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Chromatographic Studies on Rumalon®¹

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Introduction

RUMALON®, produced by Robapharm Ltd., Basle, is an extract of cartilage and red bone-marrow. It is proven to be clinically efficient in osteo-arthritis cases of various localization. In spite of the fact that RUMALON® is a tissue extract, it is possible to have some information about its chemical composition. A large number of analytical data regarding the composition of RUMALON® has been made available using chromatographic methods, e.g. paper-, thin-layer- and column-chromatography.

Our chromatographic research concerning RUMALON® consists of two parts: on the one hand, using paper-, thin-layer- and column-chromatography attempts were made to detect and determine biologically important compounds, such as *nucleic acid derivatives*, *amino acids* and *peptides directly*, on the other hand, experiments were carried out, applying different column-chromatographic methods, to fractionate the cartilage extract.

Methods

(Technical Assistance: A. Kunz)

Thin-layer chromatography of *nucleo-derivatives* (Pataki 1967) was carried out on purified cellulose layers with the following solvents: n-propanol/25% ammonia/water (6:3:1 v/v) and isopropanol/saturated ammonium sulphate/water (2:79:19 v/v). *Qualitative* analysis of *amino acids* was performed by thin-layer chromatography (Pataki 1966) on Silica Gel, containing starch, with n-butanol/acetic acid/water (4:1:1 v/v) and phenol/water (3:1 w/w). Screening of column-chromatographic fractions was also made by high-volt electrophoresis on

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Whatman No. 1-paper (buffer: 0.75 M formic acid, pH = 2; 4000 V; 55–60 mA; 45 min). *Mucopolysaccharides* were separated by *paper-chromatography* (Schleicher & Schuell 2043 BmgI; n-propanol/25% ammonia/water 40:60:5 v/v, or 9 ml 0.2 M Na₂HPO₄ + 16 ml 0.12 M NaH₂PO₄ + 75 ml water + 25 ml n-propanol) and paper electrophoresis (Whatman No. 1-paper; HCl/KCl buffer pH = 1,2; 110 V; 10–15 mA, 6 hours). *Amino acids* were detected by *Ninhydrin* (cf. Pataki 1966), *nucleo-derivatives* were visualized in UV-light (254 and 360 nm) and *mucopolysaccharides* were stained by *Toluidinblue* (Merck staining reagent Nr. 222).

Quantitative analyses of amino acids were performed according to *Moore and Stein* (Spackman, Stein and Moore 1958), using an automatic analyzer, by Dr. Curtius (Paediatr. Univ. Clinic, Zurich, Switzerland).

Fractionations of the *cartilage extract* were performed using Sephadex G-25; Sephadex G-15; DEAE-cellulose and Cellulose with an Ultrarac fraction collector and automatic registration (Recorder 6520 H) of the ultraviolet absorption (LKB, Stockholm, Sweden).

Biological assays in cell culture test (*Karzel and Domenjoz*) were carried out by Dr. Karzel (Institute of Pharmacology, University of Bonn, Germany).

Activity measurements in ³⁵S uptake test were performed according to Weigel and Jasiński (Weigel and Jasiński, 1962).

Results and Discussion

In order to detect *nucleic acid bases, nucleosides and nucleotides* in RUMALON[®], experiments have been made using our paper- and thin-layer-chromatographic techniques (Pataki, 1967; Pataki and Strasky, 1968). As shown in Figure 1 about twenty absorbing and two fluorescent spots were observed on thin-layer

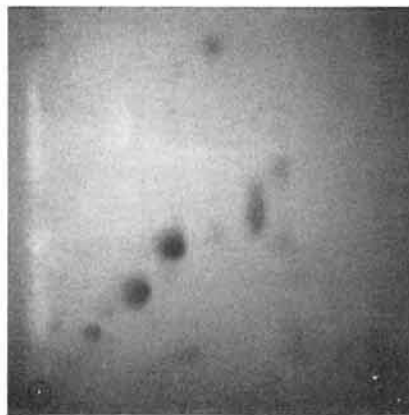


Fig. 1. Detection of nucleic acid bases, nucleosides, nucleotides and related compounds in Rumalon[®]. UV-photography. According to Pataki (1967). Some of the minor spots are not visible on the reproduction.

chromatograms. Similar results can be obtained by paperchromatography. Tentative identification of nucleo-derivatives was made by the well-known spot-strengthening technique (e.g. *Stahl, 1967; Randerath, 1965; Pataki, 1966*). The results are shown in Figure 2. As we can see, there also have been a few unknown spots detected. Further experiments to identify these compounds and to verify our findings, applying different separation and spectroscopic techniques, are now under way. The identification of *nucleic acid derivatives* in RUMALON[®] is especially important, since, as it will be shown later in this paper, these substances, among others, were detected in a biologically active fraction, obtained from the cartilage extract.

