_2^* was fairly rapidly distributed throughout the body, and appreciable amounts of S* were found in all the tissues examined 1 hr. after the injections. As in the corresponding experiments with H^*, the kidney and lung showed much higher S* contents than did the other organs and tissues.

3. Appreciable quantities of S* were excreted in the urine and/or bile after the injection of HO^* or HO_2^*, as in the case of H^*.

4. A comparison of the results of the experiments with H^*, HO^* and HO_2^* suggests that the three substances are similarly distributed throughout the body after injection, and that they all react with the tissues to form acetone- and ether-insoluble S*-containing compounds ('fixed' S*). This reaction may be partly or wholly concerned with the tissue proteins in the case of H^* and HO_2^*, but probably not with HO^*, for this compound, unlike H^* and HO_2^*, does not react or combine with proteins in *in vitro* experiments carried out under physiological conditions. The possibility of an *in vivo* conversion of HO^* into some more reactive compound is tentatively discussed.

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Studies on Mustard Gas ($\beta\beta'$-Dichlorodiethyl Sulphide) and some Related Compounds

7. THE IMMUNOLOGICAL PROPERTIES OF PROTEINS TREATED WITH MUSTARD GAS AND SOME RELATED COMPOUNDS†

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It has been shown (Berenblum & Wormall, 1939) that mustard gas (H) and its sulphone, $\beta\beta'$-dichlorodiethyl sulphone (HO_2), react with serum proteins at room temperature and at about pH 8 to give protein derivatives with a new immunological specificity. Injection of the H-treated horse serum proteins into rabbits produced antibodies which reacted with H-treated rabbit (or chicken) serum proteins, and the antibodies to HO_2-proteins showed a specificity characteristic for HO_2-proteins. These results showed that H and HO_2 had effected significant chemical changes in the protein molecule, and the absence of serological cross-reactions suggested that the two reactions might be dissimilar. Evidence was obtained that the chemical changes effected were

† This investigation formed the basis of reports to the Medical Research Council and to the Ministry of Supply in 1940.

not simply those of denaturation, oxidation, etc., but it was not possible at that time to do more than suggest, from an analogy with the results obtained by Cashmore & McCombie (1923) and Lawson & Reid (1925) with amino-acids and their esters, that new groups might have been introduced into the protein molecule as a result of reaction with the free amino-groups.

A further investigation of this problem has now been made with a view to studying, by chemical and immunological methods, the nature of the protein groups which are attacked by *H* and HO_2. In particular, it has been possible to make use of precipitin inhibition tests for this purpose, for some of the *H*- and HO_2-amino-acid derivatives required for these tests have now become available (Boursnell, Francis & Wormall, 1946). An opportunity has also been taken to investigate, by the same methods, the action of divinyl sulphide and divinyl sulphone, two compounds closely related to *H* and HO_2, on the serum and other proteins. In all these investigations the reactions have been carried out at 30–40° and pH 7·5–8, for it was intended to limit the reactions to those which might occur under physiological conditions. It was also our intention to use *H*, HO_2, etc. containing radioactive sulphur for these immunological studies, but the supplies of radio-S available to us in 1939 and 1940 were not sufficiently active for this purpose.

