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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*),

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

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.

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 1½ 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-

Table 1. *The complement activity of guinea-pig serum treated with H and divinyl sulphone at 37°*

Sample taken after (min.)	Vol.† of diluted sample tested (ml.)	Complement activity‡ of diluted serum samples									
		<i>H</i> -treated				Divinyl sulphone-treated				Control	
		A		B		C		D		E	
5	0.5	2	3	4	4	0	0	0	1	4	4
	0.3	0	1	1	3	0	0	0	0	4	4
	0.1	0	0	0	0	0	0	0	0	0	1
12	0.5	0	0	0	0	0	0	0	0	4	4
	0.3	0	0	0	0	0	0	0	0	4	4
	0.1	0	0	0	0	0	0	0	0	0	1
Haemolysis readings after (min.)		5	45	5	45	5	45	5	45	5	45

† Intermediate vol. (0.4 and 0.2 ml.) were also tested, but to save space, Tables 1 and 2 merely give the results with the extreme ends and the mid-point of the dilution range.

‡ In these tables the extent of haemolysis is recorded as follows: 4, complete haemolysis; 0, no haemolysis; 3, 2, 1, and tr. (trace), intermediate degrees of haemolysis.

Experimental details

Mixture	Guinea-pig serum (ml.)	<i>H</i> (ml.)	Divinyl sulphone (ml.)
A	2.0	0.04	—
B	2.0	0.01	—
C	2.0	—	0.03
D	2.0	—	0.0075
E (Control)	2.0	—	—

Table 2. *The complement activity of guinea-pig serum treated with divinyl sulphone and H-sulphone (HO₂) at 19°*

Sample taken after (min.)	Vol.† of diluted sample tested (ml.)	Complement activity of diluted serum samples					
		Divinyl sulphone treated		<i>H</i> -sulphone treated		Control	
		F		G		K	
5	0.5	4	4	4	4	4	4
	0.3	2	4	4	4	4	4
	0.1	0	1	0	4	2	4
20	0.5	0	1	4	4	4	4
	0.3	0	0	4	4	4	4
	0.1	0	0	0	3	1	3
75	0.5	0	0	4	4	4	4
	0.3	0	0	4	4	4	4
	0.1	0	0	0	2	1	4
120	0.5	0	0	tr.	tr.	4	4
	0.3	0	0	0	0	4	4
	0.1	0	0	0	0	2	4
Haemolysis readings after (min.)		10	30	10	30	10	30

† See footnote to Table 1.

Experimental details

Mixture	Guinea-pig serum (ml.)	Divinyl sulphone (ml.)	<i>H</i> -sulphone (g.)
F	2.0	0.03	—
G	2.0	—	0.06
K (Control)	2.0	—	—

bath at 37° with occasional shaking; prolonged or vigorous shaking was avoided, since this inactivates complement. The pH of the solution was maintained at about 7.5 by the addition, where necessary, of a small amount of half saturated Na_2CO_3 solution and the same volume of 0.9% NaCl was added to the other tubes; the amounts of H and HO_2 used were, however, so small that complete inactivation of the complement usually occurred before any change in pH could be detected. Samples (0.2 or 0.4 ml.) of the mixtures, and of a control untreated serum, were withdrawn at intervals and diluted with 9 vol. of 0.9% NaCl. Varying amounts of these diluted solutions were tested for complement activity. Attempts were also made to reactivate the inactivated complement by the addition of NH_3 -inactivated serum (containing C' 1, C' 2 and C' 3) or heated serum (C' 3 and C' 4).

H , HO_2 and divinyl sulphone have different solubilities in water, and the rate of inactivation of complement by those substances may, of course, be determined to some extent by their solubility in the serum. A few attempts were made to find a suitable solvent for these three reagents which would mix with serum and give uniform solution or suspension of the reagents, but it was found that the solvents themselves had a destructive action on the complement or on the haemolytic system. In all the experiments described here, therefore, the neat reagents were added to the serum. Where HO_2 was used, the solid was ground up with a glass-rod and the mixture stirred gently at frequent intervals. In any one experiment approximately equimolecular amounts of H , HO_2 and divinyl sulphone (mol.wt. 159, 191 and 118 respectively) were used.

RESULTS

The following experiments may be regarded as typical of many made with varying amounts of H , etc. and at different temperatures.

Exp. 1. Inactivation of complement by H and by divinyl sulphone (at 37°). The results of this experiment (Table 1) showed that the activity of complement was quickly destroyed by small amounts of H at 37° but still more rapidly by equimolecular amounts of divinyl sulphone. Complete inactivation of the complement occurred within 12 min. with H and in less than 5 min. with divinyl sulphone, under the conditions of this experiment.

