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# EFFECT OF NITROGEN MUSTARD ON SOLUBLE COLLAGEN*

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**Abstract**—The effect *in vitro* of nitrogen mustard (HN2) on physical parameters of rat tail tendon collagen and rabbit skin collagen was studied.

Intrinsic viscosities, helix-coil transition temperatures (by viscosimetry and rates of fibril formation (by turbidimetry) of HN2-treated (1 mg/ml HN2, 0·9% saline, 2 hr at 25°) and control collagens were compared. Helix-coil transition temperatures were not affected by $HN_2$ treatment. Fibril formation by both collagens was inhibited by $HN_2$, but apparently this effect was nonspecific since it was reversible by dialysis. Rat tail tendon collagen aggregates in solution were disaggregated by $HN_2$ treatment. Only 0·5 per cent of the hydroxyproline of collagen became dialyzable after $HN^2$ treatment.

THE ACTION *in vitro* of nitrogen mustard§ on a variety of biological materials has been studied by Stacey *et al.*[1] These authors found that HN2 treatment can lead to cross-linking or chain breakage in DNA, and to polymerization of bovine serum albumin. Evidence for intermolecular cross-linking of DNA by HN2 treatment was presented by Geiduschek,[2] Kohn *et al.*[3] and Doskocil and Sormova.[4] The well defined spatial order of the tropocollagen triple helix and the organized polymerization of tropocollagen into collagen fibrils seem to offer possibilities for intra- and intermolecular cross-link formation by HN2, similar to those encountered in DNA. The present communication describes experiments *in vitro* designed to study these possibilities.

Ross[5] studied the relative reactivity of the different chemical groups occurring in amino acid side chains, with HN2. He concluded that around neutrality the -COOH groups of aspartic and glutamic acid, the imidazolyl nitrogen of histidine, and the terminal sulfhydryl groups are the most reactive groups. These chemical groups are all present in mammalian collagen with the exception of terminal sulfhydryls.[6] Thus, it appears reasonable to expect that collagen might react with HN2.

## MATERIALS AND METHODS

Rat tail tendon collagen was prepared by the method of Dumitru and Garrett.[7] Rabbit skin collagen was prepared by the method of Piez *et al.*[6] The nitrogen mustard

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§ The following abbreviations are used: HN2, nitrogen mustard (methyl-*bis*[2-chloroethyl]amine); $T_m$, helix-coil transition temperature; $\eta_{sp}/c$, specific viscosity divided by concentration.

preparation used (Mustargen R, Merck, Sharp & Dohme) contained sodium chloride in addition to methyl-*bis*(2-chloroethyl)amine.

Nitrogen mustard treatment was carried out by mixing equal volumes of a solution of collagen in water (1–2 mg/ml) and a solution of HN2 (2 mg/ml in 1·8% sodium chloride). The mixture was allowed to react at 25° for 2 hr. Collagen mixed with an equal volume of 1·8% sodium chloride solution served as an untreated control. Treated and control collagen samples were first dialyzed against 50 vol. of 0·15 M acetic acid for 24 hr, then against several changes of 0·1 M sodium citrate, pH 3·5. In order to quantitate the dialyzable collagen fragments, the acetic acid dialysate was evaporated to dryness, and the residue hydrolyzed with 6 N hydrochloric acid (3 hr at 125°) and subjected to hydroxyproline assay according to Leach.[8] Hydroxyproline assay was also used to determine the concentration of the exhaustively dialyzed collagen samples. These were then volumetrically diluted with 0·1 M sodium citrate, pH 3·5, to concentrations suitable for intrinsic viscosity determination. The relative viscosities of the diluted samples were determined at 25° ± 0·01° in an Ostwald viscometer constructed according to Schachman,[9] Solution densities of buffer and of the most concentrated collagen solution were determined in a 1-ml pycnometer. Densities of the intermediate dilutions were estimated by interpolation. The helix-coil transition temperature ($T_m$) of HN2-treated and control collagen was determined viscosimetrically, essentially as described by Burge and Hynes.[10] These experiments were carried out in two different solvent systems: (1) by adding an equal volume of 0·25 M acetic acid in physiological saline directly to the HN2–collagen reaction mixture, and (2) after prolonged dialysis of the above mixture against 0·1 M sodium citrate, pH 3·5. Relative viscosities of collagen in either solvent system were determined at intervals while the temperature of the viscometer bath was raised slowly (1/8°/min) from 26° to 45°. Collagen fibril formation was determined essentially by the spectrophotometric method of Gross.[11] A collagen solution, previously dialyzed against water, was added to an equal volume of freshly prepared nitrogen mustard solution (2 mg/ml HN2–1·8% sodium chloride) or to a 1·8% sodium chloride solution. Fibril formation was followed by measuring the absorbance at 530 mμ in a spectrophotometer after various times at room temperature (25°). After about 3 hr the reaction was interrupted by the addition of 0·5 ml of 0·5 M acetic acid to each 3-ml cuvette. Unreacted HN2 and its hydrolysis products were removed by dialysis at 4° against 0·15 M acetic acid and against several changes of water. Finally, gel formation was induced again by adding 0·2 ml of 13·5% sodium chloride to 2·8 ml of the dialysed samples and warming to 25°. Fibril formation was followed spectrophotometrically as above. Fibril formation from control collagen at lowered pH was studied by substituting 0·1 M sodium acetate buffers, pH 4·3 and 4·5, in 1·4% sodium chloride for the usual 1·8% sodium chloride solution.

