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# A POSSIBILITY OF MODELLING THE *IN VIVO* ACCUMULATION OF COMPOUNDS BY CHROMATOGRAPHY

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## SUMMARY

A method for studying the interactions of proteins with various compounds by a chromatographic procedure has been developed. In principle, the protein being tested is used as the packing in a column on which tested solutes are applied and eluted with appropriate buffer systems. As exemplified for collagen, the method is capable of detecting small alterations of the protein structure and may be applicable to environmental problems.

## INTRODUCTION

In a living body, numerous interactions take place between solid biopolymers and both low- and high-molecular-weight solutes. The modelling of these interactions with adequate interpretation is not easy and methods using one of the components involved in the interaction as a column packing or chromatographic support can be made use of[1–3]. A better understanding of such interactions may help to interpret some poorly understood physiological and pathophysiological situations and to elucidate the effects of the accumulation of pollutants in organisms.

Of course, such modelling imposes various demands on the solid phase that are comparable to the demands commonly made on column packings, *e.g.*, the mechanical properties, swelling properties, grain size and shape must be within reasonable limits comparable to those used for chromatographic supports and packings[4].

To our knowledge, only collagen, the main fibrillar protein of the connective tissue, has been used up to now to demonstrate these types of interactions[1–3,5]. The rationale of a collagen-based model is based on the fact that the interactions of collagen with other molecular species may alter the amounts of nutrients reaching most of the cells in an animal's body, with obvious biological consequences.

In the present study, we have tried to obtain evidence that the alterations which occur under in vivo conditions are analogous to chemical modifications and that models of this type are justified for studying interactions and accumulatory effects in vivo. Numerous models of this type can be selected with regard to the effect

of hazardous compounds. The influence of ageing upon the capability of collagen to bind different compounds was used as a representative model.

## EXPERIMENTAL

*Preparation of cartilage collagen*

Bovine nasal septal cartilage from animals of different ages (foetal, newborn, 1-year-old adult and over 10-years-old) from a local slaughterhouse was kept frozen at −20°. The material was sliced into small pieces and subjected to a series of extractions[1]. To 10 g of the starting material 300 ml of water and 600 ml of ethanol were added. The mixture was left overnight and was then centrifuged for 30 min at 50,000 rpm. The resulting sediment was subjected to the same procedure and, after the second centrifugation, the sediment was suspended stepwise in water, absolute ethanol and diethyl ether (300 ml of each). In each step the suspension was allowed to stand overnight and centrifuged before adding the next solvent. Finally, 300 ml of 8 *M* urea which was 1.0 *M* with respect to sodium chloride were added, the mixture was allowed to stand overnight and then centrifuged and the procedure was repeated three or four times. The resulting sediment was washed with distilled water and freeze-dried. This material was homogenized in small portions in liquid nitrogen and the fraction of between 50 and 100 mesh was used for packing the chromatographic column. A light micrograph of a cluster of collagen particles from a column that had been operated for several runs is shown in Fig. 1.

*Characterization of crude collagen used for column packing*

Pronase was used for the proteolytic cleavage of collagen[6] in order to solubilize the material for further characterization. Briefly, after purification by ultrafiltration, sterilized 0.01 % pronase solution in 0.1 *M* calcium acetate solution (pH 7.18) was mixed with the cartilage sample to make the collagen to enzyme ratio 100:1. The reaction mixture was incubated at 20° for 40 h. Enzyme-treated samples were centrifuged (at 4° and 9000 rpm for 30 min) and the insoluble residue was extracted stepwise with sodium chloride–phospahte buffer (pH 7.4, ionic strength $I = 0.5$) and citrate buffer (pH 3.7). The extraction procedure was as described for skin collagen[6].

Proteoglycan complexes that were released by pronase digestion from the collagen preparation were separated from the collagen solution by DEAE-cellulose chromatography as described by Miller[7].

Additional analyses were carried out according to Bitter and Muir[8] (hyaluronic acid), Stegeman[9] (hydroxyproline), Blumenkranz and Asboe-Hansen[10] (uronic acids), Boas[11] (hexosamine) and Lowry *et al.*[12] (protein content).

