The Immunological Properties of Proteins Treated with Di-2-chloroethylmethylamine

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When proteins are treated with 'sulphur mustard' (mustard gas; di-2-chloroethyl sulphide) they undergo a slight modification of their immunological properties, and the injection of mustard gas-treated proteins into rabbits may lead to the formation of antibodies capable of reacting specifically with any mustard gas-treated protein (Berenblum & Wormall, 1939; Bournsnel, Francis & Wormall, 1946*a*). It was suggested (Berenblum & Wormall, 1939) that acquired hypersensitivity to mustard gas might be partly due to an immunological change in the tissue proteins exposed to mustard gas, i.e. to the formation of 'foreign' proteins and subsequent production of specific antibodies. This view was later supported by the induction of hypersensitivity in laboratory animals by the application of mustard gas (Holiday, 1942; Kidd & Landsteiner, 1944; Moore, 1944). More recently, Fleming, Moore & Butler (1949) have shown that in the presence of phosphate buffer at pH 7.5 or 9, mustard gas reacts with proteins to give complexes which contain residues of diethyl sulphide, phosphate and protein, and that when the mustard gas-protein complexes prepared with added phosphate at pH 8-9 are injected into rabbits they give rise to strongly precipitating antisera.

The nitrogen mustards also react with a few enzymes and other proteins. Di-2-chloroethylmethylamine ($\text{CH}_3\text{N}(\text{CH}_2\text{CH}_2\text{Cl})_2$), e.g., strongly inhibits hexokinase (Dixon & Needham, 1946), cholinesterase (Thompson, 1947; Adams & Thompson, 1948), and choline oxidase and choline acetylase (Barron, Bartlett & Miller, 1948). It has been shown that this nitrogen mustard reacts with the amino, carboxyl, sulphydryl, sulphide and pyridine groups of proteins (cf. Fruton, Stein & Bergmann, 1946; for other references see Gilman & Philips, 1946; Boyland, 1948; Philips, 1950; Karnofsky, 1950) and that it rapidly inactivates the protein components of haemolytic complement (Watkins & Wormall, 1948, 1952).

In view of the fairly widespread clinical use of the nitrogen mustards for the treatment of Hodgkin's and certain other neoplastic diseases, we decided to

study the immunological properties of proteins treated with the nitrogen mustards. One of the objects of this work was to determine whether antibodies specific for nitrogen mustard-treated proteins could be produced by the repeated injection of nitrogen mustard or nitrogen mustard proteins; any antibody response of this type might indicate the possibility of hypersensitivity occurring in patients subjected to prolonged treatment with the nitrogen mustards. It was also hoped that these immunological and chemical studies might yield information about the mode of action of the nitrogen mustards on amino-acids, peptides and proteins.

EXPERIMENTAL

Nitrogen mustard (NM). The only NM used in these experiments was di-2-chloroethylmethylamine hydrochloride (code no. HN2). After the addition of the solid to protein solutions, the mixtures were shaken gently until the solid had dissolved. When NM was intravenously injected into rabbits, the solid was quickly dissolved in a small volume of 0.9% (w/v) NaCl and the solution injected immediately.

Ovalbumin. Hen ovalbumin, prepared by $(\text{NH}_4)_2\text{SO}_4$ precipitation, was recrystallized three times (cf. Cole, 1933).

Antigens

The general method used for the preparation of the NM-treated proteins (NM proteins) was as follows. The NM was added, in portions, to the protein solution kept at 37°, and 5% (w/v) Na_2CO_3 was added to the mixture, when required, to maintain the pH at about 7.5, or in a few experiments 7.5-8.0. One-quarter of the NM was added at the start and the same amount about 4 hr. later, and this treatment was repeated the next day. On the third day the mixture was again kept for 8 hr. at 37°, this time without further addition of NM. The mixtures were kept overnight in the refrigerator during this period.

With a few exceptions (the antigens designated *A*, *C* and *E* below) the products were then dialysed against frequently changed 0.9% NaCl for 48 hr. at 4°, to remove transformation or 'hydrolysis' products of NM. Dilute HCl or Na_2CO_3 was added where necessary to bring to pH 7.4, Merthiolate (0.01%) was added as a preservative, and the solutions were stored at 0-4°. Early in these investigations we observed that the NM used has a very powerful bactericidal action, but we continued to add Merthiolate to the antigen solutions

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prepared for storage since it was considered possible that the bactericidal activity of the nitrogen mustard might be due to some of its unstable cyclic transformation products.

