# A QUANTITATIVE STUDY OF METACHROMASY IN SYNOVIAL FLUID AND MUCIN*

BY DAVID J. HAMERMAN‡ AND MAXWELL SCHUBERT

*(From the Department of Chemistry and the Study Group on Rheumatic Diseases, New York University College of Medicine, New York)*

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To localize the mucopolysaccharides in the ground substance of connective tissue, histologists have sometimes stained fixed tissue preparations with toluidine blue and noted the sites in which the dye has taken on the reddish hue characteristic of metachromasy (1, 2). It is generally agreed that high molecular weight polysaccharides which are half-sulfate esters stain metachromatically (2–5), but there is disagreement whether hyaluronate, when free of polysaccharide sulfate esters, does so.

Most workers agree that synovial fluid is a component of the ground substance of synovial tissue (6, 7). Synovial fluid contains hyaluronate (8) in a fluid medium which is thought to be a dialysate of the plasma (6). In studies employing toluidine blue, the synovial membrane and synovial fluid stained metachromatically (2, 7, 9). This reaction was destroyed by prior treatment with hyaluronidase (2). Sylvén and Malmgren (10) using azure A noted that whole, native synovial fluid appeared weakly metachromatic, and when dried and fixed, metachromasy became more pronounced.

Chemical characterization of fixed tissues by the use of special stains is subject to many pitfalls (11). Dempsey (12) has pointed out that precipitation and dehydration of the ground substance occur during fixation prior to staining. The interpretation of metachromatic staining may be particularly difficult and prompted Gersh and Catchpole (13) to use the periodate-fuchsin technique in their study of ground substance. Quantitative study of metachromasy in tissues has been impossible because of the heterogeneous nature of the material used in which concentrations of dye, chromotrope, and other components could not be controlled, and interpretations of staining have depended on visual descriptions of color.

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‡ Aided by a fellowship from the Dazian Foundation for Medical Research.

Present address: Captain, Medical Corps, Medical Nutrition Laboratory, 1819 West Pershing Road, Chicago.

Synovial fluid offers an unusual opportunity for making a study of the effect of hyaluronate in a native state on a metachromatic dye. It is a biological fluid which can be obtained as a clear solution without chemical manipulation. Related sulfated polysaccharides cannot be present in more than small amounts (3). Quantitative measurements can be made of the effect of synovial fluid on the different absorption bands of any metachromatic dye in solutions whose components are at known concentrations. Systematic variation of any of the components of the solution is possible. Furthermore the metachromatic properties found for the whole synovial fluid can be compared with those found for the mucin and the hyaluronate derived from the same synovial fluid. Such a study is here presented. The dye chosen for the study is methylene blue because it can be obtained in a purer condition than many other metachromatic dyes, and because the $\alpha$, $\beta$, and $\mu$ bands (14) which are affected by chromotropes are more sharply defined than is the case with toluidine blue.

## *Materials and Methods*

*Synovial Fluid and Derivatives.—*

1. Synovial fluid was obtained[1] from the astragalotibial joints of freshly slaughtered cattle and was clarified by filtering once or twice through glass wool in the cold. The fluid was stored at 5°C. with small amounts of thymol added as a preservative. Portions of synovial fluid that were to be used for metachromatic studies were dialyzed against several changes of distilled water for 3 or 4 days in the cold. Dialysis was carried out to remove salts which are capable of inhibiting metachromasy (14). This procedure does not seem to have been carried out in any previous studies before staining synovial fluid.

Dialyzed synovial fluid of different batches contained from 0.30 to 0.26 mg. per ml. of hexosamine and a total protein of about 10 mg. per ml. The proportion of the total hexosamine of synovial fluid that is hyaluronate-hexosamine has been reported as 40 per cent (8) and 49 per cent (15). Aliquots of various batches of synovial fluid were removed and the hexosamine in the mucin clot formed was determined. This was found to be 51, 53, and 50% of the total hexosamine. Analysis for hexosamine in the supernatant fluid in one experiment gave a figure of 0.13 mg. per ml., which accounted for all the hexosamine not precipitated as mucin. Addition of egg albumin and acetic acid to the supernatant after mucin precipitation, resulted in no further mucin clot, indicating that the remaining hexosamine was not part of an anionic mucopolysaccharide. In calculating the hyaluronate content of synovial fluid, therefore, only half of the total hexosamine was considered to be hyaluronate hexosamine. The hexosamine was taken to be 30 per cent of the hyaluronate molecule, and the period weight of the hyaluronate as 450. Concentrations of hyaluronate are expressed as milliperiods or mper. per ml.