METHODS

Preparation of the antigens. In the preparation of the *H*-protein antigens, the serum or other protein solution was stirred at 30–35° over a long period with a considerable excess of *H*, with one or occasionally two further additions of *H* during the experiment. This treatment might be regarded as drastic in the sense that the protein was exposed to a relatively large amount of *H* for a long time, but the object of these experiments and the earlier ones of Berenblum & Wormall (1939) was to determine whether or not *H* can effect serologically-detectable changes in proteins under physiological conditions of pH and temperature. The *H*-antigen solutions required for injection were extracted several times with ether to remove any remaining *H* and ether-soluble *H* derivatives, but the other *H*-antigens (*H*-rabbit serum, etc.) were not treated in this way since it was thought desirable that the use of reagents which might effect denaturation and other changes in the serum proteins should be limited as far as was possible; the possibility of unchanged *H* or some of its hydrolysis products affecting the subsequent serological tests can be excluded, since these antigen solutions were diluted with 50–6250 vol. of 0·9% NaCl for use in the tests and were tested in some cases several weeks or months after preparation. For similar reasons, no attempt was made, in general, to separate from the unchanged serum proteins or gelatin any chemically-altered proteins resulting from the action of *H*, HO_2 and divinyl sulphone, for the investigation was mainly concerned with the question whether proteins treated in this way would acquire a new immunological specificity. In a few experiments, however, the HO_2-serum proteins were separated by acid precipitation to show that it was these chemically-changed proteins which were responsible for the characteristic precipitin reactions; no acetic acid-precipitable protein was present in the *H*- or the divinyl sulphide-antigens or in the HO_2-gelatin, but this matter is discussed more fully in another paper of this series (Banks, Boursnell, Francis, Hopwood & Wormall, 1946).

For the preparation of the HO_2- and divinyl sulphone-antigens the reaction was not so prolonged as for the *H*-antigens, since it was found early in these investigations that an appreciable amount of a new protein derivative was produced in the course of a few hours under the conditions of our experiments. In the case of divinyl sulphide, the reagent was allowed to act on the protein solution over a period of several days, these experiments running parallel with the *H*-experiments. $NaHCO_3$ was added at the start and further additions of alkali (Na_2CO_3, and occasionally NaOH solutions) were made later, where necessary, to neutralize the liberated HCl in experiments with *H* and HO_2, and in order to obtain comparable conditions $NaHCO_3$ was sometimes added to the protein solutions subjected to the action of divinyl sulphone and divinyl sulphide.

H-horse serum (for injection). A mixture of 125 ml. of horse serum, 62 ml. of 0·75 M-$NaHCO_3$ and 2 ml. of *H* was stirred well at about 33° for 7–8 hr. per day on each of 5 days, with further additions of 2 ml. of *H* early on the 3rd day and 1 ml. on the 4th day; during the overnight periods this mixture, and all the other antigens described below, were kept at about 4°. Small amounts of saturated Na_2CO_3 solution were added at intervals to keep the pH about 7·6–7·8. The final product was extracted with 150 ml. of ether and then a further four times with about 100 ml. of ether each time. The aqueous solution was warmed to about 30° and evacuated several times to remove the remaining ether. Phenol was added, as a preservative, to give a concentration of 0·25% and the solution was kept at 4°.

H-rabbit serum. A mixture of 24 ml. of rabbit serum, 24 ml. of 0·5 M-$NaHCO_3$ and 0·4 ml. of *H* was stirred, as above, over a period of 5 days, with a further 0·2 ml. of *H* added on the 4th day.

H-gelatin. A mixture of 1·0 g. of gelatin dissolved in 60 ml. of hot water, 40 ml. of 0·5 M-$NaHCO_3$ and 0·6 g. of *H* (i.e. about 10 molecules of *H* per free NH_2-group in the protein) was stirred at 35–40° for 12 hr. over a period of 2 days.

HO_2-rabbit serum. A mixture of 12 ml. of serum, 12 ml. of 0·5 M-$NaHCO_3$ and 0·5 g. of HO_2 in about 10 ml. of pure ether, was stirred at about 30° for 5·5 hr. one day and 3·5 hr. the next day, with N- or 5 N-NaOH added where necessary to maintain the pH between 7·5 and 8·5. Half of the resulting solution was used for the separation of the HO_2-proteins, as follows. The solution was extracted twice with 10 ml. of ether each time, the remaining ether being removed from the aqueous solution by a current of air. 2 N-acetic acid was added to give maximum precipitation, and the centrifuged precipitate dissolved in about 10 ml. of 0·9% NaCl with sufficient N-Na_2CO_3 added to give solution. This solution was centrifuged to remove a very small amount of undissolved matter, and a second precipitation with acetic acid was made, with re-solution in 0·9% NaCl and adjustment to pH 7·5.