When most of this work had been completed, the paper by Fleming *et al.* (1949) appeared, describing the effect of phosphate on the reaction between ordinary mustard gas and proteins. Since it seemed possible that phosphate might similarly affect the reaction between NM and proteins, a few further experiments were made with antigens prepared in the presence of extra phosphate.

The following antigens were prepared by the general method described above, with occasional slight modification (detailed below). These antigen preparations contained hydrolysis products of NM. No attempt was made in the preparation of antigens *A*, *C* and *E* to remove these impurities since it was thought that the treatment involved, for example, dialysis or the precipitation of the protein complexes by ethanol, might split NM-protein linkages. Control tests showed that any hydrolysis products of nitrogen mustard likely to be present after these antigen preparations had been kept for several weeks would not in any way affect precipitin or complement-fixation tests.

Antigens for immunization

(i) *NM ovalbumin*. Preparation *A* was made by the action of NM (1.5 g.) on a solution of crystalline ovalbumin (100 ml. containing 7.6 g. of protein). Preparation *B* (extra phosphate) was made similarly, but 0.5M-phosphate buffer of pH 8.0 (50 ml.) was added to the ovalbumin solution before addition of the NM.

(ii) *NM horse serum proteins*. Preparation *C*: normal horse serum (100 ml.) was treated with NM (0.80 g.). Preparation *D* was made similarly, with 0.5M-phosphate buffer of pH 8.0 (50 ml.) added to the horse serum.

Test antigens

In the precipitin and complement-fixation tests, the following antigens were used in addition to the above-described immunizing antigens. All the test-antigen solutions had a pH of about 7.4.

(i) *NM rabbit serum proteins*. Preparation *E*: normal rabbit serum (10 ml.) was treated with NM (80 mg.). The same method was used for preparations *F* (extra bicarbonate) and *G* (extra phosphate), except that additional buffers, 0.5M-NaHCO₃-H₂CO₃ (pH 7.5) (5 ml.) and 0.5M-phosphate (pH 8.0) respectively, were added to the rabbit serum.

(ii) *NM gelatin*. Preparation *H*: NM (300 mg. added in three 100 mg. portions at intervals of a few hours) was added to a mixture of a gelatin solution (500 mg. in 30 ml.) and 0.5M-NaHCO₃ (50 ml.) and the solution kept at 37° and pH 7.5-8.0 for 48 hr. In the preparation of the 'phosphate-antigen' (*J*) 0.5M-phosphate buffer (20 ml.) was used instead of NaHCO₃.

NM-treated amino-acids. Many attempts were made to prepare NM-amino-acid derivatives for use in precipitin-inhibition tests. Details of the methods used are given in the text.

Methods

Nitrogen determinations. Total N was determined by the micro-Kjeldahl method, using the Markham (1942) distillation apparatus. The Van Slyke (1912) volumetric method was used for the determination of α -amino N.

Immunization. In view of the weak specific antibody response following the injection of proteins treated with sulphur mustards (Berenblum & Wormal, 1939; Bournnell *et al.* 1946a), we decided to use several immunization methods in our attempts to get antibodies specific for NM proteins.

(a) *Intravenous injection of NM*. Solutions of NM hydrochloride in 0.9% NaCl were injected into the ear veins of rabbits on 4 successive days. After an interval of 12 days each rabbit received a further course of four similar injections, except no. 572 which received two injections only, because of badly damaged ear veins. The amounts of NM hydrochloride injected at each injection were 0.1 mg. (rabbits nos. 523 and 563), 0.2 mg. (nos. 614 and 619) and 0.3 mg. (nos. 572 and 576) per kg. of body weight. Blood samples were taken 7 and 14 days after the final injections, and the sera were tested for antibodies capable of reacting with NM proteins.

