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EFFECT OF NITROGEN MUSTARD ON HYALURONATE*

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Abstract—The effect *in vitro* of nitrogen mustard (HN2) treatment on physical parameters of partially purified hyaluronate from human umbilical cords and of synovial fluid from patients with rheumatoid arthritis was studied.

Intrinsic viscosities, gel filtration elution patterns (Sepharose 2B) and acrylamide gel disk electrophoresis runs on HN2-treated (1 mg/ml HN2, 0·9% saline, 8 hr at 37°) and control hyaluronate were obtained. The intrinsic viscosity was reduced by 10 to 50 per cent and a typical gel filtration elution pattern showed retardation by about 10 per cent after HN2 treatment. No gross difference in disk electrophoretic pattern was noted. Similar intrinsic viscosity changes were observed after HN2 treatment of rheumatoid synovial fluids.

VARIOUS alkylating agents have long been used in the treatment of neoplastic diseases (Hall).[1] Since 1951, these drugs have also been applied on an experimental basis to the treatment of connective tissue diseases such as rheumatoid arthritis.[2–5] The rationale for the use of alkylating agents in rheumatoid arthritis appears to be connected with their potential immunosuppressive effect. However, a direct chemical action on connective tissue constituents appears possible in the case of intra-articular administration of the drugs. The effects of nitrogen mustard† on soluble collagen have been described in a previous paper.[6] The present communication describes experiments *in vitro* designed to study the direct chemical effect of nitrogen mustard on hyaluronate. A few experiments with the alkylating agent Thio-Tepa are also included because of the clinical interest of our department[5] in that agent.

MATERIALS AND METHODS

Hyaluronic acid was isolated from human umbilical cords by a modification of the method of Danishefsky and Bella[7] in which adsorption on Fuller's earth (Matsumura *et al.*[8]) was substituted for Dowex-1 chromatography. Hyaluronic acid was lyophilized and stored at 4°. It had an intrinsic viscosity of 32 dl/g and a protein content of 6%. Human synovial fluid was obtained from the knee joints of patients with rheumatoid arthritis. Prior to use it was subjected to centrifugation (30,000 *g*, 30 min) to remove tissue fragments and leukocytes. The nitrogen mustard preparation used (Mustargen R, Merck, Sharp & Dohme) contained sodium chloride in addition to methyl-*bis*-(2-chloroethyl)amine hydrochloride. Concentrations listed are those of the hydro-

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†The following abbreviations are used: HN2, nitrogen mustard (methyl-*bis*[2-chloroethyl]amine); $\eta_{sp.}$, specific viscosity; $[\eta]$, intrinsic viscosity.

chloride. Thio-Tepa (Lederle), *N*, *N'*, *N''* triethylenethiophosphoramide, a related alkylating agent, was used in one experiment.

Treatment of hyaluronic acid with HN2 or Thio-Tepa was performed as follows: One vol. of a freshly prepared HN2 solution (2 mg HN2/ml in 1·8% sodium chloride) or Thio-Tepa solution (2 mg/ml) was added to 1 vol. of hyaluronate in water (approximately 2 mg/ml) and the mixture was allowed to react at 37°. An aliquot was placed into a viscometer at the same temperature and flow-times were recorded periodically. A hyaluronate solution to which an equal volume of 1·8% sodium chloride had been added was used as a control. After a reaction time of 500 min, the samples were dialyzed at 4° for 24 hr against 50 vol. of water, then against several changes of 0·1 M sodium phosphate, pH 7·5. In order to quantitate the dialyzable hyaluronate fragments, the water dialysate was evaporated to dryness, hydrolyzed, and subjected to hexosamine assay by the Elson–Morgan reaction according to Blix.[9] Hexosamine assay was also used to determine the concentration of the dialyzed hyaluronate samples. These were then volumetrically diluted with 0·1 M sodium phosphate, pH 7·5, 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 locally according to Schachman.[10] Solution densities of buffer and of the most concentrated hyaluronate solution were determined in a 1 ml pycnometer. Densities of the intermediate dilutions were estimated by interpolation. Duplicate intrinsic viscosity determinations on the same sample usually fell within a ± 1 dl/g range of each other and differences of 2 dl/g and up between treated and control samples therefore probably represent a significant change. Synovial fluid was reacted with HN2 under the same conditions. Its intrinsic viscosity was based on hyaluronate content as determined by uronic acid assay (Dische[11]). This procedure is based on the assumptions that the viscosity of synovial fluid is entirely due to the presence of hyaluronate[12] and that no uronic acid-containing substances other than hyaluronate are present in synovial fluid. These assumptions are undoubtedly only approximately correct, but errors resulting from these assumptions would presumably affect treated and control samples alike.

