CCXIII. THE CHEMISTRY OF HEPARIN.

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(*Received May 13th, 1935.*)

HEPARIN, the anticoagulant discovered by Howell [1918], has not until recent times been readily accessible either to the research chemist or to the physiologist. Its high price has prevented a thorough study of its chemical properties and its more extensive use in physiological laboratories and in clinical practice. Thanks to the recent work of Charles and Scott [1933], fresh possibilities have been afforded in both directions. Heparin has been shown to be a common tissue constituent, and the method of its preparation has been greatly improved.

As to the chemical nature of heparin, very little is known. Howell [1928] found a hexuronic acid in his purest preparations. Schmitz and Fischer [1933] described highly purified preparations, which they believed to consist of a trisaccharide, $C_{18}H_{32}O_{17}$, containing one carboxylic group. Charles and Scott found a positive α-naphthol reaction, but the test for hexuronic acids with naphthoresorcinol was negative. Consequently the authors agree only in one respect, namely as to the occurrence of carbohydrate groups in the heparin preparations. Furthermore, there is disagreement as to the nitrogen content of the preparations. Howell found at an early stage of the preparation that the sodium cyanide test of Lassaigne proved negative, and he gives no further information as to the nitrogen content. Nor do Schmitz and Fischer pay any attention to this question. On the contrary Charles and Scott found about 2 % of nitrogen in their purest preparations, which were as pure as any hitherto prepared. Another finding in the papers of these investigators is also remarkable, namely the high ash content of the heparin samples. Howell found 37 % and Charles and Scott about the same figure. Schmitz and Fischer, who found a certain amount of ash even in the crystalline brucine salt, repeatedly discuss the possibility of molecular combination between heparin and neutral salts.

This divergence of opinion as to the composition of heparin preparations seemed to justify a reinvestigation. The author therefore prepared heparin from ox and horse liver, following the principles outlined by Charles and Scott. Only in some details was their technique modified. The crude material obtained after tryptic digestion proved to be 2 or 3 times more active than the commercial preparations. After treatment with Lloyd's reagent 2 or 3 times as recommended by Howell, no considerable further purification seemed possible. Several attempts to fractionate the preparations with barium acetate, barium hydroxide, lead acetate (basic) and glacial acetic acid did not result in more active products. In this state of purity the samples did not precipitate in aqueous solution with lead acetate or with cadmium chloride. As further purification seemed impossible, the samples were submitted to analysis.

Analysis of the heparin preparations.

The different preparations of heparin seemed to be of a rather uniform chemical composition (see Tables I and II). Furthermore the content of ash, 38–41 %, and of nitrogen, 1·63–1·84 %, corresponded with the figures given by Charles and Scott.

As Howell's statements as to the occurrence of a hexuronic acid seemed to be quite convincing, the samples were submitted to a quantitative analysis for uronic acids according to Tollens-Lefèvre. The micromethod of Dickson *et al.* [1930] was applied and somewhat modified by the author. The carbon dioxide evolved corresponded to a content of hexuronic acid of 17–19 % calculated on air-dry substance. If the high ash content is taken into account, the content of uronic acid found is quite considerable.

The presence of a hexosamine was also detected and an estimation by the method of Elson and Morgan [1933] showed between 12 and 14 %, calculated on air-dry substance. As these two constituents, hexuronic acid and hexosamine, are characteristic for chondroitinsulphuric acid, a test was made for ester sulphates. It proved to be positive. Furthermore, a certain amount of acetic acid was found on analysing for *N*-acetyl. Thus the samples seemed to contain chondroitinsulphuric acid, and to such an extent that there was hardly room for any other active organic component (see Table II). The chondroitinsulphuric acid however proved to be completely devoid of anticoagulating activity.

Table I. *Analysis of the heparin preparations.*

% of air-dry substance.

	Heparin after the first treatment with Lloyd's reagent	Heparin after a second or third treatment with Lloyd's reagent				
Preparation	...	1	2	3	4	5
Carbon dioxide (Tollens-Lefèvre)	—	—	4·60	—	—	—
	3·30	—	4·27	—	—	—
	3·40	3·92	4·16	4·11	3·99	—
	3·70	3·89	4·16	4·05	4·04	—
Average	3·50	3·91	4·30	4·08	4·02	—
Nitrogen (Kjeldahl)	2·88	1·63	1·91	1·65	1·64	1·84
Acetic acid found	—	—	3·04	—	2·19	4·13
(Friedrich-Rapoport)	—	—	3·11	—	2·29	4·11
Average	—	—	3·08	—	2·24	4·12
(Calc. for chondroitinsulphuric acid)	—	—	5·90	—	5·47	—

Table II. *Composition of the heparin preparations.*

% of air-dry substance.

