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Attempts to Prepare Heparin from Ox Liver Capsules with a Mild Method

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Heparin has been prepared almost quantitatively by a very mild method from ox liver capsules. It has, however, not been freed from a phosphorus containing component of unknown nature which seems to be firmly bound to the heparin.

The aim for the present study has been to develop a mild method for the preparation of heparin in order to obtain it in an as undamaged state as possible. Previous attempts to fractionate commercial heparin with fractionated alcohol precipitation resulted in several fractions with decreasing anticlotting activities but with almost constant sulfur contents. It would be interesting to see whether these activity differences are a result of using too violent preparation methods or if they exist *in vivo*. Efforts have been made to avoid extreme pH values, high temperatures and enzymatic degradation resulting from the presence of carbohydrate attacking enzymes possibly admixed. Acetone denaturation and zone electrophoresis have been very effective in removing the proteins.

METHODS

The ox liver capsules were removed from the livers immediately after slaughter, put into Dewar bottles with ice and transported to the Institute. There they were cleaned from other tissue by scraping with a knife, chopped, weighed and frozen in fine-ground carbon dioxide ice. The mixture was ground to a fine powder in a plate mill and left at -10°C until the carbon dioxide had evaporated. The capsule powder was treated at $+2$°C for about 5 min in a porcelain mortar with a solution 0.10 M in potassium thiocyanate and 0.05 M in potassium hydrogen phosphate (K_2HPO_4). The result was a paste which was diluted with about five times its volume of buffer solution. The pH was adjusted to 7.7—7.9, usually with 0.10 M sodium hydroxide in

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the first extraction of a capsule batch and with acetic acid diluted about twenty times in the third and following extractions. The mixture was slowly stirred over night at +2°C. After centrifugation in an International Centrifuge, Model FS, size 3, for about one hour, the supernatant was spared and the residue was stirred with the same volume buffer solution two times more in the same way as before. In some cases the residue was treated with buffer solution several times more in order to control the efficiency of the extractions. The results for two preparations are shown in Table 1. The anticlotting activities are measured with the thrombin method described by Jaques and Charles [1]. In order to avoid delicate standardizations, commercial heparin (Vitrum) has been used as standard, and the activity of this heparin dried in vacuum over P_2O_5 has been set to 100. In Table 1 the activity measured directly on the extract neutralized to 7.2 has been recounted as mg heparin.

Table 1. Yields in the extracts from two preparations. Starting weight 83 g and 120 g capsules wet weight. Yield in mg heparin.

Extract number	Yield in mg heparin	
1	32	63
2	11	16
3	2	2
4	1	4
5	1	0
Mg per kg wet weight	567	708

The three first extracts were pooled, the pH adjusted to 7.0—7.2 with acetic acid diluted tenfold, and an equal volume acetone was added, and the precipitation mixture left at room temperature over night. The precipitate was centrifuged down and treated with about ten times its volume of distilled water with a Potter-Elvehjem homogenizing apparatus. The pH was kept at 7.7—7.9 during this procedure. The insoluble proteins were removed by centrifugation in a Spinco centrifuge, Model L, rotor 20, at 20 000 *g* for one hour. The residue was washed once with a small amount of water, centrifuged again and the two supernatants were combined. The pH was adjusted to 7.0—7.2, an equal volume acetone was added, the precipitate centrifuged down at about 2 000 *g* for one hour and then washed with acetone. The precipitate was treated with a homogenizer, Spinco centrifuged, washed with water and Spinco centrifuged once more. The supernatants were combined and dialysed in a cellophane bag (Visking Co) against several changes of distilled water for about 24 h altogether. During the dialysis the pH was controlled and kept at 7.5—7.9.

The residues after the extractions and the Spinco centrifugations were pepsin incubated at 38°C at pH 2 for about 3 days, with addition of chloroform to prevent bacterial growth. Part went into solution and all except some small grains dissolved when the pH was increased to 7.5. The total anticlotting activity in this fraction corresponded to at most 10 mg heparin Vitrum per kg capsule wet weight.

The supernatants after the acetone precipitations of the extracts were collected and concentrated in air stream at about 45°C, dialysed and investiga-

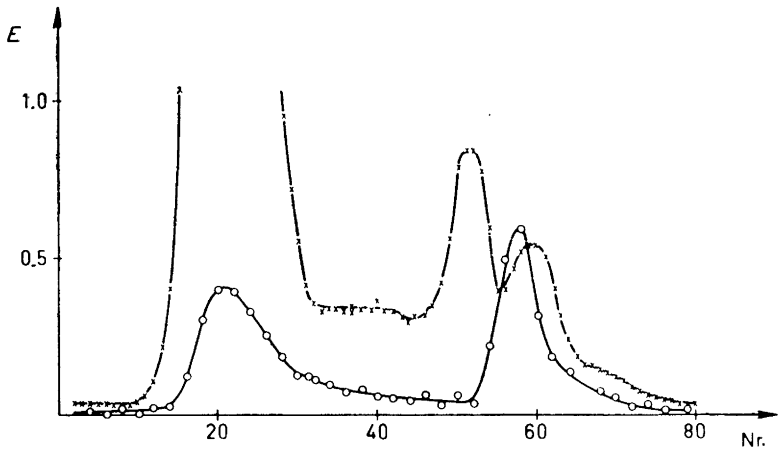

Fig. 1. Zone electrophoresis of two times acetone precipitated capsule extract: —o—o— = carbazole test curve; —x—x— = absorption at 280 mμ. Nr = number of eluated tubes, E = extinction.

ted for anticlotting activity. The activity corresponded to about 1 mg heparin Vitrum per kg capsule wet weight.