(b) *Intramuscular injection of alum-precipitated antigens*. The protein complexes were precipitated by potassium alum at pH about 6.5, as recommended by Proom (1943), and each rabbit received an intramuscular injection of 5 ml. of a suspension (containing about 17 mg. of protein/ml.) into each hind leg. A course of three injections was given, at intervals of 12-14 days, and the sera were tested 10 days after each injection.

(c) *Intraperitoneal injections*. The antigen solution (5 ml.) was injected intraperitoneally, and each rabbit, except no. 686 which died 10 days after the third injection, received five injections at intervals of 9-12 days. Precipitin tests were made on the blood serum samples taken 9 or 10 days after the third and subsequent injections.

In all, twenty-seven rabbits were immunized by methods (b) and (c), and details of the antigens and the mode of immunization are given in Table 1. The antisera were filtered through Berkefeld filters and stored in sterile tubes at 0-4°.

Table 1. *Details of immunizations with NM proteins*

(IM, intramuscular injections of the alum-precipitated antigen; IP, intraperitoneal injections.)

Group	Rabbits (nos.)	Immunizing antigen	Mode of injection
1	731, 744, 746, 747	NM horse serum (preparation C)	IM
2	734, 750, 751, 754	NM ovalbumin (preparation A)	IM
3	556, 654, 671, 708, 727	NM ovalbumin (preparation A)	IM
4	795, 809, 841	NM ovalbumin* (preparation B)	IP
5	679, 681, 686	NM horse serum* (preparation D)	IP
6	689, 719, 837, 840	NM ovalbumin* (preparation B)	IM
7	666, 692, 797, 854	NM horse serum* (preparation D)	IM

* Antigens prepared in the presence of extra phosphate.

Precipitin tests. These were made as described previously (Hopkins & Wormal, 1933). The tests with NM gelatin as test antigen were made at room temperature.

Precipitin-inhibition tests. The substance or mixture being tested for inhibitory power was dissolved in 0.9% NaCl to give a 1% (w/v) solution, or, with sparingly soluble substances, a saturated solution (at room temperature) was prepared. The solution was neutralized and varying amounts (1-4 vol.) were added to the antigen solution (1 vol.); 0.9% NaCl was then added (to give a total of 5 vol.). Antiserum (2 vol.) was added and the mixtures were shaken and kept at 37°. The extent of precipitation was noted after 15 min., 1 and 3 hr.

Complement-fixation tests. These were carried out as described previously (Berenblum & Wormald, 1939), but with a sensitized sheep erythrocyte system.

RESULTS

Intravenous injection of NM into rabbits

The sera of six rabbits which were given frequent injections of NM contained no antibodies capable of precipitating NM ovalbumin. Thus no evidence was obtained that the injection of NM leads to the production of antibodies specific for NM proteins, even when the amount injected is appreciably greater than that injected into patients suffering from Hodgkin's and some other neoplastic diseases. The amount of NM injected into man varies very considerably, but a widely adopted method is a course of injections of 0.1 mg./kg. of body weight on each of 4 successive days, with a second similar course some weeks or months later. In our experiments doses three times as large as these, with a second course after an interval of 12 days, failed to elicit the formation of antibodies specific for NM proteins.

Injection of NM proteins

Although antibodies specific for NM proteins could not be detected in the serum of rabbits following the injection of NM, the possibility of antibody production in the tissues of these injected animals cannot be excluded. We decided, therefore, to study the effect of injecting preformed NM proteins. Horse serum proteins and ovalbumin, previously treated with NM, were injected intraperitoneally or intramuscularly (as the alum-precipitated antigen) into several groups of rabbits, and the sera of these animals were tested at intervals for antibodies capable of reacting, in precipitin and complement-fixation tests, with various NM-proteins.

Some of the injected rabbits had no detectable 'NM protein-specific' antibodies in their serum at any time during the long immunization; all produced antibodies which precipitated the injected antigen or the corresponding untreated protein (horse serum proteins or ovalbumin), showing that the injected complexes were fully antigenic. Thus treatment with considerable amounts of NM at pH 7.5-8 does not destroy the antigenicity of ovalbumin or the serum proteins.