## RESULTS

Fig. 1 shows the temperature dependence of specific viscosity of HN2-treated and control rabbit skin collagen solutions. As expected, the helix-coil transition temperature ($T_m$) was found to vary with the solvent system, but in each case the HN2-treated sample showed the same $T_m$ as the corresponding control sample. $T_m$ in normal saline containing 0·125 M acetic acid was 32·6° for the control sample as well as for the treated sample, which in addition contained 0·5 mg/ml of $HN_2$ or its hydrolysis

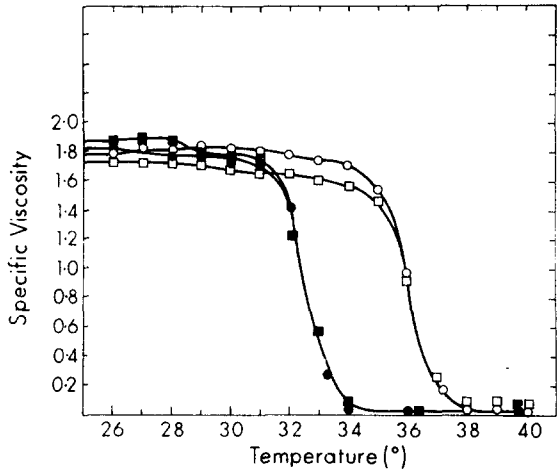


FIG. 1. Effect of HN2 treatment on the helix-coil transition temperature of rabbit skin collagen. All samples contained approximately 0·75 mg/ml acid-soluble rabbit skin collagen. ● = HN2-treated (final concentrations HN2, 0·5 mg/ml; acetic acid, 0·125 M; sodium chloride 0·9%); ■ = control (acetic acid, 0·125 M; sodium chloride 0·9%); ○ = HN2-treated and dialyzed against 0·1 M sodium citrate, pH 3·5; □ = control dialyzed against 0·1 M sodium citrate, pH 3·5.

products. $T_m$ for the same samples after dialysis against 0·1 M sodium citrate, pH 3·5, was 36·1°. Rat tail tendon collagen (treated and control) had a $T_m$ of 31·5° in the acetic acid–sodium chloride solvent.

