X. THE IMMUNOLOGICAL PROPERTIES OF PROTEINS TREATED WITH $\beta\beta'$ -DICHLORO-DIETHYLSULPHIDE (MUSTARD GAS) AND $\beta\beta'$ -DICHLORODIETHYLSULPHONE¹

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(Received 11 November 1938)

SEVERAL explanations have been put forward to account for the vesicant action of mustard gas and related compounds. Cashmore & McCombie [1923] refer to the suggestion that the effect might be due to a reaction between the mustard gas and "amino-acids present in the skin". In support of this theory might be mentioned the experiments of Clarke [1912], who showed that mustard gas combines with primary amines, those of Cashmore & McCombie [1923], who found that mustard gas and $\beta\beta'$ -dichlorodiethylsulphone give condensation products with glycine ester, and the observation that $\beta\beta'$ -dichlorodiethyl-sulphide, -sulphoxide and -sulphone react with various primary, secondary and tertiary amines [Lawson & Reid, 1925]. The last-named authors conclude that their results are consistent with the theory that the vesicant action of mustard gas is due to its reaction with constituents of the living cell.

The introduction of new groups into the protein molecule, by attachment to the free amino-groups, leads usually to a very marked change in the immunological properties of the protein. Landsteiner & Jablons [1914] and Landsteiner & Lampl [1917] found that treatment with formaldehyde renders rabbit serum proteins fully antigenic for rabbits. Although the original species specificity is not completely destroyed, the action of formaldehyde is sufficient to produce a protein derivative which is "foreign" to the body. Horsfall [1934] obtained essentially similar results with formaldehyde-treated proteins.

Amongst other reagents which react with the free amino-groups of proteins is phenyl isocyanate, and this substance destroys, almost completely, the original species specificity of serum proteins; the phenylcarb-amido-proteins produced have an immunological specificity which is characteristic for the phenylcarb-amido-grouping [Hopkins & Wormall, 1933, 1, 2; 1934]. Similar results follow the action of benzyl chloroformate on antigenic proteins [Gaunt *et al.* 1935; Gaunt & Wormall, 1938], for in this case also the acquirement of a new immunological specificity is related to the introduction of a new group attached to the free amino-groups of the original protein.

¹ The investigations described here were mainly carried out when the authors were members of the Departments of Experimental Pathology and of Physiology in the University of Leeds. A brief account of the earlier results was communicated in 1936 to the 2nd Internat. Congress for Microbiology (cf. *Report*, 1937, p. 427).

² Beit Memorial Research Fellow with a personal grant from the British Empire Cancer Campaign.

In view of these immunological investigations and the above-mentioned work on the action of mustard gas and related compounds on amino-acids, it was considered desirable to study the immunological properties of mustard gas-treated proteins. The investigations described were undertaken (a) to determine whether serological methods would enable us to detect a reaction between mustard gas and proteins, and (b) to study the general immunological properties of these mustard gas- (and sulphone)-treated proteins.

EXPERIMENTAL

For the preparation of mustard gas- (or sulphone)-treated proteins, the reagent was allowed to act on the protein solution in the presence of phosphate buffer at pH 8.0. Since mustard gas is gradually hydrolysed by water with liberation of free HCl, occasional addition of 2 *N* NaOH was sometimes necessary to maintain the pH at about 8. After some time the mixture was extracted with ether to remove unchanged mustard gas and the resulting solution used for immunization or for the serological tests. No attempt was made to separate the altered proteins from the mixture, since the sole object of early studies was to determine whether any change had occurred which could be detected immunologically.

Mustard gas-treated proteins. A mixture of 100 ml. of serum, 100 ml. of a phosphate buffer of pH 8.0 (100 ml. of 0.2 *M* KH_2PO_4 plus 93.6 ml. of 0.2 *N* NaOH) and 1 g. of NaCl was stirred at room temperature for 8 hr. on each of three successive days, after the addition of 2 ml. of mustard gas on the first and second days. During each night the mixture was kept in the ice-chest. It was finally extracted five times with 200 ml. of ether each time, the ether removed by evacuation and the pH adjusted to 7.5. The preparations used for immunization were treated with 0.05 vol. 5 % phenol.

"Sulphone"-treated proteins. These were prepared in the same way as were the mustard gas-treated proteins but the solid $\beta\beta'$ -dichlorodiethylsulphone was added in ethereal solution (two additions of 2 g. of the sulphone in about 45 ml. of ether to 100 ml. of serum). In other experiments the reaction was carried out at 30° instead of at room temperature, and the two preparations are referred to in this paper as sulphone-treated proteins (17°) and sulphone-treated proteins (30°).

Immunization. The antigens injected were all prepared from normal horse serum. The method of immunization was that recorded previously [Hopkins & Wormall, 1933, 1], all the injections being made intraperitoneally. The injected rabbits were bled after 3–7 injections. The serological reactions mentioned below were often observed after the third injection but a better response was obtained after six or seven injections.

