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Studies with ¹⁵N-Labelled Nitrogen Mustards. The Combination of Di-2-(Chloroethyl)methylamine with Proteins

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Much evidence is available that the nitrogen mustards readily react with various enzymes and other proteins. For example, hexokinase (Dixon & Needham, 1946), cholinesterase (Thompson, 1947; Adams & Thompson, 1948) and choline oxidase and choline acetylase (Barron, Bartlett & Miller, 1948) are strongly inhibited by di-(2-chloroethyl)methylamine (NM), and although this enzyme-inhibitory capacity is fairly specific, NM reacts with many widely differing proteins. It effects a slight but significant change in the immunological properties of the serum proteins and ovalbumin (Watkins & Wormall, 1952a), and it rapidly inactivates haemolytic complement (Watkins & Wormall, 1948, 1952b), a complex serum system which consists of four components which are largely or entirely of protein nature. Furthermore, there is evidence of a cross-linking effect when NM is allowed to act on proteins, and it has been shown that in the nitrogen-mustard series the presence of two halogenoalkyl groups is needed for marked biological activity (Haddow, Kon & Ross, 1948; cf. also Goldacre, Loveless & Ross, 1949; Haddow, 1949).

No precise information has yet been obtained about the nature of the particular protein groups whose reaction with NM causes inactivation of enzymes or changes in the immunological properties of proteins, but it has been shown that NM reacts with amino, carboxyl, sulphhydryl and sulphide groups of proteins (see, for example, Fruton, Stein & Bergmann, 1946, and the reviews by Gilman & Philips, 1946; Boyland, 1948; Philips, 1950; Ross, 1953; Alexander, 1954). NM has a marked alkylating capacity towards the amino and carboxyl groups of proteins (see Loveless, 1951; Ross, 1953).

Evidence of a change in certain characteristic or functional groups of a protein after its exposure to NM does not, however, necessarily establish that firm combination of the vesicant with the protein has occurred, nor does the measurement of the resulting decrease in the free amino and sulphhydryl groups of the protein afford precise quantitative evidence about the condensation of NM and protein molecules. The reaction between sulphur mustards and proteins has been studied quantitatively with

^{35}S as tracer (Banks, Bournsnel, Francis, Hopwood & Wormall, 1946), and by analogy it has often been assumed that the nitrogen mustards react similarly, with the production of fairly stable NM-protein complexes. However, it has not been established that the nitrogen and sulphur mustards both react with proteins in the same way, and indeed there is evidence that the two reactions show significant differences.

Since quantitative data on the interaction of NM and proteins is scanty, we decided to study this reaction with isotopically labelled nitrogen mustards. In the experiments described in this paper, NM labelled with ^{15}N has been prepared and used for studies of its action on various proteins. The chief objects of these investigations were to determine whether the inactivation of some enzymes and the decrease in certain functional protein groups which occur when NM acts on proteins is associated with the firm binding of NM to the protein, and to investigate the stability of any NM-protein complexes formed. With this quantitative technique the speed of the reaction between NM and proteins at 37° and pH 7.4 has also been studied, since this is important in connexion with the clinical use of NM in the treatment of some neoplastic diseases.

The work described in this paper forms part of a quantitative study, with isotopically labelled nitrogen mustards, of the fate of NM after its intravenous injection into the animal body. A short account of part of the investigations described here has already been presented (Burnop, Richards, Watkins & Wormall, 1951; Burnop, Richards & Wormall, 1952).

MATERIALS

Di-(2-chloroethyl)methylamine labelled with ^{15}N . This compound was synthesized by the following stages from potassium [^{15}N]phthalimide.

[^{15}N]Methylamine hydrochloride. This compound was prepared as described by Cox & Warne (1951). Potassium [^{15}N]phthalimide (10 g.) was placed in the larger arm of the reaction vessel, the neck of which was then constricted. The vessel was pumped out on a vacuum manifold, the potassium phthalimide was heated in an oil bath at 200° until the pressure gauge indicated that evolution of gases had ceased and then cooled. Methyl iodide (5 ml.) dried *in vacuo* over

P_2O_5 was distilled *in vacuo* into the smaller arm of the reaction vessel, which was sealed off at the constriction and removed from the vacuum manifold. The arm containing the potassium phthalimide was heated in an oil bath at 200° and the progress of the reaction followed by observing the level of the methyl iodide. The reaction had virtually ceased after 3 hr., but the heating was continued for a further 3 hr. (In some trial runs it was found that there was a latent period of up to 6 hr. before the reaction commenced, but with carefully dried reagents the reaction started as soon as the temperature was raised.) The cooled reaction vessel was opened and the *N*-methyl phthalimide which had sublimed into the top of the vessel was scraped out and transferred to a 200 ml. flask. The product (9 g.; theory, 8.7) appeared to contain a little absorbed methyl iodide. It was refluxed for 8 hr. with a mixture of water (50 ml.) and conc. HCl (50 ml.) and kept overnight at about 4° . Phthalic acid was filtered off and washed with a little conc. HCl, and the combined filtrate and washings were evaporated to small bulk under reduced pressure. The concentrated solution was filtered again and evaporated to dryness *in vacuo*, giving 3.42 g. of residue. (Theoretical yield of methylamine hydrochloride, 3.65 g.)

Di-(2-hydroxyethyl)methylamine. The methylamine hydrochloride was placed in a flask connected to a head fitted with dropping funnel and vertical condenser for distillation. The methylamine hydrochloride was dissolved in water (about 10 ml.) and 30% NaOH (10 ml.) was dropped slowly into the boiling solution. The water and methylamine were collected in a large boiling tube (cooled in ice) originally containing 2 ml. of water to cover the end of the condenser, and about 10 ml. of distillate was collected. Liquid ethylene oxide (3 ml.) was measured out at 0° in a small test tube, and allowed to evaporate slowly into the stirred methylamine solution, cooled in ice. [Abrams, Barker, Jones, Vallender & Woodward (1949) in a large-scale preparation of di-(2-hydroxyethyl)methylamine found that unless the methylamine was in excess the product contained ethers such as $\text{HO}\cdot\text{CH}_2\cdot\text{CH}_2\cdot\text{N}(\text{CH}_3)\cdot\text{CH}_2\cdot\text{CH}_2\cdot\text{O}\cdot\text{CH}_2\cdot\text{CH}_2\cdot\text{OH}$. Although the use of excess of [^{15}N]methylamine diminishes the overall isotopic yield, we adopted this plan, only 70% of the theoretical amount of ethylene oxide being used.]