HO_2-gelatin. 1·0 g. of gelatin dissolved in 33 ml. of hot water, plus 67 ml. of 0·5 M-$NaHCO_3$, plus 0·50 g. of HO_2 in 15 ml. of ether, were stirred at 30° for 5 hr.; next day a

further 0·50 g. of HO_2 in 15 ml. of ether was added and the mixture stirred for 7½ hr. Determinations of free amino-N (formol method, cf. Boursnell *et al.* 1946) showed that practically complete loss of free NH_2-groups had resulted from the action of HO_2 on the gelatin, but no acetic acid-precipitable proteins could be detected in the product.

Divinyl sulphone-rabbit serum and divinyl sulphone-gelatin. Each of the following mixtures was stirred at about 30° for 6½ hr.: (i) 10 ml. of rabbit serum plus 10 ml. of 0·5 M-$NaHCO_3$ plus 0·15 g. of redistilled divinyl sulphone (prepared as described by Alexander & McCombie, 1931) in 6 ml. of ether. (ii) 1·0 g. of gelatin dissolved in 30 ml. of hot water, plus 67 ml. of 0·5 M-$NaHCO_3$, plus 0·39 g. of divinyl sulphone in 15 ml. of ether. Formol titrations showed that the divinyl sulphone-gelatin preparation contained no significant amount of free amino-N.

Divinyl sulphide-horse serum (for injection). 1·5 ml. of freshly prepared divinyl sulphide (Bales & Nickelson, 1922, 1923) were stirred with 125 ml. of horse serum at 30° for 21 hr. over a period of 3 days, with the addition of more divinyl sulphide (0·83 ml.) about the half-way stage. The solution, which contained no acetic acid-precipitable proteins, was diluted with 60 ml. of water and extracted six times with about 60 ml. of ether each time; the aqueous solution was warmed to about 30° and evacuated several times to remove residual ether. Phenol was added to give a concentration of 0·25% as a preservative.

Divinyl sulphide-rabbit serum. A mixture of 12·5 ml. of rabbit serum, 12·5 ml. of 0·5 M-$NaHCO_3$ and 0·8 ml. of freshly prepared divinyl sulphide was stirred at 30° for 19 hr. over a period of 3 days.

Immunization. The antigens were injected into rabbits, with intervals of 7–10 days between the injections, and precipitin tests, and sometimes complement fixation tests also, were made 7–10 days after the third and subsequent injections.

With H-horse serum. Two groups of 3 rabbits each were injected with the *H*-horse serum preparation described above, 5 ml. of the solution being injected intraperitoneally each time. Tests on the antisera were made with *H*-rabbit serum as antigen.

Group (a). One rabbit gave moderately strong precipitin reactions after the 4th, 5th and 6th injections; after an interval of 2 months a second course of injections was given to this rabbit but no increase occurred in the precipitating power of the serum. The other 2 rabbits died shortly after the third injection.

Group (b). A course of 3 injections was followed by an interval of about 2 months and a further course of 5 injections. Negative or slightly positive reactions were obtained after the first course, but during the latter part of the second course the serum of one rabbit gave moderately good precipitin reactions. Another rabbit gave weak reactions and the third rabbit gave negative or very faint reactions.

With divinyl sulphide-horse serum. Three rabbits received a course of 6 intravenous injections, each of 5 ml. of the divinyl sulphide-horse serum preparation described above. After an interval of 2 months two of the rabbits received a further course of 5 intraperitoneal injections, each of 5 ml. of the antigen. None of the serum samples gave any precipitate with divinyl sulphide-rabbit serum.

With HO_2-horse serum. Some of the antisera prepared in an earlier investigation (Berenblum & Wormall, 1939) were found to have retained their precipitating power and were, therefore, used in the experiments described here.