Precipitin tests. These tests were carried out as described by Hopkins & Wormall [1933, 1].

Complement-fixation tests. Mixtures of 0.5 ml. of diluted antigen, 0.10 ml. of the antiserum (previously heated at 56° for 20 min.) and 0.10 ml. of 1:5 guinea-pig serum (in 0.9 % NaCl) were kept at room temperature for 1 hr. To each tube was then added 0.5 ml. of a 4 % suspension of sensitized ox red cells (in 0.9 % NaCl) and the tubes were immersed in a water bath at 37°. Readings were taken at various intervals. The results given in Table II are typical results obtained after 1 hr.

Results of the serological tests

The injection of mustard gas-treated horse serum into rabbits results in the production of antibodies which give precipitin reactions with the antigen injected and to a lesser extent with mustard gas-treated proteins of rabbit serum (or chicken serum). The reactions observed (Table I) were sufficiently pronounced to establish definitely that a new specificity had been acquired. The retention of the original species specificity of the serum proteins, shown by the reactions between horse serum and antisera to mustard gas-treated horse serum and between mustard gas-treated horse serum and antisera to horse serum, is not unexpected since the mustard gas-treated protein preparations undoubtedly contained unchanged serum proteins. In view of the possibility that the precipitin reactions with the mustard gas-treated proteins might be related to groups substituted in the free amino-groups of the protein, cross-tests were made with phenylcarbamido-proteins (proteins in which the free amino-groups have been replaced by $\text{NH.CO.NH.C}_6\text{H}_5$). No cross-reactions were observed, and this is perhaps not surprising in view of the sharp specificity observed when new groups are introduced into protein molecules (for a review of the literature cf. Landsteiner [1936]; Marrack [1938]). Confirmation of the reactions between antisera to mustard gas-treated horse serum and mustard gas-treated rabbit serum was obtained by complement-fixation tests, representative results of which are given in Table II.

Table I. *Precipitin reactions with antisera to mustard gas-treated proteins*

Antigen	Dilution of antigen	Immune serum against				
		Mustard gas-treated horse serum			Horse serum	Phenyl-carbamido-horse serum
		No. 498	No. 198	No. 199	No. 82	proteins No. 100
Horse serum	1:20	+	±	tr.	++	-
	1:100	++	+	±	++	-
	1:500	+±	+	±	++	-
	1:2500	+±	±	±	±	-
Mustard gas-treated horse serum	1:20	++	++±	+±	++	-
	1:100	+++	++±	+±	++	-
	1:500	++	+±	+	++	-
	1:2500	±	±	±	±	-
Mustard gas-treated rabbit serum	1:20	-	-	-	-	-
	1:100	±	±	±	-	-
	1:500	±	+	+	-	-
	1:2500	tr.	±	tr.	-	-
Mustard gas-treated chicken serum	1:20	-	-	-	-	-
	1:100	-	f. tr.	f. tr.	-	-
	1:500	±	±	±	-	-
	1:2500	f. tr.	f. tr.	f. tr.	-	-

The dilutions of antigen in Tables I, II and III refer to dilution of a solution which contains 5% of total protein. Precipitin test readings (taken after 1 hr. at 37°): - (no reaction), f. tr. (faint trace), tr. (trace), ±, +, +±, ++ etc., in increasing degrees of precipitation.

Similar results were obtained with the proteins treated with $\beta\beta'$ -dichloro-diethylsulphone. Precipitin tests (Table III) and complement-fixation tests showed that antisera to the sulphone-treated proteins are capable of reacting with sulphone-treated rabbit serum; they will not react, however, with mustard gas-treated rabbit serum. In these experiments the best reactions were given with the preparations obtained by allowing the sulphone to act on the proteins at 30°,

Table II. *Complement-fixation tests*

Antigen dilution	Antigen Immune serum	Mustard gas-treated horse serum		Mustard gas-treated rabbit serum	
		No. 498	No. 198	No. 498	No. 198
1:20		3	2	4	4
1:60		1	1	3	4
1:180		—	—	3	2
1:540		—	—	2	—
1:1,620		—	—	1	—
1:4,860		—	—	—	—
1:14,580		—	—	—	—
1:43,740		—	—	—	—
1:131,220		—	1	1	—
1:393,660		1	3	2	1
1:1,180,980		2	4	4	2
1:3,542,940		4	4	4	4
Control (NaCl)		4	4	4	4

Readings: 4 (complete haemolysis); — (no haemolysis); 3, 2 and 1 intermediate degrees of haemolysis.

Antisera: Nos. 498 and 198 (antisera to mustard gas-treated horse serum).

and it seems probable that at body temperature the chemical reaction between proteins and mustard gas (or the corresponding sulphone) may be appreciably quicker than that observed in the experiments recorded here. Further experiments are now being carried out to determine the minimum time required for mustard gas and the sulphone to produce at 37° a change in the protein molecule which can be detected by these serological methods.