Fig. 2 shows the extrapolation to infinite dilution of plots of specific viscosity/concentration vs. concentration for HN2-treated and control samples of rat tail tendon and rabbit skin collagen. Although only one extrapolation line is shown for HN2-

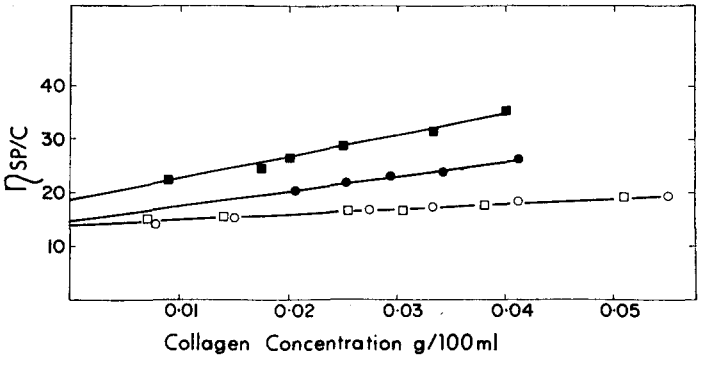


FIG. 2. Effect of HN2 treatment on the intrinsic viscosity of soluble collagen at 25°. All samples in 0·1 M sodium citrate, pH 3·5. ● = HN2-treated rat tail tendon collagen; ■ = control rat tail tendon collagen; ○ = HN2-treated acid-soluble rabbit skin collagen; □ = control acid-soluble rabbit skin collagen.

treated and control rabbit skin collagen, the two sets of values actually extrapolate to slightly, but not significantly, different values, 14·1 dl/g for the $HN_2$-treated collagen and 14·5 for the control. The intrinsic viscosity of HN2-treated rat tail tendon collagen was found to be 15·0 dl/g, while the corresponding control extrapolated to 18·8 dl/g. This value is considered to be significantly different from the three others, since duplicate intrinsic viscosity determinations usually fall within $\pm$ 1 dl/g.

Collagen fibril formation was studied under various conditions in the presence and absence of HN2. The results of these experiments, presented in the form of plots of absorbance at 530 mμ vs. time, are given in Figs. 3 and 4. Zero time in each case is the time at which $HN_2$ and sodium chloride, or sodium chloride solutions alone, were

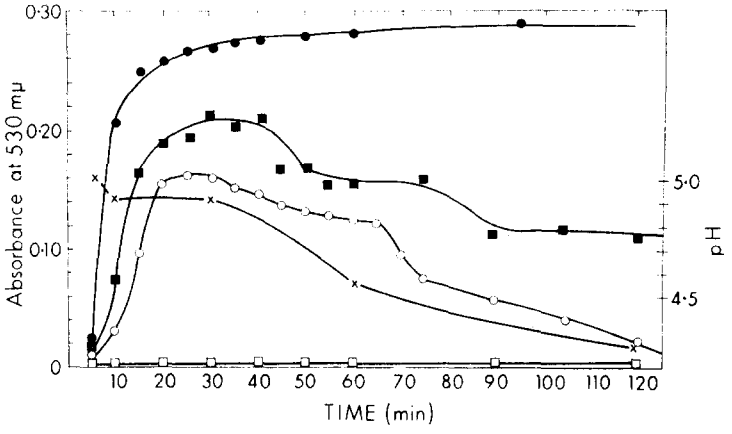


FIG. 3. Effect of HN2 on fibril formation of rat tail tendon collagen at 25°. All samples contained 0·7 mg/ml collagen in 0·9% sodium chloride solution plus HN2 as indicated below. □ = Aged HN2, 1 mg/ml; ○ = HN2, 1 mg/ml; ■ = HN2, 0·5 mg/ml; ● = control; × = pH of reaction mixture (scale at right), 1 mg/ml HN2.

added to an aqueous solution of collagen in concentrations such that the final concentration of sodium chloride was 0·9 per cent. Fig. 3 shows the effect of $HN_2$ on rat tail tendon collagen. All the samples were prepared at 4° and then brought to 25° rapidly by immersion in a water bath. The O.D.$_{530}$ of control collagen increased rapidly for about 15 min, after which only little further rise took place. With collagen containing freshly dissolved HN2 (1 mg/ml), an increase in O.D.$_{530}$ of similar duration was observed. This increase was not as steep as in the case of the control, and after a brief leveling-off period the O.D.$_{530}$ decreased again reaching essentially zero after 2 hr. While the fibril formation phase appeared to be roughly exponential, the subsequent fibril degradation phase was found to be consistently of a more complex nature. With HN2 at a level of 0·5 mg/ml, an intermediate curve was obtained, while curves at 0·1 and 0·05 mg/ml (not shown in Fig. 3) approximated the control curve closely. When an HN2 solution which had been aged at 4° for 10 days was used in 1 mg/ml concentration, fibril formation was completely inhibited. Since the hydrolysis reaction of HN2 in aqueous solution leads, among other things, to liberation of hydrogen ions and since strong acid is of course capable of dissolving collagen fibrils, it was necessary to study the pH of the reaction mixture. Consequently, pH data for the mixture containing the highest HN2 concentration are also given in Fig. 3. It will be shown (see below) that these pH changes are not of sufficient magnitude to explain the observed fibril disappearance curves.