Gel filtration of hyaluronate solutions was performed essentially as described by Barker and Young[14] on a 2·5 × 32 cm column of Sepharose 2B (Pharmacia, Uppsala, Sweden). Phosphate buffer (pH 7·5, 0·1 M) was used as an eluant. The flow rate was 15 ml per hr and 3-ml fractions were collected. The position of the elution peaks was established by analyzing aliquots of the fractions for uronic acid.[11] A partially depolymerized hyaluronate control for the gel filtration experiments was obtained as follows: A 1 mg/ml solution of hyaluronate in phosphate buffer was subjected to ultrasound in an experimental setup previously described in detail.[14] Exposure to ultrasonic vibraions of about 5 sec duration (a time derived by trial and error) yielded a hyaluronate with an intrinsic viscosity of 25 dl/g, which was considered suitable as a depolymerized control. Acrylamide gel disk electrophoresis using Tris glycine buffer, pH 8·3, and a current of 6 mA per gel for 30 min was applied in an attempt to separate new ionic species resulting from HN2 treatment of hyaluronate. Alcian blue was used to reveal the position of the bands.

RESULTS

The effect of HN2 and Thio-Tepa on the specific viscosity of hyaluronate solutions

at 37° is shown in Fig. 1. The specific viscosity of a control hyaluronate solution decreased only slightly as a result of prolonged incubation at 37°. A similar slight decrease in specific viscosity was observed in a hyaluronate solution containing 1 mg/ml of Thio-Tepa. Hyaluronate solution containing 1 mg/ml HN2 showed an initial rapid drop in specific viscosity which, over a period of a few hours, slowed down to a rate only slightly larger than that of control hyaluronate. When the HN2-treated hyaluronate solution was stored at 4° overnight and fresh HN2 was added (1 mg/ml), a drop in specific viscosity was noted comparable to the one initially observed. The fact that

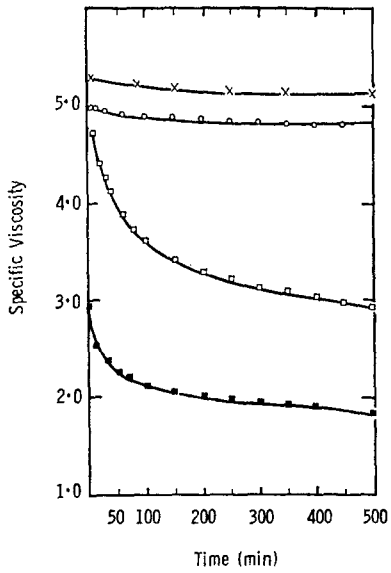

FIG. 1. Effect of HN2 and Thio-Tepa on the specific viscosity of hyaluronate solutions at 37°. Hyaluronate concentration approximately 0·8 mg/ml. ○, Thio-Tepa 1 mg/ml, 0·9% sodium chloride. ×, Control, 0·9% sodium chloride. □, HN2, 1 mg/ml, 0·9% sodium chloride. ■, HN2 twice treated (see text, final concentration, HN2 2 mg/ml, 1·8% sodium chloride).

a rapid drop in specific viscosity set in once again was interpreted as meaning that the reaction has slowed down for lack of reactive HN2 rather than for lack of reactive sites on the hyaluronate.

