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THE STUDY OF THE SULPHOMUCOPOLYSACCHARIDES OUT OF MYTILUS GALLOPROVINCIALIS (LMK)

I.A Molnar, E. Tucicov, N. Boesteanu, Y. Cristescu
The Institute of Chemical Pharmaceutical Researches
M. Mîrza
The Romanian Marine Researches Institute

ABSTRACT

The content and the properties of the sulphomucopolysaccharides (MPS) from Mytilus galloprovincialis have been studied. Ont of 1 kg dry tissue 5.9 g of partially purified mucopolysaccharide were obtained, having 3.71 % N, 1.37 % S, 2.25 % hexuronic acid, 11.7 % humidity and 9.3 % residum on calcination.

The study of the toluidine blue colouring function of the MPS concentration in the sample shows firstly the moving of the extinction maximum from 620 to 585 nm, then, on bigger MPS concentrations to 625 nm. From a pharmaceutical point of view, the anticoagulant activity in comparison with the heparine is insignificant. The preparation shows an intense antilipidic and anticholesterolemic activity on rat, against the tritonic hyperlipidemy. A protective effect against the increase of the optical density of the serum, against hyperlipidemy and hypercholesterolemy determined by Triton A 20 are noticed. The colouring properties with toluidine-blue, the very small anticoagulant action and the protective effect in experimented hyperlipidemy and hypercholesterolemy situate the MPS from Mytilus galloprovincialis in the same group with the mucopolysaccharides from the digestive tractum of cattle pork and fowl.

The tissues of the vertebrates, besides heparine, with a very intense activity in anticoagulation, also contains another group of acid sulphomucopolysaccarides, whose hypocholesterolemiant and hypolypemiand activity is similar to that of the heparine, but possesses a much losser anticoagulant activity than the heparine. The aim of our paper

is the identification of the existence of these substances in Mytilus galloprovincialis, their isolation, and the determination of some physical-chemical and pharmaceutical properties.

The first to point out the existence of these substances were Capraro, Cantone 1954; Cresseri 1956, who obtained mucopolysaccharide extracts from pork stomach mucous membrane, acting upon the lipide metabolism; then, Vressori describes the way of obtaining and the properties of these substances which contain hexuronic acid (D.glucuronic) 34,6 p.c. hexozamine (d.glucozamine) 25.5 p.c., reducing substances (d.glucose) 25.5 p.c., acetyl groupe 6.1. p.c., and sulphur 0.5 p.c. The sulphur content of the new mucopolysaccharide is smaller than that of the heparine.

A little later Bianchini 1957 prepared an acid mucopolysaccharide from pork duodenum and small intestine, which has an action upon the metabolism of the lipides, especially on the cholesterole, but its anticoagulant activity is much lesser than that of the heparine. This substance, because of its special pharmaceutical properties, is used in therapeutics under the names of Ateroid and Asclerol. The substance isolated by Bianchini containe 31.8-38.2 p.c. hexuronic acid and 24.3 p.c. hexazamine. It is not yet known if the substance obtained by the two authors are identical between them.

The works were continued by Molnar and coll. (1966 a, b, c; 1970), who determined this type sulphomucopolysaccharide content in various tissues of the digestive canal of cattle and porcines, then of fowl intestines, and determined some physical-chemical properties, such as the reaction of toluidine blue-colouring function of the mucopolysaccharide in the sample, the electroforesis migration speed, and the action on the lipide and cholesterole metabolism in a Triton-intoxicated rat.

As for Mytilus galloprovincialis, of other species of the Mytilus genus, data on their content in mucopolysaccharides cannot be found in literature.

In the present paper, we have studied the mucopolysaccharides of Mytilus galloprovincialis, common species, in the Black Sea.

MATERIAL AND METHODS

A. Preparatory works

In our experiments we have studied Mytilus galloprovincialis gathered on the 26-th of August 1973 at the Cape of Midia. The mussels were of different sized, as they are found naturally. The tissue was separated from the shells and used totally. The tissue is minced in a mincing machine with 2-3 mm diametre holes, then dried in void. The dry tissue was ungreased by successive Saxholet extraction, with ethylic ether and dichloretan. From the material thus obtained, the organic solvents are removed with a 50°C air draught. 8 volumes of of water are added to one volume of degreased tissue, and the pH is adjusted to 4.5-5.0 with hydrochloric acid, and the mixture is warmed to boiling. After cooling to 70°C, Na OH is added up to pH 6.8, ans it is mixed for two hours, the pH is modified to 8.0 with NaOH, and it is kept at 70°C for an hour, then the extract is separated from the insoluble parts. Other three extractions are done at 70°C and pH 8.0 with distilled water. The extracts united together are concentrated in void to about 1.5 times as compared to the tissue kept for extraction.