2. Mucin was prepared in the cold according to a method previously described

[1] We wish to thank Armour and Co. for their generous cooperation in providing the synovial fluid.

(16). The mucin clots from 100 to 150 ml. of synovial fluid were washed and dissolved in small volumes (20 to 30 ml.) of 0.05 M $Na_2HPO_4$. These mucin solutions were dialyzed for 2 or 3 days at 5°C. against several changes of distilled water, and were then used for analysis and metachromatic studies. If the mucin was to be reprecipitated with acetic acid, dialysis was omitted.

3. Hyaluronate was prepared from mucin (16) and had a glucosamine content of 27 per cent and a N of 4.6 per cent. Hyaluronate was also prepared by modifying a method used by Ogston and Stanier (17) in which the polysaccharide was precipitated by alcohol from synovial fluid concentrated and made alkaline. Purification involved reprecipitation and shaking with amyl alcohol and chloroform to remove protein. Comparative studies were also made with sulfur-free hyaluronate from human umbilical cords obtained by methods to be described elsewhere.

*Methylene Blue.*—

Commercial preparations were recrystallized three times from hot water. Stock solutions were made up by dissolving 25 mg. of dye in 250 ml. of water. A final concentration of $1.24 \times 10^{-5}$ mM per ml. was maintained in all solutions whose optical density was to be measured.

Most of the studies of synovial fluid were made with 1 ml. of the fluid and 0.4 ml. of the dye stock diluted to 10. This solution contained about $1.0 \times 10^{-4}$ mper. per ml. of hyaluronate and 1 mg. per ml. of protein. In a similar fashion an amount of mucin solution was taken to give an equivalent concentration of hyaluronate, 0.4 ml. of dye was added, and the mixture diluted to 10.

*Enzymes.*—

1. To 30 ml. of dialyzed synovial fluid, 6 ml. of 0.1 M phosphate buffer at pH 7.5 was added. This buffered synovial fluid was divided into two parts. Trypsin, 0.83 mg. per ml., was added to one part and both were incubated at 37°C. for 26 hours. The control and trypsin-treated fluids were then separately dialyzed against several changes of distilled water for 48 hours. Samples were removed for analyses and metachromatic studies. Ability to form a mucin clot persisted in both fluids.

2. To about 13 ml. of each of the dialyzed control and trypsin-treated synovial fluids, 2 ml. of 0.1 M phosphate buffer at pH 7.2, was added. To the trypsin-treated synovial fluid, collagenase,[2] 0.7 mg. per ml., was added, and both fluids were incubated at 38°C. for 24 hours. The solutions were again separately dialyzed, and were used for analyses and metachromatic studies. The ability to form a mucin clot persisted, although the clot was considerably reduced in amount in the trypsin- and collagenase-treated fluid. To be certain that the collagenase preparation did not attack the polysaccharide, hyaluronate was incubated with the enzyme for 36 hours. No reduction in viscosity occurred. When protein and acetic acid were added to this hyaluronate, a mucin was precipitated.

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[2] Collagenase from *Cl. histolyticum* was obtained through the courtesy of Dr. J. D. MacLennan of the College of Physicians and Surgeons, Columbia University. The enzyme was free of hyaluronidase, but contained a protease with a proteolytic spectrum similar to that of trypsin.

Parallel with the above experiments, viscosity measurements were carried out on identical solutions.

*Determination of Metachromasy.—*

The optical densities of the dye in the presence of the synovial fluid or its derivatives were measured with a Beckman spectrophotometer using 1 cm. cells. Corrections were made for the optical densities of synovial fluid or mucin in water due to opalescence, but were negligible. In conformity with previous reports (14), the optical densities were read at 570 mμ (μ band), 610 mμ (β band), and 665 mμ (α band). The molar extinction coefficient (ε) was calculated by dividing the optical density by the concentration of the dye ($1.24 \times 10^{-5}$).