Typical extrapolation plots to determine the intrinsic viscosities of treated and control hyaluronate solutions are shown in Fig. 2. The intrinsic viscosity of Thio-Tepa-treated hyaluronate was found to be somewhat higher (32·7 dl/g) than that of the untreated control (30·9 dl/g) but the difference may not be significant. The intrinsic viscosity of HN2-treated hyaluronate, on the other hand, was found to be significantly decreased (21·9 dl/g), and that of hyaluronate treated twice with separate 1 mg/ml doses of HN2 was further decreased (17·9 dl/g). Variations from one batch to the next were marked and intrinsic viscosities ranging from 28 to 15 dl/g (representing decreases of 10 –50 per cent from the control) were found after a single 500-min exposure to HN2 under standard conditions. The variability of the system is undoubtedly related to the fact, discussed below, that HN2 treatment apparently breaks only

very few bonds in the hyaluronate molecule. The location of the bonds broken therefore plays a critical role in determining the intrinsic viscosity of the product.

Analytical data on the initial dialysates of various treated and control samples of hyaluronate are listed in Table 1. The amount of hyaluronate recovered in the first dialysate, as well as the percentage of the total sample which this amount represents, is recorded. The amounts dialyzed were in most cases too small to be assayed with any

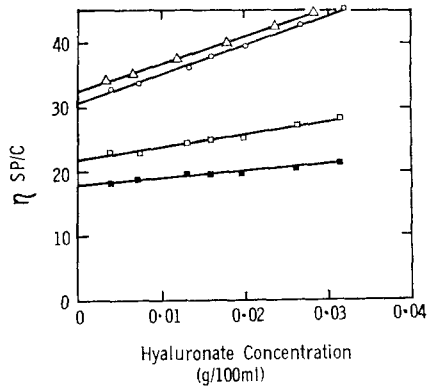

FIG. 2. Effect of HN2 and Thio-Tepa on the intrinsic viscosity of hyaluronate at 25°. All samples in 0·1 sodium phosphate buffer, pH 7·5. △, Hyaluronate, Thio-Tepa treated (1 mg/ml). ○, Control hyaluronate. □, Hyaluronate, HN2 treated (1 mg/ml). ■, Hyaluronate, twice HN2 treated (see text).

TABLE 1. DIALYZABLE FRACTIONS OF HN2 OR THIO-TEPA-TREATED AND CONTROL HYALURONATE

		Dialyzable material	
Material	Treatment	Amount* (μg)	% of Total†
Hyaluronate	Control	2·7	0·04
Hyaluronate	HN2	14·2	0·19
Hyaluronate	2 × HN2	20·9	0·28
Hyaluronate	Thio-Tepa	30·7	0·42

* Amount of hyaluronate based on hexosamine assay.

† $\frac{\text{Dialyzable material}}{\text{Dialyzable + non dialyzable material}} \times 100.$

precision. Nevertheless, it is apparent that HN2 treatment, as well as Thio-Tepa treatment, increased the amounts of dialyzable materials substantially. Viewed as a percentage of the total sample however, the dialyzable fragments always represented less than 0·5 per cent.

The elution patterns of control and HN2-treated hyaluronate from a Sepharose 2B gel filtration column are presented in Fig. 3. The ordinate represents the per cent of the total uronic acid (as O.D.$_{530}$) eluted in each fraction. This presentation eliminates differences due to variations in sample size and facilitates comparison. Treatment with HN2 did not change the volume at which hyaluronate started to elute, but the peak concentration occurred 3 tubes later than in the control sample. A similar