To the concentrated extract, another pork pancreas extract is added, is order to hydrolize, the links existing between the mucopolysaccharides and the proteins. The digestion is achieved at pH 7.5-8.0, at 37-40°C, and lasts for two days. The hydrolized solution is filtred and concentrated in void to about a quarter of the quantity of the initial volume, and is dialized on a cellophane membrane at maximum 5°C with running water for two days. During the dialize, the volume of the solution grows 2-3 times, is concentrated to the initial volume and is dialized again for two says. The dialized substance is concentrated again in void like before, the a 50 p.c. trichloracetic acid solution is added for precipitating proteins, to reaching a concentration of 10 p.c. trichloracetic acid in the mixture; after a day's rest, it is filtred. On the filtred substance, its five times acetone volume is added. The rough mucopolysaccharide sample gets

precipitated. It is filtred and dried at the room temperature. For purification, it is re-dissolved in ten times its volume in water and it is dialized with running water for two days and it is precipitated in acethone. The purified product has not the purity of that described by Bianchini, but it corresponds for the study of some physical-chemical and pharmaceutical properties.

B. Analytical works

For characterizing the product obtained, various physical-chemical determinations have been made, such as: humidity, residum on calcination, nitrogen, sulphur, anticoagulant activity, by current methods; also, a series of determinations specific of isolated mucopolysaccharide alone, according to corresponding specific techniques.

1. The Identification of the Mucopolysaccharide

Reagents: toluidine-blue solution: 0,5 g toluidine blue are dissolved in 50 ml methylic alcohol and is completed to 100 ml with 20 p.c. watery solution of glacial acid.

- 5 p.c. acetic acid watery solution
- Phosphate tampon M/30 pH = 7.0
- Whatmann paper No.1.

Way of working: 0.5 g of the analysed substance is dissolved in about 8 ml distilled water at 60°C, by adoing NaOH 0.5 N up to pH = 7.5. The volume of the solution is brought to 10 ml with distilled water. It is filtred. From the filtred solution a drop is placed on a paper strip, it is dried, then it is sprayed with toluidine-blue solution. It is left so far one minute, then the colour excess is removed by repeated washings with acetic acid solution, then it is dried. The acid mucopolysaccharide leaves a blue-violet spot on a slightly bluish environment. For identifying the spot, it is out off from the rest, and 10 ml phosphate tampon solution is added by warming upon the water bath, and its absorbtion spectre in the visible

range is determined in the 1 cm cell. By this technique, only the combination formed by the mucopolysaccharide and the toluidine blue is fixed, without either's being in excess, and the other substances are removed by washing, in this case the spectre being not misrepresented, which can happen when the reaction is achieved directly in the solution.

2. The quantitative Determination

For determining the mucopolysaccharide quantitatively, the content in hexuronic acid by the Dische method (1947) was determined, after the separation of the MPS fraction by electroforetic migration on paper, method used in other works too Molnar and coll.1966 b,c. In the case of an Ateroid-Asclerol-type mucopolysaccharide, the pure product is admitted to contain 34 p.c. hexuronic acid.

3. The Metachromatic Reaction Study

Reagents:

- Toluidine-blue watery solution 6.1 mg/l (2×10^{-5} M)
- Phosphate tampon pH = 7 M/15

Way of working: a MPS solution is prepared, exactly as previously, from which various dilutions are made.

To 14 ml toluidine-blue solution, 0.5 ml phosphate tampon and 0.5 ml of the MPS are added. To the samples obtained, the absorbtion spectre modification function of the MPS concentration in the sampls is searched.

4. The Electroforetic Migration Speed

It has been determined on paper, in the horizontal system, in borate tampon, according to a technique described previously. (Molnar; Winter, 1966 c.).

