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Note

Molecular distribution within a 1% collagen gel column

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This paper details the construction of a collagen gel chromatographic column, the interactions of several types of injected tracers with the gel, and the effects of varied buffer conditions on the column. The work reported is preliminary to a major program to establish properties of a series of collagen-containing gels of increasing concentration and compositional complexity, to be used as models for analysis of tracer distribution in interstitial tissue, hence we are interested in establishing a stable range of buffer conditions about physiological values, in classifying interactions, and comparing findings with those obtained from a hyaluronate gel column[1].

This type of column may have potential as a separation medium for substances that interact specifically with collagen, *e.g.*, fibronectins and fibronectin peptides[2].

MATERIALS AND METHODS

Minced Achilles tendon from calves, 12–16 weeks old, was diluted with 0.5 *M* acetic acid to a final pH of 2.45 and pepsinized. Temperature was maintained at 4° throughout so that denaturation would not occur. Insoluble collagen was removed by filtration of the preparation through glass wool, and the remaining collagen was precipitated with sodium chloride (5%, w/w). The precipitate was redissolved in 0.5 *M* acetic acid and dialyzed against distilled water, and the solution was lyophilized. Assay of the resulting material indicated a total carbohydrate content of 0.3%[3], and the amino acid content was similar to published results on calf skin collagen[4]. Electrophoretic analysis of this material on 5% polyacrylamide[5] showed characteristically high molecular weight in regions $\alpha 1$, $\beta 11$, $\alpha 2$, and $\beta 12$.

This collagen was then cross-linked by a procedure based on that of Öbrink and Wasteson[6], as follows. To a mixture of 2.0 g of lyophilized collagen and 400 ml of solution (0.14 *M* sodium chloride, 24 m*M* phosphate buffer, pH 7.4) at 4° was added 5.0 ml of 25% aqueous glutaric dialdehyde solution (G 400-4; Aldrich, Milwaukee, Wisc., U.S.A.). The reaction mixture was stirred for 5 min and allowed to stand for two days at 4°. The cross-link thus formed consisted of two Schiff bases connecting a pair of lysine residues on two neighboring molecules; rather than a single Schiff base connecting the two lysine residues, as in naturally cross-linked collagen. Since cross-links are formed at lysine residues and the lysine content of our material was 22 residues/1000 residues, the degree of cross-linking was necessarily less than about 2%.

The gel was subsequently granulated in a blender, and gel particles ranging in size from 250 to 500 μm were separated by successive filtration through 250- and 500-μm screens. The gel slurry was packed into a 40.9 × 1.6 cm column. The resulting gel concentration (1%) was determined through lyophilization of a measured volume of packed column. At an intermediate point during the study the column had to be repoured because of equipment failure. The new version was checked against the old by elution of standard molecules. Repouring the column did not affect the partition coefficients of species applied to both versions of the collagen column by more than the reproducibility (±3%) obtained on a column. We felt that it was important to demonstrate this matter owing to the relative heterogeneity of the gel particles.

To the column were applied the tracers bovine serum albumin (BSA) (part of the sample from previous studies[1,7]), fluorescein isothiocyanate dextrans (FITC-dextrans) from Pharmacia and Dr. Kirsti Granath*, Ficolls from Dr. Granath*, and the proteins ovalbumin, bovine chymotrypsinogen A and bovine ribonuclease A from Pharmacia. Column void volumes were measured by the elution volume of tobacco mosaic virus (TMV), and the total volume was taken to be the elution volume of tritiated water.

The column was operated in the ascending flow mode with a flow-rate of 3.3 ml/h under each of the following 0.01 *M* phosphate buffer conditions: 0.4 *M* sodium chloride, pH 8.2 and 6.7; 0.15 *M* sodium chloride, pH 8.2, 7.3, and 6.7.

Partition coefficients, K_{av}, were calculated from the elution data for the applied species according to eqn. 1:

$$K_{av} = (V_e - V_0)/(V_t - V_0) \quad (1)$$

where V_0 is the void volume, V_t the total volume of the column, and V_e is the elution volume of the sample species[8].

RESULTS AND DISCUSSION

The following observations can be made with respect to elution data plotted in Fig. 1.

First, the dependence of K_{av} on variation of pH and ionic strength about the physiological values is small. The largest effect, *e.g.*, was seen with FITC-dextrans (Fig. 1). When the sodium chloride concentration was high (0.4 *M*), reduction of pH (from 8.2 to 6.7) increased K_{av} of FITC-dextrans by 10%. Deyl *et al.*[9] have reported elution from a collagen gel column of naturally occurring negatively charged polyelectrolytes with a pH gradient (pH 3.4 to 10). FITC-dextrans are not considered charged, on the basis of gel filtration, but there may be a slight negative charge on some of the fluoresceins[10].

Secondly, the relative volume available to a given net negatively charged or neutral molecule (K_{av}) was several times larger in the 1% collagen gel than in the

* Dr. Kirsti Granath, Department of Polymer Chemistry, Pharmacia (Uppsala, Sweden), supplied us with narrow dispersion fractions with measured number-average and weight-average molecular weights