The solution was kept overnight and then distilled, the lower-boiling fractions being removed under reduced pressure (water pump) with a bath temperature up to 100° . The viscous residue was then distilled at 5 mm. pressure, yielding 3.18 g. of distillate, representing a 49% yield of di-(2-hydroxyethyl)methylamine from the phthalimide.

Di-(2-chloroethyl)methylamine hydrochloride. The above diol was dissolved in trichloroethylene (5 ml.) and run slowly into a gently boiling mixture of thionyl chloride (5 ml.) and trichloroethylene (15 ml.). The mixture was refluxed for 15 min., and cooled to room temp.; the solvent was removed

in vacuo and the dry crystalline residue was recrystallized from acetone. The resulting NM-HCl (3.66 g., containing 33 atoms % of ^{15}N and representing a 36% yield based on the phthalimide) had m.p. 109° . The mother liquors were treated with 1 g. of NM-HCl of normal ^{15}N content, and on concentration a further crop of NM-HCl (1.36 g., 2.54% based on phthalimide) was obtained, which contained 6.17 atoms % of ^{15}N .

In trial runs with materials containing normal abundance of ^{15}N , the NM-HCl obtained was identified by mixed m.p. with authentic material prepared from compounds of normal isotope abundance by the method of Hanby & Rydon (1947), and by conversion into the picrate (m.p. 133°).

Proteins

Ovalbumin. Hen ovalbumin was prepared by $(\text{NH}_4)_2\text{SO}_4$ precipitation, and recrystallized three times (Cole, 1933). For one experiment the ovalbumin was prepared by precipitation with saturated Na_2SO_4 solution, and recrystallized three times (Kekwick & Cannan, 1936).

Bovine-plasma albumin. Crystallized bovine-plasma albumin was used (Armour Laboratories: fraction V).

Pepsin (crystalline). This was supplied by Armour Laboratories.

Zein and gliadin. Samples of these proteins were kindly supplied by Professor A. C. Chibnall.

Casein. 'Acetic acid precipitated casein' (British Drug Houses Ltd.) was used.

Protamine sulphate. This was obtained from L. Light and Co. Ltd.

Horse-serum γ -globulins. These were separated from horse serum as described by Kekwick (1940) and were reprecipitated once with 12% Na_2SO_4 .

Rabbit-serum proteins. Freshly separated whole rabbit serum.

Haemoglobin. This was prepared from horse blood as described by Dudley & Lovatt-Evans (1921).

Acetylated proteins. Proteins were acetylated as described by Fraenkel-Conrat, Bean & Lineweaver (1949), but less acetic anhydride was used than was recommended by these authors (about 0.7 compared with 1.2–2.4 ml./g. of protein). To 25 ml. of protein solution (approx. 6%) 10 g. of sodium acetate was added and the solution was diluted with water to 30 ml. The bulk of this solution (25 ml.) was cooled in ice and acetic anhydride (0.9 ml.) was added with constant slow stirring. The acetylated protein solution was dialysed against running tap-water overnight and then against distilled water to remove as much acetate as possible.

Buffer solutions. Various buffers were used, the choice depending on the nature of the experiment. When a wide range of pH was to be covered, with constancy in the buffer-salt concentration, Universal buffer (buffer solution S₁, Baird and Tatlock) was used. This solution is prepared by adding sodium hydroxide solution to diethylbarbituric acid (Johnson & Lindsay, 1939). NaHCO_3 – H_2CO_3 buffer solutions were made by passing CO_2 into 0.75M- NaHCO_3 until the solution had a pH of about 7.4. NaH_2PO_4 – Na_2HPO_4 buffers were prepared according to Sørensen (1912) and acetate buffers as described by Walpole (1914).

METHODS

Formation of NM-protein complexes. The following general method was used for the preparation of nitrogen

mustard-treated proteins (NM-proteins). The protein solution was treated with either 0.1N- NaOH or 0.1N- HCl to bring it to the required pH, and buffer solution of the same pH was added. The NM was then added, either as the solid hydrochloride or as a freshly prepared solution of this salt in 0.9% (w/v) NaCl , or water, and the mixture was kept at 37° for 2 hr. with occasional shaking, then at about 4° overnight, and the pH of the mixture was checked with a glass electrode. The NM-protein complexes in these solutions were then freed from NM and its hydrolysis products either by precipitation with 4 vol. of methanol or by prolonged dialysis against frequent changes of 0.9% NaCl . The methanol-precipitated complexes were washed four times with 1 vol. of methanol and then dried *in vacuo* over H_2SO_4 .

Total nitrogen and ^{15}N determinations. These were made as described by Francis, Mulligan & Wormald (1954). The ratio $^{15}\text{N}:^{14}\text{N}$ was determined on a Consolidated Nier isotope ratio mass spectrometer, Model 21–201, and a correction was made for the percentage of air present in the sample. Samples containing more than 5% of air were rejected.

Amino-nitrogen determinations. Excess of neutralized 20% formaldehyde solution was added to the neutralized sample (pH 7) and the amino N was calculated from the amount of 0.05N- NaOH required to bring this mixture to pH 9 (assessed with phenolphthalein).