*Chromatographic separation*

This was performed on a 20 × 1 cm stainless-steel column packed by pouring in the collagen suspension. As soon as the first portion (5 ml) had settled, pressure was introduced using a double-piston pump operated at 30 atm. Before operation, the column was equilibrated with 0.01 *M* sodium citrate buffer (pH 3.4). Samples were applied as aqueous solutions (3000 mg, dry weight); excess of the sample (overloaded column) was washed out with 0.01 *M* sodium citrate buffer (pH 3.4). Adsorbed material was eluted with a pH gradient ranging from 3.4 to 7.3 (0.01 *M* sodium citrate

Fig. 1. Light micrograph of a cluster of collagen fibres. This material served as the column packing for six runs. Note the presence of cells (fibroblasts). Magnification 300×.

buffer) followed with a linear sodium chloride gradient (0–1.0 *M*) at pH 7.3 (0.01 *M* sodium citrate buffer). The amount of glycosaminoglycans and proteoglycans was determined by measuring the hexuronic acid concentration of the eluate (4-ml fractions were collected at a flow-rate of 1 ml/min). With γ-globulin the UV absorbance at 280 nm was considered to be indicative of the eluate concentration, while the absorbance at 440 nm was applied with actinomycin C and absorbance at 250 nm with penicillin G. All measurements were carried out on a Unicam SP-700 spectrophotometer.

*Substances tested*

Chondroitin 6-sulphate, hyaluronic acid and γ-globulin were obtained from Miles Laboratories (Elkhardt, Ind., U.S.A.) and penicillin G and actinomycin C from Serva (Heidelberg, G.F.R.) Skin proteoglycans were prepared by the method of Miller[7].

## RESULTS AND DISCUSSION

The properties of collagen packings differ depending on the age of the animal from which the material was prepared. With advancing age of the animal, the material became more resistant towards proteolytic cleavage, *i.e.*, more reactive groups were involved in crosslinks (Table I). Although the preparation procedure removed most of the non-collagenous material, a certain amount of proteoglycans remained bound to collagen (Table II).

TABLE I

THE EXTRACTIBILITY OF COLLAGEN FROM DIFFERENT SOURCES BY PRONASE

40 h, 20°, pronase to collagen ratio 1:100 (w/w).

| *Type of preparation (animal)* | *Collagen (mg per 100 mg of total collagen)* | | | |
|---|---|---|---|---|
| | *Pronase extract* | *0.45 M NaCl extract after pronase digestion* | *Citrate extract after pronase extraction* | *Total* |
| Calf | 39.263 | 9.052 | 5.694 | 54.009 |
| 1-year-old cow | 27.557 | 16.838 | 6.284 | 49.076 |
| >10-years-old cow | 16.818 | 5.763 | 2.962 | 25.543 |

TABLE II

PROTEOGLYCANS BOUND TO COLLAGEN PACKINGS AND RELEASED BY PRONASE DIGESTION

Conditions as in Table I.

| *Type of preparation (animal)* | *Total protein (mg per 100 mg of collagen)* | *Uronic acids (mg per 100 mg of collagen)* | *Hexosamine (mg per 100 mg of collagen)* |
|---|---|---|---|
| Calf | 1.8 | 0.30 | 0.1 |
| 1-year-old cow | 7.5 | 1.32 | 1.1 |
| >10-years-old cow | 5.6 | 0.98 | 0.4 |

If the column packing is prepared from foetal collagen and chondroitin 6-sulphate is used as the solute, then all material is eluted from the column at pH 7.3 and a salt concentration of 0.25 *M*. If the column packing is prepared from a newborn individual, a small portion of the material loaded appears as a fast-moving peak. With increasing age, the binding capacity of the slowly moving peak decreases and the amount of material eluted in the fast-moving peak increases. This fast-moving peak is usually not very sharp and its maximum appears at about pH 6–7. In column packings obtained from animals aged more than 10 years most of chondroitin 6-sulphate is eluted in the fast-moving peak (Fig. 2).

