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SOME FACTORS AFFECTING THE INTERACTION OF HYALURONIC ACID WITH BOVINE-PLASMA ALBUMIN

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SUMMARY

In veronal buffer at pH 8.6, hyaluronic acid and bovine plasma albumin may form a complex which is evident in electrophoretic patterns as a peak intermediate between those for the components. This peak is called the Pi complex.

The present study was undertaken to determine the effects of relative viscosity of the solution, intrinsic viscosity of the hyaluronic acid and the relative concentrations of the components on Pi complex formation. With solutions in which the ratio of albumin to hyaluronic acid was low, all the albumin was bound to hyaluronic acid to form a Pi complex. In solutions that contained higher ratios of albumin to hyaluronic acid, the binding capacity of hyaluronic acid was apparently exceeded and free albumin was demonstrated in the solution. In contrast, some hyaluronic acid remained free even in the presence of an excess of albumin. Complex formation seemed to be directly related to the intrinsic viscosity of the hyaluronic acid.

INTRODUCTION

Previous work in this laboratory using electrophoretic analysis demonstrated that purified hyaluronic acid will combine with bovine plasma albumin. The electrophoretic patterns showed a peak with intermediary mobility, termed the Pi peak[1,2]. The interaction took place readily at pH 8.6 in veronal buffer and under certain conditions at pH 7.4 in phosphate buffer. This type of complex was frequently observed in normal human synovial fluids and postmortem human synovial fluids classed as normal, but was rarely found in synovial fluid from patients with rheumatic disease[3,4].

The present studies were undertaken in an effort to determine more quantitatively the composition of the Pi complex and to establish the effects of the relative viscosity of the solution, of the intrinsic viscosity of the hyaluronic acid and of the relative concentrations of albumin and hyaluronic acid.

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EXPERIMENTAL

Materials

Hyaluronic acid was prepared from bovine synovial fluid, collected from the astragalo-tibial joint with Merthiolate as preservative, by a modification of the electrodialysis method of ROSEMAN AND WATSON[5]. The hyaluronic acid was stored at −10° as a gel or as the lyophilized material. Prior to use, a weighed sample was dissolved overnight with stirring at 2–4° in veronal buffer (pH 8.6) $\mu = 0.1$. A small amount of Celite was added, and the solution was stirred for approx. 30 min. The solution was centrifuged at 25000 rev./min (43000 × *g*) for 30 min, and the supernatant fluid was decanted. This procedure gave a product that contained less than 2 % protein by the Lowry phenol method. The crystalline bovine plasma albumin was obtained from the Armour Laboratories. The buffers used were veronal (pH 8.6) $\mu = 0.1$ and phosphate (pH 7.3) $\mu = 0.2$ (containing NaCl $\mu = 0.15$).

General procedures

For studies of the combining ratio of albumin and hyaluronic acid in the Pi complex, solutions of albumin and of hyaluronic acid were mixed in different proportions and dialyzed for 18–48 h at 0–4° against veronal buffer prior to electrophoretic analysis. The shorter periods of dialysis were used for experiments in which minimal degradation of the hyaluronic acid was desired. In every case the electrical resistance of the dialyzed product was the same as that of the buffer against which it was dialyzed. Electrophoretic analyses were carried out in a 6-ml cell in a Perkin-Elmer Tiselius type apparatus in veronal buffer. An aliquot of the reaction mixture was analyzed for protein by the Lowry phenol method[6] or by the biuret reaction[7]. Hyaluronic acid was determined by analysis for uronic acid by the carbazole method using glucurone as a standard[8]. The viscosity of the solution was determined with a No. 200 or 400 Cannon-Manning semi-micro viscometer at 30.00°. Limiting intrinsic viscosity was determined by measurement of the specific viscosity of serial dilutions of aliquots of the hyaluronic acid solution and plotting specific viscosity divided by concentration of hyaluronic acid against specific viscosity of the solution. This plot gave essentially a straight line.