Molecular ratio calculations. In calculating the molecular ratios the following molecular weights have been used: ovalbumin, 40500 (Pederson, 1943); bovine-plasma albumin, 70000 (Adair, 1925); casein, 33600 (Burk & Greenberg, 1930); gliadin, 27000 (Krejci & Svedberg, 1935); zein, 40000 (Watson, Arrhenius & Williams, 1936); pepsin, 37000 (Philpot & Eriksson-Quensel, 1933); horse haemoglobin, 67000 (Svedberg & Fåhræus, 1926); horse γ -globulins, 167000 (Svedberg, 1937); an arbitrary mean molecular weight of 100000 was used for the mixed serum proteins. An N content of 16.0% was assumed for all the proteins studied. The nitrogen-mustard contents of the NM-protein complexes are expressed as moles of NM/mole of protein; this method of recording the results was found most satisfactory in view of the appreciable and varying amounts of inorganic salts present in the methanol-precipitated complexes.

Since NM-HCl is very hygroscopic, it was found more satisfactory to determine the relative amounts of NM and protein used from the ^{15}N content of the initial mixture than from the weights of NM and protein used.

RESULTS

Combination of NM with proteins

Effect of varying amounts of NM on its combination with ovalbumin and plasma albumin. Following our observations that NM is capable of combining firmly with various proteins (Burnop *et al.* 1951) we decided to study its combination with ovalbumin and plasma albumin by the use of widely varying relative amounts of NM.

The results of these experiments (Figs. 1, 2) showed that with an increase in the amount of NM used, in relation to the protein, there is an increase in the amount which is bound in both proteins. With ovalbumin, a maximum combination is obtained of

about 5 moles of NM/mole of protein for the complexes precipitated by methanol. The majority of these bound nitrogen-mustard groups are firmly bound to the ovalbumin, and prolonged dialysis removes only one-fifth to one-third.

The results with plasma albumin are similar to those obtained with ovalbumin, but with relatively large amounts of NM the amount of vesicant combining is slightly greater. These complexes con-

Fig. 1. Combination of nitrogen mustard (NM) with ovalbumin in phosphate buffer, pH 7.4. Ovalbumin solution, 10 ml. containing 240–940 mg. of protein; 0.5M-phosphate buffer, 3 ml., or 6 ml. with quantities of NM-HCl greater than 40 mg.; NM-HCl, 8–154 mg. The mixtures were kept at 37° for 2 hr. and overnight at 0–4°. O, Methanol-precipitated products; Δ, products obtained by dialysis against 0.9% NaCl at 0–4° for 96 hr.

Fig. 2. Combination of NM with bovine-plasma albumin in phosphate buffer, pH 7.4. Bovine albumin, 200 mg. in 3 ml. of 0.9% NaCl; 0.5M-phosphate buffer, 3 ml.; NM-HCl, 1.7–11.0 mg. O, Methanol-precipitated products; Δ, dialysed products.

tained less loosely attached NM than did the NM-ovalbumin complexes, and in some as much as 90% of the NM present in the precipitated NM-albumin complexes was not removable by long dialysis.

Combination of NM with different proteins. Experiments were carried out to determine the capacity of various widely differing proteins to combine firmly with NM. It was not possible to obtain precisely the same experimental conditions in all cases, but the general object in most of the experiments was to use an NM:protein molecular ratio of 4–5 and to carry out the reaction at about pH 7.4. With some proteins additional tests were made at other pH values, and also with much higher NM:protein molecular ratios, so that some idea might be obtained about the maximum capacity of these proteins to bind NM.

The reactions were carried out as for NM-ovalbumin and NM-plasma albumin, with slight modifications to deal with solubility properties of some of the proteins. In a few cases (noted below) the complexes could not be precipitated by methanol or similar solvents, and the analyses had to be carried out on the dialysed NM-protein solutions, or as otherwise stated.

The results of these experiments (Table 1) show that all the widely differing proteins tested react readily with NM at pH 7–8 to form complexes in which the NM is firmly bound. At pH 4.1 only a small amount of NM combines with pepsin, this result being very similar to those obtained with ovalbumin and plasma albumin at pH 4 (see below). Where the reactions were studied under similar conditions (at pH 7.4, with amounts of NM used equivalent to an NM:protein molecular ratio of about 4 or 5 and with the NM-protein complexes precipitated by methanol) these complexes usually contained an average of 1.5–2.5 moles of NM/mole of protein; slightly higher values were obtained with pepsin and lower values with protamine. Where both the methanol-precipitated and the dialysed NM-complexes of any one protein were examined, the NM contents of the latter were usually appreciably lower than those of the former, but occasionally there was little or no difference. When large excesses of NM were used, the amount of vesicant bound to ovalbumin, plasma albumin and serum globulin was 5–6 moles/mole of protein in the methanol-precipitated products.

Combination of ovalbumin with hydrolysed NM. The nitrogen mustards have been shown (Golumbic, Stahmann & Bergmann, 1946; Hanby & Rydon, 1947) to undergo a series of reactions in aqueous solution which lead to the formation of various products, solutions of which have an acute convulsive effect and remain toxic indefinitely (Boylard, 1946; Anslow, Karnovsky, Jager & Smith, 1947). It was therefore decided to study the

Table 1. *Combination of di-(2-chloroethyl)methylamine (NM) with various proteins*

Protein solutions: ovalbumin, protamine, plasma albumin, haemoglobin and γ -globulins, 1–2% solutions in water or 0.9% NaCl plus phosphate buffer (pH 7.4; 0.1–0.2M); serum proteins, undiluted rabbit serum; casein, solution in 0.9% NaCl (pH 8.5) adjusted to pH 7.4 with 0.1N-HCl, centrifuged, and mixed with phosphate buffer (final concn.: protein 4%, buffer 0.1M); pepsin, 2% solution in water plus phosphate buffer (pH 7.4, 0.024M) or acetate buffer (pH 4.1, 0.4M); gliadin and zein, 3% solutions in ethanol–water (7:3) mixed with 0.4 vol. of Universal buffer (pH 8). **Reaction with NM.** NM-HCl was added in the quantities shown in the Table, either as solid or in solution in water or ethanol–water (7:3) (zein and gliadin). Drops of 0.1N-NaOH were added as required (protamine) to keep the mixture at approximately constant pH. **'Purification' of products:** NM-gliadin and NM-zein could not be 'purified' by precipitation with water or acetone, and analyses were carried out only on the dialysed products. The preparation of NM-pepsin at pH 7.4 could not be precipitated by methanol or ethanol, and was isolated by evaporating the solvent *in vacuo*, grinding the residue with acetone to remove NM and its hydrolysis products, and drying *in vacuo*. All other preparations were 'purified' by methanol precipitation and by dialysis. **pH Measurements.** The pH values in this Table are those recorded by glass-electrode measurements on samples of the mixtures at the end of the reaction, with corrections applied to experiments with zein and gliadin.