As criteria for metachromasy we have followed the principles set forth before (14). The changes in optical density of all three bands have been taken into account, and only that substance is considered to be metachromatic which simultaneously causes an elevation of the μ band and a depression of the α and β bands. The rise of the μ band should exceed 30 per cent, and with strong chromotropes will exceed 65 per cent. The α and β bands should fall at least 30 per cent, and in intense metachromasy, the fall of the α band may exceed 50 per cent.

Sylvén and Malmgren (10) consider the γ band (which, following Michaelis (18), we have termed the μ band) to be the chief indication of metachromasy. This is reasonable since the μ band does not appear unless chromotropes are present. However, appearance of the μ band is always associated with a fall in the α and β bands. A single number which combines the changes of the α and μ bands can be used as a measure of metachromasy (19) by calculating the ratio of ε at 570 mμ to ε at 665 mμ. For the dye alone this ratio, *R*, is 0.17, but presence of chromotropes will cause it to rise to values four to five times greater.

A spot test was useful as a visual guide to the chromotropic properties of a substance. A small amount of the solid, or 2 or 3 drops of the solution to be tested, was placed on a spot plate, and 1 or 2 drops of water and 2 drops of dye added. A control with water and dye was always run.

*Analytic Methods.—*

Nitrogen was determined by the Kjeldahl method; glucosamine by the method of Schloss (20). Viscosities were determined in an Ostwald viscometer immersed in a constant temperature bath at 38°C. Flow times for water were about 10 seconds.

## RESULTS

*Metachromasy of Synovial Fluid.—*

*1. Effect of Added Hyaluronate.*—Neither the whole native synovial fluid nor dialyzed synovial fluid in dilutions of 1:10, 1:25, 1:50, and 1:100 produced metachromasy with methylene blue. Over this range the hyaluronate concentration was $1 \times 10^{-4}$ to $1 \times 10^{-6}$ mper. per ml., and simultaneously the protein concentration was 0.1 to 0.01 mg. per ml. In no case was there any significant effect on α, β, or μ bands. These and subsequent results are summarized in Table I.

To determine whether the milieu of the whole dialyzed synovial fluid was in itself "antimetachromatic," sulfur-free hyaluronate from umbilical cord, or hyaluronate from synovial fluid was added to such fluid to give a total final concentration of $2 \times 10^{-4}$ mper. per ml., including the hyaluronate naturally present. Metachromasy was produced by free added hyaluronate in the environment of whole synovial fluid, the $R$ value rising to twice that of the control fluid.

TABLE I

*Summary of Extinction Values of Methylene Blue in Synovial Fluid, Mucin, and Hyaluronate*

| Solution | Hyaluronate concentration | Protein concentration | $\epsilon \times 10^{-4}$ | | | $R = \frac{\epsilon 570}{\epsilon 665}$ |
|---|---|---|---|---|---|---|
| | | | 570 mμ | 610 mμ | 665 mμ | |
| | *mper. per ml.* $\times 10^4$ | *mg. per ml.* | | | | |
| Dye blank | 0 | 0 | 0.78 | 2.24 | 4.67 | 0.17 |
| Synovial fluid 1:10 | 1.0 | 1.0 | 0.81 | 2.46 | 4.79 | 0.17 |
| Synovial fluid 1:50 with added hyaluronate | 1.5 | 0.20 | 1.27 | 2.0 | 3.31 | 0.39 |
| Synovial fluid 1:10 + alkali | 1.0 | 1.0 | 1.03 | 2.08 | 3.56 | 0.29 |
| Synovial fluid + alkali after dialysis | 1.0 | 1.0 | 0.84 | 2.39 | 4.70 | 0.18 |
| Synovial fluid after treatment with proteolytic enzymes | 1.0 | 0.28 | 0.84 | 2.42 | 4.76 | 0.17 |
| Mucin, once precipitated | 1.0 | 0.20 | 1.36 | 1.62 | 2.28 | 0.60 |
| Mucin, twice precipitated | 1.0 | 0.15 | 1.32 | 1.59 | 2.19 | 0.60 |
| Sulfur-free hyaluronate (umbilical cord) | 1.0 | — | 1.39 | 1.66 | 2.46 | 0.56 |
| Hyaluronate (synovial fluid) | 1.3 | 0.007 | 1.40 | 1.53 | 2.12 | 0.66 |

*2. Effect of Alkali.*—The addition of alkali has been thought to dissociate hyaluronate from protein in mucin solutions (16). The addition of approximately 0.05 ml. of concentrated ammonium hydroxide to 1 ml. of synovial fluid diluted 1:10 resulted in metachromasy. The effect was reversible, and after dialysis, the fluid was no longer metachromatic.