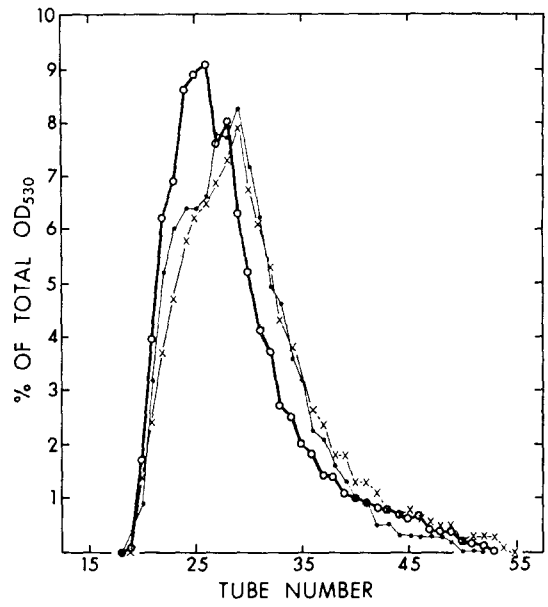

FIG. 3. Elution pattern of HN2-treated and control hyaluronate from a Sepharose 2B gel filtration column. The ordinate represents the per cent of the total uronic acid (as O.D. 530) eluted in each fraction. See text for details. ○, Control hyaluronate, $[\eta]$ = 31 dl/g. ×, HN2-treated hyaluronate $[\eta]$ = 23 dl/g. ●, Ultrasonically depolymerized control hyaluronate $[\eta]$ = 25 dl/g.

slight shift in peak position was observed during elution of hyaluronate which had been treated with ultrasound to obtain a preparation of an intrinsic viscosity comparable to the one of the HN2-treated sample.

Acrylamide gel disk electrophoresis was used in an attempt at demonstrating the presence of new ionic species resulting from HN2 treatment of hyaluronate. Untreated hyaluronate showed two bands on electrophoresis, which presumably indicated the presence of a contaminating polysaccharide in the preparation used. However, neither the HN2-treated sample nor the mixture of control and treated samples showed any new bands or any broadening or fading of the existing bands.

The results of intrinsic viscosity determinations on seven rheumatoid synovial fluids treated with HN2 and on corresponding control fluids are listed in Table 2. These were obtained from extrapolation plots of η_{sp}/c vs. concentration at 5 concentrations. Good fits on linear extrapolation plots were obtained in all but one case. The values from the control synovial fluid of patient W. D. showed a curvilinear relationship of η_{sp}/c vs. concentration. Replotting on a logarithmic scale did not entirely remedy the situation, and the value listed for the intrinsic viscosity of this synovial fluid must be considered as only a rough approximation since it was obtained by curvilinear extrapolation. Also listed in Table 2 are calculated values of the mean molecular weights of the hyaluronate in treated and control synovial fluids as well as the ratio of these two values. Molecular weights were obtained by applying the empirical equation proposed by Laurent *et al.*[15] which relates intrinsic viscosity and molecular weight of hyaluronate. These values are obviously affected by the same

TABLE 2. EFFECT *IN VITRO* OF NITROGEN MUSTARD ON THE HYALURONATE IN SYNOVIAL FLUIDS FROM PATIENTS WITH RHEUMATOID ARTHRITIS

	Intrinsic viscosity*		Molecular weight† ($\times 10^6$)		
Patient	Control	HN2-treated	Control	HN2-treated	Ratio ‡
P. K.	69·7	54·4	6·0	4·4	1·4
W. D.	58·1	45·1	4·8	3·4	1·4
A. D.	47·9	41·9	3·7	3·1	1·2
G. M.	42·3	30·4	3·2	2·1	1·5
I. G.	37·1	31·1	2·7	2·1	1·3
L. D.	23·2	20·4	1·5	1·2	1·2
H. S.	23·1	19·9	1·5	1·2	1·2

* Intrinsic viscosity of hyaluronate in dl/g; hyaluronate concentration based on uronic acid assay of synovial fluid.

† Calculated from the intrinsic viscosity by means of Laurent's[41] empirical relation $[\eta] = 0{\cdot}036 \times M^{0{\cdot}78}$.

‡ Ratio control/treated molecular weights.

uncertainties as the intrinsic viscosity values and are indeed somewhat higher than most values reported in the literature, but they illustrate the extent of the depolymerization of hyaluronate by HN2 better than the viscosity data.

