Med. Pharmacol. exp. *15:* 344–350 (1966)

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Analytical Investigations of the Cartilage Extract in Rumalon®

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The important role of the collagen and mucopolysaccharide metabolism and its alterations in rheumatic conditions has become more and more obvious. The restorative influence on these changes by biological or synthetic drugs is the subject of numerous investigations. Interesting clinical and experimental results concerning a cartilage bone marrow extract (*Rumalon®*) led us to fractionate the cartilage extract in order to separate and investigate the effective and essential components.

Methods

The cartilage extract contained in Rumalon® was kindly supplied by Robapharm AG, Basle, Switzerland.

Qualitative identification of amino acids, sugars and amino sugars was performed by paper chromatography on Schleicher und Schüll paper No. 2043 b Mgl. with the following solvent systems: n-butanol + acetic acid + water (4 + 1 + 1) and isopropanol + acetic acid + water (70 + 15 + 15). The staining reactions were done with: ninhydrin, isatin, aniline phthalate and Ehrlich reagent.

The high molecular fraction was isolated by dialysis, gel filtration, ultrafiltration, electrophoresis and by precipitation. The reparation on Sephadex columns using a variety of buffers was performed with a Gilson Medical fraction collector with automatic registration of the ultraviolet absorption.

Paper electrophoresis was performed on Schleicher und Schüll No. 2043 b paper strips in the 110 volts Elpho apparatus by *Grassmann and Hannig,* using the following buffers: Na-barbiturate buffer pH 8.6; HCl-KCl buffer pH 1.0; phosphate buffer pH 6.4; glycine buffer pH 10.0.

The staining procedure was carried out with solutions of: Azocarmine B, Amindoblack 10 B, Gentianaviolet, Toluidineblue.

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* Most of the equipment used in this study was kindly granted by the Stiftung Volkswagenwerk.

Received: February 4, 1966.

Ion echange chromatography was applied for the separation and quantitative determination of the amino acids. Analysis of the hexosamine content was carried out with the Elson-Morgan reaction. A Zeiss-Spektralphotometer type PMQ II was used for all photometric and colorimetric measurements.

Experiments and Results

Precipitation.

Our first experiments showed that the cartilage extract contains a broad spectrum of free amino acids. After hydrolysis with hydrochloric acid a marked increase in the content of amino acids was found, indicating that bound amino acids are present in the cartilage extract. Adding cetylpyridinium chloride to the extract we yield a voluminous white precipitate. Short heating of the cartilage extract with the addition of buffered acid ethanol also resulted in a voluminous white precipitate, which after purification and drying in vacuum gave a light white powder, easily soluble in water. This precipitate we designated as 'fraction DAK-16'.

Gel filtration.

In consideration that the above-mentioned procedure of isolation of the fraction DAK-16 might have influenced or denaturated the substance, we tried to fractionate the cartilage extract by gel filtration with Sephadex G-25. By many different buffer systems (best results were obtained with HCl-KCl buffer pH 1.0) the extract could be separated into three ultraviolet absorbing peaks (Figure 1). Further investigations showed that the first peak mainly contains DAK-16, the second one mainly free amino acids and nucleo derivatives and the third peak m-cresol (which is added to the cartilage during manufacturing of the extract). The substance in peak No. 1 is excluded by Sephadex G-25 and appeared in the void volume of the column.

Electrophoresis.

If the cartilage extract was subjected to 110 volts paper electrophoresis with different, above-mentioned buffers, a high molecular fraction could be detected, only stainable with toluidine blue or with gentiana violet but not with azocarmine B or amidoblack 10B. A solution of the precipitate 'fraction DAK-16' gave