The sera of seven of the twenty-seven injected rabbits gave, at some stage in their immunization, weak but significant precipitin reactions with NM proteins different from those used for immunization. For example, the injection of NM horse serum proteins occasionally gave rise to antibodies which

Table 2. *Precipitin reactions with antisera to proteins treated with nitrogen mustard (NM)*

(Precipitin reactions are recorded, as follows: -, (no reaction); tr., (trace); ±, +, +±, ++ etc., in increasing degrees of precipitation.)

Antigen	Concentration of antigen solution (%)	Antisera against			
		NM ovalbumin		NM horse serum proteins	
		No. 751	No. 754	No. 746	No. 747
NM ovalbumin	0.25	+++	+++	-	-
	0.05	+++	+++	-	-
	0.01	++	++	±	-
	0.002	-	tr.	-	-
NM horse serum proteins	0.25	-	-	+±	+±
	0.05	±	-	++±	++±
	0.01	±	±	+	+
	0.002	-	-	tr.	-
NM rabbit serum proteins	0.25	-	-	-	-
	0.05	tr.	tr.	±	±
	0.01	-	-	tr.	tr.
	0.002	-	-	-	-
Ovalbumin	0.25	+±	+±	-	-
	0.05	±	±	-	-
	0.01	tr.	tr.	-	-
	0.002	tr.	tr.	-	-
Horse serum proteins	0.25	-	tr.	+±	+±
	0.05	tr.	-	++±	++±
	0.01	-	-	+±	+±
	0.002	-	-	tr.	-

gave precipitates with NM ovalbumin or NM rabbit serum proteins, but not with NM gelatin. Typical results of these reactions are given in Table 2. No evidence was obtained that the specific antibody response depended on the particular NM protein injected or on the mode of immunization. The results with the NM protein antigens prepared in the presence of phosphate were not significantly different from those obtained with antigens prepared without added phosphate.

Complement-fixation tests fully confirmed this observation that antibodies specific for NM proteins were occasionally produced in the injected rabbits. Typical results (Table 3) showed that the sera of some of the rabbits injected with NM ovalbumin reacted with heterologous NM proteins, e.g. NM horse serum proteins. No complement fixation was obtained when normal horse serum proteins were used as the antigen in these tests with antisera to NM ovalbumin.

Table 3. *Complement-fixation tests with antisera to NM ovalbumin*

(Mixtures of 0.5 ml. of the diluted antigen (of concentration recorded in column 1), 0.10 ml. of antiserum (previously heated at 56° for 20 min. to inactivate any complement present) and 0.10 ml. of dilute guinea pig serum (1 vol. of serum plus 4 vol. of 0.9% NaCl) were kept at room temperature for 1 hr. After the addition of 0.5 ml. of a 3% suspension of sensitized sheep erythrocytes to each tube, the mixtures were shaken and kept at 37°, and the extent of haemolysis was noted at intervals. The readings recorded in this table were those after 1 hr. Degrees of haemolysis; 0, none; tr., trace; 1, 2 and 3, increasing degrees of haemolysis; 4, complete haemolysis.)

Concentration of antigen (NM horse serum proteins) (mg./100 ml.)	Degree of haemolysis	
	Antiserum no. 750	Antiserum no. 734
250	4	4
83	4	4
28	3	4
9.3	2	3
3.1	tr.	2
1.0	0	1
0.34	0	0
0.11	0	0
0.038	0	0
0.013	0	tr.
0.004	tr.	1
None (control)	4	4

Attempts to obtain specific inhibition of the reaction between NM proteins and their antibodies

Our immunological and chemical evidence suggests that the slight change in the immunological properties of proteins effected by NM is due to the combination of NM and the protein. It is conceivable, therefore, that a suitable NM amino-acid or NM peptide might be found which would

specifically inhibit the NM protein antigen-antibody reaction, for example the precipitin reaction between NM ovalbumin and antibodies to NM horse serum globulins. Specific inhibition of this type would, of course, provide useful information about the nature of the protein groups to which the NM becomes attached.