Precipitin and complement-fixation tests. These tests were carried out as described previously (Hopkins & Wormall, 1933, and Berenblum & Wormall, 1939, respectively), the antigens for these tests being prepared from rabbit serum or from gelatin, to exclude the species factor. The concentration of antigen, in the tables, refers to dilution (with 0·9% NaCl) of a solution of the antigen containing approx. 5% of protein. Unless otherwise stated, the precipitin readings given in the tables were those recorded after 1 hr. at 37°. Precipitin tests with the gelatin derivatives were made at room temperature.

Precipitin inhibition tests (cf. Hopkins & Wormall, 1934). The preparation of the amino-acid derivatives '*H*-glycine' (1:4-thiazan-4-acetic acid), 'HO_2-glycine' (1:4-sulphonazan-4-acetic acid) and 'HO_2-alanine' (1:4-sulphonazan-4-propionic acid) is described elsewhere (Boursnell *et al.* 1946).

RESULTS

In confirmation of the previous findings (Berenblum & Wormall, 1939), antisera to *H*-proteins were obtained by the injection of *H*-horse serum into rabbits, but, as in the earlier work, the precipitating power of the serum was not very high; the antisera were definitely weaker than those produced by injection of HO_2-treated serum. In addition, some of the rabbits failed to produce at any time during the prolonged immunization period more than a very weak antibody response towards *H*-proteins, though they invariably produced powerful precipitins for untreated horse serum proteins.

Precipitin tests, a few of which are given in Table 1, showed that the antisera to *H*-horse serum gave reactions with *H*-rabbit serum proteins and other *H*-serum proteins, but not with *H*-gelatin or with divinyl sulphide-treated rabbit serum. Antisera to HO_2-horse serum reacted with HO_2-rabbit serum, other HO_2-serum proteins and also with divinyl sulphone-rabbit serum and divinyl sulphone-gelatin (Tables 1 and 2); excellent precipitates were obtained with HO_2-rabbit serum proteins which had been separated from unchanged proteins by two precipitations with acetic acid. As in the earlier work (Berenblum & Wormall, 1939), no significant cross-reactions between *H*- and HO_2-proteins and their antisera were obtained, i.e. antisera to *H*-proteins did not give precipitates with HO_2-proteins.

Since divinyl sulphone-gelatin (and other divinyl sulphone-proteins) readily reacted with antisera to HO_2-proteins, the relative rates of action of HO_2 and divinyl sulphone on serum proteins were compared by means of (*a*) the precipitin reaction, and (*b*) precipitation of the new protein derivatives by acetic acid. The results of this experiment (Table 3) showed that divinyl sulphone acts much more rapidly on serum proteins than does HO_2, under the conditions of our experiment. With divinyl sulphone, appreciable changes in the serum proteins were effected in

Table 1.† *Precipitin reactions with antisera to H-treated and HO_2-treated proteins*

Antigen	Dilution of antigen	Immune serum against: *H*-treated horse serum (No. 456)	Immune serum against: HO_2-treated horse serum (No. 404)
H-rabbit serum	1 : 100	tr.	–
	1 : 500	+±	–
	1 : 2500	+	–
HO_2-rabbit serum	1 : 100	–	±
	1 : 500	–	++
	1 : 2500	– or tr.	+±
H-gelatin	1 : 100	–	–
	1 : 500	–	–
	1 : 2500	–	–
	1 : 12,500	–	–
Horse-serum	1 : 100	+++	±
	1 : 500	+++	±
	1 : 2500	+	tr.

† In this and later tables in this paper, precipitin reactions are recorded as follows: – (no reaction), f.tr. (faint trace), tr. (trace), ±, +, +±, ++, etc. in increasing degrees of precipitation.