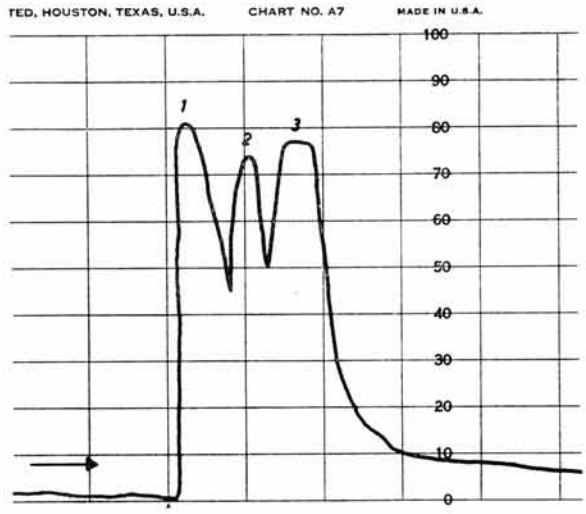

Fig. 1. Fractionation of the cartilage extract by gel filtration with Sephadex G-25, UV-registration at 265 nm.

the same results on electrophoresis and had the same properties as the cartilage extract. The substance in the peak No. 1 of Sephadex G-25-fractionation of the cartilage extracts showed the same behaviour and pattern on electrophoresis and for this reason could be identified as the fraction DAK-16. In Fig. 2 there are two electrophoretic separations, run for four hours with HCl-KCl buffer pH 1.0 and 110 volts, stained with toluidine blue. The first strip shows the electropherogram of a solution of DAK-16, and the second strip the genuine cartilage extract.

Properties and characterization of the fraction DAK-16.

Experiments with dialysis and ultrafiltration indicated that DAK-16 is non-dialyzable and cannot pass an ultrafilter, due to its molecular size. The estimation of the molecular weight of DAK-16 by ultracentrifuge resulted in 55 000 $\pm$ 10 000. Up to the present time we are unable to explain whether the broad dispersion of the molecular weight is due to the presence of different substances or due to the different size of molecules of DAK-16, broken or split during the extraction procedure. As mentioned above DAK-16, isolated by precipitation, gel filtration or dialysis, furnishes a uniform, slightly tailing zone on paper electrophoresis

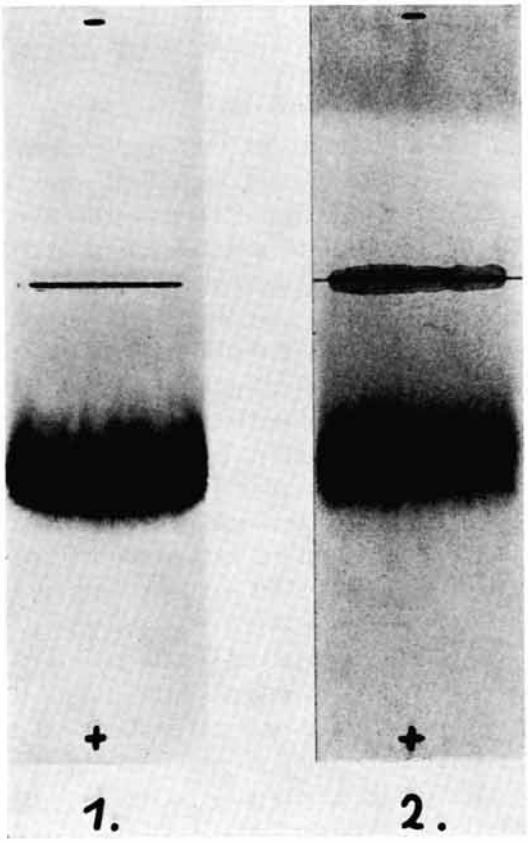

Fig. 2. Paper electrophoresis of DAK-16 (No. 1) and cartilage extract (No. 2).

with different buffers. The high molecular zone can be made visible by toluidine blue or by gentiana violet staining. This fact indicates the mucopolysaccharide character of the substance.

After hydrolysis with hydrochloric acid the fraction DAK-16 can be split into the following constituents:

Taurine, hydroxyproline, asparagic acid, threonine, serine, phosphoserine, glutamic acid, proline, citrulline, glycine, alanine, cystine, valine, methionine, isoleucine, leucine, tyrosine, phenylalanine, lysine, histidine, arginine, hexosamine and uronic acid.