5. The Pharmaceutical Study of the Hypocholesterolemiant and Hypollipemiant Actions

The determination were achieved according to techniques described in a previous paper (Molnar; Simionovici; Tucicov; Georgescu; Winter 1966 a). Adult male rats, after a 24 hours fast, were divided in three groups of 10 animals each. The first group (intoxicated) received an intravenous shot of a 25 p.c. Triton A-20 solution "Serva" (polyoxyethylene p-isoethylphenol) in distilled water in a dose of 500 mg/body Kg. The second group received a Triton shot under the same conditions, followed immediately by an intraperitoneal injection of mucopolysaccharide in the dose of 89 mg MPS (corresponding to 12 mg hexuronic acid in 10 ml water) of the raw preparation, and of 89 mg MPS (corresponding 70 20 mg hexuronic acid) of the purified preparation in 10 ml water/body kg. The third group was constituted of witness animals, which were not treted in any way. The animals were kept fasting for another 18 hours, after which they were immolated for having their blood taken from them. The blood was taken on citrate and centrifugated. In the plasma thus obtained, the optic density, the total lipemy and the cholesterolemy were determined.

The optic density is spectrophotometrically determined at 610 mm in the 1 cm vat, the lipemy after the Bloor modified method to a standard curve established with tributyrine, and the cholesterolemy after the Liebermann - Burghardt method.

For each determinatio, the hindering effect (named the protective effect) of the tritonic hyperlipidemy was calculated in percentage according to the following formula:

$$E\ \% = 100 - \frac{T - C}{I - C} \times 100, \text{ where}$$

E % - percentage protective effect

T - average value obtained from the animale protected with MPS (value of extinction, total lipides, cholesterole)

C - average value obtained from the untreated witness animals

I - average value obtained from the animals which received only Triton.

RESULTS AND DISCUSSIONS

The dry tissue contains 10.38 p.c. total lipides. Achieving the extraction of the acid mucopolysaccharide by the method described, out of 1 kg dry degreased tissue, 30.1 g raw preparation, respectively 5.9 g purified preparation were obtained. The preparation obtained has not the degree of purity corresponding to that obtained by Bianchini, but it is similar to those obtained by Molnar and col., out of various tissues of the digestive tube from cattle, hog and fowls, ans is qualitatively good for establishing certain physical-chemical and phyarmaceutical properties. The following analytical data have been obtained for the two preparations:

Table 1

Analytical results obtained with MPS samples

	Raw MPS	Purified MPS
Loss on drying at 105°C %	8,7	11.7
Residum on calcination, %	18.5	9.3
Total N, %	5.86	3.71
Total S, %	0.94	1.37
Hexuronic acid, %	1.34	2.25
Anticoagulant activity corresponding to I U Heparine/mg	0.02	0.02
Electroforetic migration speed U	5.0-5.4	5.0-5.4
Metachromatic reaction on toluidine-blue paper	intensely violet	intensely violet
Maximum of extinction = nm	585	585

The identification of mucopolysaccharide achieved according tothe technique described at point 1, shows the maximum of extinction for

the two samples is situated at 585 nm, and the spectres are identical. In comparison with a mucopolysaccharide sample prepared by us from pork pancreas, a surprising similarity is seen between the absorbtion spectres (fig.1) which proves that the structures of these mucopolysaccharides, coming from totally different species are very much related.