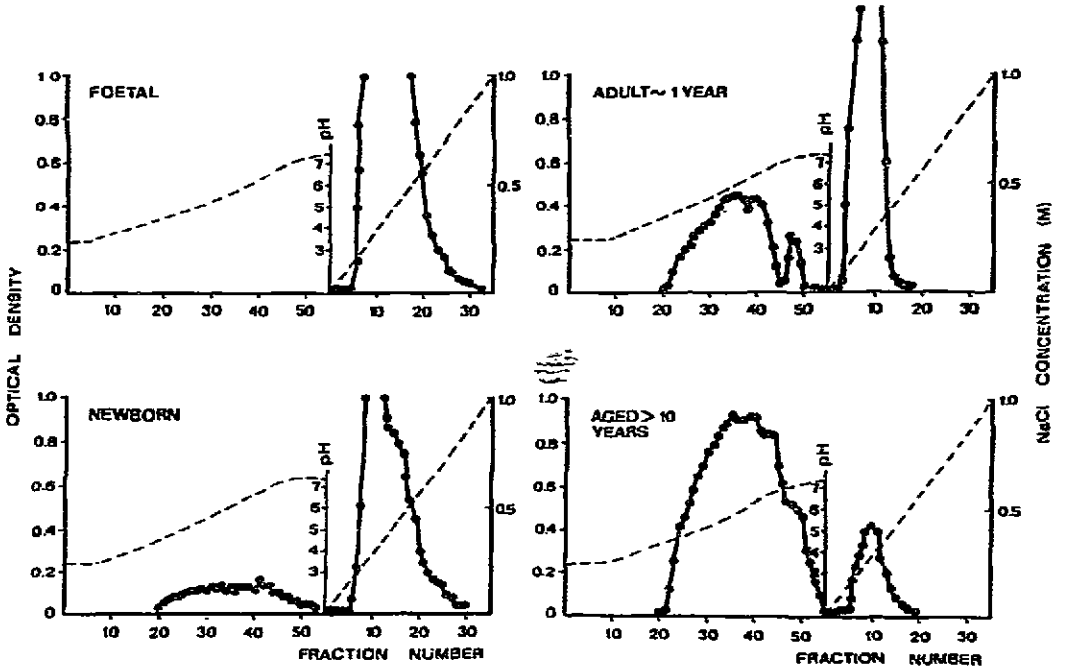


Fig. 2. Chromatographic profile of chondroitin 6-sulphate on collagen. Column packing prepared from animals of different ages (650 mg, dry weight); amount applied, 3000 mg as hexuronic acid. Chondroitin 6-sulphate was applied to the column equilibrated with 0.01 *M* sodium citrate buffer (pH 3.4) and eluted with a pH gradient as indicated (broken line in the right-hand side of each figure). Subsequently (left-hand side of each figure) a linear sodium chloride gradient (0–1.0 *M*) was introduced (also indicated by broken line).

Analogous ontogenetic relations are also observed when γ-globulin, hyaluronic acid or proteoglycans are used as the solutes (Figs. 3, 4 and 5). Also, peptides that are foreign to the organism, such as actinomycin C and penicillin, behave as indicated in Figs. 6 and 7. The relative proportions of the slow- and fast-moving peaks differ in the individual solutes. With hyaluronic acid, the slow-moving peak is small. If the column packing is prepared from aged animals, the retention volume of the fast-moving peak is shifted to lower values and the maximum appears at about pH 5.