*3. Effect of Proteins.*—Although hyaluronate concentration was optimum at a dilution of 1:10, the thought arose that the proteins of synovial fluid might inhibit metachromasy. A study was made of the effects produced by the addition of dialyzed egg albumin or gelatin to chondroitin sulfate, sulfur-free hyaluronate from umbilical cord, and hyaluronate from synovial fluid. The chromotrope and dye concentrations were kept constant, and increasing amounts of protein were added. Controls were run with similar concentrations of the protein and dye alone, and the rise in optical density above the dye blank noted. These values were subtracted from the corresponding optical densities of the test solutions. The results are shown in Table II. Both gelatin and albu-

min were effective in almost completely suppressing metachromasy at a concentration of 0.1 per cent, and gelatin abolished it completely at 1 per cent.

It is evident, therefore, that proteins present in synovial fluid at a concentration of 0.1 per cent are capable of almost completely suppressing metachromasy. To reduce this suppressive effect of the proteins in synovial fluid, proteolytic enzymes were used as the method least likely to alter the hyaluronate. Following this, metachromatic properties of the fluid were again investigated.

TABLE II

*The Effects of Added Gelatin and Albumin on the Metachromasy Produced by Chromotropes*

| Chromotrope | Gelatin | Albumin | $\epsilon \times 10^{-4}$ | | | $R$ |
|---|---|---|---|---|---|---|
| | | | 570 mμ | 610 mμ | 665 mμ | |
| | *mg. per ml.* | *mg. per ml.* | | | | |
| Chondroitin sulfate | 0 | 0 | 1.3 | 1.37 | 1.88 | 0.69 |
| | 0 | 0.1 | 1.23 | 1.54 | 2.33 | 0.52 |
| | 0 | 1.0 | 0.80 | 1.94 | 3.78 | 0.21 |
| | 0.1 | 0 | 1.31 | 1.50 | 2.22 | 0.59 |
| | 1.0 | 0 | 0.67 | 1.96 | 3.96 | 0.17 |
| | 10.0 | 0 | 0.75 | 2.22 | 4.70 | 0.16 |
| Sulfur-free hyaluronate (umbilical cord) | 0 | 0 | 1.39 | 1.66 | 2.46 | 0.56 |
| | 0 | 0.25 | 1.29 | 1.60 | 2.38 | 0.54 |
| | 0 | 0.50 | 1.19 | 1.69 | 2.80 | 0.42 |
| | 0 | 0.75 | 1.05 | 1.81 | 3.01 | 0.35 |
| | 0 | 1.0 | 0.90 | 1.83 | 3.48 | 0.26 |
| Mucin (once precipitated) containing 0.22 mg. per ml. of protein | 0 | 0 | 1.36 | 1.46 | 1.85 | 0.73 |
| | 0 | 0.3 | 1.23 | 1.51 | 2.29 | 0.54 |
| | 0 | 0.5 | 1.09 | 1.75 | 2.91 | 0.37 |
| | 0 | 1.0 | 0.72 | 2.04 | 3.96 | 0.18 |

Ammonium sulfate could not be used to remove the proteins, since it has been shown (16) that it precipitates hyaluronate along with the protein.

*4. Effect of Enzymes.*—By the use of trypsin and collagenase, and removal of the products of enzymatic action by dialysis, the protein content of whole synovial fluid was reduced to below a third of its original value. There was no significant reduction in the viscosity of enzymatically treated synovial fluid as compared to controls, indicating little alteration in the "native state" of the hyaluronate in synovial fluid (Table III). Heating synovial fluid to 75°C. for 3 minutes to denature the proteins did not increase the subsequent degree of proteolysis by the enzymes. Apparent decrease in milligrams of N per milliliter of control synovial fluid is due to dilution with buffer and dialysis.