Attempts were made to isolate products formed by the action of NM on various amino-acids, and in these experiments, as in the preparation of NM protein antigens, we kept the conditions as near as possible to the physiological, namely temperatures not exceeding about 37° and a pH of 7.5–8.5. Fruton, Stein & Bergmann (1946) describe the reduction of free amino nitrogen which occurs when solutions of various amino-acids are shaken with NM at pH 8–8.5 and 25°, and they isolated from NM plus phenylalanine a reaction product to which they gave the formula

Our efforts to prepare this pure substance have, unfortunately, been unsuccessful, and so have our attempts to obtain pure NM derivatives of glycine, lysine and cysteine. The method we used was essentially that described by Fruton, Stein & Bergmann (1946), and although with all these amino-acids we observed a considerable reduction (24–41%) in the free amino nitrogen and a complete loss of free SH when cysteine was used, we were unable to separate crystalline reaction products; in some instances we attempted, without success, to separate the NM amino-acid derivatives as copper salts, a method found satisfactory for a mustard gas derivative of glycine (Boursnell, Francis & Wormald, 1946b). Since, however, the relatively crude products differed from the original amino-acids in total nitrogen and free amino-nitrogen contents, it was decided to find out whether they specifically inhibited the NM protein antigen-antibody reaction.

In a further search for inhibitory substances and as part of a more general study of the action of NM on proteins, we investigated the action of NM on nucleic acid and glutathione.

(a) Yeast nucleic acid (British Drug Houses) was purified by dissolving the sodium salt in water and precipitating the acid at pH 1.5–3.0. After it had been washed successively with ethanol and ether and dried at 50°, the product (0.5 g.) was dissolved in 0.5M-NaHCO₃-H₂CO₃ buffer of pH 7.5 (20 ml.) and mixed with a freshly prepared neutralized solution of NM (0.25 g.) in about 4 ml. of water. The mixture was kept at room temperature for 24 hr. and three fractions were separated by treatment with N-HCl: (A) a light-brown solid (0.43 g.) precipitated over the range pH 2–4, (B) an almost white solid (0.06 g.) precipitated at pH 1–2, and (C) a white solid (0.03 g.) precipitated from the mixture at pH 1, after the removal of fraction (B), by adding excess of ethanol. All these fractions were washed with ethanol and then with ether, and finally dried *in vacuo*. They

were not sufficiently pure for complete analysis, but N and P determinations were made on the two largest fractions (*A*) and (*B*); these had N/P ratios of 2.06 and 1.95 respectively, compared with a ratio of 1.79 for the untreated purified nucleic acid.

(b) A freshly prepared solution of NM (400 mg.) in 0.5M-NaHCO₃-H₂CO₃ buffer of pH 7.5 (5 ml.) was added to a solution of glutathione (300 mg.) in NaHCO₃ buffer (5 ml.) and the mixture was kept at room temperature. After 30 min. there was no free SH detectable by nitroprusside, and in the course of 48 hr. there was a 48% reduction in the free amino nitrogen of the solution. The mixture was then concentrated to a syrup *in vacuo* over H₂SO₄ at room temperature, and the crystalline material which separated was rubbed with acetone to give a flocculent powder which was washed with acetone and dried. The remaining syrup was kept for some time *in vacuo* over conc. H₂SO₄, and the gelatinous solid obtained was rubbed with acetone, giving a powder which was washed with acetone and dried. In view of the inability of these products to inhibit the NM protein serological systems, no attempts have so far been made to purify and identify them.

Precipitin inhibition tests with the above-mentioned NM-treated amino-acids, nucleic acid and glutathione showed that these products had no specific inhibitory effect on the NM protein antigen-antibody system. Where inhibition occurred it was of a non-specific nature, for the product concerned inhibited unrelated precipitin reactions. No inhibition was obtained with some 'hydrolysis' products of NM (namely di-2-hydroxyethylmethylamine, 2-chloroethyl-2-hydroxyethylmethylamine and the dimer, *NN'*-di(2-chloroethyl)-*NN'*-dimethylpiperazinium chloride), or with the following NM derivatives; a crystalline compound formed by the interaction of NM and di-2-hydroxyethylmethylamine (Fruton, Stein, Stahmann & Golumbic, 1946) and the hexamethylenetetraminium derivative formed by the action of NM on hexamethylenetetramine (Gurin, Deluva & Crandall, 1947).