The results obtained have to be judged from the viewpoint that collagen, when used as a column packing, behaves as an amphoteric ion exchanger. Therefore, it may

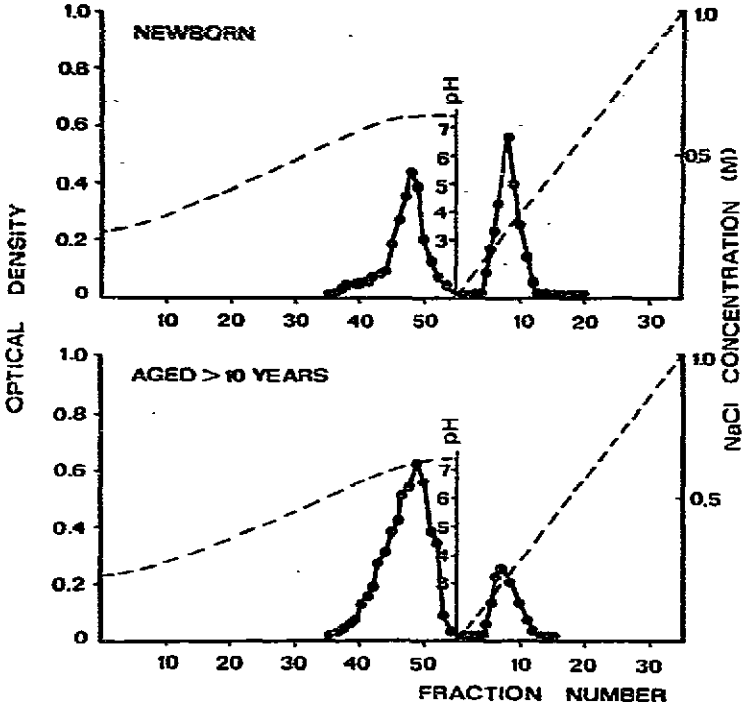


Fig. 3. Chromatographic profile of $\gamma$-globulin on collagen. Column packing prepared from animals of different ages. Separation conditions as in Fig. 1.

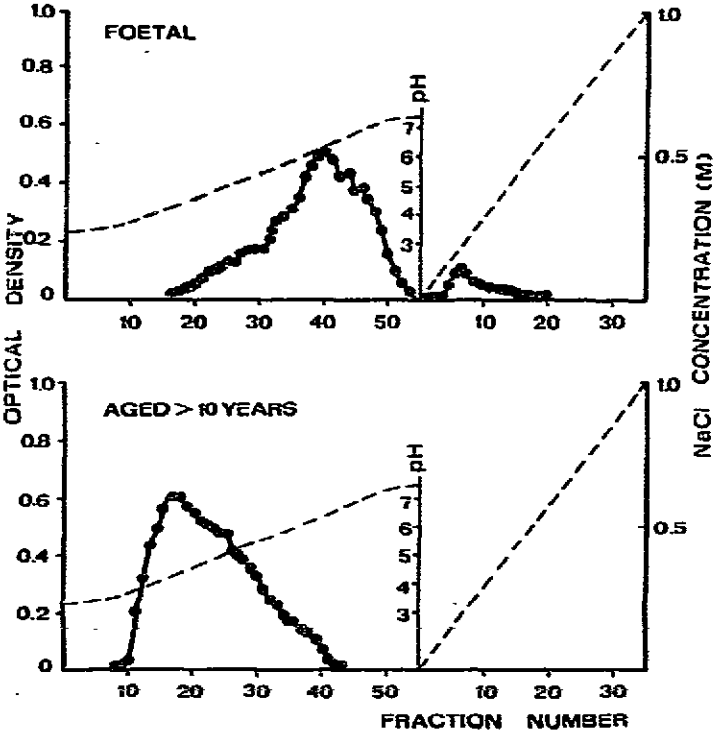


Fig. 4. Chromatographic profile of hyaluronic acid on collagen. Packing prepared from animals of different ages. Separation conditions as in Fig. 1.

