The Action, *in vitro* and *in vivo*, of some Nitrogen Mustards on Haemolytic Complement

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It has been shown (Bournsnel, Francis & Wormal, 1946) that haemolytic complement is rapidly inactivated by small amounts of sulphur mustard (di-2-chloroethyl sulphide; code no. H) and divinyl sulphone. Since the nitrogen mustards have been widely used during recent years in the treatment of some neoplastic diseases, we decided to study the action, *in vitro* and *in vivo*, of a typical nitrogen mustard (di-2-chloroethylmethylamine; code no. HN 2) on the haemolytic complement of man and some other animals.

Haemolytic complement is a complex system containing four functionally distinct components, and it is generally agreed that complement plays an important role in natural immunity and resistance to infection. Although it has not been definitely established that a low blood complement in an animal is associated with a low resistance to infection, many investigators have reported low blood complement titres in some infectious diseases (for the literature see the reviews by Osborn, 1937; Ecker, Seifter & Dozois, 1945; Ecker, 1948). If the therapeutic use of the nitrogen mustards, involving a course of intravenous injections of the drug, were to result in a severe diminution of the blood complement, this loss might have serious ill effects.

A considerable amount of information is available about the separation of the four components of complement, and about their stability and general chemical properties, but the mode of action of this complex system is still unknown. It is a relatively labile system associated largely with certain serum globulins, and the inactivation of any one component, for example by mild oxidation or by denaturation, leads to complete loss of haemolytic, bacteriolytic and bactericidal effects. Pillemer & Ecker (1941a) and Heidelberger (1941) have suggested the use of C'1, C'2, C'3 and C'4 as symbols for the 'midpiece', 'endpiece', and third and fourth components respectively, and this nomenclature is used in the present paper.

The main object of the present investigations was to determine whether the clinical use of HN 2 and related nitrogen mustards is likely to lower seriously

the blood-complement level of the injected individuals. Our *in vitro* tests soon showed that HN 2, like the sulphur mustard (H), rapidly destroys haemolytic complement, and the investigations were therefore extended to include a more general study of the anticomplementary action of various nitrogen mustards and some related compounds. It was hoped that these experiments would yield useful information about (a) the chemical properties of individual components of complement, and (b) the nature of the action of nitrogen mustard on proteins and related compounds. Some of the results of these investigations have been reported briefly elsewhere (Watkins & Wormal, 1948). Since the work described here was completed, Southam & Goldsmith (1951) have confirmed our observation that complement is inactivated by nitrogen mustard *in vitro*, and they have also found that no depression of human complement titre occurs following the clinical use of HN 2 and X-rays.

EXPERIMENTAL

MATERIALS

Di-2-chloroethylmethylamine (code no. HN 2). This was prepared, as the hydrochloride, by the method of Hanby & Rydon (1947).

Di-2-hydroxyethylmethylamine. This compound (Light and Co. Ltd.) was freshly distilled under reduced pressure immediately before use.

NN'-Di-2-chloroethyl-NN'-dimethylpiperazinium chloride. Prepared as described by Hanby & Rydon (1947). Melting point of picrate, 205°.

2-Chloroethyl-2'-hydroxyethylmethylamine. This was isolated via the picrate from a mixture of reaction products, as described by Hanby & Rydon (1947). Melting point of mercurichloride, 197°.

Haemolytic complement

Fresh serum. Normal guinea pig serum was used as the source of complement for most of the experiments on the *in vitro* action of nitrogen mustard on haemolytic complement.

Freeze-dried serum. Human, rabbit and guinea pig sera were dried and kept for use as standards for the experiments on the effect of the injection of di-2-chloroethylmethylamine on the serum haemolytic-complement level in man, rabbit and guinea pig. Samples of bulked serum (0.50 or 1.0 ml. in each tube) were freeze-dried as described by

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Greaves (1946) and were sealed under vacuum. The sera were reconstituted in distilled water when required, and were used only on the day on which the tube had been opened. As far as could be judged from a comparison of the complement activity of various standards prepared at different times, no marked loss of activity occurred when the dried standards were kept at room temperature for up to at least 6 months.

Complement lacking C'1 and C'2. Serum was heated at 56° for 30 min. and then diluted with 9 vol. 0.9% (w/v) NaCl.

Complement lacking C'3. Zymosan, the insoluble carbohydrate of yeast used for the specific inactivation of the third component of complement, was prepared by the method of Pillemer & Ecker (1941*b*). The amount of carbohydrate required to inactivate the third component was determined for each sample of serum in a preliminary experiment. The carbohydrate was suspended in distilled water, heated on a boiling-water bath for 30 min. and then centrifuged; the supernatant solution was removed and the residue allowed to cool. Guinea pig serum (1 vol.) was added to this residue and the suspension was kept in a water bath at 37° for 2 hr. with frequent shaking. After the addition of 0.9% NaCl (9 vol.) the suspension was centrifuged and the supernatant solution was separated and its pH was brought to 7.5.

Complement lacking C'4. Complement deficient in the fourth component was prepared by the method of Gordon, Whitehead & Wormall (1926). Undiluted serum (1 ml.) was treated with 0.2 ml. of 0.2 N-NH₃, and the mixture was kept at 37° for 1.25 hr. At the end of this time, 0.16 ml. of 0.2 N-HCl and 8.6 ml. 0.9% NaCl were added.

Separation into 'midpiece' and 'endpiece' fractions. Guinea pig serum (1 vol.) was diluted with CO₂-saturated water (9 vol.) and a slow stream of CO₂ was passed through the mixture which was cooled in ice-water during this process. After 20 min. the mixture was centrifuged and the precipitate ('midpiece' fraction) was washed with 0.02 M-phosphate solution of pH 5.4, and finally dissolved in 10 ml. 0.9% NaCl; the pH of the solution was adjusted to 7.5 with 0.1 M-NaHCO₃. The original supernatant solution ('endpiece' fraction) (10 vol.) was treated with 10% (w/v) NaCl (1 vol.) and the CO₂ was removed *in vacuo*; 0.1 M-NaHCO₃ was added to bring the pH to 7.5.