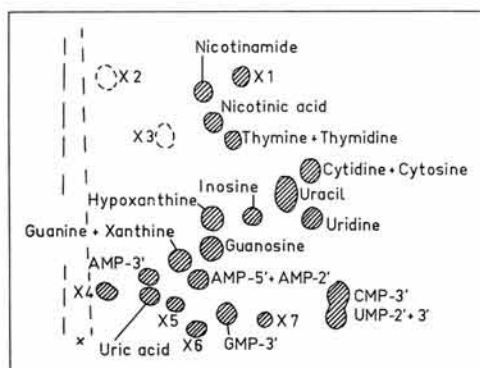


Fig. 2. Chromatographic identification of nucleic acid bases, nucleosides, nucleotides and related compounds in Rumalon[®] (schematic).

Quantitative analyses of *amino acids* were made by column-chromatography, using an automatic analyzer according to *Moore and Stein (Spackman, Stein and Moore, 1958)*. The results are shown in Table I. It is especially worthy of note that the amounts of *amino acids increase* after hydrolysis, that means not only *free amino acids* but also *bound amino acids* are present in RUMALON[®]. This fact is not a surprise, since it was possible to isolate a highmolecular mucopolysaccharide-polypeptide complex of cartilage extract (*Kalbhen, 1966; Karzel, Kalbhen and Domenjoz, 1966*). We are of the opinion that RUMALON[®] also contains other polypeptides, since a number of spots on thin-layer chromatograms cannot be ascribed to free amino acids. The presence of

TABLE I

Free and bound amino acids in Rumalon® determined by column chromatography, according to Spackman, Stein and Moore

Compound	before hydrolysis	after hydrolysis	Compound	before hydrolysis	after hydrolysis
Taurin	2.41	2.31	Cystathionine	0.08	0.15
Urea	2.17	1.66	Methionine	0.22	2.25
Methioninsulfoxide	0.56	0.38	Isoleucine	0.74	4.49
Hydroxyproline	0.45	5.84	Leucine	1.68	13.04
Aspartic acid	1.37	18.31	Tyrosine	1.00	4.36
Threonine	0.54	12.24	Phenylalanine	0.98	6.43
Serine	0.97	7.33	β -Alanine	0.07	1.41
Asparagine + Glutamine	0.37	—	β -Hydroxylysine ¹	traces	0.50
Sarcosine	traces	traces	γ -Aminobutyric acid	—	0.44
Proline	2.22	14.43	Ornithine	0.04	0.05
Glutamin acid	8.39	31.72	Ethanolamine	0.56	0.94
Citrulline	0.36	traces	Lysine	0.38	—
Glycine	4.32	21.55	Lysine	1.54	11.30
Alanine	4.99	13.89	1-CH ₃ -Histidine	0.08	0.68
α -Aminobutyric acid	0.37	0.51	Histidine	0.34	7.11
Valine	1.34	8.01	Tryptophane	0.16	2.31 ²
1/2 Cystine	0.09	traces	Creatinine	57.37	19.56
			Arginine	0.53	11.00

¹ One of the β -Hydroxylysines is the allo modification.

² These values are probably too high, maybe due to the presence of compounds which were formed by hydrolysis.

polypeptides will now be investigated, since, as it will be pointed out later in this paper, a biologically active fraction of the *cartilage extract* probably contains some peptides, besides other substances.