DISCUSSION

The potential usefulness of alkylating agents in the treatment of the rheumatic diseases is usually assumed to be associated with immunosuppression. Nevertheless, a direct chemical action also appears possible, particularly in the case where alkylating agents are injected directly into the joint cavity and, therefore, get into immediate contact with connective tissue constituents such as hyaluronate. Obviously, the significance of the present studies *in vitro* would be enhanced if it could be shown that the conditions of the reaction *in vitro* are similar to those encountered in a joint. These conditions are much too complex to be simulated *in vitro*. Nevertheless, the joint space is a relatively isolated area, and excess joint fluid is usually aspirated prior to injection of HN2 so that the injected HN2 solution should, initially, not be diluted considerably. The present study was done on the basis of the undiluted medication. The most serious difficulty in trying to extrapolate work *in vitro* with a highly reactive substance such as HN2 to the situation *in vivo* is the problem of competition of unrelated nucleophilic substances for the available HN2. There is no entirely satisfactory way of handling this problem. Nevertheless, it would appear that the study of the effect of HN2 on the hyaluronate in synovial fluid might approximate physiological conditions with respect to competing substances.

HN2 has long been recognized as an extremely reactive substance capable of reacting with nucleic acids, proteins, etc.[16,17] The studies of Ross[18] on the relative reactivity of the amino acid residues in proteins indicate that around neutrality the —COOH groups of aspartic and glutamic acids are highly subject to reaction with HN2. The —COOH groups of hyaluronic acid, which have similar dissociation characteristics, might be expected to react readily with HN2 as well. Alkylation of —COOH groups of hyaluronate would be expected to lead to different ionic species with altered electrophoretic mobilities. Since alkylation is likely to be a random process, it would probably lead to an electrophoretically heterogeneous mixture. Acrylamide

gel disk electrophoresis, a sensitive although only qualitative method, was used in an attempt to obtain evidence of alkylation of the —COOH groups of hyaluronate after HN2 treatment. No band broadening was observed, which would have been evidence of alkylation. Consequently, it was concluded that no massive alkylation of —COOH groups had taken place although, of course, alkylation of a small number of groups cannot be excluded by this method.

The most obvious result of the treatment of hyaluronate with HN2 reported here is a drop in intrinsic viscosity. This lowering of the intrinsic viscosity can be most simply explained on the basis of depolymerization of the hyaluronate. However, since HN2 is a bifunctional reagent, thus potentially capable of crosslink formation, other possible explanations cannot be ruled out *a priori*. It appeared desirable, therefore, to confirm the viscosity data by an independent measure of the size of the hyaluronate molecule, namely gel filtration on Sepharose 2B. The data obtained showed a retardation of about 10 per cent of the hyaluronate elution peak after HN2 treatment compared with that of control hyaluronate, and no detectable difference in peak positions between a hyaluronate sample which had been mechanically depolymerized with ultrasound and a HN2-treated sample of comparable intrinsic viscosity. These findings are consistent with a depolymerizing action of HN2 on hyaluronate. Depolymerization would be expected to eventually lead to dialyzable fragments. This was found to be true, although even after treating hyaluronate twice with HN2, less than 0·5 per cent of the hyaluronate became dialyzable, an amount which is probably not significant. A similar amount of dialyzable hyaluronate was produced by treatment with Thio-Tepa even though this treatment did not lead to a decreased intrinsic viscosity.

HN2 in aqueous solution is known to undergo a series of hydrolytic reactions which result in the liberation of hydrogen ions among other things. This results in a rapid decrease in pH of the hyaluronate–HN2 reaction mixture to 4·2 followed by a further slow decrease to 3·5. Since it was intended to study the effect of HN2 on isolated hyaluronate in the absence of competing nucleophilic centers, the use of buffers appeared undesirable. Consequently it became important to rule out the possibility that ordinary acid hydrolysis might be reponsible for the observed viscosity changes. Exposure of hyaluronate to pH 3·35 for 500 min under standard conditions yielded a product with an intrinsic viscosity of 29·3 dl/g, a value not significantly different from that of the control. Acid hydrolysis therefore does not appear to be a significant factor.