	Heparin after the first treatment with Lloyd's reagent	Heparin after a second or third treatment with Lloyd's reagent				
Preparation	...	1	2	3	4	5
Moisture	15·00	15·60	13·90	13·84	10·90	10·15
Ash	37·90	38·40	40·80	41·09	45·15	41·05
Hexuronic acid (found)	15·45	17·26	18·98	18·00	17·74	—
Hexosamine less H_2O (calc. 1 mol. per mol. uronic acid)	12·82	14·32	15·74	14·93	14·71	—
Acetic acid less H_2O (calc. 1 mol. per mol. uronic acid)	3·34	3·74	4·11	3·89	3·83	—
Maximum amount of protein calc. from the N content	11·00	2·41	3·38	2·19	3·06	—
	95·51	91·73	96·91	93·94	95·39	—

The ash was proved to consist of magnesium sulphate. As to its origin, it is evident that the magnesium originates from the Lloyd's reagent. A certain amount of sulphate could also be extracted with water from the adsorbent and precipitated by 1·5 vols. acetone. This amount however was not nearly sufficient

to explain the high ash content of the heparin samples, particularly as it was found that only a small amount of sulphur could be precipitated with barium chloride. Furthermore, some samples were reprecipitated 3 times by 1·5 vols. acetone. The heparin samples showed a sulphur content of about 11 %, calculated on air-dry substance. Only 3·5–4 % was precipitated directly with barium chloride in slightly acidic solution. The remainder, about 7·5 %, could be precipitated only after acid hydrolysis (see Table III).

In view of this high content of ester sulphates particular attention was paid to the possibility that the chondroitinsulphuric acid might prevent the precipitation of the barium sulphate. The free sulphates were first precipitated with barium chloride in 0·1*N* HCl solution. After ½-1 hour the precipitate was collected in a Neubauer-Gooch microcrucible. Then the solution was left standing for 24 hours at room temperature, without any formation of a new precipitate. On adding a definite amount of sodium sulphate (corresponding to 27·2 mg. $BaSO_4$), a precipitate immediately formed, which was collected after an hour, about the theoretical amount of $BaSO_4$ (28·2 mg.) being recovered. The same experiment was repeated several times with the same result. When the solution was boiled, however, a precipitate formed which for two samples agreed well with the total amount of ester sulphates.

Further and definite proof of the correctness of this analysis was obtained by means of dialysis in a collodion sac and electrodialysis through a parchment membrane. In both cases only the free sulphates were found in the outer and anode liquor, and about the theoretical amount of ester sulphates in the inner liquor.

Seeing that 7·5 % sulphur requires about 28 % ash, calculated as magnesium sulphate, the high ash content of the heparin preparations of previous investigators apparently originates from the ester sulphates. In a sample with 11 % S the corresponding figure is 42 %. The ash content of the author's heparin preparations agreed well with this figure. There was also a striking correspondence in the ash content of the purest heparin preparations referred to later with the figures calculated for the ash from the S content.

Table III. *The partition of sulphur in the heparin preparations.*

% of air-dry substances.

Preparation	1	2	3	4	5
Total sulphur	—	—	—	12·36	11·11
	10·68	11·15	11·20	12·46	11·02
	10·60	11·18	11·15	12·19	10·82
Average	10·64	11·17	11·18	12·34	10·98
S in free sulphates	—	—	—	4·74	4·09
	3·88	3·40	3·86	4·79	4·09
	3·62	3·52	3·85	4·72	4·27
Average	3·75	3·46	3·86	4·75	4·15
Total S less S in free sulphates	6·89	7·71	7·32	7·59	6·83
Calc. for 2 atoms S per mol. chondroitin	5·75	6·26	5·94	5·85	—
Calc. for 2·5 atoms S per mol. chondroitin	7·13	7·83	7·43	7·32	—
Calc. for 3 atoms S per mol. chondroitin	8·55	9·39	8·91	8·78	—

As is evident from Table II, the samples analysed here consisted of chondroitin and ash almost exclusively. The fairly accurate Tollens-Lefèvre method allows such a conclusion to be drawn, particularly as the hexosamine content was almost theoretical and acetic acid was found to be present. This being the case, it will be necessary to assign the ester sulphates to the chondroitin, where

already about 3 % sulphur is fixed as chondroitinsulphuric acid. Consequently the question of a chondroitinpolysulphuric acid must be considered.