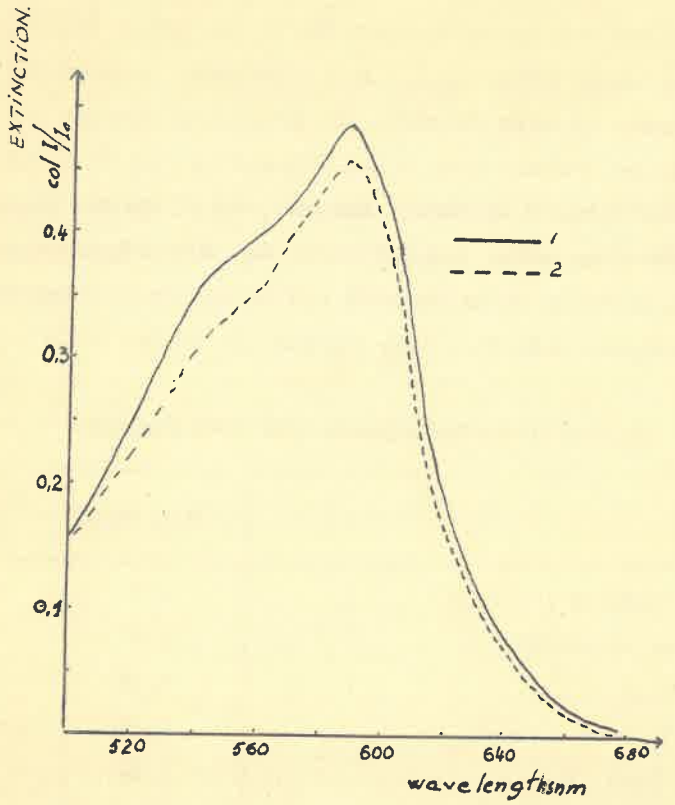

Fig.1. Reaction for the identification of the mucopolysaccharides
1- MPS from Mytilus galloprovincialis; 2- MPS from pork pancreas

On studying the metachromatic reaction with toluidine-blue in the function of the MPS content in the sample for the purified sample, there has been visually stated the appearance of a violet shade whose intensity increases with the MPS concentration in the sample, reaching a maximum, after which a second reaction appears and the colours of the samples pass gradually to light blue. This colour modification was quantitativele searched

spectrophotometrically between 500-700 nm. In the first stage of the reaction the moving of the maximum of extinction of the toluidine-blue to shorter wave-lengths is noticed, this being known under the name of metachromatic reaction; in the second stage the maximum of extinction moves to longer wave lengths. At a concentration of 3 mg MPS in the 15 ml sample, maximum of extinction is at 580 nm, and on concentrations stronger than 24-32 mg MPS in the sample, at 625 nm, slightly moved to longer wave lengths in comparison with 620 nm, the maximum of extinction of the toluidine-blue (fig.2).

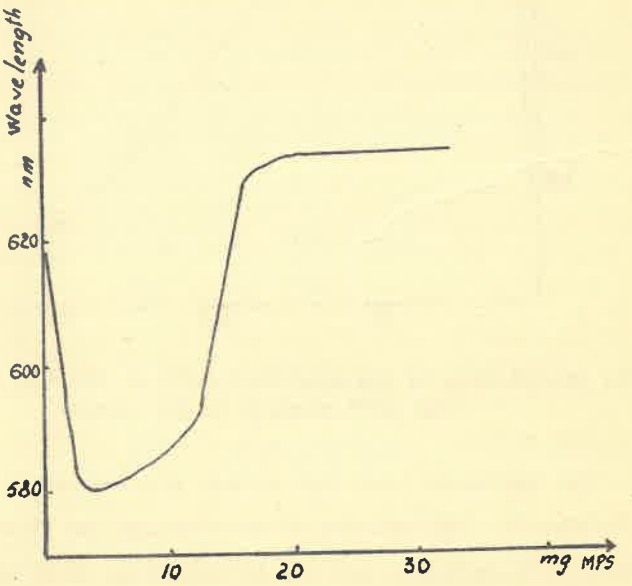

Fig.2. The modification of the position of the maximum of extinction depending on the MPS concentration in the 15 ml sample

Following the extinction modifications at 620 nm, the wave lengths corresponding to the maximum of extinction of the toluidine-blue in the first stage of the reaction, a decrease of the value of the extinction is stated, while in the second stage an increase is seen. The reaction can be best traced by the modifications of the extinction values ratios for the two wave lengths correspondingly chosen function of the MPS content in the

sample. The extinction ratios for the 580 and 630 nm wavelengths are shown in fig. 3. Here, in the first stage an increase, and in the second a decrease of the ratio is noticed.

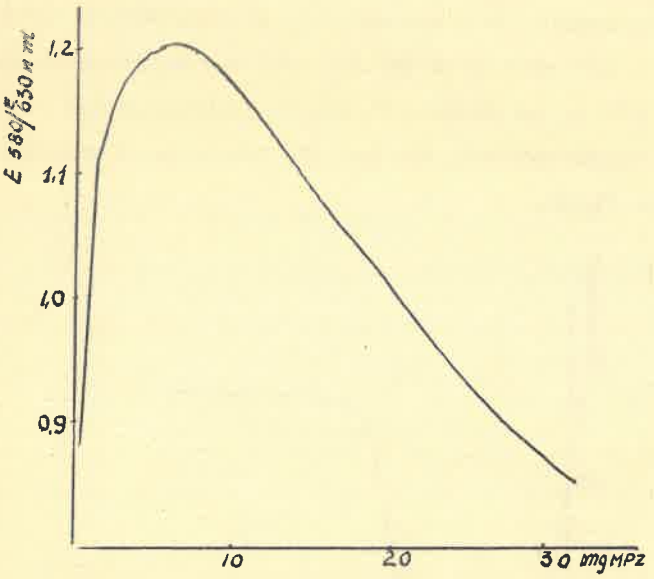

Fig. 3. The modification of the extinction ratio at 580/630 on depending on the MPS quantity in the sample