Table 2. *Precipitin reactions with antisera to HO_2-treated horse serum*

Antigen		Antigen dilution 1:100	1:500	1:2500	1:12,500
HO_2-Divinyl sulphone-	rabbit serum	f.tr.	+±	+±	
		f.tr.	+±	+±	
HO_2-Divinyl sulphone-	gelatin	f.tr.	tr.	+±	+
		–	tr.	+	±

less than 25 min., these changes being detectable by acid-precipitation and also by the precipitin reaction; a change of the same order was not observed in the serum treated with HO_2 until well over 90 min. from the start of the reaction, but in the final stages of the reactions (after about 3½ hr.) there was no significant difference between the precipitates obtained with the two mixtures.

Precipitin inhibition tests, which are of considerable value in giving information about the nature of the immunologically dominant groups in chemically altered and conjugated proteins (cf. reviews by Landsteiner, 1936, and Boyd, 1943) were then carried out to determine whether or not *H*- and HO_2-amino-acid derivatives can inhibit the antigen-antibody reactions with *H*- and HO_2-proteins. Typical results of these tests are given in Table 4. The HO_2-protein antigen-antibody reaction was completely or almost completely inhibited by HO_2-glycine or HO_2-alanine, and very slightly by *H*-glycine. The *H*-protein antigen-antibody reaction on the other hand was only partially inhibited by *H*-glycine. Where inhibition occurred, it was specific, for no inhibition was obtained when glycine was used instead of its *H*- or HO_2-derivative, and none of the amino-acid derivatives tested had any inhibitory effect on an unrelated precipitin system (e.g. horse serum + antiserum to horse-serum proteins).

Table 3. *Precipitin and acetic acid-precipitation tests with HO_2-treated* (A) *and divinyl sulphone-treated* (B) *rabbit serum*

Time after start (min.)	Acetic acid pptn. A	Acetic acid pptn. B	Precipitin reactions† A 1 : 500	A 1 : 2500	B 1 : 500	B 1 : 2500
20	–	±				
25			–	–	+	+
45	f.tr.	+				
60	tr.	+	–	–	+±	+
90	tr.	+±	±	tr.	++	+
155	+	++	+±	+	++	++
210	++	++	++	++	++	++

† Precipitin reaction with an antiserum to HO_2-horse serum.

Experimental details

	Rabbit serum	0·5 M-$NaHCO_3$	Sulphone (in 6 ml. pure ether)
A	10 ml.	10 ml.	0·25 g. HO_2
B	10 ml.	10 ml.	0·15 g. divinyl sulphone

Mixtures A and B were stirred vigorously, under exactly similar conditions, at 32°. Samples of each were withdrawn at intervals and tested for acid precipitability (0·5 ml. of A or B plus 2 or more drops of 2 N-acetic acid to give maximum precipitation) and for ability to react with an antiserum to HO_2-horse serum (1 ml. of A or B extracted twice with 9 ml. of ether each time, the ether removed and the aqueous solution diluted with 0·9% NaCl to the concentration required).

The results of the tests with divinyl sulphide-treated serum were entirely negative; no antibodies other than those against untreated horse serum could be detected in the serum of rabbits injected with the divinyl sulphide-treated horse serum, nor did divinyl sulphide-treated rabbit serum give precipitates with antisera to *H*-proteins or HO_2-proteins.

DISCUSSION

The production of antibodies capable of reacting specifically with *H*-rabbit serum proteins and with other *H*-proteins, following the injection of *H*-horse serum, is an indication that a significant alteration in the protein molecule has been effected by *H*. Analysis of *H*-proteins and the results of the precipitin inhibition tests described in this paper show that this alteration is partly or perhaps wholly due to the introduction of new groups into the protein. The specific antibody response in the injected rabbits was, however, not always as good as that normally found in immunization with serum proteins, nor was it as good as that in the animals injected with HO_2-proteins; antibodies to unchanged horse serum, and to *H*-horse serum were invariably produced in good