In previous studies two different active principles in RUMALON® have been demonstrated. *Karzel* and *Domenjoz* (*Karzel* and *Domenjoz*, 1963) have found a stimulating effect of RUMALON® on the production of mucopolysaccharides by cells cultivated *in vitro*. *Weigel* and *Jasiński*, 1962) have shown, on the other hand, that the *in vitro* uptake of ³⁵S in the rat cartilage is significantly increased by RUMALON®. There is no doubt that the stimulation of mucopolysaccharide production in tissue cultures can be ascribed to the highmolecular substances contained in RUMA-

LON[®] (Karzel, Kalbhen and Domenjuz, 1966; Kalbhen, Karzel and Domenjuz, 1966; Kalbhen, 1966; Kalbhen, Karzel, Pritz and Domenjuz, 1967; Karzel, Kalbhen and Domenjuz, 1967). This highmolecular fraction, designed as DAK-16, has been isolated, subsequently by Kalbhen *et al.* (Kalben, 1966; Kalbhen, Karzel and Domenjuz, 1966) either by precipitation or by gel-chromatography on Sephadex G-25 coarse (Fig. 3) and has been found to be active in tissue cultures (Fig. 4). The lowmolecular substances contained in RUMALON[®] have been found, on the other hand, inactive in this biological assay. Investigations in our Biochemical Department have shown that the lowmolecular fraction of RUMALON[®] (cf. Fig. 3) is capable of activating the *in vitro* uptake of ³⁵S in the cartilage. Subsequently, we have found that the substances with lowmolecular weight can be more efficiently separated using Sephadex G-25 fine and longer columns (Fig. 5). The partial resolution of the highmolecular fraction into two peaks is probably due to some adsorption effects which can be observed on high cross-linked Sephadex, especially if water is used as eluent. Finally, we would like to mention that about 5–10% of the material applied to the column cannot be recovered with water, and, as indicated in Figure 5, solvent change is necessary to elute this part of the extract. This phenomenon can most probably be ascribed besides adsorption to the ion-exchange properties of Sephadex. The activity of the lowmolecular fractions, according to Figure 5, in ³⁵S test is now being investigated; they are, of course, inactive in tissue cultures.

DAK-16, the highmolecular part of RUMALON[®], is excluded even by Sephadex G-200, which indicates a much higher molecular weight as was found by ultracentrifugation, namely $55\,000 \pm 10\,000$ (Kalbhen, 1966). As already mentioned, a partial resolution of this fraction into two peaks can be observed on Sephadex G-25 fine. Very recently Kalbhen *et al.* (Kalbhen, Karzel, Pritz and Domenjuz, 1967; Karzel, Kalbhen and Domenjuz, 1967) were able to fractionate DAK-16 into three fractions by ion-exchange-chromatography on Dowex-1 \times 2. The activity measurements in tissue cultures are, as far as we are aware, not yet completed. We were also able to obtain three fractions using Sephadex G-15. DAK-16 has been characterized as a mucopolysaccharide-polypeptide complex (Kalbhen, 1966; Kalbhen, Karzel and Domenjuz, 1966), and it has been subsequently found that the peptide part is

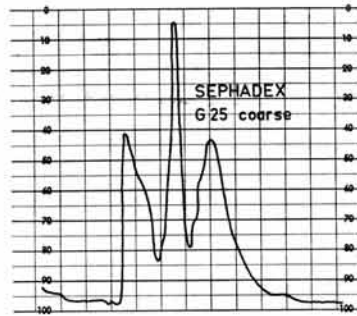


Fig. 3. Isolation of a highmolecular mucopolysaccharide-polypeptide complex of cartilage extract by gel-chromatography on Sephadex G-25 coarse, according to Kalbhen *et al.* (Kalbhen, 1966; Karzel, Kalbhen and Domenjcz, 1966).

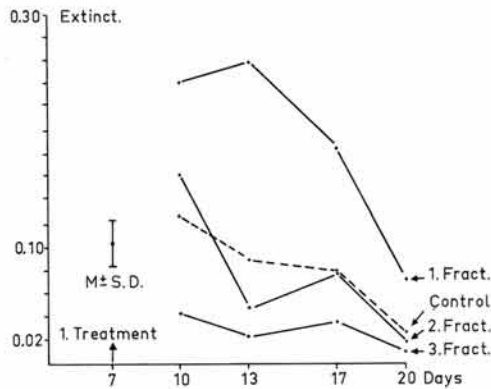


Fig. 4. Action of the fractions of a cartilage extract (cf. Fig. 3) on the mucopolysaccharide content in culture medium of fibroblasts according to Karzel *et al.* (Karzel, Kalbhen and Domenjcz, 1966).