Protein	pH	Molecular ratio (NM:protein)		
		Mixture	Precipitated complex	Dialysed complex
Ovalbumin	7.4	4.0	2.3	1.0
	7.4	23.2	5.0	3.7
Casein	7.4	4.2	2.5	2.3
Protamine	about 7*	5.0	1.2	1.2
Serum proteins (rabbit)	7.5	7.0	5.5	4.6
Pepsin	7.4	4.5	3.3	2.6
	4.1	5.0	0.3	0.3
Bovine-plasma albumin	7.4	4.0	1.6	1.3
	7.4	24.0	5.9	5.1
Haemoglobin (horse)	7.2	3.4	1.7	1.0
	7.0	15.3	7.2	4.1
γ -Globulins (horse serum)	7.4	7.9	1.1	0.5
	7.0	52	5.1	3.7
Zein	about 8†	4.8	—	1.5
	about 8†	5.2	—	1.2
Gliadin	about 8†	3.7	—	1.1
	about 8†	5.0	—	1.5

* pH varied during the reaction.

† Measured values were about 7.5 but are recorded as 'about 8' since pH meter readings in dilute ethanol are about 0.5 unit below the actual values (Mizutani, 1925; Albert & Goldacre, 1943).

combination of the hydrolysis products of NM with ovalbumin during the various stages of the hydrolysis in aqueous solution.

A solution of NM in 0.25M-phosphate buffer (pH 7.4) containing 0.63 mg. of NM-HCl/ml. was prepared and kept at about 20°; samples (7.5 ml.) were removed at intervals and immediately added to ovalbumin solution (50 mg./ml. in dilute phosphate buffer) to give a molecular ratio of 4.9 moles of NM/mole of protein. The mixtures were kept at 37° for 2 hr. and the NM-proteins were then precipitated with methanol.

The results (Table 2) showed that when NM hydrolyses in aqueous solution its capacity to combine with ovalbumin is lost only very slowly. In fact, if the vesicant solution is kept for 30–60 min. before it is added to the protein, there is often a significant increase in the amount of NM (or its hydrolysis products) combining firmly with the

Table 2. *Combination of 'hydrolysed' di-(2-chloroethyl)methylamine with ovalbumin*

NM was dissolved in phosphate buffer, pH 7.4, and after being kept at 20° for the times stated, samples of this solution (equivalent to 4.9 moles of NM/mole of protein) were added to ovalbumin solutions; the NM-protein complexes were precipitated with methanol after 2 hr. at 37°.

Period of hydrolysis of NM (hr.)	Nitrogen-mustard content of the precipitated NM-ovalbumin complex (mole of NM/mole of ovalbumin)
0	0.80
0.25	0.82
0.50	0.92
1.0	0.86
2	0.76
4	0.66

protein. In a further experiment it was shown that when kept for 7 days at 18°, NM solutions retain over half their original capacity to combine with ovalbumin.

Combination of NM with acetylated proteins. Experiments have been carried out to determine whether there is any reduction in the capacity of plasma albumin (or ovalbumin) to react with NM when the protein is acetylated. The reaction of NM with these proteins has been studied over the pH range 3–10.

In a typical experiment acetylated bovine-plasma albumin solution (4 ml. containing 116 mg. of protein, in which 47 % of the free amino groups of the original protein had been blocked) was mixed with Universal buffer solution (4 ml.) of about the required pH and 0.9 % NaCl (3 ml.). Similar solutions of bovine-plasma albumin, treated with sodium acetate equivalent in amount to that used in the acetylation process, and dialysed for the same time as the acetylated proteins, were used as controls. NM-HCl (1.13 mg. in 1 ml. of water, i.e. about 4.9 moles of NM/mole of protein) was added and the mixtures were kept for 2 hr. at 37° and overnight at 0–4°, after which samples of the mixtures were dialysed against 0.9 % NaCl. The acetylated proteins gave a small amount of precipitate during the treatment with NM at 37° and also during any subsequent dialysis to remove unchanged NM or its hydrolysis products; these precipitates were removed by centrifuging. The pH of the protein-buffer mixture was determined, by glass electrode, before the NM was added and also at the end of the reaction. No appreciable change in the pH of the

mixtures occurred during the reaction, except in the most alkaline solution, where the pH fell from 10.6 to 9.6, the latter figure being used for the results recorded in Fig. 3.

These results showed that over the pH range 5–8 the acetylated protein combines with appreciably more NM than did the original plasma albumin. At pH 10, however, the non-acetylated protein showed the higher capacity for binding NM (85 % of the added NM, compared with 60 % bound by the acetylated albumin), and many other experiments have confirmed this observation that when plasma albumin is acetylated it loses part of its capacity to react with NM over the pH range 8.5–10. (In all experiments made at about pH 7.4, however, acetylated plasma albumin combined with more NM than did the original protein.)

In other experiments with ovalbumin similar results have been obtained. For example, acetylation of this protein with the blocking of 60 % of the free amino groups led to a very marked increase in the capacity of the protein to combine with NM at about pH 7.4, the complexes precipitated by methanol containing 3.6 and 2.3 moles of NM/mole of protein for the acetylated and original proteins respectively.