The maximum value was noticed at a content ot 8 mg purified MPS in the sample. The spectres of the combinations given by MPS in various quantities in samples, with the toluidine-blue in comparison with the toluidine blue are shown in fig. 4.

Comparing the absorbtion spectres obtained in the first stage of the reaction, the so called metachromatic reaction obtained with mucopolysaccharides of various extractions, heparine from cattle lungs (Biofarm), MPS from pork duodenum (Biofarm), MPS from fowl intestine (prepared at I. C. C. F.), with the preparation obtained by us, it can be seen that the mucopolysaccharides obtained from Mytilus galloprovincialis, pork duodenum and hen intestine have a common characteristic, that is the maximum of extinction at 580-585 nm, while the maximum of extinctic of the combination formed with the heparine is found at shorter wavelengths (see fig. 5).

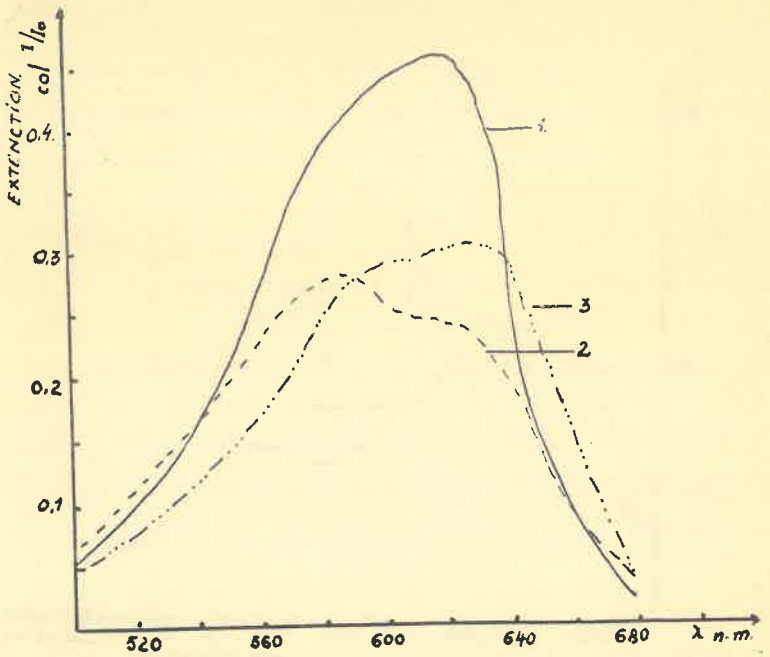

Fig. 4. The absorbtion spectre of the toluidine-blue in the presence of the mucopolysaccharide from Mytilus in various quantities
1- A.T. (toluidine blue solution); 2- A.T. and 8 mg MPS in the sample; 3- A.T. and 32 mg MPS in the sample

Furthermore, comparing the absorbtion spectres in the second stage of the reaction in the case of the mucopolysaccharides from pork duodenum, fowl intestine (Molnar, 1966 c, 1970) and Mytilus, the maximums are slightly moved towards longer wave-lengths in comparison with the maximum of the toluidine-blue, while in the case of the heparine, the two maximums coincide in the wave-lengths, only the values of the extinctions and the aspects of the spectres are different.

The electroforetic migration speed is of 5.0-5.4 and coincides with the migration speeds previously found by Molnar and Winter (1966 c) for the mucopolysaccharides from the digestive tube of cattle, hog, and hen intestine.

The result of the pharmaceutical control of the raw and purified MPS are to be seen in the table below.