Reduction of viscosity of hyaluronic acid

To determine the effect of the intrinsic viscosity of hyaluronic acid on Pi complex formation, highly polymerized hyaluronic acid was degraded by ascorbic acid by the following procedure. Two samples of approx. 100 mg each of lyophilized hyaluronic acid with an intrinsic viscosity of 42 dl/g were weighed and dissolved at 2–4° overnight with stirring in 10 ml of phosphate buffer. A small amount of Celite was added. The solution was stirred for approx. 30 min, and then centrifuged at 43000 × *g* for 30 min. 5 ml of ascorbic acid solution (0.32 mmole/l) was added to the supernatant and the viscosity was measured at 30.00°. When the viscosity became relatively constant, another 5 ml of freshly prepared ascorbic acid solution was added. The decrease in viscosity was again followed until the relative viscosity was 8.6 and 8.0 centipoises, respectively. The samples were combined and dialyzed against veronal buffer for 18–24 h. The combined sample had an intrinsic viscosity of approx. 9 dl/g. This sample was concentrated by dialysis against a 25 % solution of polyvinylpyrrolidone to a

relative viscosity of 31 centipoises. Albumin equal to 300 mg/100 ml was added to 10 ml of the concentrate. This solution was dialyzed against veronal buffer for 18 h and analyzed electrophoretically.

Hyaluronic acid with an intrinsic viscosity of 42 dl/g was degraded by heating at 100° in a 10-ml volumetric flask in veronal buffer. Viscosities were determined before, during, and after degradation. Albumin was added to the degraded samples of hyaluronic acid and the solution was dialyzed 40–64 h before electrophoresis.

RESULTS

Variations of concentration of albumin

When hyaluronic acid of high intrinsic viscosity (64 dl/g) was mixed with bovine plasma albumin, and an electrophoretic analysis was made of the solutions, the ascending patterns showed three components when the concentration of albumin was sufficient. This is shown in Fig. 1 E. The absolute mobilities were 7.9, 8.4 and $8.9 \cdot 10^{-5}$ $cm^2\ V^{-1}\ sec^{-1}$ for albumin, the Pi complex and hyaluronic acid, respectively. Patterns 1A and 1B are those for hyaluronic acid with a mobility of 9.1 and the plasma albumin with a mobility of 6.6. Patterns 1C and 1D, for lower albumin concentrations, show only two components, but the slowest moving component had a mobility greater than that for albumin alone. At the lowest concentrations, all of the albumin seemed to be present as the Pi complex.

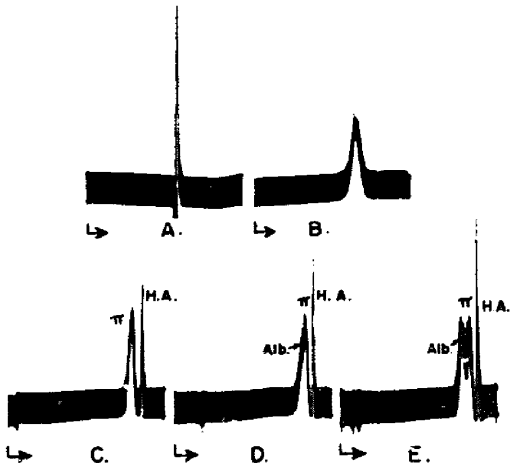

Fig. 1. The Pi peak: a complex of hyaluronic acid with albumin. A, 160 mg hyaluronic acid/100 ml with time of 195 min; B, 250 mg bovine plasma albumin/100 ml; C, 114 mg hyaluronic acid per 100 ml + 250 mg bovine plasma albumin/100 ml; D, 98 mg hyaluronic acid/100 ml + 256 mg bovine plasma albumin/100 ml; E, 132 mg hyaluronic acid/100 ml + 306 mg bovine plasma albumin/100 ml. Time: B–E 225 min. Veronal buffer (pH 8.6), $\mu = 0.1$.