The content of hexosamine was around 25%, that of total amino acids between 25 and 30%. Separation and quantitative analysis of the amino acids on ionexchange columns showed an especially high content of: alanine, glycine, glutamic acid, hydroxy-

proline, and proline. This fact indicates that the peptide part of the fraction DAK-16 may be of a collagen type.

Gel filtration of fraction DAK-16.

In order to prove the uniformity of the fraction DAK-16 we tried separations by gel filtration with Sephadrex types: G-75, G-100, and G-200 with different buffers and with urea (to prevent aggregations). 100 mg DAK-16 were dissolved in 2-3 ml buffer and submitted on Sephadex columns with a bed size of 75 cm length and 2 cm diameter. The buffers used were: HCl-KCl buffer pH 1.0, phosphate buffer pH 6.4 and phosphate buffer pH 6.4 + 2 mol urea. Using the three mentioned gel types in all experiments two ultraviolet absorbing peaks were noticed. The first peak always appeared within the void volume of the colum, and contained the high molecular substance, while the second peak contained free amino acids. Fig. 3 (Sephadex G-75) and Fig. 4 (Sephadex G-200) show the typical elutions of DAK-16 by $^1/_{15}$ mol phosphate buffer pH 6.4, optical density measurement at 260 nm.

The free amino acids within the second peak are presumably split off from DAK-16 by hydrolysis. Our finding that the high molecular fraction is excluded even by Sephadex G-200 indicates a much higher molecular weight as was found by the ultracentrifuge method. This phenomenon may be due to aggregation. On the other hand it is well known that polysaccharides do not follow a normal pattern in gel filtration.

Discussion

Our analytical investigations concerning the cartilage extract in *Rumalon®* have shown that this extract contains low molecular components (free amino acids, nucleo derivatives) as well as a high molecular fraction, which can be considered as a mucopolysaccharide-peptide complex. Until now it has not been known, how many and what sort of constituents of the cartilage extract are essential for the clinical effect, though in our laboratory we could demonstrate that the high molecular fraction DAK-16 has a specific influence on fibroblast cells in tissue culture (*Karzel and Domenjoz, Karzel* et al.). As mentioned by *Weigel* (personal communication) the characteristic effects on sulfate uptake in rat

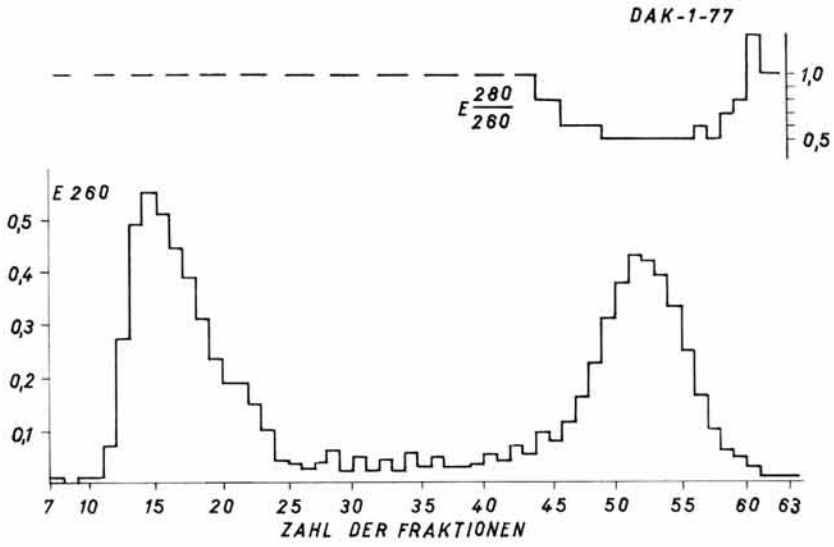

Fig. 3. Fraction DAK-16 on gel filtration with Sephadex G-75.