Speed of the reaction. In all the experiments described above the NM was allowed to act for 2 hr. on the protein before the unchanged NM and its hydrolysis products were removed by dialysis or by adding methanol. It was realized, however, that the reaction between the vesicant and proteins is quite rapid (cf. Watkins & Wormall, 1952*a, b*), and since the speed of the reaction is of interest in connexion with the distribution of the drug after intravenous injection it was decided to study the rate at which added NM becomes firmly attached to the protein molecule. Ovalbumin was used as the protein in these experiments, in order that a reliable estimate could be made of the molecular ratio of NM:protein in the 'purified' product.

In a preliminary experiment, an NM:protein molecular ratio of 12:1 being used, the NM-HCl (24 mg.) was dissolved in 0.5M-phosphate buffer (pH 7.4, 6 ml.) and added immediately to 20 ml. of ovalbumin solution containing 382 mg. of protein. The mixture was well shaken and kept at 18°. The NM-ovalbumin complexes were precipitated by methanol from 5 ml. samples of the mixture at intervals.

The results showed that the combination of the vesicant with the protein had practically reached a maximum in 15 min., the value then being 4.08 moles compared with 4.16 moles/mole of protein at the end of 60 min. After the first hour there was no significant increase in the amount of NM bound to the protein, and the complexes separated from the mixture after 24 hr. contained only 3.48 moles of NM/mole of ovalbumin.

Fig. 3. Combination of bovine-plasma albumin (Δ) and acetylated bovine-plasma albumin (○) with NM. The NM-protein complexes were dialysed against 0.9 % NaCl.

A second experiment was carried out with a smaller NM:protein ratio (4.9:1) in which the NM-HCl was added directly to a solution of ovalbumin in phosphate buffer (pH 7.4) and samples were removed at frequent intervals, as shown in Table 3.

The results of this experiment showed that appreciable amounts of NM combine with proteins immediately after the mixing of the reagents. Under the conditions of this experiment maximum combination occurred in about 1 hr., but nearly half this maximum was reached in about 2 min. Of the NM which was firmly bound to the protein after 1 hr. about one-quarter became dissociable when the reaction was allowed to proceed for a further 23 hr., but no significant additional loss of bound NM occurred when the mixtures were kept for a further 3 days at 20°.

Although the reaction between NM and ovalbumin is essentially complete in 1 hr., this might not be true for the reaction with other proteins. It was decided therefore, as a general rule in the investigations described here, to allow the vesicant and the proteins to react for 2 hr. before separating the NM-protein complexes.

Effect of pH on the reaction. Ovalbumin and bovine-plasma albumin solutions were treated with 0.1N-NaOH or 0.1N-HCl to bring them to the required pH, mixed with Universal buffer solution of the same pH and treated with NM-HCl (3.6 moles of NM/mole of protein). After allowing the reaction to proceed for 2 hr. at 37° and overnight at 0-4° the pH of the solutions was checked with a glass electrode and the reaction products were separated by methanol precipitation. The pH values recorded in Figs. 4 and 5 are the values determined at the end of the reaction, since an appreciable fall in pH had

occurred in the more alkaline solutions during the reaction (a fall of 1-2 units for solutions initially at pH 8-11).

The results (Figs. 4, 5) show that there is little or no combination of NM with either protein below pH 4, but above pH 5 the amount combining increases rapidly with rise of pH. About two-thirds of the added NM was bound to ovalbumin and 87 % to plasma albumin, at pH 9.

Fig. 4. Effect of pH on the combination of NM with ovalbumin. Ovalbumin (260 mg. in 2 ml. of solution) plus 3 ml. of buffer (Baird and Tatlock S₁, see text) plus 4.7 mg. of NM-HCl in 1 ml. of freshly prepared solution. Reaction occurred for 2 hr. at 37° and overnight at 0-4°. Products were precipitated with methanol.

Table 3. Rate of reaction of nitrogen mustard di-(2-chloroethyl)methylamine with ovalbumin

NM was added to the ovalbumin solution (4.9 moles of NM/mole of protein) at pH 7.4 and 20°, and the NM-complexes were precipitated by adding methanol to samples withdrawn at intervals.

Time after adding NM	Composition of precipitated complexes (mole of NM/mole of ovalbumin)
0	0.39
2 min.	0.60
5 min.	0.81
7 min.	0.88
10 min.	0.91
15 min.	1.01
30 min.	1.06
1 hr.	1.33
2 hr.	1.24
6-5 hr.	1.16
24 hr.	1.01
92 hr.	0.98

Fig. 5. Combination of NM and bovine-plasma albumin at various pH values. The NM-protein complexes were precipitated by methanol. Experimental details are as for Fig. 4, but 4 ml. of buffer was used.

Effect of phosphate. Phosphate has been found to have a marked effect on the action of sulphur mustard on proteins, with phosphate groups entering the final mustard gas-protein complexes (Fleming, Moore & Butler, 1949). Although the addition of phosphate does not lead to any increase in the relatively slight serological changes which antigenic proteins undergo when they are treated with NM (Watkins & Wormald, 1952*a*), this does not exclude the possibility that phosphate might affect the amount of NM which can combine with a protein. A comparison was therefore made of the relative effects of phosphate and bicarbonate when used as buffers in the reaction between NM and ovalbumin.

A mixture of ovalbumin solution (2 ml., containing 172 mg. of protein) and 0.9% (w/v) NaCl (3 ml.) was buffered with 4 ml. of either 0.5M-phosphate (pH 7.5) or 0.75M-bicarbonate (pH 7.4). A freshly prepared solution of NM (1 ml. containing 4.15 mg. of NM-HCl, i.e. 5.3 moles of NM/mole of protein) was added to each mixture, the solutions were kept at 37° for 2 hr., then overnight at about 4°, and the NM-proteins were separated by methanol precipitation.

The complex prepared in the presence of bicarbonate contained considerably more NM than the one prepared in the presence of phosphate (1.6 and 0.9 mole of NM/mole of protein respectively). Thus phosphate is not only not essential for the combination of NM and proteins, but it probably competes with the protein for the added NM.

Effect of other inorganic salts. In view of the capacity of proteins to form complexes with various buffer and other anions, and the consequent competitive effect of some inorganic salts on the binding of dyes, etc., by proteins (see, for example, the reviews by Klotz, 1949; Scatchard, 1952) it was thought desirable to study the effect of some anions on the combination of NM with proteins.