The effect of variations in the albumin concentration on the formation of the Pi peak is also shown in Fig. 2. A series of electrophoretic patterns were obtained for solutions which contained approx. 150 mg/100 ml hyaluronic acid having an intrinsic viscosity of 46 dl/g. The albumin concentration was decreased progressively from 480 mg/100 ml (A) to 360 mg/100 ml (B), 280 mg/100 ml (C) and 250 mg/100 ml (D). At the greatest concentration of albumin, large albumin and hyaluronic acid peaks were observed in addition to the intermediate Pi peak. For the solutions which contained progressively smaller concentrations of albumin, the size of the albumin peak decreased with essentially no change in the size of the hyaluronic acid or Pi peaks. The solution that contained only 250 mg of albumin (ratio bovine plasma

albumin: hyaluronic acid, 1.74:1) gave a pattern (D) which had no definitive albumin peak. Essentially all the albumin had reacted to form the Pi complex.

Variations in concentration of hyaluronic acid

The effect of variation of the hyaluronic acid concentration at an approximately constant albumin concentration (73–103 mg/100 ml) is shown in Fig. 3. At the lowest concentration (55 mg/100 ml of hyaluronic acid and 100 mg/100 ml of albumin (A)),

Fig. 2. The effect of varying the albumin content with a constant hyaluronic acid content on the Pi peak formation. A, 153 mg hyaluronic acid/100 ml + 480 mg bovine plasma albumin/100 ml η rel. 44 centipoises; B, 153 mg hyaluronic acid/100 ml + 360 mg bovine plasma albumin/100 ml η rel. 44 centipoises; C, 146 mg hyaluronic acid/100 ml + 280 mg bovine plasma albumin/100 ml η rel. 46 centipoises; D, 144 mg hyaluronic acid/100 ml + 250 mg bovine plasma albumin/100 ml η rel. 45 centipoises. Veronal buffer (pH 8.6) μ = 0.1. The intrinsic viscosity of the hyaluronic acid was 46 dl/g.

no definitive Pi peak was observed in the electrophoretic pattern. However, the albumin peak was asymmetric with an increase in mobility of the leading edge as shown in Fig. 3A. A small but definitive Pi peak was observed (3B) for a solution that contained 89 mg/100 ml of hyaluronic acid and 76 mg/100 ml of albumin. With an increase of the concentration of hyaluronic acid to 113 mg/100 ml and 189 mg/100 ml as shown in Figs. 3C and 3D, the electrophoretic patterns showed hyaluronic acid and Pi peaks but no peak for albumin. Thus, an excess of hyaluronic acid caused all of the albumin to be complexed. Even in solutions that contained low concentrations of hyaluronic acid, however, a peak having the mobility of "free" hyaluronic acid appeared in the electrophoretic patterns.

Electrophoretic analyses were made of a series of solutions that contained approx. 125–300 mg/100 ml of hyaluronic acid which had been degraded earlier by heat

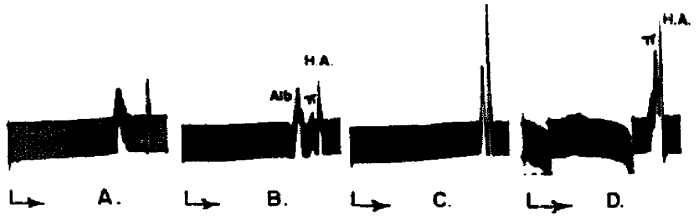

Fig. 3. Effect of hyaluronic acid concentration on Pi peak formation. A, η rel. 6 centipoises hyaluronic acid 55 mg/100 ml + bovine plasma albumin 105 mg/100 ml; B, η rel. 14 centipoises hyaluronic acid 89 mg/100 ml + bovine plasma albumin 76 mg/100 ml; C, η rel. 43 centipoises hyaluronic acid 113 mg/100 ml + bovine plasma albumin 73 mg/100 ml; D, η rel. 189 centipoises hyaluronic acid 194 mg/100 ml + bovine plasma albumin 100 mg/100 ml. The intrinsic viscosity of the hyaluronic acid was 42 dl/g. Veronal buffer (pH 8.6), μ = 0.1.