Fig. 4 shows the effect of HN2 on acid soluble rabbit skin collagen. The control fibril formation curve for rabbit skin collagen was quite similar to the one for rat tail tendon collagen. The fibril formation curve obtained with rabbit skin collagen in the presence of HN2 was also similar to the corresponding one obtained with rat tail tendon collagen, except that the rabbit skin collagen showed a steeper and more

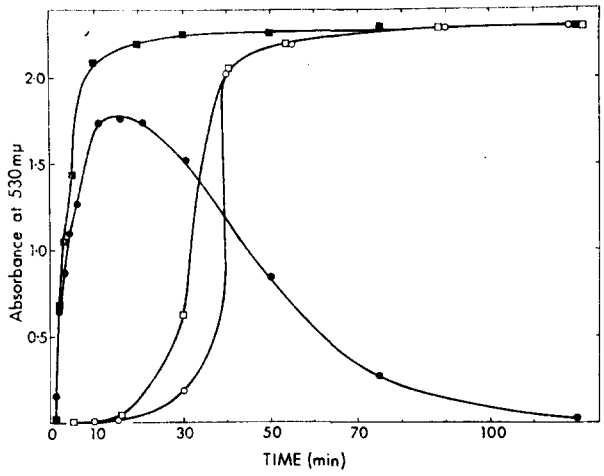


FIG. 4. Effect of HN2 on fibril formation of acid-soluble rabbit skin collagen at 25°. All samples contained 1·5 mg/ml collagen in 0·9% sodium chloride solution. ● = 1 mg/ml HN2 added; ○ = HN2-treated, after dialysis; ■ = control; □ = control after dialysis.

symmetrical fibril disappearance phase. Curves of treated and control collagen after dialysis were identical, indicating that the effect of HN2 could be reversed by dialysis. The lag period preceding the fibril formation phase from dialyzed samples is presumably due to the fact that the samples were allowed to warm to 25° slowly.

The effect of lowering the pH of untreated collagen solutions to 4·5 and 4·3 on fibril formation is shown in Fig. 5. Lowering the pH to 4·5 did not significantly affect the final O.D.$_{530}$ values of either rat tail tendon or rabbit skin collagen, although the rate of fibril formation was reduced in the rabbit collagen preparation. Lowering