METHODS

Haemolytic system. Varying amounts of diluted serum, or other mixture being tested for complement activity, were measured into a series of tubes, and 0.9% NaCl was added, where necessary, to bring the total volume to 1.0 ml. in every tube. A 3% suspension (1.0 ml.) of sensitized washed sheep cells was added to each tube and the tubes were immersed in a water bath at 37° for 30 min. Mayer, Osler, Bier & Heidelberger (1946, 1948) have shown that Ca⁺⁺ and Mg⁺⁺ are essential constituents of the haemolytic system, and in experiments involving dialysis or fractionation of the serum we therefore ensured that there were optimum concentrations of these two ions in the final mixtures.

Measurement of complement activity. In the experiments on the *in vitro* action of nitrogen mustard on complement the extent of haemolysis was estimated visually after 10, 20 and 30 min., and was recorded as follows: 4 (complete haemolysis); 3, 2, 1 and tr. (trace) (intermediate degrees of haemolysis); 0 (no haemolysis).

For the determination of the complement level in rabbits, guinea pigs and human subjects before and after the injection of nitrogen mustard, the degree of haemolysis was estimated colorimetrically. After the mixtures had been kept at 37° for 30 min. they were centrifuged and the colour intensity of the liberated haemoglobin in the supernatant solution was measured, after suitable dilution, in a photoelectric colorimeter (Evans Electroselenium Ltd., portable model) using a green Ilford Spectrum filter no. 624. The results were expressed as a percentage of the reading given by a mixture showing complete haemolysis. A graph was then constructed from the values for the percentage haemolysis given by different volumes of serum. The amount of undiluted serum (*v*) required to produce 50% haemolysis was read off from the curve, and the complement content of the serum was recorded in arbitrary '50% units'/ml. (1.0/*v*). This method is essentially similar to that described by Mayer, Eaton & Heidelberger (1946) and Kent, Bukantz & Rein (1946).

In experiments involving a comparison of the serum complement levels of an animal on different days, the results were expressed as the ratio of the number of 50% units/ml. in the tested serum to the number of 50% units/ml. in the 'standard' freeze-dried serum. When small amounts of serum only were available, or when many titrations had to be carried out on the same day, it was not always possible to titrate the sera over the whole range of partial lysis. For these determinations, therefore, the 50% unit of complement activity was obtained from a single analysis using the conversion factors calculated by Mayer, Osler *et al.* (1946) from the von Krogh equation $x = K(y/(1-y))^{1/n}$ which expresses the S-shaped haemolysis curve.

Since rabbit and human sera frequently contain non-specific haemolysins, these sera were subjected to one absorption with sheep cells before complement determinations were made on them. Packed washed sheep cells (0.2 ml.) were added to the serum (1.0 ml.) and the mixtures were kept at room temperature for 10 min., after which the cells were removed by centrifuging.

RESULTS

IN VITRO ACTION OF NITROGEN MUSTARDS ON HAEMOLYTIC COMPLEMENT

Effect of HN 2 on guinea pig, human and rabbit complement

The action of di-2-chloroethylmethylamine on guinea pig serum complement at 37° and pH about 7.5 has been studied.

The crystalline HN2 hydrochloride was dissolved in 0.9% NaCl and varying amounts of this solution were immediately added to 0.5 ml. samples of guinea pig serum, with 0.9% NaCl to give a total volume of 1.5 ml. in each tube. Phenol red (1 drop of 0.04%) was added to each mixture and each was then kept at 37° with occasional gentle shaking. The pH was maintained at 7.5 by adding dilute NaOH where necessary, and 0.9% NaCl was added to the other tubes to compensate for the volume change. Samples (0.10 ml.) were withdrawn from each mixture at intervals and diluted with 0.9% NaCl (0.90 ml.). The complement activity of each diluted sample was then determined as follows: measured volumes (0.5, 0.2 and 0.1 ml.) were

added to tubes containing 0.5 ml. of a 3% suspension of sensitized sheep cells, 0.9% NaCl was added to give a total volume of 1.0 ml. in each tube, and haemolysis readings were taken after 10, 20 and 30 min. at 37°.

The results showed (Table 1) that partial inactivation of complement occurred in 10 min. when 0.5 ml. of guinea pig serum was treated at 37° with 2.5 mg. of the vesicant, and complete inactivation was effected in 35 min. by 1.25 mg. At room temperature inactivation was less rapid, but the complement activity of 0.5 ml. of guinea pig serum was almost completely destroyed by treatment with 1.0 mg. of HN 2 for 1 hr., and with 10 mg. about half the activity had disappeared within 15 min.

Other experiments showed that the haemolytic complements of human and rabbit sera are similarly inactivated by treatment with HN 2.

Effect, on complement, of 'hydrolysed' HN 2 and of the individual products of this 'hydrolysis'

The nitrogen mustards undergo a series of reactions in aqueous solution (Hanby, Hartley, Powell & Rydon, 1947; Golumbic, Fruton & Bergmann, 1946), and experiments were therefore carried out to determine the complement-inactivating power of 'hydrolysed' nitrogen mustard and some of the individual reaction products. The inactivating power of a 0.25% solution of HN 2 in

Table 1. *The action of HN 2 on guinea pig complement at 37°*

HN ₂ added to 0.5 ml. of guinea pig serum (mg.)	Volume of diluted sample tested (ml.)	Complement activity* of the samples taken after				
		10 min.	30 min.	60 min.	120 min.	180 min.
0 (control)	0.50	4	4	4	4	4
	0.20	4	4	4	4	4
	0.10	2	2	2	2	1
0.25	0.50	4	4	4	2	2
	0.20	4	3	2	tr.	0
	0.10	2	tr.	0	0	0
1.25	0.50	4	1	0	0	0
	0.20	3	0	0	0	0
	0.10	1	0	0	0	0
2.50	0.50	4	0	0	0	0
	0.20	2	0	0	0	0
	0.10	0	0	0	0	0

* Haemolysis readings taken after 30 min.