treatment at 100° to a relative viscosity of between 29 and 7 centipoises. The results are presented in Fig. 4. The solutions represented by Figs. 4A and 4B contained 127 mg/100 ml and 158 mg/100 ml of hyaluronic acid that had been degraded to relative viscosities of 24 centipoises and 29 centipoises, respectively. Small Pi peaks were present in the patterns for both solutions. The solutions represented by Figs. 4C, 4D and 4E contained progressively larger concentrations of hyaluronic acid which had been degraded to progressively smaller relative viscosities. Pi peaks could not be detected in these electrophoretic patterns. The results of this series of determinations suggested that in solutions of low relative viscosity (7–13 centipoises), Pi peak formation can not take place at concentrations of hyaluronic acid as high as 139–306 mg/100 ml. Since the decrease in relative viscosity was achieved by physically degrading the hyaluronic acid, the results did not indicate whether the failure to obtain a Pi peak was due to the low relative viscosity of the solution or to the low intrinsic viscosity of the hyaluronic acid.

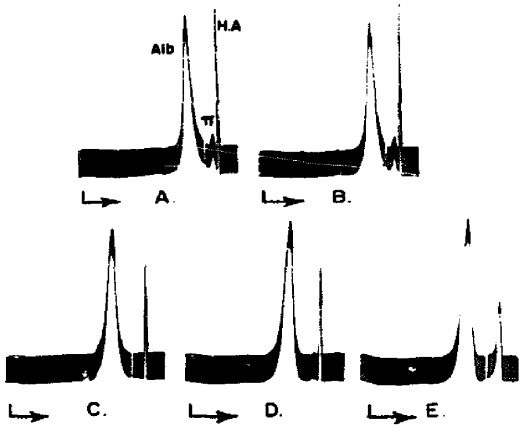

Fig. 4. The effect of relative viscosity of hyaluronic acid degraded by heat on the Pi peak formation. A, 127 mg hyaluronic acid/100 ml degraded from η rel. of 35 centipoises–24 centipoises + bovine plasma albumin 500 mg/100 ml; B, 158 mg hyaluronic acid/100 ml degraded from η rel. of 64–29 centipoises + bovine plasma albumin 440 mg/100 ml; C, 139 mg hyaluronic acid/100 ml degraded from η rel. of 40–13 centipoises + bovine plasma albumin 480 mg/100 ml; D, 169 mg hyaluronic acid per 100 ml degraded from η rel. of 136–12 centipoises + bovine plasma albumin 500 mg per 100 ml; E, 306 mg hyaluronic acid/100 ml degraded from η rel. of 1043–7 centipoises + bovine plasma albumin 620 mg/100 ml. Veronal buffer (pH 8.6), $\mu = 0.1$.

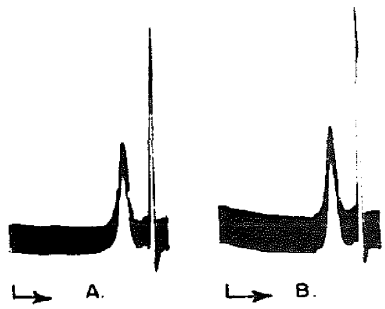

Fig. 5. The effect of concentration of hyaluronic acid degraded by ascorbic acid on the Pi peak formation. A, 226 mg hyaluronic acid/100 ml degraded from an intrinsic viscosity of 42 dl/g to 9 dl/g and concentrated to η rel. of 31 centipoises + bovine plasma albumin 300 mg/100 ml; B, 421 mg hyaluronic acid/100 ml degraded from an intrinsic viscosity of 42 dl/g to 4 dl/g and concentrated to η rel. of 36 centipoises + bovine plasma albumin 309 mg/100 ml. Veronal buffer (pH, 8.6) $\mu = 0.1$.