Table III. *Precipitin reactions of antisera to "sulphone"-treated proteins*

Antigen	Dilution of antigen	Immune serum against				Mustard gas-treated horse serum No. 493
		Sulphone-treated horse serum				
		No. 403	No. 404	No. 406	No. 408	
Horse serum	1:20	tr.	tr.	—	—	tr.
	1:100	±	±	±	tr.	+
	1:500	tr.	tr.	tr.	tr.	±
	1:2500	—	tr.	f. tr.	—	tr.
Sulphone-treated horse serum (30°)	1:20	++	++ ±	±	+	f. tr.
	1:100	++	++ ±	+	+	±
	1:500	+	+ ±	+	+	+
	1:2500	tr.	±	tr.	tr.	tr.
Sulphone-treated rabbit serum (30°)	1:20	—	tr.	—	—	—
	1:100	tr.	+	f. tr.	f. tr.	—
	1:500	+	+	f. tr.	tr.	—
	1:2500	+	+	tr.	±	—

Immune sera nos. 403 and 404 were obtained with the sulphone-treated horse serum (30°) and nos. 406 and 408 with sulphone-treated horse serum (17°).

DISCUSSION

The results of the experiments described here show that mustard gas and $\beta\beta'$ -dichlorodiethylsulphone effect a change in proteins which can be detected by immunological methods. The change is sufficient to convert the protein into derivatives which may be considered "foreign" to the homologous animal. Some of the more characteristic features of the vesicant action of mustard gas are the smallness of the effective dose, the delayed action and the fact that people who have been badly "gassed" are liable to become hypersusceptible to mustard gas.

These facts are compatible with the view that the biological mechanism of the reaction may be related to protein sensitivity, but whether the present results have any bearing on this problem cannot be decided without further investigation.

The identity of the chemical changes effected in the protein molecule by mustard gas and the sulphone has not yet been established, but it seems probable that they are concerned with the free amino-groups of the protein. Cashmore & McCombie [1923], using glycine ester, obtained products which suggest reactions of the following types:

Lawson & Reid [1925] obtained similar results but found that although amino-acids condense readily with the sulphone they do not react with the sulphide (mustard gas). In these experiments of Cashmore & McCombie and those of Lawson & Reid, the conditions of the reaction were much more drastic than those employed by us. In the investigations described here, however, it has been necessary to avoid high temperatures and the use of strong alkalis or acids, in order that the antigenic power of the proteins might not be destroyed. Furthermore, it was the intention to reproduce as far as possible reactions which might take place when mustard gas is allowed to come into contact with the animal body. In spite of these differences it seems probable that the protein changes detected by the serological tests are similar to those occurring in amino-acids and their esters. The differences between the actions of mustard gas and of $\beta\beta'$ -dichlorodiethylsulphone [Cashmore & McCombie, 1923] would readily explain the failure, in our experiments, of proteins treated with mustard gas and those with the sulphone to give serological cross-reactions (cf. Table III).

In connexion with these studies of the action of mustard gas on proteins there are several other observations which appear to be pertinent. Arising out of the work on the anticarcinogenic action of mustard gas [Berenblum, 1935], Berenblum *et al.* [1936] studied the effects of mustard gas and $\beta\beta'$ -dichlorodiethylsulphone on the respiration and glycolysis of minced Jensen sarcoma tissue *in vitro*. They found that these compounds reduce the glycolysis of the tumour material more than its respiration. Jány & Sellei [1935] also noted that mustard gas inhibits glycolysis by *B. coli* and by Ehrlich rat sarcoma. Peters [1936] has shown that $\beta\beta'$ -dichlorodiethylsulphone has an inhibitory effect, similar to that of iodoacetic acid, on the respiration of brain tissues; this inhibition is exercised on the change pyruvate $\rightarrow$ oxidation products, and the sulphone has no appreciable effect on the conversion, in this tissue, of lactate into pyruvate. The inactivation of certain enzymes in the tissues studied by these various authors may possibly be due to the same change which produces an alteration in the immunological properties of the protein. Investigation from both aspects may, therefore, throw some light on the nature of the action of mustard gas and related compounds on the animal body.

SUMMARY

1. $\beta\beta'$ -Dichlorodiethylsulphide (mustard gas), when allowed to act on horse-serum proteins at room temperature, produces a change in the immunological properties of these proteins. These derivatives produce in the rabbit antibodies which are capable of reacting with mustard gas-treated rabbit serum.

2. $\beta\beta'$ -Dichlorodiethylsulphone-treated horse-serum proteins produce antibodies which will react with sulphone-treated serum proteins, but not with the mustard gas-treated proteins.

3. The possible chemical basis of this change in immunological properties may be an interaction between the mustard gas (or sulphone) and the free amino-groups of the protein molecule.

The authors would like to take this opportunity of thanking the Medical Research Council for a grant to one of them (A. W.) to cover part of the expenses incurred in this work.

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