PRECIPITATION OF COLLAGEN FIBRILS IN VITRO BY PROTEIN POLYSACCHARIDES

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There is considerable evidence that collagen fibrils do not arise intracellularly, but are formed by the aggregation of a soluble collagen secreted by connective tissue cells (Porter and Pappas, 1959; Goldberg and Green, 1964; Revel and Hay, 1963). In vitro soluble collagen (tropocollagen), when warmed to 37° in physiological saline at pH 7.3, aggregates into fibrils which show "native" banding in the electron microscope. The packing arrangement in the aggregates and their rate of formation are greatly modified by serum a_1-glycoprotein and various metabolites which might be expected to be present in the extracellular space (Gross, 1956; Gross and Kirk, 1958). However, the major extracellular soluble component in many connective tissues is an acid mucopolysaccharide-protein complex which is also synthesised and secreted by connective tissue cells (Green and Hamerman, 1964; Jackson, 1965).

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This paper reports the precipitation of a fraction of tropocollagen as "native" fibrils on the addition of sulphated acid mucopolysaccharide-proteins (proteinpoly-saccharides).

Materials

Chondroitin sulphate-proteins were prepared from extracts of bovine nasal cartilage and intervertebral discs by cesium chloride density gradient centrifugation (Franek and Dunstone, 1967). Dermatan sulphate-proteins were prepared from hot urea extracts of the fibrous residues of bovine heart valves, skin and tendon (Toole and Lowther, 1966; Lowther, Toole and Meyer, 1967). Hyaluronate-protein was prepared from extracts of bovine heart valves by density gradient centrifugation (Meyer and Lowther, manuscript in preparation) and from bovine synovial fluid by ultrafiltration (Preston, Davies and Ogston, 1965).

Protein-free chondroitin sulphate, dermatan sulphate, and keratan sulphate were prepared by proteolytic digestion as described before (Toole and Lowther, 1966; Lowther and Baxter, 1966).

Tropocollagen was prepared from 0.1M acetic acid extracts of bovine embryo skin by precipitation with NaCl at acidic and neutral pH, followed by dialysis against 0.01M Na_2HPO_4 (Green and Lowther, 1959). The final precipitate was dispersed in acetic acid.

All materials were dialysed against three changes of 0.14M NaCl/0.008M phosphate buffer, pH 7.3 and centrifuged at approx. 120,000 g for 3 hours before use.

Methods

Precipitation was detected by measurement of turbidity

in a spectrophotometer at 400 mμ (Wood and Keech, 1960). Proteinpolysaccharide and tropocollagen were mixed at 4° and turbidity measured immediately. Acid mucopolysaccharide concentration was determined by uronic acid analyses (Bitter and Muir, 1962) and collagen concentration by hydroxyproline analyses (Serafini-Cessi and Cessi, 1964). Electron microscopy was performed with specimens prepared by depositing dilute suspensions of fibrils on copper mesh grids and staining with neutral 1.5% phosphotungstic acid.

Results

Several sulphated proteinpolysaccharides give a precipitate with tropocollagen immediately after mixing either at 4° or 37°. For convenience this reaction has been studied at 4° where no spontaneous precipitation of collagen occurs. Chondroitin sulphate-protein prepared from cartilage or intervertebral discs and dermatan sulphate-protein from heart valves, skin or tendon give this reaction. These compounds also react with acid-extracted tropocollagens from rat skin and guinea pig skin granuloma and with neutral salt-extracted tropocollagen from bovine embryo skin. The precipitates obtained were sedimented by centrifugation and examined in the electron microscope. Fibrils with banding of periodicity 550 to 650 Å were observed. Hyaluronate-proteins give no precipitate with tropocollagen suggesting that the precipitation process is dependent on the sulphation of the proteinpolysaccharide. Also other aspects of the structure of the protein complexes appear to be important since protein-free chondroitin sulphate and dermatan sulphate derived from these complexes do not form precipitates.

When tropocollagen was mixed with increasing amounts

of a sulphated proteinpolysaccharide, the resultant turbidities increased until a point was reached where the further addition of proteinpolysaccharide gave no further increase in absorbance, The ratio of tropocollagen to proteinpolysaccharide at this point is defined as the "limiting ratio" It varied in magnitude according to the mode of preparation of the proteinpolysaccharide, but was normally in the range 3.5 to 7.5. The ratio could also be determined by titrating a constant concentration of proteinpolysaccharide with varying amounts of tropocollagen and was reproducible for given batches of the two reactants.

Precipitates obtained at the limiting ratio with cartilage chondroitin sulphate-protein and heart valve dermatan sulphate-protein were sedimented by ultracentrifugation (approx. 120,000 g for 1.5 hours). The supernatants were assayed for acid mucopolysaccharide and collagen and the composition of the pellets calculated (Table 1). The ratio of collagen to proteinpolysaccharide varied between 3.6 and 4.7. Up to 96% of the total proteinpolysaccharide was found to occur in these pellets, however, the amount of collagen sedimenting varied between 55 and 69% of the total (Table 1).

On addition of further proteinpolysaccharide to the supernatants from these experiments no further precipitation was obtained suggesting that two fractions of tropocollagen, differing in their ability to react with proteinpolysaccharide, were present in the original solution. Wood (1962) has found that tropocollagen consists of two fractions, "nucleus-forming" and "growth" collagens. The former is involved in the formation of a small aggregate essential

Table 1 Precipitation of tropocollagen by proteinpolysaccharide at the limiting ratio*.

AMPS-P** preparation.	Percentage of total TC precipitated by AMPS-P.	Ratio of TC to AMPS-P in pellet.
Chondroitin sulphate-protein (Batch 1)	68	3.6
Chondroitin sulphate-protein (Batch II)	62	3.9
Chondroitin sulphate-protein (Batch II)	69	4.3
Dermatan sulphate-protein	55	4.7

* Concentrations of reactants in original mixtures; tropocollagen, 1200µg/ml; proteinpolysaccharide, 200-320µg/ml. The variation in the latter was due to the different limiting ratios of reactants obtained for different batches and types of proteinpolysaccharide.

** Abbreviations: AMPS-P, proteinpolysaccharide; TC, tropocollagen.

for the subsequent accretion of tropocollagen to form fibrils. We have obtained evidence that this "nucleus-forming" collagen is contained in the fraction of collagen which reacts instantaneously with sulphated proteinpolysaccharides (Toole and Lowther, 1967).

Since this precipitation of fibrils by proteinpolysaccharides takes place from dilute solutions of tropocollagen (e.g. 150 µg/ml) and as proteinpolysaccharides are present in the extracellular space of most connective tissues, we conclude that the formation of fibrils from newly secreted tropocollagen within grooves on the cell membranes (Porter

and Pappas, 1959; Goldberg and Green, 1964; Revel and Hay, 1963) depends on this reaction.

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