In order to study this problem, two solutions of hyaluronic acid that contained 226 mg/100 ml and 441 mg/100 ml were degraded with ascorbic acid to an intrinsic viscosity of 9 dl/g and 4 dl/g, respectively. After concentration by dialysis to a relative viscosity of 31 centipoises for the first and 36 centipoises for the second, and adding approx. 300 mg of albumin to each, the solutions were analyzed electrophoretically. The results are presented in Fig. 5. In Fig. 5A a small Pi peak may be present at the

base of the hyaluronic acid peak. There is no evidence of a Pi peak in Fig. 5B. These observations provide strong support for the concept that albumin combines only with highly polymerized hyaluronic acid. The relative viscosity of the solutions appears to be of importance primarily as it reflects the intrinsic viscosity of the polysaccharide.

DISCUSSION

Hyaluronic acid and bovine plasma albumin form a complex under the conditions of Tiselius electrophoresis at pH 8.6 and ionic strength 0.1, in veronal buffer[2]. This is shown as a peak, called the Pi peak, in the ascending pattern which migrates between the hyaluronic acid and albumin peaks. The earlier work suggested that highly polymerized hyaluronic acid was required for the formation of the Pi complex since it could not be produced from some samples of hyaluronic acid and treatment of synovial fluids with hyaluronidase caused its disappearance[1,9].

The present work demonstrates the influence of the degree of polymerization as measured by the intrinsic viscosity on Pi complex formation. Whereas formation of the Pi complex was demonstrated with highly polymerized hyaluronic acid, degraded hyaluronic acid had little or no capacity to form the complex, even in solutions with relative viscosities equal to those that contained undegraded hyaluronic acid.

TABLE I

THE RELATIONSHIP OF INTRINSIC VISCOSITY TO THE COMBINING RATIO OF HYALURONIC ACID AND PLASMA ALBUMIN

Figure	*Intrinsic Viscosity (dl/g)*	*Hyaluronic acid (mg/100 ml)*	*Bovine plasma albumin (mg/100 ml)*	*Bovine plasma albumin/hyaluronic acid*
1-C	64	114	250	2.19
2-D	46	144	250	1.74
3-C	42	113	73	0.65

Free albumin existed in solutions of undegraded hyaluronic acid only when present in excess of the amount required for saturation of the polysaccharide. On the assumption of an equality of negative charges in hyaluronic acid with positive charges in albumin at pH 8.6, the combining ratio can be calculated from the data of TANFORD[10]. Bovine albumin has 80 positively charged groups at pH 8.6 for a molecular weight of 65000. Hyaluronic acid has one ionized carboxyl group for each repeating unit of 379, assuming complete ionization. An equivalence of charges would be given by 2.17 g of albumin and 1.00 g of hyaluronic acid.

The electrophoretic patterns in Figs. 1 to 3 were used to estimate the amount of albumin required to saturate the negative charges of hyaluronic acid. The results given in Table I show that the amount of albumin seems to approach the calculated ratio of 2.17 when the hyaluronic acid has a high intrinsic viscosity, but may decrease for hyaluronic acid of lower intrinsic viscosity. However, a concentration effect may also be involved as indicated by patterns 2D and 3C.

In addition, the patterns always showed a sharp peak corresponding to that for free hyaluronic acid even when an excess of albumin was present. This suggests that

the hyaluronic acid samples contained a fraction which could not complex with albumin. Electrophoretic patterns of synovial fluid have always shown peaks corresponding to free hyaluronic acid and have protein concentrations considerably above those apparently required for saturation. Synovial fluids thus appear to contain some hyaluronic acid which does not complex with albumin. The polydispersity of the hyaluronic acid of synovial fluids has not been studied directly, but vitreous humor is known to be polydisperse[11].

The relation of the Pi complex of synovial fluids to other soluble complexes[12-16] formed under other conditions has not yet been clarified. In any case, the pH and ionic strength would provide major influences on the type of complexing.

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