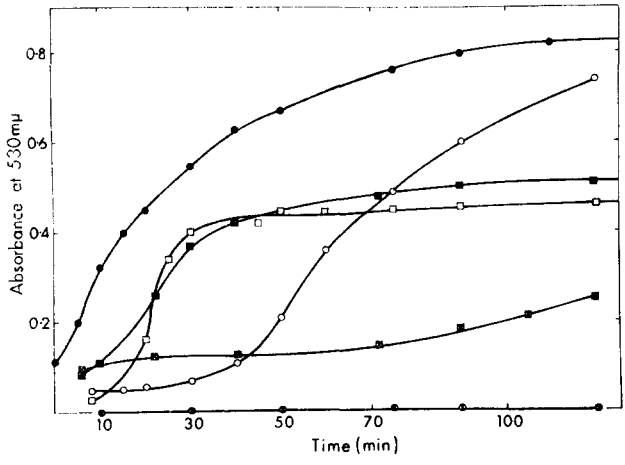


FIG. 5. Effect of pH on fibril formation of rat tail tendon collagen and of acid-soluble rabbit skin collagen at 25°. ● = Unbuffered rabbit skin collagen (0·9% sodium chloride); ○ = rabbit skin collagen, pH 4·5 (0·05 M sodium acetate buffer, pH 4·5, 0·7% sodium chloride); ⊗ = rabbit skin collagen, pH 4·3 (0·05 M sodium acetate buffer, pH 4·3, 0·7% sodium chloride); ■ = unbuffered rat tail tendon collagen (0·9% sodium chloride); □ = rat tail tendon collagen, pH 4·5 (0·05 M sodium acetate buffer, pH 4·5, 0·7% sodium chloride); ⊠ = rat tail tendon collagen, pH 4·3 (0·05 M sodium acetate buffer, pH 4·3, 0·7% sodium chloride).

the pH to 4·3, on the other hand, prevented fibril formation completely in the rabbit skin preparation and decreased it considerably in the rat tail tendon preparation.

Analyses of the initial dialysates of control and HN2-treated rat tail tendon collagen for dialyzable hydroxyproline gave values of 12 and 20 $\mu$g hydroxyproline, representing 0·8 and 1·3 per cent of the total. Thus, HN2 treatment resulted in an increase in dialyzable hydroxyproline of 0·5 per cent.

## DISCUSSION

Nitrogen mustard has long been recognized as an extremely reactive substance capable of alkylating many chemical groupings in a large number of biologically important substances. Since HN2 is a difunctional reagent, it was reasonable to assume that it might form cross-links between suitably spaced reactive groups on DNA, protein, etc. This view was supported by the finding, based on physical-chemical studies, that cross-linking of DNA and protein appears to result from HN2 treatment.[1] Subsequent work by Brookes and Lawley,[12] Geiduschek,[2] Doskocil and Sormova[4] and Kohn *et al.*[3] has shown that DNA can be cross-linked by HN2.

The situation in the case of proteins is not as well understood. The well defined spatial order of the tropocollagen triple helix and the organized polymerization of tropocollagen into collagen fibrils would seem to offer possibilities for crosslink formation by HN2 similar to the ones encountered in DNA. Cross-linking can take place only if reactive groups are available and spaced in such a fashion that the HN2 molecule can bridge the gap between the two reactive groups. It is known that a relatively large number of aspartyl and glutamyl side chains and a smaller number of histidyl side chains are present in collagen.[6] Most of these groups are titratable,[13] thus presumably reactive with HN2 at neutral or slightly acid pH.[5] It is not known, however, if the steric relations of these groups would permit cross-linking either intra- or intermolecularly. The possibility exists, of course, that individual amino acid side chains might be alkylated without any effect on the macromolecular properties of the tropocollagen molecule. We have not addressed ourselves to this aspect of the problem on the premise that the most important physiological alkylation reactions would be the ones affecting either the stability of the tropocollagen molecule or its polymerization into collagen fibrils, since collagen appears to have a largely structural function in the body.

The effect of collagen cross-linking on the helix-coil transition temperature has been discussed in detail by McBride and Harrington.[14] While crosslinking can result in either a decrease or an increase of $T_m$, it appears likely that cross-links introduced by HN2 under our experimental conditions would lead to an increase in $T_m$. This has been found to be the case in the aldehyde cross-linked collagens.[15] Our data show no change in transition temperature as a result of HN2 treatment. This observation suggests the absence of intramolecular cross-linking due to HN2. However, it does not rule out the possibility of HN2 cross-links close to already existing natural cross-linkages. Attempts to show the presence of new molecular species qualitatively by acrylamide gel disk electrophoresis according to Nagai *et al.*[16] failed to show any gross changes.