The dialysates were controlled for activity after concentration as described and redialysing in extra fine porous bags (Visking Co) but no heparin was found.

The activity not extracted with buffer and the activity lost in the precipitations correspond together to about 1 or 2 % of the total amount heparin activity in the capsules.

The dialysed crude heparin was subject to zone electrophoresis as described by Porath [2,3] with a cellulose powder, described by Flodin and Kupke [4], as supporting medium. The cellulose column was 70 cm long and 4.5 cm in diameter. The buffer used was 0.10 M potassium phosphate at pH 6.8. As DNP-leucine moves about half as fast as heparin at this pH and has a strong yellow colour, a little of this substance was added to make it possible to see how far the electrophoresis had proceeded. The sample was made only 0.03 M in phosphate buffer, which had a zone sharpening effect. This effect could be increased further by adding 5 ml distilled water to the sample. The starting zone contained about 50 ml solution. Each electrophoresis took about 80 h with a current of 0.1 A. After this time the zones were eluated with buffer in test tubes in a fraction collector changing tube every fifth minute. 15 ml was collected in each tube. One ml was taken from every second tube and tested for hexuronic acid with the Dische carbazole method [5]. All fractions were measured in a Beckman spectrophotometer, model DU, at 280 mμ.

A typical run is shown in Fig. 1. The first peak in the ultraviolet absorption curve is mainly due to nucleic acids, the second to DNP-leucine, and the third to the bulk of the proteins. In the carbazole reaction curve, the first peak (tubes 16—32) contained all the anticlotting activity. This fraction was

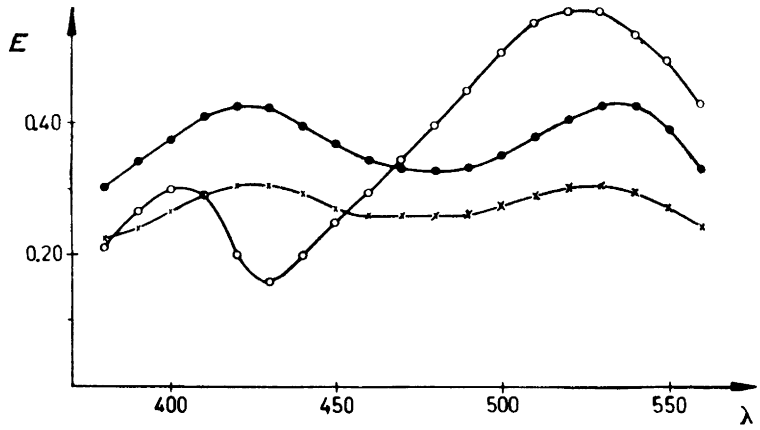

Fig. 2. Spectrophotometric absorption diagrams of carbazole tests on heparin = —o—o—; glycogen = —●—●—; the substance in peak 2 = —×—×—. E = extinction, λ = wavelength in mμ.

collected. Absolute alcohol was added until the alcohol concentration was 25 %. The precipitate, which formed after some hours, was discarded since a test for hexuronic acid was negative. Acetone was added to the supernatant to 50 %. The precipitate formed was centrifuged down, redissolved in a small volume of water and dialysed against distilled water in order to remove the precipitated phosphates.

The second zone (tube 53—64 in Fig. 1) was precipitated by adding 1 ml 4 M sodium chloride per 25 ml solution and two volumes alcohol. The precipitate formed was dried over P_2O_5 in vacuum and analyzed with the carbazole method. The absorption curve was compared with those of heparin and glycogen. The nitrogen content was 6.5 %. The data obtained indicated that this zone contained about 60 % glycogen and 40 % proteins.

The dialysate from zone one was run once more in zone electrophoresis. The result is shown in Fig. 3, which shows carbazole tests on the eluate. After this second electrophoresis the heparin containing zones from several preparations were pooled, dialysed against several changes of distilled water, and precipitated by adding 1 ml 4 M sodium chloride per 25 ml dialysate and two volumes alcohol. The precipitate was washed with alcohol and acetone and dried in vacuum over P_2O_5. The weight was 114 mg.