In view of the clinical use of the nitrogen mustards and the possibility that a similar inactivation might be produced *in vivo*, tests were made to determine the lowest concentration of HN 2 which would destroy the complement activity of serum. Varying amounts of a freshly made 0.1% solution of HN 2 hydrochloride in saline were added to 0.5 ml. samples of guinea pig serum, and complement determinations were made on the mixtures at intervals. The results showed that the haemolytic-complement activity of the serum was completely destroyed in 6 hr. by treatment at 37° with 0.2 mg. of HN 2, and that appreciable inactivation was brought about in this time by as little as 0.05 mg.

Treatment of haemolysin with amounts of HN 2 appreciably greater than those used to inactivate complement did not decrease the activity of the serum, and exposure of sensitized or unsensitized sheep cells to corresponding amounts of the vesicant did not influence their subsequent haemolysis by complement. The inhibitory effect of nitrogen mustard on the haemolysis of sensitized red cells by complement must, therefore, be due to the destruction or inactivation of one or more of the components of complement.

normal saline which had been kept for 26 hr. at 37° and pH 7.5 was compared with that of a freshly prepared solution of the same strength. The results showed that the ability of the hydrolysed material to inactivate complement was very considerably less than that of an equivalent amount of unhydrolysed HN 2. Only slight inactivation was effected in 180 min. by a volume of the hydrolysed HN 2 solution containing the equivalent of 2.5 mg. of the unhydrolysed amine, whereas comparable inactivation was effected in 60 min. by a tenth of the volume of freshly prepared HN 2 solution.

Further experiments were made to determine how rapidly the power to inactivate complement is lost when aqueous solutions of HN 2 are kept at 37°.

Samples (10 mg.) of HN 2 were dissolved in 0.9% NaCl (2.0 ml.) and the solutions were kept at 37° for varying lengths of time, the pH being maintained at about 7.5. Measured volumes of these solutions of hydrolysed HN 2 were added to guinea pig serum (0.5 ml.), and 0.9% NaCl was added to give a total volume of 1.5 ml. in each tube. The mixtures were kept at 37°, the pH again being maintained at 7.5. Samples were taken from each mixture at intervals and diluted with 9 vol. of 0.9% NaCl. The complement activity of these diluted samples was then measured.

The results (Table 2) showed that hydrolysis for 30 min. slightly reduced the inactivating power of the nitrogen mustard, but even with hydrolysis for 2.5 hr. there was not complete loss of complement-inactivating capacity.

Di-2-hydroxyethylmethylamine in concentrations of up to 125 mg./0.5 ml. guinea pig serum effected no destruction of the complement activity, and this hydrolysis product of di-2-chloroethylmethylamine is apparently not the agent responsible for the complement-inactivating power of hydrolysed

Action of some compounds in protecting complement against inactivation by HN 2

HN 2 and its half-hydrolysis product, 2-chloroethyl-2'-hydroxyethylmethylamine, both undergo cyclization to form ethyleniminium ions and, since both these substances inhibit complement action, the possibility arises that it is the ethyleniminium ions which are responsible for the inactivation. In order to test this possibility, substances which are known to react with ethyleniminium ions were

Table 2. *The action, on complement, of previously 'hydrolysed' HN 2*

'Hydrolysed' HN 2 added* (mg.)	Duration of prior hydrolysis of HN 2 (min.)	Volume of diluted sample tested (ml.)	Complement activity† of the mixture of serum and hydrolysed HN 2 after	
			20 min.	60 min.
1.0	0	0.50	4	0
		0.20	1	0
		0.10	0	0
1.0	30	0.50	4	2
		0.20	2	0
		0.10	0	0
1.0	150	0.50	4	4
		0.20	3	1
		0.10	tr.	0
5.0	0	0.50	0	0
		0.20	0	0
		0.10	0	0
5.0	30	0.50	0	0
		0.20	0	0
		0.10	0	0
5.0	150‡	0.50	3	0
		0.20	0	0
		0.10	0	0
0 (control)	0	0.50	4	4
		0.20	3	3
		0.10	tr.	tr.

* Amount added to 0.5 ml. of guinea pig serum.

† Haemolysis readings taken after 30 min.

‡ Hydrolysed for 45 min. at 20° and a further 105 min. at 37°.

nitrogen mustard solutions. Similarly, it was found that the piperazinium dimer, *NN'*-di-2-chloroethyl-*NN'*-dimethylpiperazinium chloride, was without action on complement at concentrations comparable with those of HN 2 which rapidly produce inactivation. The chlorohydrin, 2-chloroethyl-2'-hydroxyethylmethylamine, on the other hand, showed some capacity for reducing the complement activity of guinea pig serum, although the inhibition produced was less than that given by a fresh HN 2 solution. Thus, a solution containing 2.5 mg. of the chlorohydrin hydrochloride effected complete inactivation of the complement in 0.5 ml. guinea pig serum in 120 min. at 37°, whereas the freshly prepared HN 2 solution of the same strength gave almost complete inactivation in 10 min.

introduced into the complement-nitrogen mustard system. Table 3 shows the results of an experiment in which 0.1N-sodium thiosulphate (1 vol.) was mixed with a freshly prepared 0.5% (w/v) HN 2 solution (1 vol.), and the mixture was added at once to guinea pig serum. In the presence of this concentration of thiosulphate the nitrogen mustard failed to inactivate complement. Control tests showed that in these concentrations sodium thiosulphate alone did not affect the haemolytic power of guinea pig serum, showing that this substance is not anti-complementary or lytic *per se*. Treatment with lower concentrations of sodium thiosulphate did not completely prevent inactivation of complement, although the amounts added were in excess of those calculated to 'neutralize' all the

ethyleniminium ions liberated from the HN2 used.

The results suggest that under these conditions complement and thiosulphate compete for reaction with the ethyleniminium ions. The inactivation of complement by 2-chloroethyl-2'-hydroxyethyl-methylamine was similarly prevented by adding sodium thiosulphate. Hexamethylenetetramine and phosphate ions were also found to protect complement against inactivation by nitrogen mustard, although in equimolar concentrations they were not as effective as thiosulphate ions. This is in accordance with the degree of avidity with which hexamethylenetetramine and thiosulphate react with ethyleniminium ions, as shown by Fruton, Stein, Stahmann & Golumbic (1946).

when added to rabbit serum in concentrations up to $1.5 \times 10^{-3}M$, had no effect on the haemolytic-complement activity of the serum, nor did it protect the complement against inactivation by HN2.