Table 4. *Influence of some anions on the combination of di-(2-chloroethyl)methylamine and bovine-plasma albumin*

Bovine-plasma albumin (150 mg.) in 2.5 ml. of 0.9% NaCl (series A) or water (series B); 0.5M-buffer (2 ml.); NM-HCl (1.5 mg. with phosphate buffer, 1.8 mg. with acetate buffer) in 1 ml. of 0.9% NaCl (A) or water (B). Reaction at 37° for 2 hr. and overnight at 0-4°.

Buffer	NM content of methanol-precipitated complexes (mole of NM/mole of bovine-plasma albumin)	
	(A)	(B)
Phosphate, pH 7.4	1.03	1.02
Phosphate, pH 5.6	0.62	0.56
Acetate, pH 5.6	0.53	0.43

Solutions of bovine-plasma albumin in water and in 0.9% NaCl were mixed with phosphate or acetate buffer solutions, and a freshly prepared solution of NM-HCl in either water or 0.9% NaCl was then added.

In these experiments (Table 4) added sodium chloride had no significant effect on the amount of NM combining with bovine-plasma albumin at pH 7.4 (phosphate buffer). At pH 5.6, however, the addition of chloride led to a small increase (about 10%) in the amount of NM combining with the protein in a phosphate solution, and a larger increase (about 20%) when acetate buffer was used. It is significant, in view of the possible role of phosphate in the reaction between sulphur mustard and proteins, that in these mixtures at pH 5.6 more NM combined with the albumin in phosphate than in acetate buffer solution.

Stability of NM-protein complexes

Stability at various pH values. A series of experiments was carried out to determine the stability of the linkages between NM and protein when the NM-protein complexes were kept at 37° for 24 hr.

Bovine-plasma albumin solution (1.8 g./10 ml. of 0.9% NaCl) was mixed with 4 ml. of 0.5M-phosphate buffer (pH 7.4) and 17.8 mg. of NM-HCl was added; 0.9% NaCl was then added to bring the volume to 25 ml. The mixture was kept at 37° for 2 hr., stored at 0-4° overnight and dialysed for 72 hr. at about 4° against 0.9% NaCl. Samples (2 ml. containing 2.02 moles of NM/mole of protein) of the dialysed solution were taken and the pH was adjusted with 0.1N-NaOH or 0.1N-HCl to cover the range pH 4.7-8.7, at intervals of about 0.4 unit. Universal buffer solution (4 ml.) of the appropriate pH was added to each sample and the solution diluted to 10 ml. with 0.9% NaCl. The solutions were kept at 37° for 24 hr. and then dialysed against four successive portions of buffer-saline mixture (3:1, v/v) for a total of 6 hr. A sample (5 ml.) was removed and the remainder of the protein solution was dialysed for a further 36 hr. The results showed that under these conditions the NM-albumin complexes lost only a small part of the attached NM. These losses of bound NM were about 10% in the acid and neutral solutions and about 15% at pH 8.0-8.7, and no further loss occurred when dialysis was continued for a further 36 hr.

Two similar experiments were made with ovalbumin. In the first experiment 12.8 mg. of NM-HCl was added to 20 ml. of ovalbumin solution (approximately 4% protein) plus 5 ml. of 0.5M-phosphate buffer (pH 7.4), and in the second 15 mg. of NM-HCl was added to 10 ml. of ovalbumin solution (8.6% protein) plus 5 ml. of 0.5M-phosphate buffer. The NM-proteins obtained contained respectively 0.78 and 0.8 mole of NM/mole of ovalbumin.

Samples of these solutions were brought to the pH values recorded in Table 5, buffer of the appropriate pH was added and the mixtures were kept at 37° for 24 hr. and subsequently dialysed for 36 hr. at about 4°.

The results (Table 5) showed that the loss of bound NM was appreciably less at pH 5-6 than it was at pH 7-8. It ranged, in Expt. 1, from 18% at pH 5.3 to 40% at pH 7.8, and from 20% at pH 5.1 to 50% at pH 7.2 in Expt. 1. This marked loss of NM by the NM-ovalbumin complexes over the range 5-7, in contrast with the relatively slight loss by NM-serum albumin under similar conditions, may conceivably be partly due to the lability of ester links between NM and phosphate groups in the former complexes (pK_2 for phosphoric acid, 7.2) since the ovalbumin molecule is known to contain one molecule of phosphoric acid (Cohn & Edsall, 1943).

Stability towards protein precipitants. Experiments with different protein precipitants were carried out to determine whether the nature of the precipitant had any effect on the amount of NM present in the precipitated NM-protein complex. Three precipitating agents were used: (a) methanol (4 vol.); (b) trichloroacetic acid (10%, w/v; 4 vol. added to 1 vol. of NM-protein solution, with vigorous stirring during the addition); (c) sodium tungstate solution (10%, w/v; 1 vol. was added, and 0.66N-H₂SO₄ was then added dropwise until precipitation was apparently complete. The mixture was allowed to stand for 10 min. before centrifuging). All the precipitates were washed three times with the precipitant, and then dried *in vacuo* over conc. H₂SO₄.

NM-ovalbumin and the NM-protein complexes of the mixed proteins of horse serum were prepared as described above. Ovalbumin solution (15 ml., containing 1.07 g. of protein) was mixed with Universal buffer solution (5 ml.), 0.9% NaCl (5 ml.) and NM-HCl (17.5 mg.); 9.3 mg. of NM-HCl was added to 20 ml. of horse serum and buffer solution (5 ml.).

The results (Table 6) indicate that the amounts of NM present in the precipitates obtained with the three greatly differing protein precipitants did not vary considerably. With both proteins the highest NM content was obtained with the methanol precipitates, and the lowest for the complexes precipitated by trichloroacetic acid. Methanol may precipitate a little of the inorganic matter present in the mixtures, and this may explain the slightly higher results with this precipitant, since the precipitated inorganic matter may carry down NM or some of its hydrolysis products.