The content of ester sulphates in the purified heparin preparations is given in Table III. The figures are to be considered somewhat uncertain, since there is no proof of the quantitative recovery of the free sulphates. Repeated analyses gave figures slightly deviating from those given in the table, although without impairing the results. The amount of ester sulphates corresponded to about 2·5 atoms of S per mol. of chondroitin. Seeing that, both in the hexosamine and in the hexuronic acid, there are three hydroxyl groups available for esterification, a tri- or di-sulphuric acid would be the most likely to occur. Levene [1925] tentatively assigns the sulphur group of the chondroitinsulphuric acid to C_6 of the hexosamine. 2·5 atoms of S per mol. of chondroitin would require that one of the sulphate radicals combines with 2 mols. of chondroitin. A more plausible explanation would therefore be that the samples contain a mixture of different chondroitinsulphuric acids. In spite of the similarity in the chemical properties of these acids it was possible by means of alkaloids to separate a fraction having the composition of a chondroitintrisulphuric acid and showing an increased heparin activity, whereas the chondroitinsulphuric acid isolated from the mother liquor showed a lower sulphur content.

The properties of the purified heparin preparations.

The preparations of heparin analysed showed the properties described by the previous investigators. No precipitate was obtained with metal salts, except with basic lead acetate, which also precipitates chondroitinsulphuric acid.

For the study of the solubility of the different salts of heparin and of chondroitinsulphuric acid they were electrodialysed with a parchment membrane to almost neutral reaction of the cathode liquor. The free acids were then neutralised in small portions with alkaline solutions of the different metals.

The free acids are soluble in an excess of methyl alcohol, ethyl alcohol, acetone and glacial acetic acid. The sodium, potassium and ammonium salts, when dissolved in several volumes of these liquids only flocculate after addition of a certain amount of foreign electrolytes. A solution of the calcium salt shows no opalescence on the addition of 1 vol. of methyl alcohol, slight opalescence with 1 vol. of ethyl alcohol and flocculates with 1·5 vols. of acetone. The barium salts are readily precipitated in these conditions. The magnesium salt needs for its precipitation the addition of some sodium chloride, which is extensively used in the preparation and purification of heparin.

These properties are common to heparin and chondroitinsulphuric acid, as is the precipitability of their salts by means of glacial acetic acid and basic lead acetate. In one respect, however, there is a difference. Heparin flocculates on the addition of an excess of barium hydroxide whereas chondroitinsulphuric acid remains in solution. This property however has been proved by the previous investigators to be of little or no value in the purification of heparin.

A noteworthy difference was found in their behaviour towards alkaloids, particularly in the solubility of their brucine salts. The electrodialysed solutions were neutralised with solutions of the alkaloid bases in methyl alcohol. Morphine and cinchonine gave no precipitate. With quinine, both heparin and chondroitinsulphuric acid flocculated in the cold, but with brucine only the heparin precipitated. After removal of the brucine from this precipitate a preparation was obtained showing a content of hexuronic acid, hexosamine and ester sulphates calculated for a chondroitintrisulphuric acid.

The separation of a chondroitintrisulphuric acid from the purified heparin preparations by means of alkaloids.

On adding morphine, cinchonine, brucine and quinine, or their salts to a neutral solution of heparin, a precipitate was obtained only with quinine and its hydrochloride. When the precipitate was collected and the base was removed with sodium hydroxide and chloroform, and the heparin precipitated with acetone in the usual way, the content of ester sulphates proved to be considerably higher than in the initial material. It corresponded well with the calculated content for a chondroitintrisulphuric acid.

Seeing that chondroitinsulphuric acid also gives a sparingly soluble quinine salt on cooling and freezing the solution, the precipitate obtained with quinine must be considered as less specific. Brucine proved more useful, for the pure brucine salt of heparin is insoluble in water, whereas chondroitinsulphuric acid does not give any precipitate with brucine under the same conditions. A brucine salt of heparin stated to be crystalline was also prepared by Schmitz and Fischer.