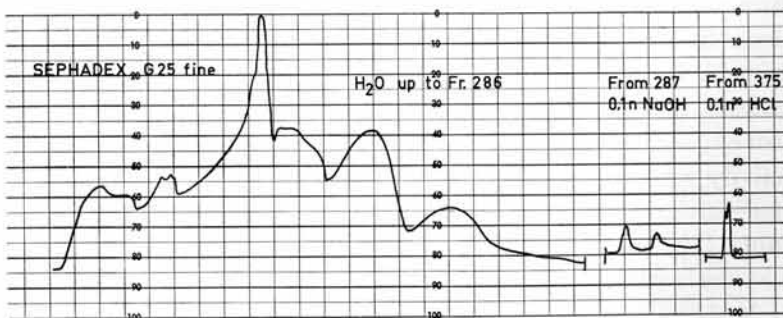


Fig. 5. Fractionation of cartilage extract on Sephadex G-25 fine.

important as being active in tissue culture test (*Kalbhen, Karzel, Pritz and Domenjoz, 1967*).

In order to obtain some evidence on the chemical nature of substances which are active in ^{35}S -incorporation test, we are investigating the lowmolecular substances of RUMALON[®] in further detail. Some of our results have already been mentioned at the beginning of this paper. The fractionation of the cartilage extract on DEAE-cellulose (OH-form) is shown in Figure 6. As we can see two UV-positive peaks can be observed. The second UV-positive peak contains, for instance, all the *nucleo-derivatives*. Thin-layer-chromatography and high-volt electrophoresis have shown that the fractions I, II, III and IV contain ninhydrin-positive material, e.g. amino acids, amines, peptides. Fraction V has given on Silicagel thin-layers, in solvents suitable for the analysis of amines, amino acids and peptides, only one spot at the origin, which indicates the high molecular weight of this substance. As a matter of fact, further investigations have shown that this substance, or these substances, can be detected by Toluidinblue, that means it is, or they are, mucopolysaccharide-like compounds. Since no UV-absorption could be observed, this, or these, mucopolysaccharides do not contain any peptide chain and cannot be identical with DAK-16. As already mentioned, the peptide part seems to be a requisite for the activity in tissue cultures. We would, therefore, assume that fraction V shows no activity in this biological assay. Dr. *Karzel* was kind enough to investigate our fraction V, and found, in fact, no activity. Activity measurements by ^{35}S -uptake assay have been carried out in our Biochemical Department. The results are shown in Table II. These preliminary results indicate that fraction III exhibits an activity, whereas the other fractions show no activity at all. Of course the sum of fractions I to V, as given in the table, shows approximately the same activity as fraction III. The difference between the activity of the starting material and fraction III (cf. Table II) is, as a statistical analysis proved, not significant. In order to prove the significance of the activity which has been found in fraction III, measurements were made on greater experimental material; the results are shown in Table III. Since fraction III contains *amino acids* and *nucleic acid derivatives*, an artificial mixture of amino acids found in RUMALON[®] (cf. Table I) has been made and activity measurement by ^{35}S -uptake assay has been carried out. As Table IV shows,

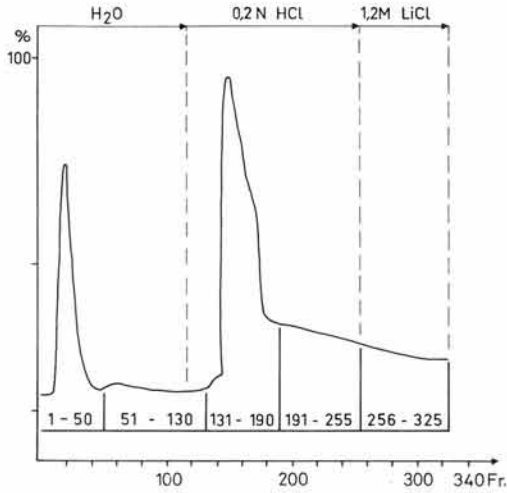


Fig. 6. Fractionation of cartilage extract on DEAE-Cellulose.