The resulting synovial fluid, with hyaluronate concentration at 1.0 to 1.4 $\times$ $10^{-4}$, and protein reduced to a point where almost no interference would be expected, still failed to show metachromasy, as shown in Table I.

*Metachromasy of Mucin.—*

Dialyzed solutions of mucin, whether derived from once or twice precipitated material, were intensely metachromatic. Table I shows examples. It is also

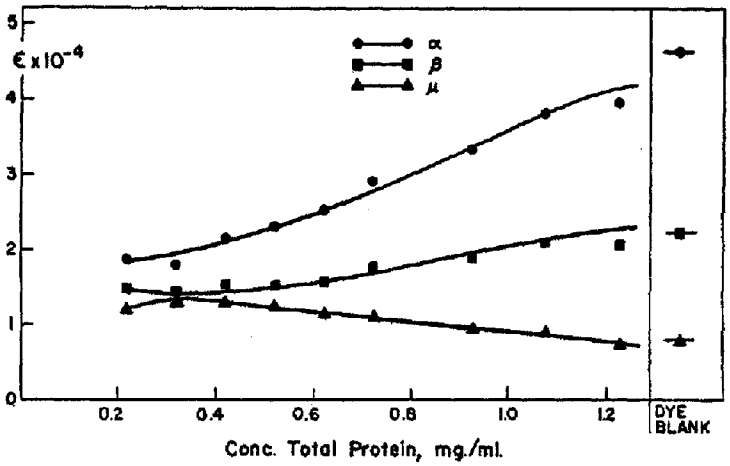


FIG. 1. The effects of progressive addition of albumin on the metachromasy of mucin solution.

TABLE III

*Effects of Proteolytic Enzymes on Synovial Fluid*

| | Time | Relative viscosity | Mucin clot | N | Maximum protein |
|---|---|---|---|---|---|
| | *hrs.* | | | *mg. per ml.* | *mg. per ml.* |
| Control synovial fluid for A | 0 | 2.4 | + | 1.6 | 10 |
| | 24 | 2.3 | + | 1.5 | 9.4 |
| A. Synovial fluid treated with trypsin | 0 | 2.3 | + | 1.6 | 10 |
| | 24 | 2.1 | + | 0.74 | 4.9 |
| Control synovial fluid for B | 0 | 2.0 | + | — | — |
| | 24 | 2.0 | + | 1.08 | 6.8 |
| B. Trypsin-treated synovial fluid further treated with collagenase | 0 | 1.8 | + | — | — |
| | 24 | 1.6 | ± | 0.36 | 2.4 |

evident that at optimum hyaluronate concentrations, the protein content of mucin solutions was considerably lower than that of native synovial fluid diluted 1:10, but not lower than that of protease-treated synovial fluid.

To test the effects of added protein on the metachromasy of mucin egg albumin was added in increasing amounts, as already described. The curves of Fig. 1 show the progressive return of the band heights of the originally metachro-

matic mucin solution to those of the dye alone. There was complete abolition of metachromasy at a protein concentration of 1.0 mg. per ml.

Addition of alkali can cause metachromasy to appear in mucin solutions whose metachromasy has been abolished by the addition of protein but has no effect on strongly metachromatic mucin solutions.

*Metachromasy of Hyaluronate.—*

Sodium hyaluronate prepared from synovial fluid was intensely metachromatic at a concentration of $1.3 \times 10^{-4}$. At similar hyaluronate concentrations, mucin and hyaluronate solutions show a like degree of metachromasy, as would be expected if the polysaccharide were "free" in both. Sulfur-free umbilical cord hyaluronate was compared with hyaluronate from synovial fluid, and their metachromatic effects were similar.

## DISCUSSION

This study has shown that synovial fluid is not metachromatic even when the protein content has been reduced to levels found in mucin solutions. This observation, together with the fact that metachromasy appeared when sulfur-free hyaluronate was added to synovial fluid, suggests that the native hyaluronate of synovial fluid may be in a form incapable of producing metachromasy. This form may be either a compound of the polysaccharide with protein or some other substances, or it may be a condition of the hyaluronate in which some part of the anionic groups is not free. The fact that metachromasy is evident in mucin and in hyaluronate derived from synovial fluid indicates that in their preparation some alteration of the hyaluronate molecule has occurred to make it metachromatic.