Exp. 2. Inactivation of complement by divinyl sulphone and HO_2 (at 19°). The results of this experiment (Table 2) showed that HO_2 also inactivates complement but at a very slow rate compared with the inactivation by divinyl sulphone. Experiments carried out at 37° showed a similar difference, with, as might be expected, much quicker inactivation by both reagents.

Exps. 3, 4. Attempts to reactivate H -inactivated and divinyl sulphone-inactivated complement by the addition of complement inactivated by NH_3 or by heat. In these experiments an attempt was made to determine whether inactivation by H and by divinyl sulphone is confined to one or two of the components of complement or whether it is of a more general

nature. The amounts of H and divinyl sulphone used in some of these experiments (cf. Exp. 4) were much smaller than those used in the other experiments described in this paper; in this way relatively slow inactivation of complement was effected, in the hope that evidence might be obtained of a preferential inactivation of some of the components. For various reasons it was not possible for us to make an extensive study of this problem, for example by the use of the separated components, but a sufficient number of tests were made to suggest that, in the initial stages, the C' 1 and C' 2 components (those more readily destroyed by heat) may be more rapidly destroyed than the others. In Exp. 4 (Table 4) for example, and to a lesser extent in Exp. 3 (Table 3), reactivation of H -inactivated or divinyl sulphone-inactivated serum was sometimes obtained by the addition of NH_3 -inactivated serum (containing C' 1, C' 2 and C' 3) but little or none by the addition of heated serum (C' 3 and C' 4). When larger amounts of H and divinyl sulphone were used however, and the reaction allowed to continue for 55 min. (Table 3), the inactivated sera could not be reactivated by NH_3 -inactivated or by heat-inactivated serum.

DISCUSSION

The destruction of complement activity by H and by divinyl sulphone is probably without significance as far as the action of H on the animal body is concerned, but this inactivation is of interest in that it provides a further example of a reaction between H and substances of physiological interest under conditions similar to those found in the animal body, i.e. 37° and pH 7.5.

Inactivation of complement by H or by divinyl sulphone is very rapid, and small amounts of the reagent will effect complete loss of the haemolytic complement activity of guinea-pig serum in a few minutes. The evidence produced in this paper taken in conjunction with other investigations with various proteins, suggests that complement inactivation by H (or divinyl sulphone) might be due largely to its action on certain protein components of complement. Of the four known components, two at least (C' 1 and C' 2) appear to be proteins, and there is evidence that one, or possibly both, of the other two components may also be of protein nature; C' 1 and C' 2 are, however, the most sensitive to destruction by heating at 56°. Thus it is perhaps not surprising that complement inactivation by H or by divinyl sulphone appears to be concerned in the early stages with a destruction of the activities of C' 1 and C' 2; there is, however, at the same time a general inactivation of all the components, and it is difficult to choose conditions which will produce a differential inactivation. The inactivation of complement by H or by divinyl sulphone is, therefore, by

Table 3. *Attempted reactivation of H- and divinyl sulphone-inactivated sera*

Sample taken after (min.)	Diluted sample (ml.)	NH ₃ -inactivated serum† (ml.)	Heated serum† (ml.)	Complement activity				
				H-treated		Divinyl sulphone-treated		Control
				A	B	C	D	
12	0.5	—	—	0	0	0	0	4
	0.2	—	—	0	0	0	0	3
	0.2	0.2	—	1	3	1	1	—
	0.2	—	0.2	0	0	0	0	—
55	0.25	—	—	0	0	0	0	4
	0.25	0.25	—	0	0	0	0	—
	0.25	—	0.25	0	0	0	0	—
Haemolysis reading after (min.)				30				

† These inactivated sera were separately devoid of complete complement activity, but a mixture of the two was fully active, since the heated serum supplied the fourth component (C' 4) missing from the NH₃-inactivated serum.
Experimental details. Mixtures A, B, C, D and E as in Exp. 1 (temp. 37°).