The final purification was made by dissolving the crude heparin in 26 ml distilled water and 20 ml of a buffer 0.05 M in sodium phosphate, pH 6.8, and 0.20 M in sodium chloride. Three fractions were obtained by adding absolute alcohol to 25, 54 and 66 %. The fraction before 25 % and the supernatant after 66 contained no hexuronic acid and were discarded. The fractions between 25 and 54 % and 54 and 66 % were washed with alcohol and acetone, dried and weighed. The fraction between 25 and 54 % weighed 104 mg. The fraction between 54 and 66 % weighed 3 mg, had an anticlotting activity of 8

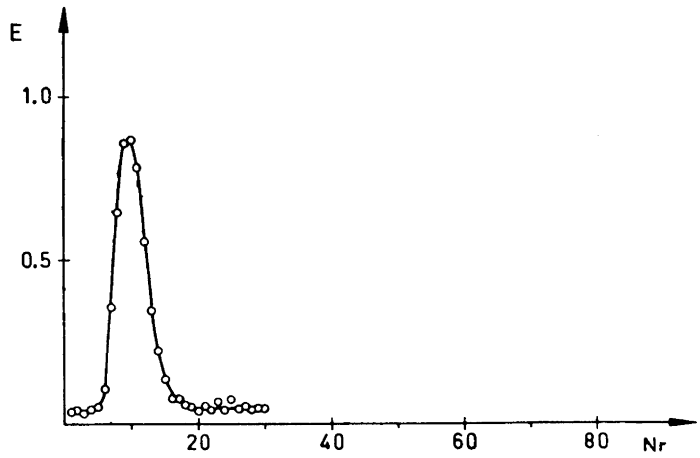

Fig. 3. Reelectrophoresis curve of the tubes No. 16—36 in the carbazole curve from the experiment shown in Fig. 1. Nr, *E* and —o—o— the same as in Fig. 1.

and a colour yield in the carbazole test of 36 % of that of commercial heparin (Vitrum).

The fraction between 25 and 54 % was subject to the following investigations, the results of which are tabulated in Table 2.

The ultraviolet absorption at 260 and 280 mμ was measured in a Beckman spectrophotometer, Model DU, in a 1 cm cuvette in order to get an estimation of the content of nucleic acid and proteins.

Ninhydrin tests were performed by taking some drops of a 3 % solution of the fraction, adding some drops of 0.2 % ninhydrin and heating in a water bath for 10 min.

The sulfur content was measured according to Ottoson and Snellman [6].

The anticlotting activity was measured.

The colour yield in per cent of the colour yield of the same weight commercial heparin (Vitrum) was measured with the carbazole method [5]. This measurement does not necessarily give even a relative value for the hexuronic acid content since the colour yield per mole hexuronic acid depends on how it is bound. Glucuronic acid in heparin gives a colour about 50 % more intense than that of free glucuronic acid.

Phosphorus was determined using a modification of a method described by Scheel [7]. In order to determine whether the phosphorus was organically bound or was a coprecipitation of inorganic phosphorus, tests were made without hydrolysis in sulfuric acid-perchloric acid mixture. The procedure exhibits a strong reaction for inorganic phosphorus in about one tenth of the phosphorus quantity present in the sample taken for analysis. The test was, however, negative.

One mg heparin was tested with the fuchsin-sulfurous acid test for aldehydes according to Feulgen, Boguth and Andresen [8]. The positive reaction for capsule heparin could, however, not be transferred by shaking into octyl alcohol.

To 1 mg heparin in 2 ml water 2.5 ml 0.1 % anthrone solution in concentrated sulfuric acid was added. The colour caused by hexoses is clearly visible for a few micrograms of glucose and strong for 50 μg micrograms.

Table 2. Data for capsule heparin and commercial heparin (Vitrum).

Determination	Capsule heparin	Heparin Vitrum
UV absorption at 260 mμ 1 mg per ml	0.304	0.360
UV absorption at 280 mμ 1 mg per ml	0.171	0.300
Ninhydrin test	negative	negative
Sulfur content, %	10.05	13.1 [a]
Anticlotting activity	112	100
Colour yield in the carbazole test	84	100
Phosphorus, %	1.10	—
Inorganic phosphorus	nil.	—
Fuchsin-sulfurous acid test	positive	negative
Anthrone test for hexoses	very weak	very weak

[a] Reprecipitated heparin Vitrum contains 12.2 % sulfur.

The results in Table 2 indicate that capsule heparin contains a component missing in the commercial heparin. Efforts to remove this component by refluxing a sample of heparin with ether-ethanol 5:1 or benzene-ethanol 5:1 on a water bath had no effect on the phosphorus content or the colour yield after 20 h refluxing. Shaking a heparin solution with phenol or reprecipitating it as barium salt has also without effect.

DISCUSSION

On comparing the commercial heparin and the capsule heparin here prepared, it seems probable that this heparin contains organically bound phophorus. The results indicate that this substance is not nucleic acid or a nucleotide, a protein or peptide or some hexose compound. The phosphorus content gives an equivalent weight of about 500.

The ratios of activity to sulfur content and to colour yield in the carbazole test seems to be almost the same as the corresponding ratios for one of the fractions of commercial heparin obtained by fractionated alcohol precipitations [9]. The heparin prepared has not yet sufficed for fractionation studies.

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