The stability of the NM protein complexes

It was not possible in these investigations to determine the number of NM residues present in the NM protein complexes, and indeed this could only be achieved in later investigations with ¹⁵N-labelled NM (Burnop, Richards, Watkins & Wormall 1951). A few preliminary qualitative experiments were made, however, to determine the stability of the linkages between the NM and proteins, and whether the treatment of serum proteins with NM produces protein derivatives which are precipitated, as are the corresponding mustard gas sulphone proteins, by the addition of dilute acid.

Dialysis of NM horse serum proteins for 4 days at 4° against frequently changed 0.9% NaCl, or their maintenance in acid or alkaline solution (over the range pH 3-9) for 16 hr. at 37° and subsequent

dialysis against 0.9% NaCl at 4°, effected no significant loss of the power of these NM proteins to give precipitates with antisera to NM ovalbumin. A slight loss of precipitability occurred in solutions at pH 11 kept for 16 hr., but this reduction was most probably due to denaturation rather than removal of NM groups, since there was a corresponding decrease in the capacity of the NM protein complex to precipitate antibodies to untreated horse serum globulins.

With regard to precipitation by acid, it was found that the NM serum proteins, prepared by the action at pH 7.5 of either freshly prepared aqueous solutions of NM or NM solutions which had been allowed to hydrolyse (for example, at 37° for 4 hr. followed by 18 hr. at 4°), could not be precipitated by adding dilute acid. In this respect, therefore, the action of NM resembles that of 'sulphur mustard' and differs from that of mustard gas sulphone.

DISCUSSION

The extensive work of Landsteiner and his colleagues (for the literature, see Landsteiner, 1936, 1945) has shown that many simple organic compounds produce, when introduced into the animal body, a condition of hypersensitivity because they react with the body proteins to form immunologically foreign conjugates. Induced hypersensitivity to ordinary mustard gas has been explained in this way (Berenblum & Wormall, 1939), and if NM combines firmly with proteins the risk of producing allergic reactions by injecting nitrogen mustard must be considered.

Our investigations show that when the serum proteins and ovalbumin are treated with nitrogen mustard under physiological conditions of pH and temperature, these proteins undergo modifications in their immunological properties. The complexes formed produce antibodies specific for NM-treated proteins in some, but not all, of the injected rabbits. This alteration in the immunological properties of the proteins is, however, very slight, since specific antibody formation was only occasionally produced. NM undoubtedly combines firmly with proteins, as was indicated by the investigations described here and more fully established by our quantitative studies with nitrogen mustard labelled with ¹⁵N (Burnop *et al.* 1951), and it is surprising that the specific antibody production following the injection of NM-treated protein is not stronger and more frequent. The specific antisera produced by the injection of sulphur mustard-proteins were not very potent (Berenblum & Wormall, 1939; Bournsnel *et al.* 1946*a*), but they were more powerful than the antisera to NM proteins produced in the experiments described here, and they were obtained in a higher percentage of injected animals.

This infrequency of specific antibody production following the injection of NM proteins may be due to the fact that relatively few NM residues become attached to each protein molecule, and since some of these groups can be detached by long dialysis (Burnop *et al.* 1951), the injected NM proteins may soon lose many of their determinant NM groups. Another possible explanation is that NM might act on proteins mainly by a cross-linking action. Haddow, Kon & Ross (1948) showed that the cytotoxic activity of a series of halogenoalkylarylamines could be related to the presence of two halogenoalkyl groups in the molecule, and Goldacre, Loveless & Ross (1949) suggested that cross-linking might account for the action of the nitrogen mustards in producing chromosome abnormalities (see also Loveless & Revell, 1949; Loveless, 1951). According to this view two reactive groups are required in the NM molecule to permit it to react at two distant points either on the same fibre or on two contiguous fibres. Cross-linking of this type might explain why NM-treated proteins differ so little immunologically from the unchanged protein, for with a cross-linking mechanism there would be no characteristic determinant grouping projecting from the NM protein molecule.