Action of HN2 on the individual components of complement

Experiments were carried out to determine whether all four components of complement are sensitive to the action of nitrogen mustard or whether the vesicant would prove a useful reagent for the specific inactivation of one or two components. Guinea pig serum samples which had been treated in such a way as to destroy specifically one or two complement components were added to other

Table 3. *The effect of sodium thiosulphate on the inactivation of complement by HN2*

Solution added* to complement		Volume of diluted sample (1:10) tested (ml.)	Complement activity† of sample taken after		
			30 min.	60 min.	120 min.
0.25 % HN2 in 0.9 % NaCl	0.1	0.5	4	3	2
		0.2	2	1	0
		0.1	0	0	0
	0.5	0.5	1	0	0
		0.2	0	0	0
		0.1	0	0	0
	1.0	0.5	0	0	0
		0.2	0	0	0
		0.1	0	0	0
0.25 % HN2 in 0.05 N-Na ₂ S ₂ O ₃	0.1	0.5	4	4	4
		0.2	2	2	2
		0.1	tr.	tr.	0
	0.5	0.5	4	4	4
		0.2	2	2	2
		0.1	tr.	0	0
	1.0	0.5	4	4	4
		0.2	2	2	2
		0.1	tr.	0	0
Control (untreated complement)	—	0.5	4	4	4
		0.2	2	2	2
		0.1	tr.	0	0

* To 0.5 ml. guinea pig serum.

† Haemolysis readings after 30 min.

In view of the reaction of HN2 with the α -amino groups of amino-acids (Fruton, Stein & Bergmann, 1946), glycine was tested for its capacity to prevent the inactivation of complement by the vesicant. The results showed, however, that glycine has no protective action in this respect.

The inhibition, by nitrogen mustard, of enzymes concerned with choline metabolism has been attributed (Barron, Bartlett & Miller, 1948) to the similarity in structure between choline and the ethyleniminium ions formed by the nitrogen mustards. We decided, therefore, to determine whether choline has any action on complement, and whether it can protect complement against inactivation by HN2. It was found that choline,

samples which had been treated with HN2, and the mixtures were tested for full complement activity.

Typical results of an experiment in which ammonia-treated serum (lacking C'4) and heated serum (lacking C'1 and C'2) were added to serum which had been inactivated by treatment with 2 vol. of an 0.25 % (HN2) solution for 35 min. at 37° are shown in Table 4. Partial reactivation occurred when the ammonia-treated serum was added to the HN2-treated serum, but there was no reactivation when heated guinea pig serum was added to the latter. This suggests that the fourth component (C'4) is less rapidly inactivated by HN2 than one or both of the heat-labile com-

ponents C'1 and C'2. Further experiments showed that longer treatment with HN2 gave an inactive serum which was not activated by the addition of ammonia-treated serum (deficient in C'4).

Other experiments showed that the addition of serum lacking complement component C'3 (i.e. guinea pig serum treated with an insoluble carbohydrate of yeast) to HN2-inactivated complement (guinea pig serum treated with 1 vol. of 0.25% HN2 at 37°) gave a mixture with significant complement activity, if the treatment with HN2 was short (e.g. 15–30 min.). Longer treatment with HN2 under these conditions resulted in complete loss of C'3 activity.

of nitrogen mustard-inactivated complement could be restored by treatment with this reagent. Samples (0.2 ml.) of guinea pig serum which had been treated with an equal volume of a 0.25% HN2 solution for various lengths of time were mixed with 0.1N-sodium thiosulphate (0.2 ml.) and the mixtures were tested for complement activity after they had been kept for 10, 30 and 60 min. at room temperature. The results showed that the sodium thiosulphate had effected no reactivation of the HN2-inactivated complement.

It has been suggested (Fruton, Stein & Bergmann, 1946; Loveless & Revell, 1949) that the nitrogen mustards react with proteins by esterification of

Table 4. *Attempted reactivation of HN2-inactivated complement with (a) NH₃-treated (lacking C'4) and (b) heated serum (lacking C'1 and C'2)*

(For experimental details see pp. 366 and 369. The HN2-treated serum, NH₃-treated serum and heated serum used in these tests corresponded, respectively, to 1:5, 1:10 and 1:10 dilutions of the original guinea pig serum. Where necessary, 0.9% NaCl was added to make a total volume of 1.0 ml. for each mixture, and 0.5 ml. of a 3% suspension of sensitized sheep erythrocytes was then added to each tube.)

HN2-treated serum (ml.)	NH ₃ -treated serum (ml.)	Heated serum (ml.)	Complement activity of the mixtures*	
			(a)	(b)
0.5	—	—	0	0
0.5	0.2	—	0	1
0.5	0.5	—	tr.	3
0.5	—	0.2	0	0
0.5	—	0.5	0	0
0.2	—	—	0	0
0.2	0.2	—	0	1
0.2	0.5	—	0	3
0.2	—	0.2	0	0
0.2	—	0.5	0	0
—	0.2	0.2	2	4

* Haemolysis readings after (a) 10 min. (b) 30 min.

Several experiments were made to determine whether HN2-inactivated complement can be activated by adding 'midpiece' or 'endpiece' fractions of complement. It was found that when the HN2 was allowed to act for only a short time, the addition of 'endpiece', but not 'midpiece', to the HN2-inactivated serum gave a mixture with complement activity. In a few experiments the difference in the degree of reactivation effected by the two fractions was not very marked, but where a difference was observed, it was always in the same direction. An experiment designed to determine the comparative rates of inactivation of the four components by HN2 indicated that C'2 is the component which is most rapidly destroyed, and that C'1, C'3 and C'4 disappear at approximately the same rate.