Some depolymerization of the polysaccharide may occur in the preparation of mucin, but this would not account for the appearance of metachromasy. It is chiefly high polymers that are metachromatic and there is evidence that the higher the state of polymerization the greater the degree of metachromasy. As would be expected, hyaluronate that has been depolymerized by hyaluronidase is not metachromatic (2).

Three views concerning the state of hyaluronate in synovial fluid are presented in Table IV. By ultrafiltration a hyaluronate-protein complex has been isolated from synovial fluid (15, 17) with a viscosity equal to that of the original starting material. Preparation of hyaluronate by means other than ultrafiltration has led to products of lowered viscosity (3, 16). The fact that Ogston and Stanier's product had a viscosity equal to that of the native material suggests that the combination of protein and polysaccharide is present in, and determines the properties of, the original synovial fluid.

The evidence of this study supports the view of other workers that hyaluronate is not free in synovial fluid. (1) Native synovial fluid, with a viscosity

higher than the mucin, or the hyaluronate derived from it, is not metachromatic. (2) Addition of alkali has been noted to "free" the hyaluronate and to produce a fall in viscosity (16). It also causes synovial fluid to become metachromatic. Thus the altered state of the hyaluronate is correlated with fall in viscosity and appearance of metachromasy. (3) Mucin produced from synovial fluid is degraded (17) and mucin solutions are intensely metachromatic.

There is a similarity in the conception of the "native state" of hyaluronate in synovial fluid and Gersh's (21) concept of "highly polymerized" ground substance. By his definition polymerization is meant to include "carbohydrate-protein complexes which are aggregated to form submicroscopic structures." By the use of histologic technique that includes freeze-drying of tissues, he

TABLE IV

*Summary of Views on the State of Hyaluronate*

| | Meyer | Ropes *et al.* | Ogston and Stanier |
|---|---|---|---|
| Synovial fluid | Hyaluronate and protein | Hyaluronate-protein and protein | Hyaluronate-protein and protein |
| Mucin solutions | Anionic hyaluronate + Cationic protein | Hyaluronate-protein (50 per cent protein) | Anionic hyaluronate + Cationic protein |
| Ultrafiltered hyaluronate complex | — | — | Hyaluronate-protein (25 per cent protein) |

finds that "highly polymerized" ground substance shows little or no metachromasy with toluidine blue.

Metachromasy has sometimes been exclusively ascribed to polysaccharide sulfate esters (4). More recently, Sylvén and Malmgren (10) found that dried films of sulfur-free umbilical cord hyaluronate, stained with azure A, were metachromatic, but in dilute solutions, a metachromatic color failed to appear. Meyer (3) stated that 1 per cent solutions of vitreous hyaluronate stained metachromatically with toluidine blue. He further concluded that in tissues, hyaluronate is never at sufficient concentration to produce metachromasy (22).

Under the experimental conditions described here sulfur-free hyaluronate from umbilical cord and from synovial fluid produced metachromasy at a concentration of 0.005 per cent.

It must be emphasized that the conclusions to be drawn from this study cannot be directly compared with histologic work. Metachromasy has been studied in dilute salt-free solutions with much lower concentrations of dye, chromotrope, and protein than are usual in fixed tissue preparations.

## CONCLUSIONS

Spectrophotometric measurements on synovial fluid and solutions of mucin and hyaluronate in the presence of methylene blue showed that:

1. Dialyzed synovial fluid was not metachromatic.
2. Albumin and gelatin at a concentration of 1 mg. per ml. inhibited the metachromasy of strong chromotropes.
3. Reduction of the protein of synovial fluid by the use of proteolytic enzymes still did not make the synovial fluid chromotropic.
4. Mucin solutions, with a protein content equal to that of protease-treated synovial fluid, were intensely metachromatic.
5. Sulfur-free hyaluronate produced intense metachromasy.

The evidence presented indicates that in its native state in synovial fluid hyaluronate is either bound or its anionic groups are not entirely free.

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