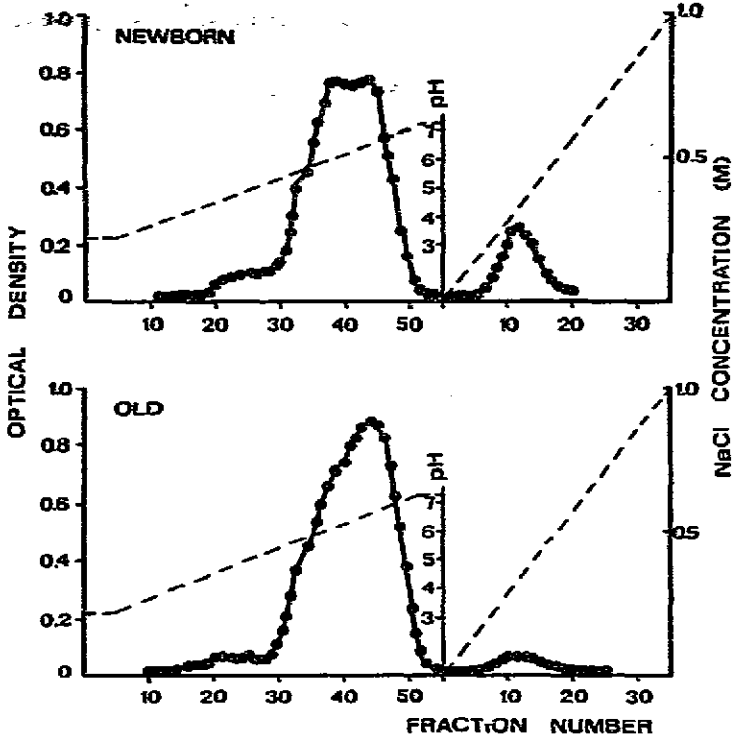


Fig. 5. Chromatographic profile of proteoglycans on collagen. Column packing prepared from newborn and old (>10 years) animals. Separation conditions as in Fig. 1.

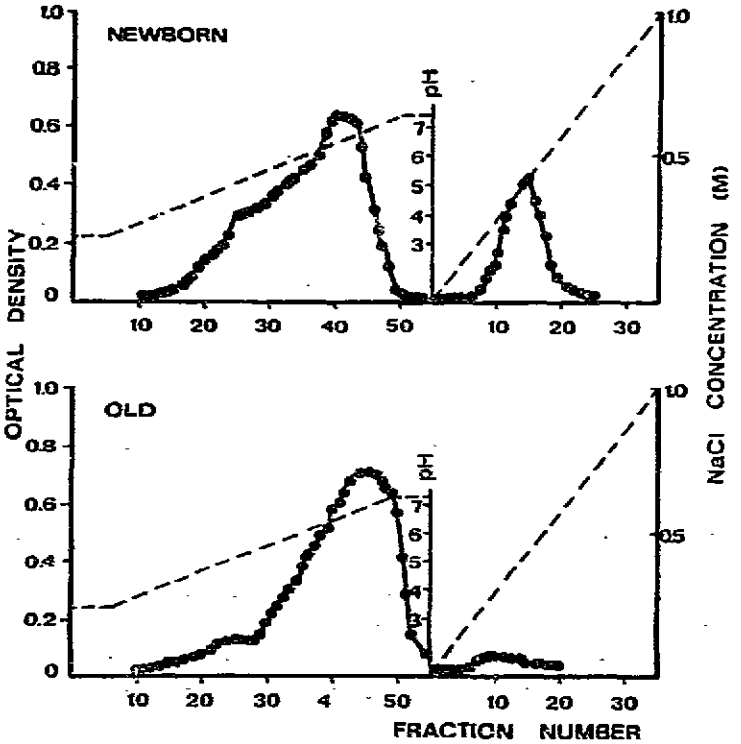


Fig. 6. Chromatographic profile of actinomycin C on collagen. Column packing prepared from newborn and old (>10 years) animals. Separation conditions as in Fig. 1.