Intermolecular cross-linking, i.e. cross-linking between adjacent tropocollagen molecules, might either enhance or diminish fibril formation and the stability of fibrils, depending on the location of the cross-link. If one assumes that the treatment sub-

sequent to reaction with HN2, namely dialysis against dilute acetic acid and citrate buffer at 4°, is sufficiently mild so as not to break cross-links formed by HN2, then intermolecular cross-linking should manifest itself by the presence of collagen dimers. Intrinsic viscosities of HN2-treated rat tail tendon collagen were consistently and significantly lower than those of untreated controls. These data might be interpreted as being indicative of side-by-side aggregation as a result of crosslinking by HN2. However, since the intrinsic viscosity of the untreated collagen was substantially higher than most of the values given in the literature,[17–19] the possibility was entertained that the rat tail tendon collagen might be partly aggregated and that HN2 had led to disaggregation. The tendency of neutral salt-soluble collagen to aggregate has been described before.[20,21] An attempt to obtain a monomeric tropocollagen preparation by subjecting rat tail tendon collagen to the purification method of Piez *et al.*[6] did not alter the intrinsic viscosity of the control solution. Consequently, experiments with acid-soluble rabbit skin collagen were carried out, since it has less of a tendency to aggregate. The intrinsic viscosities of treated and control samples of this collagen did not differ significantly from each other or from the one of HN2-treated rat tail tendon collagen. Thus, it appears that HN2 treatment does not yield intermolecular cross-links in collagen, but rather has a tendency to disaggerate already formed aggregates. This observation was further confirmed by studies on collagen fibril formation. Interestingly enough, freshly prepared HN2 did not seem to inhibit fibril formation markedly. However, the process of fibril formation was reversed after less than half an hour. This, along with the observation that no fibril formation occurred in the presence of an aged nitrogen mustard solution, led to the concept that nitrogen mustard *per se* did not inhibit fibril formation, but rather that the hydrolysis products of HN2 interfered with fibril formation. According to Golumbic *et al.*,[22] HN2 in aqueous solution goes through a whole series of reactions leading to two reactive ethyleneimonium compounds and several inactive compounds. The most abundant products in aged HN2 solutions are *N,N'*-dimethyl-*N,N'bis*(2-chloroethyl)piperazinium salt (31 per cent), and methyl-2-chloroethyl-2-hydroxyethyl amine (49 per cent). The formation of this latter compound is accompanied by the liberation of hydrogen ions. Strong acid is, of course, capable of dissolving collagen fibrils. Since it was impractical to buffer the HN2–collagen reaction mixture adequately, it became important to establish whether the acidity of the reaction mixture was sufficient to prevent fibril formation. The pH of the reaction mixture did not change during the first 30 min of the reaction, a period during which the change from fibril formation to fibril dissolution had already taken place. Even after 60 min the pH of the reaction mixture had dropped to only 4·55, a value at which fibril formation was not inhibited in control samples. Thus, it would appear that the inhibition of fibril formation by HN2 is not primarily due to hydrogen ions, but to one of the other hydrolysis products of HN2. It appears possible, however, that acid liberated by HN2 hydrolysis plays a role in the final phase of fibril dissolution. The fact that the effect of HN2 can be abolished by subsequent dialysis suggests that this effect may be one of nonspecific competition for ionic polymerization sites on the collagen molecule rather than one of covalent bond formation.

It appears desirable to find out experimentally which of the hydrolysis products of HN2 prevent collagen fibril formation. However, none of these products, with the exception of *N,N-bis*(2-hydroxyethyl)methylamine, is easily available. The latter

compound was qualitatively tested for its effect on fibril formation by rabbit skin collagen. At a concentration of 0·63 mg/ml, it failed to prevent formation of a solid collagen gel.

Various alkylating agents have long been used in the treatment of neoplastic diseases.[23] Since 1951, these drugs have also been applied on an experimental basis and with variable success to the treatment of connective tissue diseases such as rheumatoid arthritis.[24–28] The rationale for the use of alkylating agents in rheumatoid arthritis appears to be connected with their potential immunosuppressive effect. The possibility of a direct chemical action of HN2 on connective tissue constituents has been considered here. Such a possibility appears particularly likely in the case of intra-articular administration of the drug where high initial concentrations, of the order of magnitude tested here, occur. It is interesting to note that Peacock[29] suggested that joint stiffness in rheumatoid arthritis may be related to deposition of new collagen. If this is so, an agent such as HN2 capable of depolymerizing collagen might be beneficial. While the relatively nonspecific nature of the action of HN2, which does not appear to involve alkylation, would make any beneficial effect short-lived, it would possibly also tend to minimize competition from reactive alkylation sites on other tissue constituents

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