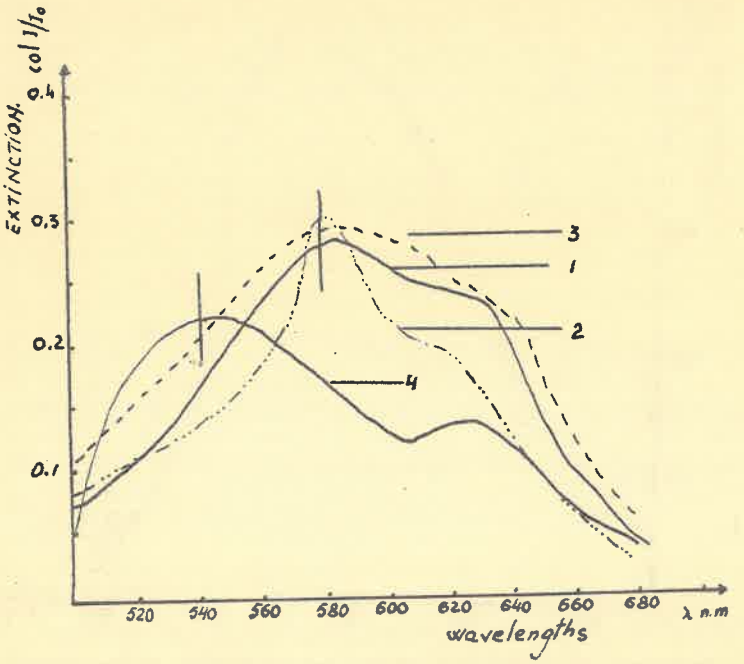

Fig. 5. The absorbtion spectre of the toluidine-blue in the presence of various mucopolysaccharide
1- MPS from Mytilus galloprovincialis; 2- MPS from fowl intestine;3-MPS from pork duodenum; 4- Heparine

Table 2

The antilipemic effect

A. Optic density

L o t	Number of animals	D.O.	P	Protective effect, %
Intoxicated with triton	20	0.94±0.068	-	-
Witness	20	0.058±0.003	-	-
Intoxicated and treated with Raw MPS	20	0.35±0.015	0.01	67.0
Intoxicated and treated with purified MPS	20	0.49±0.027	0.01	51.2

Table 2 (continuation)

B. Total lipemy

Lot	Number of animals	Total lipides mg in 100 ml	P	Protective effect %
Intoxicated with Triton	20	406.0±26.7	-	-
Witness	20	72.9±15.1	-	-
Intoxicated and treated with raw MPS	20	210.0±15.0	0.01	58.8
Intoxicated and treated with purified MPS	20	227.6±20.9	0.01	53.5

Table 3

The anticholesterolemic effect

Lot	Number of animals	Cholesterole in mg in 100 ml	P	Protective effect
Intoxicated with Triton	20	171.5±10.7	-	-
Witness	20	41.2± 6.6	-	-
Intoxicated and treated with raw MPS	20	84.5±16.9	0.01	66.7
Intoxicated and treated with purified MPS	20	67.0±13.0	0.01	80.2

From these results one can see that both the raw and the purified MPS present an intense action in comparison with the hyperlipidemy and the hypercholesterolemy of the Triton-20 intoxicated rats. This protection is similar to that described by Bianchini for mucopolysaccharides obtained from pork duodenum and intestine, and by Molnar and col. from various tissues of the digestive tube from cattle, pork and fowls.

The physical-chemical and pharmaceutical characteristics studied show a great similarity between the mucopolysaccharides extrated from Mytilus galloprovincialis and those from pork, cattle and fowl.

CONCLUSIONS

The dry of Mytilus galloprovincialis contains a sulpho mucopolysaccharide which has physical-chemical properties very close to those from the cattle and pork duodenum, this intenstine, stomach and pancreas, and from the fowl intestine, such as electroforetic migration speed, reaction of colouring with toluidine-blue, with which all this mucopolysaccharides have a characteristic maximum of extinction to 585 nm,

From the view point of the pharmaceutical activity, the mucopolysaccharide from Mytilus has an intense protective activity against hyperlipidemy and hypercholesterolemy, experimented on the rat intoxicated with Triton A-20. The intensity of the protective effect is similar to those described for the mucopolysaccharides obtained from the digestive tube tissues of cattle, pork and fowl. Another common feature of these mucopolysaccharides is that they present only an insignificant anticoagulant effect, almost negligeable, by which they are different from the heparine.

It is interesting that mucopolysaccharides with physical-chemical and pharmaceutical propertie so close to those from mammals and fowl can be found in mollusca.

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