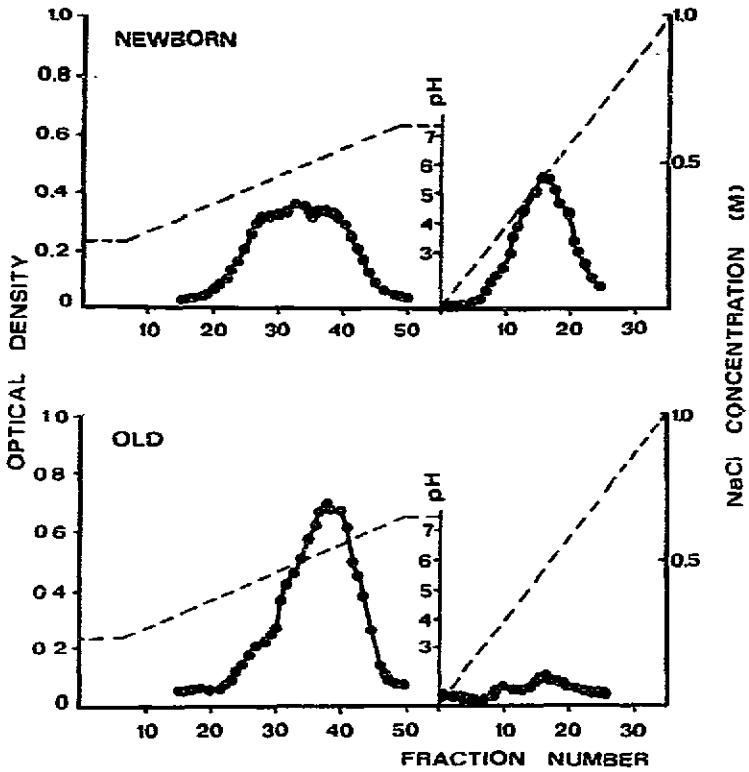


Fig. 7. Chromatographic profile of penicillin G on collagen. Column packing prepared from newborn and old (>10 years) animals. Separation conditions as in Fig. 1.

happen that the variation in the dissociation of the amphoteric counter ions allowing selective desorption may be compensated for by variations in the charge of the functional groups of the exchanger, which increase the sorption, and *vice versa*.

When glutaraldehyde-crosslinked collagen gels were used[3,5], the elution patterns of chondroitin 6-sulphate varied considerably with ionic strength and pH. This result indicates that ionic interactions are involved in this type of chromatography, as in conventional gel chromatography this type of interaction would not be expected. The influence of ionic strength is greater than that of pH. The effect of ionic strength seems to be eliminated at $I = 0.4$. Hanada and Anan[2] state that "it should be pointed out that the physiological ionic strength and pH are in a range where small changes in the environment seem to exert a great influence on the interaction". Also, chromatographic investigations of the collagen–glycosaminoglycan interaction give superior results to those obtained on this interaction in an electric field, where the interpretation of the results may be ambiguous. The same statement can be made for the antibiotics tested.

The fact that the compounds tested are eluted from the column in two discrete peaks indicates the existence of two different ways of binding these solutes to the collagenous matrix. At acidic pH values the column packing should behave as a weak cation exchanger and basic groups in amino acid side-chains should be dissociated.

It has been shown by other workers that some of these side-chains, namely those of lysine, are converted into crosslinks of the Schiff base type, which would result in a decreased binding capacity if the number of residues reacted is sufficiently large (for a review, see ref. 13). If the number of residues reacted is small than there should be no difference in the chromatographic profiles. In our experiments, however, the peak that occurs during the pH gradient elution is increased when the packing is prepared from older animals, which indicates that the occurrence of lysine-derived crosslinks is not decisive for the binding capacity of the column at acidic pH values. There are two other possibilities that could explain the altered behaviour of the column: (a) crosslinks introduced into the structure cause conformational changes and perhaps changes in the assembly of individual protein molecules into fibres, thus making other regions available for the tested solutes and; (b) the collagenous component present binds different amounts of non-collagenous components, mainly proteoglycans, with the same result as in the first instance. Our results indicate that both mechanisms participate in the age-dependent changes of the collagenous matrix.

The method of studying *in vivo* interactions of different compounds with proteins and perhaps with other naturally occurring compounds appears to be widely applicable. The ability of the method to indicate small changes in structure appears to be adequate, as demonstrated in the case of the ageing collagen structure and its capability to bind different high- and low-molecular-weight compounds.

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