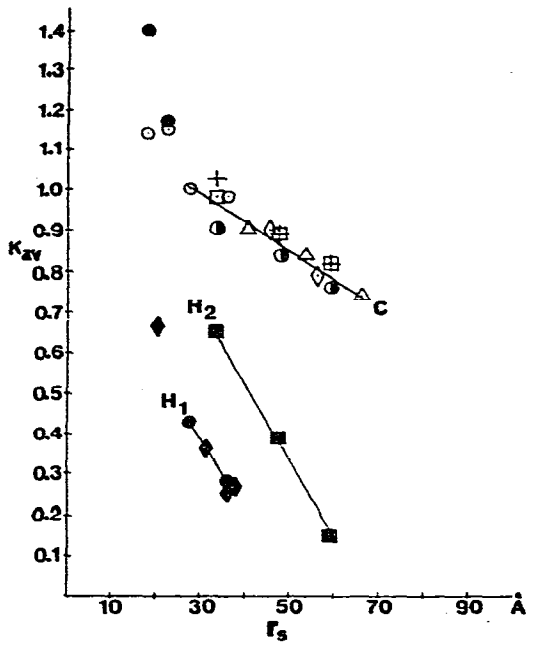

Fig. 1. Relationships between K_{av} values and Stokes radii (r_s) of the species applied to the columns. Line C is the regression line ($r = -0.98$) for data from polysaccharides (□ = FITC-dextran; △ = dextran; ◇ = Ficoll) eluted from the 1% collagen gel column with 0.15 *M* NaCl, pH 7.3. Not included in the regression were the points for FITC-dextrans eluted from the collagen column with 0.4 *M* NaCl, pH 6.7 (+) and with 0.4 *M* NaCl, pH 8.2 (◑), and the points (○) for proteins eluted from collagen with 0.15 *M* NaCl. pH 7.3. Points (●) correspond to proteins eluted from the 1.5% hyaluronate column with 0.15 *M* NaCl, pH 7.3. The protein points at K_{av} values greater than 1.0 represent chymotrypsinogen A and ribonuclease A which have a net positive charge at pH 7.3. The line H_1 connects the points for BSA and ovalbumin, which have a net negative charge. The line H_2 connects points (■) corresponding to FITC-dextrans eluted from the hyaluronate column with 0.15 *M* NaCl, pH 7.3. The points (◆) represent data from Laurent[16] for negatively charged globular proteins on a similar hyaluronate column and are included for verification.

1.5% hyaluronate gel (Fig. 1). The maximum excluded volume, that for TMV, per gram of matrix material was 33.6 ml/g in the collagen column, compared with 39.0 ml/g in the hyaluronate column.

Thirdly, chymotrypsinogen A (p*I* 8.8–9.6, ref. 11) had a similar adsorptive interaction with hyaluronate and collagen, whereas ribonuclease A (p*I* 8.8, ref. 11) had a much stronger interaction with hyaluronate than with collagen.

Fourthly, the correlation between Stokes radius and K_{av} for ovalbumin (p*I* 4.7, ref. 11) and BSA (p*I* 5.85, ref. 11) compares with that for the polysaccharides on collagen, as shown by the fit with line C. This situation also holds for proteins and polysaccharides on Sephadex gels[12]. This similarity in correlation does not hold on the hyaluronate gel for ovalbumin and BSA (line H1) and FITC-dextrans (line H_2). This may indicate a charge repulsion of the ovalbumin and BSA on the negatively charged hyaluronate.

The value of maximum excluded volume, 33.6 ml/g, obtained on the collagen column lies below the range, 53–70 ml/g, reported for polymeric collagen by Pearce

and Laurent[13]. Their experiment involved an equilibrium technique with centrifugation, which may possibly have compressed their collagen sample.

Buffer concentrations ranging from 0.15 to 0.4 *M* in sodium chloride and pH in the range 6.7–8.2 were tested, because these ranges cover both physiological values and the values of pH and ionic strength observed in hemorrhagic shock[14,15].

Altered interstitial distributions of water and Na^+ *in vivo* in hemorrhagic shock have been postulated[14], and collagen is a major interstitial component. We looked for corresponding alterations in the distribution of larger molecules, both charged and uncharged, and water with changes in pH and ionic strength in the *in vitro* collagen column but did not find any major shifts in the ranges studied.

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REFERENCES

1 M. Shaw and A. Schy, *Biophys. J.*, 17 (1977) 47.
2 E. Engvall, E. Ruoslahti and E. J. Miller, *J. Exp. Med.*, 147 (1978) 1584.
3 M. Dubois, K. A. Gilles, J. K. Hamilton, P. A. Rebers and F. Smith, *Anal. Chem.*, 28 (1956) 350.
4 G. N. Ramachandran, *Treatise on Collagen*, Vol. 1, New York, Academic Press, 1967, p. 536.
5 B. Goldberg, E. H. Epstein and C. J. Sherr, *Proc. Nat. Acad. Sci. U.S.*, 69 (1972) 3655.
6 B. Öbrink and A. Wasteson, *Biochem. J.*, 121 (1971) 227.
7 M. Shaw, *Biophys. J.*, 16 (1976) 43.
8 T. C. Laurent and J. Jillander, *J. Chromatogr.*, 14 (1964) 317.
9 Z. Deyl, M. Adam, J. Musilová and K. Macek, *J. Chromatogr.*, 148 (1978) 283.
10 L. Beadling, Pharmacia Fine Chemicals, Uppsala, Sweden, personal communication.
11 P. G. Righetti and T. Caravaggio, *J. Chromatogr.*, 127 (1976) 1.
12 T. C. Laurent and K. A. Granath, *Biochem. Biophys. Acta*, 136 (1967) 191.
13 R. H. Pearce and T. C. Laurent, *Biochem. J.*, 163 (1977) 617.
14 G. T. Shires, J. N. Cunningham, C. R. F. Baker, S. F. Reeder, H. Illner, I. Y. Wagner and J. Maher, *Ann. Surg.*, 176 (1972) 288.
15 D. S. Campion, L. J. Lynch, F. C. Rector, N. Carter and G. T. Shires, *Surgery* 66 (1969) 1051.
16 T. C. Laurent, *Biochem. J.*, 93 (1964) 106.