Table 4. *Attempted reactivation of H- and divinyl sulphone-inactivated sera*

Samples taken after (min.)	Diluted sample (ml.)	NH ₃ -inactivated serum† (ml.)	Heated serum† (ml.)	Complement activity							
				H-treated				Divinyl sulphone-treated			
				P		Q		R		S	
20	0.5	—	—	4	4	4	4	0	2	4	4
	0.3	—	—	2	4	4	4	0	1	1	2
	0.1	—	—	0	0	0	0	0	0	0	0
33	0.5	—	—	tr.	tr.	4	4	0	0	3	4
	0.3	—	—	0	0	2	4	0	0	1	2
	0.1	—	—	0	0	0	0	0	0	0	0
	0.3	0.2	—	2	4	4	4	3	4	4	4
	0.3	0.4	—	2	4	4	4	4	4	4	4
	0.3	—	0.2	0	1	4	4	1	2	4	4
	0.3	—	0.4	tr.	2	4	4	3	3	4	4
70	0.5	—	—	0	0	—	—	0	0	—	—
	0.3	—	—	0	0	—	—	0	0	—	—
	0.3	0.2	—	0	0	—	—	tr.	3	—	—
	0.3	0.4	—	0	1	—	—	2	4	—	—
	0.3	—	0.2	0	0	—	—	0	0	—	—
	0.3	—	0.4	0	0	—	—	0	1	—	—
Haemolysis readings after (min.)				10	30	10	30	10	30	10	30

† See footnote to Table 3.

Experimental details

Mixture	Guinea-pig serum (ml.)	H (ml.)	Divinyl sulphone (ml.)
P	4.0	0.01	—
Q	4.0	0.002	—
R	4.0	—	0.0075
S	4.0	—	0.0015

Temp. 37°.

no means comparable with that effected by such agents as NH₃ or yeast preparations, for with the latter reagents the inactivation is much more specific.

The investigations described here were of a preliminary nature, and for various reasons it has not been possible for us to continue this work. It is hoped, however, that a further study of this problem will be made, and that it will be possible to decide whether or not H and divinyl sulphone effect inactivation of complement in exactly the same way. Our own investigations, and those of other authors, suggest that these two reagents differ considerably in the way in which they react with the protein

molecule, and it is probable that they differ in their action on complement.

The combination of *H*-sulphone (HO_2) and divinyl sulphone with proteins is mainly concerned with a reaction of the sulphone with the free amino-groups of the protein, and it is possible that inactivation of complement by these two reagents is effected by this means. In the case of *H*, however, the reaction with proteins is apparently largely concerned with the carboxyl and iminazole groups (Ball, 1942). The rate of combination of protein with *H* is somewhat slower than that with HO_2 (Banks *et al.* 1946*b*), and it may seem rather surprising that complete inactivation of complement was effected, under the conditions of the experiments described in this paper, more rapidly with *H* than with an equivalent amount of HO_2 . Complement inactivation might, however, be the result of the action of *H* or HO_2 on a few essential groups in one or more of the components of complement, and the rate of inactivation may bear no relationship to the total amount of *H* or HO_2 which has combined with the serum proteins.

Another point which we intend to investigate further is the significance of the SH groups of the serum proteins as far as complement activity is concerned. *H*, HO_2 and divinyl sulphone all readily react with SH groups, and a reaction of this type might account for part at least of their inactivating

action on complement. In this connexion it is of interest to note that Ecker, Pillemer, Martiensen & Wertheimer (1938) have suggested that complement may possess SH groups, and that inactivation by oxidation might be due to their conversion to disulphide ($-S-S-$) groups.

SUMMARY

1. Small amounts of mustard gas (*H*) rapidly inactivate complement. The inactivation is not due to pH changes.
2. The inactivation appears to affect all the components of the complement. There is some indication, however, that the relatively heat-labile components (C' 1 and C' 2) are more rapidly inactivated than the other two components.
3. Divinyl sulphone similarly inactivates complement, but even more rapidly than does *H*.
4. *H*-Sulphone ($\beta\beta'$ -dichlorodiethyl sulphone) inactivates complement, but relatively slowly compared with *H* and divinyl sulphone.

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The Polarographic Estimation of Steroid Hormones

3. COMPARISON OF NORMAL VALUES FOR URINARY 17-KETOSTEROIDS BY POLAROGRAPHIC AND COLORIMETRIC METHODS

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In the work of Wolfe, Hershberg & Fieser (1940) referred to in a previous communication (Barnett, Henly & Morris, 1946), a comparison was made between the polarographic and the Zimmermann (1935, 1936) methods for the estimation of neutral urinary 17-ketosteroids. Most of the colorimetric assays (for

example, those supplied by Dr Talbot) were significantly higher than the polarographic results, whilst those obtained by another group were lower. Wolfe *et al.* consider that this was due to the high correction factor applied by this group to their Zimmermann values. They do not give any figures for the