Effect of salt precipitation on NM-ovalbumin. Since ovalbumin can be readily obtained crystalline with the aid of Na₂SO₄, we decided to attempt the

crystallization or precipitation of NM-ovalbumin with the Na₂SO₄ technique to determine whether this process leads to rupture of the NM-ovalbumin linkages.

Ovalbumin precipitated with Na₂SO₄ was freed from this salt by dialysis, and the product (5 ml. containing 0.33 g. of ovalbumin) was treated with NM-HCl (11.3 mg.) in 0.5M-phosphate buffer (2 ml.) and the mixture kept at 37° for 2 hr., and at 0-4° overnight. The solution was then dialysed against distilled water to remove uncombined NM and its 'hydrolysis' products, and brought to pH 7.4. A sample (5 ml.) of this solution was warmed to 34° and saturated Na₂SO₄ solution (5 ml., pH 7.4), also at about 34°, was added dropwise with slow mixing. The resulting precipitate was centrifuged off, redissolved in about 5 ml. of water and the precipitation with Na₂SO₄ repeated on part of the solution.

Table 5. *Stability of di-(2-chloroethyl)methylamine-ovalbumin at various pH values*

Samples of NM-ovalbumin complex in buffer solutions of various pH values were kept at 37° for 24 hr. and then dialysed against 0.9% NaCl buffered at the appropriate pH. Other experimental details are given in the text.

pH	NM content (mole/mole of protein) of the dialysed complexes	
	Expt. 1	Expt. 2
	Before keeping at 37°	
	0.78	0.80
	After keeping at 37°	
5.1	—	0.64
5.3	0.65	—
5.7	—	0.62
6.2	0.55	0.58
6.5	—	0.54
6.8	0.50	0.40
7.2	—	0.40
7.8	0.47	—
8.7	0.47	—

Table 6. *Stability of di-(2-chloroethyl)methylamine-proteins to various precipitants*

NM-protein complexes (either NM-horse-serum proteins or NM-ovalbumin, prepared with 3.61 and 3.08 moles of NM/mole of protein respectively) in Universal buffer, pH 7.4, were separated from uncombined and hydrolysed NM by precipitation with various protein precipitants as described in the text.

Precipitant	NM content of precipitated complex (moles of NM/mole of protein)	
	Horse-serum proteins	Ovalbumin
Methanol	2.21	1.25
Sodium tungstate	2.12	1.17
Trichloroacetic acid	2.02	0.98

Analysis of the products showed that there was no loss of NM resulting from the first precipitation of the NM-ovalbumin, both the original complex and the precipitated complex containing 1.33 moles of NM/mole of ovalbumin, but with a second precipitation the molecular ratio fell to 1.01.

DISCUSSION

These results have shown that NM reacts rapidly under physiological conditions of temperature and pH with all the proteins studied. The reaction is so rapid that it is highly probable that when the drug is injected intravenously into man for therapeutic purposes, much of the NM combines firmly with the plasma proteins and remains in the bloodstream for an appreciable time. Part of the attached NM may be only loosely bound to the proteins. For example, the complexes separated from mixtures of NM and ovalbumin showed a maximum NM content 1–2 hr. after the drug had been added to the protein, and complexes separated 6–24 hr. later had appreciably lower NM contents (Table 3). However, about three-quarters of the NM bound to the protein at the 1 hr. stage was still firmly bound 92 hr. later, and in general the NM-protein complexes are relatively stable after the initial small loss of NM. NM-plasma albumin and NM-ovalbumin complexes show only partial loss of bound NM when they are kept in neutral solution at 37° for 24 hr., and it appears probable that most of the NM-protein complexes formed immediately after the intravenous injection of NM will persist in this form for many hours. Even if the injected NM undergoes 'hydrolysis' in the blood or tissue fluids before reacting with protein it will still retain most of its capacity to combine with the body proteins, since aqueous NM solutions lose this capacity very slowly (Table 2). Although phosphate ion apparently competes with protein for combination with NM, hydrolysis of NM in the presence of phosphate for several hours does not appreciably impair its ability to react with protein. Thus any NM-phosphate compounds which may be formed are probably freely dissociable.

Direct information about the precise fate of NM in the animal body and the duration of its retention in the vascular system cannot as yet be obtained with ^{15}N -labelled NM. The toxicity of the drug is too great to permit the injection of sufficient NM to allow the ^{15}N to be measured after its dilution with very large amounts of protein nitrogen, but we hope to obtain more precise information about the behaviour of NM in the animal body in our experiments with NM labelled with ^{14}C or ^3H or both. With NM labelled with ^{14}C in the methyl group, Skipper, Bennett & Langham (1951) have found that the drug is extensively fixed in most body

tissues, and these results are in agreement with our observations that NM combines rapidly and firmly with many widely differing proteins.

The results described in this paper do not provide positive information about the protein groups which are responsible for the binding of nitrogen mustard, but they show that the free amino groups are not essential for this reaction. For example, it has been found that zein and gliadin, proteins which contain very little or no lysine, combine with almost as much NM as do proteins rich in this amino acid. Furthermore, plasma albumin and ovalbumin treated with sufficient acetic anhydride to give fairly extensive acetylation of the free amino groups combine with more NM than do the non-acetylated proteins (Fig. 3). [The method used effects the acetylation of only the amino groups in a number of proteins (Olcott & Fraenkel-Conrat, 1947), though free SH groups may also react (Fraenkel-Conrat *et al.* 1949).] Thus protein groups which react rapidly with NM are apparently unattacked by acetic anhydride. Banks *et al.* (1946) found that blocking the free amino groups of a protein with phenyl isocyanate did not reduce the capacity of the protein to combine with mustard gas. It is also of interest that whereas Fruton *et al.* (1946) found that NM effects little reduction of the free amino groups of ovalbumin during the first 20 min. of the reaction, we have found that there is extensive combination of the drug with the protein during this period. Thus groups other than amino groups (e.g. carboxyl and imidazole) are concerned with the rapid action of NM on proteins. It is also possible that NM combines with phosphoric acid groups in some of the proteins studied here, e.g. casein and probably ovalbumin.