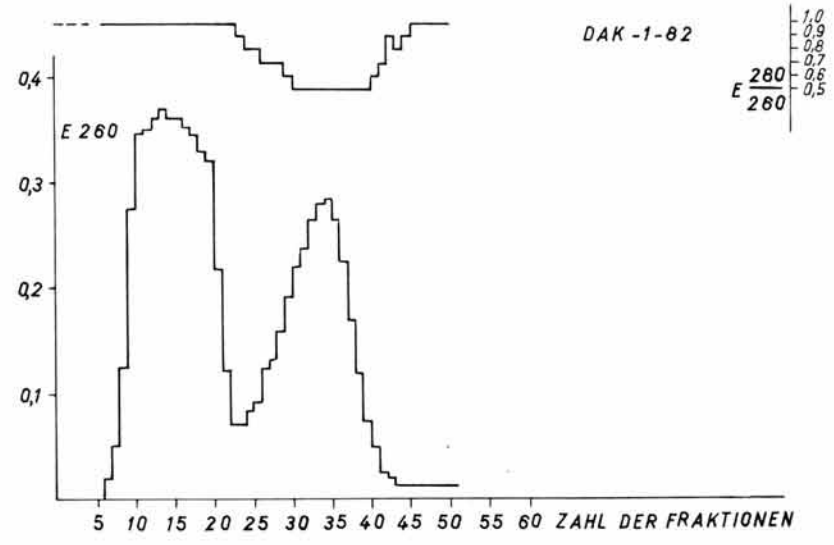

Fig. 4. Gel filtration of fraction DAK-16 with Sephadex G-200.

costal cartilage as reported in the literature (*Weigel and Jasiński*, 1962) can be ascribed to the low molecular fraction. Further research on other effective components of the extract and on the mode of action are in progress.

Summary

Analytical experiments on the components of the cartilage extract in Rumalon® are reported. The extract was separated by different methods in a low and a high molecular fraction. The low molecular fraction contains free amino acids and nucleo derivatives. The high molecular fraction is uniform on electrophoresis and can be considered as a mucopolysaccharide-peptide complex with an average molecular weight of 55 000. The peptide part seems to be of collagen type.

Zusammenfassung

Analytische Untersuchungen über die stoffliche Zusammensetzung des Knorpelextraktes im Rumalon® werden beschrieben. Mit verschiedenen Methoden gelingt es, aus dem Extrakt eine hochmolekulare und eine niedermolekulare Fraktion abzutrennen. Während der niedermolekulare Anteil freie Aminosäuren und Nukleoderivate enthält, besteht die hochmolekulare Fraktion aus einem elektrophoretisch einheitlichen Mucopolysaccharid-Peptid-Komplex mit einem durchschnittlichen Molekulargewicht von 55 000. Die Aminosäurenzusammensetzung weist auf einen kollagenen Charakter des Peptidanteils hin.

Résumé

On donne des résultats des analyses de composants de Rumalon dans des extraits de cartilage. L'extrait a été repéré au moyen de différentes méthodes dans une fraction de poids moléculaire basse et dans une fraction de poids moléculaire élevée. La fraction de poids moléculaire basse contient des acides aminés libres et des dérivés nucléiques. La fraction de poids moléculaire élevée est uniforme à l'électrophorèse et peut être considérée comme un complexe mucopolysaccharide-peptide avec un poids moléculaire moyen de 55 000. La partie de peptide semble être de type collagène.

References

Karzel, K. und *Domenjoz, R.:* Stimulation der Mucopolysaccharidproduktion in Gewebekulturen durch einen biologischen Faktor. Med. exp. *9:* 385 (1964).

Karzel, K.; Kalbhen, D. A. and *Domenjoz, R.:* Influence of a cartilage bone marrow extract on the mucopolysaccharide metabolism of cells cultured in vitro. Med. exp. *14:* (1966) (in press).

Weigel, W.: Robapharm AG, Basle: personal communication.

Weigel, W. und *Jasiński, B.:* Über die Beeinflussung des Knorpelstoffwechsels durch einen Knorpel-Knochenmark-Extrakt. Path. Microbiol. *25:* 400 (1962).

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