Table 4. *Precipitin inhibition tests*

Antigen	Antiserum against	Inhibitory substance (drops)		Precipitin reaction A	Precipitin reaction B
H-rabbit serum	*H*-horse serum	NaCl		+	+
		H-glycine	1	±	+
			2	±	±
			4	tr.	±
		HO_2-glycine	1	+	+
			2	+	+
			4	±	+
HO_2-rabbit serum	HO_2-horse serum	NaCl		+	+ +
		H-glycine	1	+	+ +
			2	+	+ ±
			4	±	+
		HO_2-glycine	1	tr.	±
			2	–	tr.
			4	–	–
HO_2-gelatin†	HO_2-horse serum	NaCl		+ ±	+ ±
		H-glycine	1	+ ±	+ ±
			2	+	+ ±
			4	+	+
		HO_2-glycine	1	tr.	tr.
			2	–	–
			4	–	–

A and B: readings after 15–45 and 60–90 min. respectively, depending on the potency of the antisera.

† Tests with divinyl sulphone-gelatin as antigen gave similar results.

Experimental details

Antigens. Solutions containing approx. 0·05% protein (*H*-rabbit serum) and 0·01% protein (HO_2-rabbit serum, HO_2-gelatin and divinyl sulphone-gelatin) in 0·9% NaCl were found most suitable for these tests.

Inhibitory substances. Neutralized 0·02 M-solutions in 0·9% NaCl.

Tests. 1 drop of antigen solution plus 1–4 drops of the 'inhibitor' solution plus 0·9% NaCl where necessary to make a total of 5 drops. 2 drops of antiserum were then added to each tube.

strength, showing that treatment with *H* had not impaired the antigenic power of the proteins. This relatively weak production of antisera specific for *H*-proteins would seem to suggest (*a*) that the number of new determinant groups introduced was not sufficient to effect a pronounced change in the immunological properties of the proteins, or (*b*) that the protein groups which are affected by *H* are not concerned with serological specificity, or (*c*) that the introduced groups are not of the type to determine the specificity of the antibodies formed in the animal, i.e. they may not be 'dominant' in this respect. It seems rather unlikely that (*b*) and (*c*) offer the true explanation in view of the fact that almost any pronounced chemical change in an antigenic protein alters its serological specificity, but they cannot be excluded; it should be remembered that some azo-proteins produce antisera which react with the immunizing antigen but not with azo-antigens made from other proteins (cf. Landsteiner, 1936, p. 103).

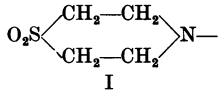
With regard to the first suggestion, there is evidence that although a few new groups introduced into each molecule of protein antigen may give the antigen the power to produce antibodies capable of reacting specifically with the new group, many more may have to be introduced into another protein before it will give precipitates with the antiserum. Hooker & Boyd (1932), for example, found that azo-proteins prepared from diazotized arsanilic acid and casein gave precipitates with an anti-arsanilic anti-serum only when about 13 or more groups were introduced into each casein molecule (assuming a mol.wt. of 96,000), and Haurowitz (1936) in similar investigations with rabbit globulin azoproteins showed that more than 10 introduced or hapten groups were needed (assuming a mol.wt. of 100,000 for the globulin azoprotein). In the case of the *H*-serum proteins used in the immunological experiments described here, we were not able to determine the number of *H* molecules or residues attached, but subsequent work with *H* containing radio-S (Banks *et al.* 1946) suggests that there might have been as many as 20 such groups in the *H*-protein antigens, assuming a mean mol.wt. of 100,000 for the serum proteins. Thus it seems unlikely that the relatively weak capacity of *H*-proteins to give precipitates with antisera to *H*-proteins was due to a deficiency of hapten groups.

The failure of *H*-gelatin to react with our antisera to *H*-proteins is also difficult to explain, but experiments with 'radio'-*H* (*H**) suggest that there are fewer S*-containing groups in *H**-gelatin than in *H*-*serum proteins prepared under similar conditions (Banks *et al.* 1946). HO_2-gelatin, on the other hand, readily reacts with HO_2-protein antisera, and this is a further instance of the marked difference between the actions of *H* and HO_2 on proteins.