Attempted reactivation of complement inactivated by HN2

In view of the strong affinity of sodium thiosulphate for ethyleniminium ions, experiments were carried out to determine whether the activity

of carboxyl groups. We therefore attempted to regenerate the complement activity of HN2-inactivated serum by treatment with an esterase. A preparation obtained from rat liver was found to be without haemolytic action on sensitized sheep cells and to have no marked destructive action on complement at dilutions at which it was still actively hydrolytic for ethyl butyrate. Treatment of HN2-inactivated rabbit serum with the esterase preparation for 1 hr. did not restore complement activity. The sole object of this experiment was to determine whether the rat-liver enzyme preparation could reverse the inactivation of complement effected by HN2, and the result does not militate against the view that the carboxyl groups of the complement proteins are esterified by the drug.

There is evidence that some of the linkages by which nitrogen mustard is bound to proteins are unstable, and it seemed possible that nitrogen mustard residues might be removed from nitrogen mustard complexes by dialysis or by treatment with very dilute alkali. HN2-inactivated serum was

dialysed against 0.9% sodium chloride, and in other experiments the inactivated serum was treated with a weakly alkaline buffer (pH 9.0), in the presence of 10% sodium chloride (to protect complement against inactivation by alkali (Pillemer & Ecker, 1941c)), and subsequently dialysed. In all these experiments there was no restoration of the complement activity.

The activity of complement which has been destroyed by oxidation can in certain instances be regenerated by reduction, and it has been suggested (Ecker, Pillemer, Martiensen & Wertheimer, 1938) that inactivation by oxidizing agents might be due to the conversion of thiol groups to disulphide groups. The nitrogen mustards react with sulphhydryl groups *in vitro* and experiments were therefore carried out to determine whether reducing

attempt was made to reactivate the serum with potassium cyanide.

Cuprous oxide (15 mg.) was added to 3 ml. of dilute guinea pig serum (1 vol. of serum plus 9 vol. of 0.9% NaCl), and the mixture was shaken gently for 5 min. and then allowed to stand for 45 min. at room temperature; the suspension was then centrifuged to remove the solid. A freshly prepared 1% HN2 solution (0.2 ml.) was added to the Cu_2O -treated serum (1 ml.) and also to a sample of untreated guinea pig serum (0.1 + 0.9 ml. of 0.9% NaCl). The mixtures were kept for 30 min. at 37°, and 0.1 N- $\text{Na}_2\text{S}_2\text{O}_3$ (0.2 ml.) was then added to each mixture to prevent further reaction of the nitrogen mustard with complement. After a further 5 min. at 37° a sample (0.5 ml.) of each solution was added to 0.01 M-KCN (0.25 ml.); the mixtures were kept for 15 min. at room temperature and 0.5 ml. of a sensitized sheep-cell suspension (0.5 ml.) was added to each tube, and the extent of haemolysis after 10 and 30 min. was noted.

Table 5. *Attempt to reactivate, with KCN, guinea pig serum complement inactivated with Cu_2O and subsequently treated with HN2*

(The volumes given in this table refer to ml. of serum diluted 1:10. Where necessary, 0.9% NaCl was added to bring the volume of the mixture to 0.75 ml. before the sensitized red cells were added.)

Untreated guinea pig serum (ml.)	HN2-treated serum (ml.)	Cu_2O -treated serum (ml.)	Cu_2O - and HN2-treated serum (ml.)	KCN (0.01 M) (ml.)	Complement activity of the mixture*	
					(a)	(b)
0.5	—	—	—	—	4	4
0.5	—	—	—	0.25	4	4
—	0.5	—	—	—	0	tr.
—	0.5	—	—	0.25	0	tr.
—	—	0.5	—	—	0	0
—	—	0.5	—	0.25	2	4
—	—	—	0.5	—	0	0
—	—	—	0.5	0.25	0	tr.

* Haemolysis readings after (a) 10 min. (b) 30 min.

agents could reactivate complement which had been inactivated by HN2. No reactivation occurred when ascorbic acid or potassium cyanide was allowed to act on the inactivated guinea pig serum at pH 7.4 for various lengths of time.

Role of sulphhydryl groups in complement inactivation by HN2

The failure of ascorbic acid or potassium cyanide to restore complement activity to HN2-treated sera indicates that the inhibition is not due solely to a reversible oxidation of SH groups, but this evidence does not exclude inactivation due to an irreversible reaction with these functional groups. It has been shown (Ecker *et al.* 1938) that sera treated with cuprous oxide, which reacts specifically with SH groups to form mercaptides, lose their capacity to haemolyse sensitized cells, but subsequent addition of dilute potassium cyanide or hydrogen sulphide completely restores complement activity. In the following experiment serum was treated with cuprous oxide to protect the sulphhydryl groups, allowed to react with HN2 and then an

The results (Table 5) show that complement inactivated with cuprous oxide and subsequently treated with HN2 is not reactivated by potassium cyanide. The fact that HN2 inactivates complement even when the sulphhydryl groups of the latter have been protected by cuprous oxide indicates that complement groups other than sulphhydryl are concerned in the inactivation by HN2.

Effect, on complement, of various nitrogen mustards and related compounds

Aromatic nitrogen mustards. In view of the interesting observations of Haddow, Kon & Ross (1948) on the cytotoxic action of a series of aromatic nitrogen mustards, we decided to examine the complement-inactivating power of typical members of this series. Samples of the following compounds (four aromatic nitrogen mustards and one related compound with a single 2-chloroethyl side chain) were kindly supplied by Dr Ross; *NN'*-di-(2-chloroethyl)-*p*-anisidine, *NN'*-di-(2-chloroethyl)-*p*-toluidine, *NN'*-di-2-chloroethyl-2-naphthylamine, *p*-chloro-*NN'*-di-(2-chloroethyl)aniline and *N*-ethyl-*N*-2-chloroethylaniline.

Guinea pig serum (0.2 ml.) was added to 10 mg. samples of each of these compounds, and the solid was ground with a glass rod. The mixtures were kept for 2 hr. at 37°, and then centrifuged to remove the sparingly soluble aromatic nitrogen mustards. The complement activity of the supernatant solutions was then determined.