Our attempts to obtain crystalline characterizable NM derivatives of various amino acids have so far been unsuccessful, possibly because the products were almost invariably mixtures of two or more NM-amino acid compounds, and in this respect the cross-linking action of the nitrogen mustards may be a contributory factor. With the aid of the labelled NM it has been possible, however, to obtain an approximate measure of the amount of NM which combines firmly with various amino acids. For example, cysteine treated with NM at pH 7 showed a complete loss of free SH groups and a very marked reduction in free amino groups, and gave a mixture which yielded an oil containing 1.3 moles of NM/mole of cysteine. From reaction mixtures of NM and either arginine or histidine in alkaline solution amorphous products were obtained, each of which contained compounds whose average NM:amino acid molecular ratio was about 2:1. It has also been found that NM combines firmly with various di- and tri-peptides, and that the histidyl group is particularly important in this

reaction; for example, complexes obtained from histidylhistidine contained nearly 3.5 moles of NM/mole of dipeptide.

However, this reaction with histidyl groups is not the sole mode of combination of NM and proteins, for in our tests with a wide range of proteins we have found that there is no relationship between the capacity of a protein to bind NM and its histidine content. Furthermore, all the histidyl groups in a protein do not react, for the total amount of NM which can be bound by many proteins is equivalent to only a small fraction of the histidine residues.

The amount of NM bound by proteins varies with the pH at which the reaction is carried out. As the results in Figs. 4 and 5 show, there is no significant combination of the vesicant with either ovalbumin or plasma albumin at pH 4, presumably because the NM is present as a relatively stable 'ammonium' salt. As the pH is raised and the total negative charge on the protein molecule is increased, the possibility of nucleophilic attack by the protein on the α -methylene group of the 2-chloroethyl side chain of the free nitrogen mustard is enhanced, thus a gradually increasing fraction of the added NM becomes firmly attached to the protein. In some experiments at pH 9, for example, 87% of the added NM was bound by plasma albumin and 68% by ovalbumin, the amounts of NM added in these experiments being respectively 3.8 and 3.6 moles/mole of protein. From these results, and other evidence, it appears that the reaction between NM and plasma albumin (or ovalbumin), like that between NM and haemolytic complement (Watkins & Wormall, 1952b), is due to ethyleneiminium ions produced by intramolecular quaternization of the NM.

The maximum number of NM residues which can be introduced into a protein molecule is usually between five and six, and since at least four times as many sulphur-mustard residues can be introduced into serum albumin and other proteins (Banks *et al.* 1946), the two mustards evidently differ considerably in their reaction with proteins. The fact that only a few NM residues can be introduced into a protein may explain why there is only a very slight change in the immunological specificity of antigenic proteins when they are treated with NM (Watkins & Wormall, 1952a), and a further contributing factor may be the removal, by dialysis, of part of the NM after the NM-protein complexes have been injected into rabbits. There is also the possibility that any nitrogen-mustard groups which have been concerned with the cross-linking of protein molecules may not act as serologically dominant or characteristic groups, but this possible significance of cross-linking actions of the nitrogen and sulphur mustards in relation to changes in the immunological properties of proteins is being investigated further.

SUMMARY

1. The microsynthesis of di-(2-chloroethyl)-methylamine (NM) hydrochloride labelled with ^{15}N is described.

2. With this labelled NM the combination of the vesicant with proteins under various conditions has been studied quantitatively, the NM-protein complexes being freed from unchanged NM or its hydrolysis products by prolonged dialysis or precipitation with methanol.

3. Considerable amounts of NM are rapidly bound by ovalbumin and bovine-plasma albumin at pH 7.4 and 37°; when excess of NM is used, the amounts bound to these proteins are respectively about 5 and 5.5 moles of NM/mole of protein.

4. Greater amounts of NM are bound by these proteins at higher pH values, less in acid solution and no combination occurs at pH 4.

5. The reaction between NM and proteins is very rapid, and a considerable amount of the added NM is bound to the protein in a few minutes.

6. The NM which combines with proteins at pH 7.4 is firmly attached, and prolonged dialysis of the complex at about 4° removes only part (sometimes less than one-fifth) of the NM. When the protein complexes are precipitated by trichloroacetic acid or sodium tungstate there is only a small loss of bound NM.

7. Some NM-protein complexes are only moderately stable at 37°. NM-plasma albumin complexes show only a small loss of NM when kept in solution at 37° for 24 hr. at pH values between 3 and 8.7, but NM-ovalbumin lost 40–50% of its NM when kept in neutral or slightly alkaline solution, and nearly 20% at about pH 5.

8. All the other proteins tested (casein, pepsin, haemoglobin, γ -globulins, protamine, zein and gliadin) firmly bind added NM at pH 7.4 and 37°. Where the maximum binding capacity of the protein for NM was determined, it was similar to that of the plasma albumin.

9. The free amino groups of the protein are not essential for the reaction with NM, since acetylated ovalbumin and bovine-plasma albumin react with slightly more NM at pH 5–8 than do the non-acetylated proteins.

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The Determination of the Structure of Unsaturated Fatty Acids on a Micro Scale with the Gas-Liquid Chromatogram

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It has been pointed out in previous publications (James & Martin, 1956; James & Wheatley, 1956) that some information on the structure of unsaturated acids can be obtained from a study of their times of emergence from a gas-liquid chromatogram column. In general, the more double bonds in the molecule the more rapidly the acid moves, mono- and di-unsaturated acids running between the

corresponding saturated acid and its next lower homologue when the column stationary phase is a saturated paraffin hydrocarbon (e.g. Apiezon M vacuum stopcock grease). The position of the double bond also affects the chromatographic behaviour: e.g. methyl-9:10-octadecenoate has a retention volume (James & Martin, 1956) relative to methyl myristate of 4.75, methyl-6:7-octadecenoate 4.87