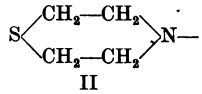
The action of HO_2 on proteins appears to be concerned mainly with the free amino-groups, and although there are possibly other changes, such as loss of SH, the dominant immunological change is due to the replacement of NH_2 by 1:4-sulphonazan groups. This chemical change, like the action of phenyl *iso*cyanate and benzylcarbonyl chloride, produces serum protein derivatives which are precipitated by dilute acetic acid and which have a new serological specificity characteristic for the grouping introduced; at the same time there is considerable loss of the original species specificity. Divinyl sulphone, $(CH_2{:}CH)_2SO_2$, acts on proteins to give products which are identical, chemically and immunologically, with the corresponding HO_2-compounds; these results are not inconsistent with

the view put forward later by Ford-Moore (1940) (cf. also Ford-Moore & Lidstone, 1940), that HO_2 is converted into divinyl sulphone before it reacts with amino-acids.

The precipitin inhibition tests show quite definitely that whereas the HO_2-amino-acid derivatives, containing the 1:4-sulphonazan group (I), strongly inhibit the HO_2-protein precipitin reaction, the H-protein precipitin reaction is only partially inhibited by H-glycine, a compound having the 1:4-thiazan structure (II).



I



II

These results, and the absence of significant serological cross-reactions between H-proteins and HO_2-proteins, suggest that H and HO_2 differ very considerably in their action on proteins, and they support the view that H does not act mainly, if at all, on the free amino-groups. Maximum specific inhibition in tests of this type is usually effected by compounds whose structures most closely resemble that of the introduced group plus the protein group to which it is attached, e.g. the best inhibition of the phenylcarbamido-protein precipitin reaction is obtained with the phenylcarbamido-derivatives of lysine and ϵ-amino-n-hexoic acid (Hopkins & Wormall, 1934), but unfortunately the H-derivative of lysine was not available for the tests described here. A possible explanation for the partial nature of the inhibitory effect of H-glycine on the H-protein precipitin reaction is that H enters the protein molecule in two or more different places, and that there is more than one type of specific antibody in the corresponding antiserum, or that the antibodies have a multiple specificity, but as yet there is no serological evidence of this. It should be noted, however, that a very slight precipitation of HO_2-proteins was obtained occasionally with an H-protein antiserum, but this may have been due to a general similarity between the H groups in H-proteins and the sulphonazan group. The slight inhibition of the HO_2-protein precipitin reaction by H-glycine can be explained in a similar manner.

There is also the possibility that the changes in the immunological properties of proteins effected by H may be due to changes in the acid groups of the protein, for these groups, or the parts of the protein molecule carrying these groups, appear to have a significant influence on serological activity (cf. Landsteiner, 1936, pp. 32, 105), and later investigations have shown that H reacts with COOH groups of proteins (Northrop, 1942; Ball, 1942).

Divinyl sulphide, $S(CH{:}CH_2)_2$, has no significant action on proteins similar to those of H, HO_2 and divinyl sulphone, and no change in the immunological properties or in acid-precipitability occurs in serum proteins subjected to the prolonged action of this compound. Thus, although HO_2 probably undergoes transformation to divinyl sulphone before acting on amino-acids and proteins, it seems very unlikely that H combines with proteins by a similar mechanism.

The distinct change in immunological properties of proteins caused by H, i.e. the production of a protein 'foreign' to the animal from which the serum or other protein was derived, supports the view that the acquired hypersensitivity to H of some individuals exposed to the gas might be due to an immunological reaction of this type (cf. Berenblum & Wormall, 1939). These results also suggest that it should be possible to induce hypersensitivity to H in laboratory animals, and it is of interest to note that Holliday (1942), Moore (1944) and Kidd & Landsteiner (1944) have later achieved this.

SUMMARY

1. A further study has been made of the immunological properties of proteins treated with mustard gas (H) and the corresponding sulphone, $\beta\beta'$-dichlorodiethyl sulphone (HO_2), and in addition the action of divinyl sulphone and divinyl sulphide on proteins under the same conditions, 30–40° and pH about 8, has been investigated.