Under the conditions of these experiments the aromatic nitrogen mustards tested were without action on complement. These compounds have a very low solubility in water and consequently a very much slower rate of hydrolysis than the aliphatic nitrogen mustards, but even when kept for 21 hr. at 37° aqueous suspensions gave supernatant solutions devoid of complement-inactivating capacity.

2-Chloroethyldimethylamine. Since compounds with a single 2-chloroethyl side chain are ineffective as tumour-inhibitory or carcinogenic agents, we decided to determine whether complement inactivation could be effected by a monofunctional nitrogen mustard.

examine typical diepoxides (kindly provided by Dr Ross) for their action on complement. Treatment of guinea pig serum (0.5 ml.) with different volumes (0.1–1.0 ml.) of 0.02M solutions of di-2:3-epoxypropyl ether, 1:2:3:4-diepoxybutane and 1:2:5:6-diepoxydiallyl for periods up to 2 hr. failed to inactivate complement, but when the experiments were repeated using 0.5M solutions of these diepoxides, there was considerable diminution in complement activity. The results obtained with 1:2:5:6-diepoxydiallyl are given in Table 6. Solutions (0.5M) of the diepoxides which had been kept for 20 hr. at 37° still retained some capacity to inactivate complement. The results of tests in which complement fractions lacking one or more components were added to serum which had been inactivated by treatment with 1:2:3:4-diepoxybutane indicated that the diepoxides have no selective action on any one component of complement.

Table 6. *The action of 1:2:5:6-diepoxydiallyl on guinea pig complement*

1:2:5:6-Diepoxydiallyl added* (mg.)	Volume of diluted† sample tested (ml.)	Complement activity of samples taken after							
		15 min.		30 min.		60 min.		120 min.	
—	0.5	4	4	4	4	4	4	4	4
	0.2	2	4	2	4	2	3	1	3
	0.1	0	2	0	2	0	2	0	tr.
5.7	0.5	4	4	4	4	4	4	3	4
	0.2	2	4	2	3	1	3	0	3
	0.1	0	2	0	1	0	0	0	0
28.5	0.5	4	4	3	4	3	4	0	1
	0.2	2	3	tr.	3	0	2	0	0
	0.1	0	tr.	0	0	0	0	0	0
57.0	0.5	4	4	2	4	tr.	2	0	0
	0.2	1	3	0	2	0	0	0	0
	0.1	0	0	0	0	0	0	0	0
Haemolysis readings after (min.)		10	30	10	30	10	30	10	30

* To 0.5 ml. guinea pig serum.

† Equivalent to a 30-fold dilution of the original serum.

Varying amounts (0.1–0.3 ml.) of an 0.02M solution of 2-chloroethyldimethylamine were added to samples (0.3 ml.) of fresh guinea pig serum, and the mixtures were kept at 37°. Tests made on samples withdrawn at intervals up to 2 hr. showed that no inactivation of complement had occurred; control tests showed that the complement activity of 0.3 ml. of serum was completely destroyed in 30 min. at 37° by 0.3 ml. of 0.02M-HN2 and partially destroyed by 0.1 ml. The experiment was repeated using 0.2M-2-chloroethyldimethylamine, but this larger amount gave only slight inactivation after 3 hr. at 37°.

Diepoxides. In view of the similarity in the cytological effects of the nitrogen mustards and diepoxides (Loveless & Revell, 1949) we decided to

THE EFFECT OF THE INJECTION OF HN2 ON THE SERUM HAEMOLYTIC-COMPLEMENT ACTIVITY IN MAN, RABBIT AND GUINEA PIG

Experiments on guinea pigs

HN2 hydrochloride (5 mg.) was dissolved in sterile saline (5 ml.) immediately before use. The solution was injected into the heart, except in the case of guinea pig no. 2 which received an intravenous injection into the ear. Blood was taken from each of the eight guinea pigs before and at a suitable interval (1–6 hr.) after the injection. The samples were obtained by pricking an ear vein with a needle and squeezing out drops of blood which were collected in a small tube (2 × $\frac{1}{4}$ in.). About 0.2 ml. of serum was obtained by this method, and the complement activities of these samples were determined and expressed in 50% units/ml.

The results (Table 7) showed that the intravascular injection of HN2, in amounts of 1.33–2.86 mg./kg. of body weight, had no marked effect on the haemolytic-complement activity of the serum of the injected guinea pigs during a period of 1–6 hr. after the injection.

Experiments on rabbits

Five rabbits were given intravenous injections of HN2 (a 0.1 % solution of the hydrochloride in sterile 0.9 % NaCl), and the doses given ranged from 0.5 to 2.0 mg./kg. body weight. One rabbit received one injection only, three had two injections and one had three injections, with intervals of

Table 7. *Complement activity of serum of guinea pigs before and after injection with HN2*

Guinea pig no.	HN2 injected (mg./kg. body wt.)	Blood sample taken	Complement activity (50 % units/ml.)
1	2.66	Before injection	129
		1 hr. 20 min. after injection	144
2*	1.82	Before injection	130
		2 hr. 50 min. after injection	130
3	1.33	Before injection	110
		1 hr. 15 min. after injection	116
4	2.86	Before injection	135
		2 hr. 30 min. after injection	126
5	2.22	Before injection	145
		4 hr. after injection	136
6	2.15	Before injection	142
		4 hr. after injection	136
7	1.54	Before injection	110
		1 hr. after injection	143
		6 hr. after injection	156
8	2.44	Before injection	185
		1 hr. 15 min. after injection	185
		6 hr. after injection	196

* The HN2 was injected intravenously into this guinea pig; the other animals received intracardiac injections.