In order to study the effect of HN2 on hyaluronate under more nearly physiological conditions, seven rheumatoid synovial fluids were subjected to HN2 treatment. Again, the main effect was a reduction in intrinsic viscosity of the hyaluronate. This effect was most marked in the more viscous synovial fluids. However, calculation of the approximate molecular weights of the hyaluronate by means of the empirical formula of Laurent *et al.*[15] revealed that the result of HN2 treatment is, on the average, somewhat less than one break per hyaluronate molecule over the whole range of viscosities studied. Similarly calculated ratios of control to treated molecular weights in the isolated hyaluronate system fell into the range from 1·2 to 2·5. Thus, HN2 may be somewhat less effective in synovial fluid than in the isolated hyaluronate preparation, but the difference is not substantial.

The fact that hyaluronate is easily depolymerized by such agents as ascorbic acid, thiols or metallic ions has long been known. These reactions have recently been

investigated by Herp *et al.*[19] All of these depolymerizing agents are believed to react by formation of free radicals from water. It is conceivable that HN2 might act similarly.

The hyaluronate in rheumatoid synovial fluid is generally less polymerized than that from normal individuals[20,21] although there is considerable overlap of the size ranges. Thus, even though HN2 shows a definite chemical action hyaluronate, leading to less highly polymerized molecules, this reaction may not be of benefit to the patient.

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REFERENCES

1. T. C. HALL, *New England J. Med.* **226**, 129 (1962).
2. C. JIMENEZ DIAZ, E. LOPEZ GARCIA, A. MERCHANTE, and J. PERIANES, *J. Am. med. Ass.* **147**, 1418 (1951).
3. N. M. SHUTKIN, *J. Bone. Jt Surg.* **33A**, 265 (1951).
4. A. L. SCHERBEL, *Cleveland. Clin. Q.* **24**, 71 (1957).
5. J. ZUCKNER, J. UDDIN, R. H. RAMSEY, A. M. AHERN, G. E. GANTNER, JR. and R. W. DORNER *Ann. rheum. Dis.* **25**, 178 (1966).
6. R. W. DORNER, and J. UDDIN, *Biochem. Pharmac.*, **17**, 975 (1968).
7. I. DANISHEFSKY, and A. BELLA, *J. biol. Chem.* **241**, 143 (1966).
8. G. MATSUMURA, M. DE SALEGUI, A. HERP and W. PIGMAN, *Biochim. biophys. Acta* **69**, 574 (1963)
9. G. BLIX, *Acta Chem. Scand.* **2**, 467 (1948).
10. H. K. SCHACHMAN, *Meth. Enzym.* **4**, 32 (1957).
11. Z. DISCHE, *J. biol. Chem.* **167**, 189 (1947).
12. K. MEYER, *Physiol. Rev.* **27**, 335 (1947).
13. S. A. BARKER and N. M. YOUNG, *Carbohydrate Res.* **2**, 363 (1966).
14. R. W. DORNER, G. E. GANTNER JR., J. UDDIN and J. ZUCKNER, *Arthritis Rheum.* **8**, 368 (1965).
15. T. C. LAURENT, M. RYAN and A. PETRUSZKIEWICZ, *Biochim. biophys. Acta* **42**, 476 (1960).
16. K. A. STACEY, M. COBB, S. F. COUSENS and P. ALEXANDER, *Ann N.Y. Acad. Sci.* **68**, 682 (1958).
17. K. W. KOHN, C. L. SPEARS and P. DOTY, *J. Molec. Biol.* **19**, 266 (1966).
18. W. C. ROSS, *Ann. N. Y. Acad. Sci.* **68**, 669 (1958).
19. A. HERP, T. RICKARDS, G. MATSUMURA, L. B. JAKOSALEM and W. PIGMAN, *Carbohydrate Res.* **4**, 63 (1967).
20. C. W. CASTOR, R. K. PRICE, and M. J. HAZELTON, *Arthritis Rheum.* **9**, 783 (1966).
21. E. A. BALAZS, D. WATSON, I. F. DUFF and S. ROSEMAN, *Arthritis Rheum.* **10**, 357 (1967).