2. As in the previous investigation (Berenblum & Wormall, 1939) antisera to H-proteins were obtained by the injection of H-treated horse serum. The precipitin reaction between H-rabbit serum proteins and antisera to H-horse serum was only partially inhibited by an H-glycine derivative (1:4-thiazan-4-acetic acid).

3. The precipitin reaction between HO_2-proteins and their antisera was completely or almost completely inhibited by HO_2-glycine (1:4-sulphonazan-4-acetic acid) and by HO_2-alanine, and slightly inhibited by H-glycine. These results suggest that the action of HO_2 on proteins is largely concerned with the free NH_2-groups of the protein.

4. Divinyl sulphone reacts more rapidly than does HO_2 on serum and other proteins, to give compounds which react, chemically and immunologically, exactly like HO_2-proteins.

5. No immunological evidence could be obtained of any action between divinyl sulphide and serum proteins.

6. The absence of serological cross-reactions between H-proteins and HO_2-proteins, and the results of the inhibition tests, offer strong support for the view that H and HO_2 differ very considerably in their action on proteins.

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Studies on Mustard Gas ($\beta\beta'$-Dichlorodiethyl Sulphide) and some Related Compounds

8. THE ACTION OF MUSTARD GAS, DIVINYL SULPHONE AND $\beta\beta'$-DICHLORODIETHYL SULPHONE ON COMPLEMENT†

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Complement, which appears to play an important role in many of the defence mechanisms of the animal body, is a complex system consisting of at least four factors (for a review of the literature, cf. Osborn, 1937). At least two of these factors [C′1 and C′2 in the nomenclature suggested by Pillemer & Ecker (1941) and Heidelberger (1941)] have properties which suggest that they are of protein nature; the other two however, the third and the fourth components (C′3 and C′4), are not destroyed when the serum is heated at 56° for 30 min., but are destroyed by yeast (Coca, 1914; cf. also von Dungern, 1900) or yeast preparations, and by NH_3 (Gordon, Whitehead & Wormall, 1926) respectively. Various suggestions have been made that one or other of these components is of enzymic nature, but there is no general agreement on this point.

Complement is readily inactivated by processes which affect the state of the serum proteins, and since mustard gas (*H*), $\beta\beta'$-dichlorodiethyl sulphone (HO_2) and divinyl sulphone react with a variety of proteins (for a review of the literature, cf. Banks, Boursnell, Francis, Hopwood & Wormall, 1946*a*, *b*), it was decided to study the action of these substances on the haemolytic complement system. It was hoped that this investigation might yield useful information about the reaction between *H*, etc. and certain proteins, and perhaps also about the properties and nature of complement.

† The investigation described here was the subject of a report to the Ministry of Supply in October 1941.

METHODS

Serum. Fresh guinea-pig serum was used in all experiments.

Heated serum (deficient in C′1 and C′2). Serum heated at 56° for 30 min. and then diluted with 9 vol. of 0·9% NaCl.

NH_3-inactivated serum (deficient in C′4; cf. Gordon, Whitehead & Wormall, 1926). 1 ml. of serum plus 0·2 ml. of 0·2 N-NH_4OH, kept at 37° for 1¼ hr., neutralized with 0·2 N-HCl and diluted to 10 ml. with 0·9% NaCl.

Haemolytic system. 0·5 ml. of a 4% suspension of sensitized washed ox red-cells plus varying amounts of the diluted inactivated sera, with 0·9% NaCl added where necessary to make the total volume 1 ml. The tubes containing these mixtures were immersed in a water-bath at 37°, and the extent of haemolysis was recorded at frequent intervals.

Inactivation of complement by H, etc. Fresh guinea-pig serum was treated with *H*, HO_2 or divinyl sulphone in stoppered pyrex tubes, and the mixtures kept in a water-