Table 8. *Complement activity of serum of rabbits before and after injection with HN2*

Rabbit no.	HN2 injected (mg./kg. body wt.)	Blood sample taken	Complement activity (50 % units/ml.)
506	0.5	Before injection	12.5
		20 min. after injection	11.1
722	0.5	Before injection	9.1
		20 min. after injection	8.4
506*	0.5	Before injection	10.0
		1 hr. after injection	8.4
722*	0.5	Before injection	6.7
		1 hr. after injection	7.1
723	0.5	Before injection	12.5
		1 hr. 30 min. after injection	12.5
510	0.5	Before injection	20.0
		1 hr. 30 min. after injection	16.7
723*	1.0	Before injection	7.4
		3 hr. 40 min. after injection	7.2
510*	1.0	Before injection	12.1
		3 hr. 40 min. after injection	11.5
723*	2.0	Before injection	6.7
		2 hr. 20 min. after injection	7.1
		5 hr. after injection	7.1
		24 hr. after injection	7.7
767	2.0	Before injection	6.9
		2 hr. 20 min. after injection	6.9
		5 hr. after injection	6.9
		24 hr. after injection	7.4

* 1 week after the previous injection.

1 week between the injections. Blood samples were taken from each rabbit, from the ear not used for the injection, before and at various intervals after the injection. The complement activities of the sera were determined and recorded in terms of 50% units/ml. Parallel titrations were also made with a freeze-dried bulked rabbit serum which was kept as the 'standard' for all the tests in this series, and in this way it was possible to compare the serum complement activity on any one day with the activities recorded for the same rabbits at earlier and later stages of the experiments. The rabbits injected with the largest amount of HN2 (2.0 mg./kg. body weight) showed some loss of appetite about 6 days after the injection.

The results of these experiments (Table 8) showed that there was no consistent change in the complement titre of the blood 20 min.–24 hr. after the injection of relatively large amounts of HN2; the changes observed were within the normal variation in serum complement level. Furthermore, the values for the ratio of the complement activity of the tested serum to the activity of the 'standard' dried rabbit serum showed that the serum complement activity 6 or 7 days after the injection, even with a dose as large as 2.0 mg. of HN2/kg. body weight, was not significantly lower than the level before the injection.

Observations on man

Serum samples from patients receiving intravenous injections of HN2 during the period December 1948–June 1949 were examined for complement activity. The patients normally received four injections of 0.1 mg. HN2/kg. body weight, given daily or every other day. Blood was taken by venepuncture immediately before the first injection and on the day following the final injection. Complement titrations were carried out on a 'standard' freeze-dried preparation of human serum, in parallel with the titrations on the patient's serum. A typical pair of curves for a patient's serum (case no. 4, before injection) and the reconstituted 'standard' serum, are given in Fig. 1; the dotted lines indicate the amount of serum required for 50% haemolysis.

The complement activities of the serum samples taken from 18 patients, before and after injection, are summarized in Table 9; the age and disease of the patients are included where this information is available. These results show that there is no general fall in the serum complement activity of man following a course of nitrogen mustard injections customarily given for the treatment of certain neoplastic and related conditions. The blood complement activity of man varies appreciably from day to day, and the variations cannot be attributed solely to variations in the haemolytic system used in the tests, as shown by the typical results recorded in April–June 1949, for five normal individuals (Table 10). The variations observed with the subjects injected with HN2 were thus not outside the range of variation for subjects who have not been injected with the drug.

DISCUSSION

The nitrogen mustards have important biological effects, some of which can be attributed to reactions of the drug with proteins and related compounds. HN2, a typical nitrogen mustard, reacts with amino, carboxyl, sulphhydryl and sulphide groups of amino-acids and peptides, and it inactivates several enzymes (see, for example, Fruton, Stein & Bergmann (1946); Dixon & Needham (1946); Thompson (1947); for other references see Boyland (1949); Philips (1950); Karnofsky (1950)). Quantitative information about the reaction of HN2 with proteins at pH 7.5 has been obtained more recently with the aid of the ^{15}N -labelled drug, and it has been found that part of the HN2 which is bound to proteins is very firmly attached (Burnop, Richards, Watkins & Wormal, 1951).

The inactivating action of HN2 on haemolytic complement reported here can most readily be explained by assuming that the drug reacts with essential groups in the complement system. The nitrogen mustard proteins produced are not *per se* anti-complementary, and we have evidence from our immunological investigations (Watkins & Wormal, 1952) and other studies with HN2, that even if the drug effects denaturation of proteins this change is not the cause of the altered immunological specificity resulting from the action of HN2 on proteins. As yet, however, it is not possible to relate the change in immunological specificity, or the inactivation of haemolytic complement, or the inactivation of certain enzymes, to specific changes in functional protein groups.

Since complement is readily inactivated by low concentrations of HN2 at 37° and pH 7.5, the possibility of a serious depletion of the blood complement following intravenous injection of the drug into man could not be dismissed without further investigation. The doses usually given to man (a series of four or five intravenous injections, each of about 0.1 mg. of HN2 hydrochloride/kg. of body weight) will give considerably lower concentrations of HN2 in the plasma than are required to completely inactivate complement *in vitro*, though significant destruction of complement might be expected when larger doses are injected (e.g. up to 1.0 mg./kg. over a period of 4–7 days, or a single injection of 0.4 mg./kg.). Furthermore, there is the possibility that the anti-complementary action of HN2 might continue for a long time after the injection of the drug, for we have found that 'hydrolysed' HN2 possesses significant complement-inactivating power; there is also the possibility that HN2 may have a cumulative anti-complementary effect, since it is cumulative in relation to toxicity (Karnofsky, 1950).

Fig. 1. Complement titration curves for human sera. *A*, serum from patient no. 4 before injection with HN2; *B*, standard dried human serum.