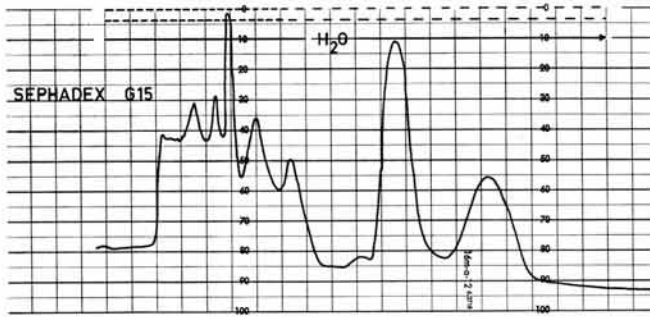


Fig. 7. Fractionation of cartilage extract on Sephadex G-15.

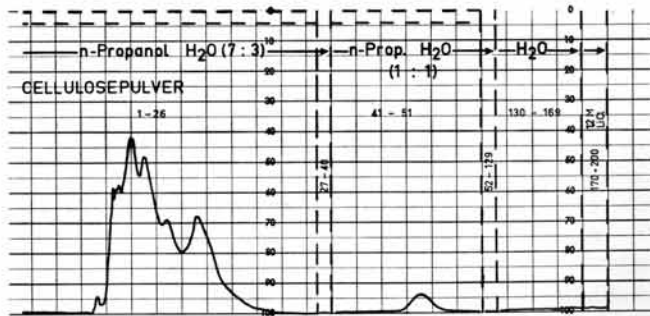


Fig. 8. Fractionation of cartilage extract on cellulose.

TABLE II

Stimulation of in vitro uptake of ^{35}S in the rat cartilage by cartilage extract and its fractions according to Figure 6

cpm/100 mg dry substance	
Control	937 \pm 100
Cartilage extract	1236 \pm 270 ¹
Fractions 1-55 (I)	850 \pm 234
Fractions 56-160 (II)	762 \pm 164
Fractions 161-190 (III)	1109 \pm 170 ^{1, 2}
Fractions 192-220 (IV)	858 \pm 128
Fractions 221-315 (V)	901 \pm 150
Fractions 1-315 (I-V)	1141 \pm 211 ²

} Mean values
± standard deviations

¹ These two mean values are identical according to t-test.

² These two mean values are identical according to t-test.

TABLE III

Statistical analysis of ^{35}S uptake by fractions of a cartilage extract (compare text and Tab. II)

cpm/100 mg dry substance ¹	
Control	29 700 \pm 1040 ^{2, 3}
Fractions 1-315 (I-V)	32 950 \pm 2470 ²
Fractions 161-190 (III)	33 125 \pm 3050 ³

} Mean values
± standard deviations

¹ n = 12.

^{2, 3} The differences are significant (p < 0.0005).

TABLE IV

Effect of an artificial amino acid mixture (cf. Tab. I) on the in vitro uptake of ^{35}S in the rat cartilage

cpm/100 mg dry substance	
Control	2096
Amino acids ¹	2118
Amino acids ²	2294
Rumalon [®]	3258

¹ Concentration as given in Table I.

² Concentration five times more than given in Table I.

such an artificial amino acid mixture does not exhibit any activity. This finding indicates that the activity cannot be ascribed to the amino acids contained in RUMALON®. Similar experiments with nucleic acid derivatives are in progress.

Finally, experiments were made (one of them has been shown in Figure 4) to fractionate the cartilage extract: we have used Sephadex G-25 fine (Fig. 5), Sephadex G-15 (Fig. 7) as well as cellulose (Fig. 8). Chemical and biological characterization of these fractions is now being investigated and will be reported soon.

Summary

Using thin-layer chromatography, a great number of *nucleic acid derivatives* was detected in RUMALON®. Quantitative analyses of *amino acids before and after* hydrolysis have proven the presence of *bound amino acids*, besides *free amino acids*. The fractionation of the cartilage extract in RUMALON® by column-chromatography on DEAE-Cellulose, cellulose, Sephadex G-25 fine and Sephadex G-15 is reported. One of the fractions isolated on DEAE-cellulose has been found capable of activating the *in vitro uptake* of ³⁵S in the cartilage. Preliminary characterization of this fraction was carried out by thin-layer chromatography and high-volt electrophoresis: *nucleic acid derivatives*, as well as *ninhydrin-positive* compounds, e.g. amino acids and possibly peptides, were found. An artificial mixture of amino acids which are present in RUMALON® was found, on the other hand, *inactive* in ³⁵S-uptake assay.

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