Whatever the explanation, it appears that the injection of NM or of NM-treated proteins into rabbits rarely leads to the production of antibodies specific for NM proteins and this is supported by clinical evidence. There are apparently few, if any, cases of allergic reactions in man attributable to the intravenous injection of the nitrogen mustards. Furthermore, Hartman, Mangun, Feely & Jackson (1949) found no evidence of antigenic effects when man and dogs were given repeated injections of plasma to which NM had been added as a preservative. On the other hand, Moore & Rockman (1950) have recently found that skin hypersensitivity to one of the nitrogen mustards (di-2-chloroethyl-methylamine) can be induced in man by a single NM burn or preferably two successive burns with a week's interval between the applications of NM; no evidence was obtained of simple cross-reactions between sulphur mustards and NM in these sensitivity tests. These observations of Moore & Rockman suggest that the possibility of occasional production of hypersensitivity to NM should not be overlooked.

Nitrogen mustards such as di-2-chloroethyl-methylamine react with many groups in the protein molecule (cf. reviews by Philips, 1950, and Karnofsky, 1950) and they degrade deoxyribonucleic acid solutions as shown by a decrease in viscosity (Gjessing & Chanutin, 1946; Butler, Gilbert &

Smith, 1950; Butler & Smith, 1950). It is not known, however, which parts of the protein molecule are primarily concerned when NM effects its characteristic biological actions, including the slight serological change described in this paper. Our attempts to obtain information on this point by serological inhibition tests have so far been unsuccessful, for we have not been able to obtain any NM amino-acid derivative or any similar preparation which will specifically inhibit the reaction between NM proteins and their antibodies. With the aid of NM labelled with ^{15}N and ^{14}C we are making a further study of the action of NM on proteins and tissue constituents, and it has now been possible to determine the amount of NM which combines firmly under physiological conditions with the serum and other proteins (Burnop *et al.* 1951). Quantitative data about the combination of NM and nucleic acids has also been obtained (Burnop, Richards & Wormald, 1952), and it is hoped that these quantitative studies will throw some light on the immunological results reported here.

SUMMARY

1. Antibodies capable of reacting with NM (nitrogen mustard)-treated proteins could not be detected in the sera of rabbits which had received a series of intravenous injections of di-2-chloroethyl-methylamine, one of the nitrogen mustards frequently used therapeutically.

2. The sera of rabbits which had been given a course of injections of NM-treated proteins (horse serum proteins or ovalbumin treated with NM at 37° and pH 7.5–8.0) occasionally contained antibodies capable of reacting specifically with NM proteins, including NM-treated rabbit serum proteins.

3. This specific antibody production was, however, very weak and infrequent. It is concluded that the risk of hypersensitivity following frequent exposure to NM or a series of intravenous injections of the drug, is slight, at least with the rabbit.

4. The complexes formed when NM acts on serum proteins or ovalbumin are comparatively stable, and the NM protein does not lose its serological specificity as a result of long dialysis. Attempts to obtain a NM amino-acid compound capable of specifically inhibiting the NM protein antigen-antibody reaction have been unsuccessful.

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The Determination of Plasma Fibrinogen by the Clot-Weight Method

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The clot-weight technique is a convenient method for the determination of plasma fibrinogen. The result is obtained as fibrin with sufficient accuracy for clinical purposes. Nevertheless, certain possible causes of error appear not to have received attention and these have been investigated.

METHODS

Plasma. 9 vol. of human blood are received from a paraffined syringe into 1 vol. of sodium citrate solution containing 3.8 g./100 ml. and the mixture is centrifuged at 2000 rev./min. for 30 min.

Technique of fibrinogen determination. The citrated plasma is diluted with an approximately equal vol. of 0.025 M-CaCl₂ solution and allowed to stand at 37°. The fibrin is wound off

on to a wooden applicator until no further clot forms, washed in distilled water, rolled off the rod, drained between filter papers, pulled out thin (when of sufficient bulk), dehydrated first in acetone and then in air at 105° for 3 hr., and weighed.

From the haematocrit of the citrated blood the calculation is made as follows:

$$\text{Plasma fibrinogen content} = \frac{\text{clot weight}}{\text{sample volume}} \times \frac{\text{volume of citrated plasma in 10 ml. citrated blood sample (from haematocrit)}}{\text{volume of citrated plasma} - \text{volume of citrate}}.$$

This correction ignores the variable effect of the citrate upon the cell-plasma fluid partition, but error from this cause is thought to be small compared with differences between persons.