Table 9. Complement activity of serum samples from human subjects before and after injection with HN 2

Case no.	Age (yr.)	Disease	Serum sample	Complement activity of serum (50% units/ml.)		Ratio (X/Y)
				Subject (X)	Standard (Y)	
1	32	Mikulicz's syndrome	Before injection	38.5	18.5	2.1
			After injection	45.4	13.5	3.4
2	50	Hodgkin's disease	Before injection	76.9	27.0	2.8
			After injection	55.5	16.7	3.4
3	66		Before injection	47.0	17.0	2.8
			After injection	55.5	17.0	3.3
4	42	Reticulosis	Before injection	55.5	19.6	2.8
			After injection	66.5	21.3	3.1
5	28	Hodgkin's disease	Before injection	83.4	27.0	3.1
			After injection	66.7	21.3	3.1
6	55	Carcinomatosis	Before injection	66.7	21.3	3.1
			After injection	62.7	20.4	3.1
7	42		Before injection	43.4	10.6	4.1
			After injection	77.0	17.2	4.5
8			Before injection	23.8	10.6	2.2
			After injection	55.5	17.5	3.2
9	31	Lymphadenoma	Before injection	45.5	12.8	3.5
			After injection	59.0	15.6	3.8
10	54	Carcinoma	Before injection	47.6	7.0	6.8
			After injection	50.0	6.7	7.4
11	27	Hodgkin's disease	Before injection	76.9	7.0	10.9
			After injection	66.7	10.6	6.3
12	22	Hodgkin's disease	Before injection	50.0	14.3	3.5
			After injection	83.4	18.5	4.5
13	47	Lymphadenoma	Before injection	37.0	9.8	3.8
			After injection	55.5	16.1	3.5
14	46	Lymphatic leukaemia	Before injection	62.5	9.6	6.5
			After injection	66.7	10.9	6.1
15	37	Lymphadenoma	Before injection	47.6	9.6	5.0
			After injection	52.6	10.9	4.8
16	66		Before injection	52.6	12.3	4.3
			After injection	50.0	10.9	4.6
17	13	Sarcoma	Before injection	43.5	9.6	4.5
			After injection	55.5	14.5	3.8
18			Before injection	62.5	12.1	5.2
			After injection	66.7	11.0	6.1

The results of our investigations have shown, however, that the nitrogen mustard therapy normally adopted does not involve any risk of a significant destruction of the blood complement. It seems probable that the drug combines with various blood and other tissue proteins and that the proteins of complement are not selectively attacked. In other investigations we have found that the risk of producing hypersensitivity to HN 2 by repeated

indicate a difference in the mode of complement inactivation by the two groups of compound, since the chemical reactivity of the diepoxides is considerably less than that of HN 2. Thus the inactivation of complement by HN 2, or by the diepoxides, may be associated with a cross-linking mechanism of the type suggested by Goldacre, Loveless & Ross (1949) in respect to the production of chromosome abnormalities by the nitrogen mustards.

Table 10. *Complement activity of serum samples taken at weekly intervals from normal subjects*

Subject	Serum sample	Complement activity of serum (50% units/ml.)		Ratio (X/Y)
		Subject (X)	Standard (Y)	
A.W.	1	38.4	10.9	3.5
	2	38.4	12.5	3.1
	3	41.7	10.0	4.2
H.G.R.	1	25.6	10.9	2.4
	2	43.6	12.5	3.5
	3	38.5	10.0	3.9
W.M.	1	30.3	10.9	2.9
	2	33.3	11.5	2.9
	3	27.8	10.0	2.8
R.L.F.T.	1	38.4	10.9	3.5
	2	25.6	12.5	2.0
	3	41.7	10.0	4.2
W.M.W.	1	27.8	12.8	2.2
	2	41.7	9.9	4.2
	3	29.4	10.0	2.9

injections of the drug is not serious (Watkins & Wormall, 1952), but in this case the chief factor concerned is probably the lability of many of the linkages between the nitrogen mustard and the body proteins.

Part of the HN 2 which is readily bound by proteins at pH 7.5 can be removed by dialysis (Burnop *et al.* 1951), but dialysis does not restore the activity of HN 2-inactivated complement. All our efforts to reverse the inactivating effect of HN 2 on the whole complement system have been unsuccessful, but we have not yet explored the possibility of reversing the effect of the drug on the individual separated components. This inactivation of complement is apparently of a fairly specific character, for amounts of HN 2 considerably greater than that required to inactivate complement have no detectable destructive action on the erythrocyte-sensitizing antibody of haemolytic serum.

The diepoxides, which have been used for effecting cross linking between carboxyl groups of wool fibres and which have cytological effects similar to those produced by the nitrogen mustards, are much less powerful inactivators of complement than is HN 2. For the destruction of complement the diepoxides we have tested have to be used in molar concentrations about 25 times that required in the case of HN 2. However, this difference does not necessarily

SUMMARY

1. Haemolytic complement is rapidly inactivated *in vitro* by the nitrogen mustard di-2-chloroethyl-methylamine (HN 2), at 37° and pH 7.5. All four components of complement are inactivated.

2. When aqueous solutions of HN 2 are kept, most of the complement-inactivating capacity is lost. Of the 'hydrolysis' products tested, only 2-chloroethyl-2'-hydroxyethylmethylamine showed complement-inactivating power, and this was low compared with that of HN 2.

3. Thiosulphate protects complement against inactivation by HN 2 or by 2-chloroethyl-2'-hydroxyethylmethylamine. Less marked protection is afforded by phosphate and hexamethylenetetramine. On this evidence it is suggested that complement inactivation by HN 2 is due to ethyleniminium ions.

4. The nature of the inactivation has not been established, but reaction of the HN 2 with sulphydryl groups of complement is not the sole cause of inactivation. Attempts to reverse the inactivation effected by HN 2 have failed.

5. Three diepoxides known to effect cross linking of protein molecules inactivate complement, though they are much less effective in this respect than is HN 2. These results, and others with a

monofunctional nitrogen mustard, are not inconsistent with the view that cross linking between protein molecules is an important factor in complement inactivation by the nitrogen mustards.

6. Five aromatic nitrogen mustards tested had no destructive action on complement, possibly because of their low solubility in water.

7. The intracardiac or intravenous injection of HN2 into guinea pigs or intravenous injection into rabbits (single injections of 0.5–2.8 mg./kg. body weight) caused no significant fall in the serum haemolytic-complement activity of these animals.

8. The serum complement activity of 18 patients receiving nitrogen mustard therapy has been

studied before and after the course of intravenous injections of HN2 (four injections, each of 0.1 mg./kg. body weight, over a period of 4–8 days). This drug treatment caused no depression of the serum complement activity.

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