In order to obtain the pure brucine salt, the positive ions of the heparin samples, as well as the free sulphates were removed by electrodialysis. When the strongly acid solution of the inner cell was neutralised with brucine dissolved in methyl alcohol, only a slight opalescence appeared. When the solution was kept in the cold and frozen 2 or 3 times during the course of a week a considerable amount of precipitate separated. The brucine salt settled out in spherical masses much like crystals of crude leucine, without any other visible particles in the microscopic field. On boiling with sufficient water, the precipitate dissolved and could be purified by repeating this procedure.

As no certain conclusion can be drawn from the analysis of the brucine salt, the precipitate and the mother-liquor were analysed after removal of the brucine.

The material recovered from the insoluble brucine salt again showed a content of about 20 % of uronic acid and a sulphur content corresponding within 3–4 % to the calculated figures for a chondroitintrisulphuric acid. The hexosamine content, determined with the Ehrlich reagent as recommended by Elson and Morgan also agreed with the theoretical figure.

The substance recovered from the mother-liquor after removal of the insoluble brucine salt showed about the same or a slightly higher content of uronic acid and hexosamine, and a sulphur content somewhat lower than would be the case in a chondroitindisulphuric acid.

The use of brucine in isolating the trisulphuric acid is thus very valuable. If the same experiment is repeated with electrodialysed chondroitinsulphuric acid, only a trace of brucine salt settles out when the solution is frozen. The substance recovered from this precipitate in one experiment was only 2·4 % of the amount of chondroitinsulphuric acid recovered from the mother-liquor.

After this fractionation of the heparin samples by means of brucine, the heparin activity proved to be increased in the material recovered from the brucine precipitate and decreased to 1/3–1/4 in the material obtained from the mother-liquor (see Table VI).

The purest preparations of heparin thus obtained showed rather more than 10 times the activity of the commercial preparations of Kahlbaum and of Hynson, Westcott and Dunning, Baltimore (see Table VII).

Experimental.

The crude heparin was prepared from ox and horse liver as described by Charles and Scott. The material was first autolysed with toluene at 40° for 48 hours. The yield varied greatly as well in amount as in activity. It was not possible to make a thorough study of these details. A description of the details of the preparation and purification of the heparin will therefore be postponed until more experience has been gained.

The crude heparin was about 2 or 3 times as strong as the commercial preparations (see Table IV). In the first treatment with Lloyd's reagent about 70 % of the material was removed, without any considerable loss of activity, the product now being about 7–8 times as strong as commercial heparin (Kahlbaum). When this product was treated once or twice more with the adsorbing agent, the activity was but slightly increased. A considerable amount of contaminating material was however removed, as will be seen from Table II. The amount of protein possibly present was reduced from 11 to 2–3 %, as calculated from the N content of the samples, possible small amounts of ammonium salts being neglected. (Preparations Nos. 1–3 of Table II were obtained from the first preparation of the same table.)

The analysis of the material thus obtained is given in Tables I and II.

Table IV. *Time of coagulation, in hours, of ox blood at room temperature.*

mg. heparin per 100 ml. of blood (air-dry substance)	10	5	2·5	1·25	0·62	0·31	0·16
Heparin (Hynson, Westcott and Dunning.) Lot No. 126	5·5	5·0	2·5	0·5	—	—	—
	5·5	5·0	2·5	0·5	—	—	—
	5·5	5·5	1·5	0·75	—	—	—
	5·5	2·5	1·5	0·75	—	—	—
Heparin (Kahlbaum)	5·0	5·0	2·5	1·5	—	—	—
	5·5	5·5	2·25	0·75	—	—	—
	5·0	2·5	1·5	0·75	—	—	—
	5·5	2·25	1·0	0·75	—	—	—
Crude heparin	—	—	5·5	2·5	0·5	25 mins.	—
	—	—	5·5	1·5	0·75	35 ,,	—
	—	—	5·5	2·25	1·25	25 ,,	—
	—	—	2·5	1·5	0·5	20 ,,	—
Heparin after the first treatment with Lloyd's reagent	—	—	—	5·5	3·25	1·0	0·25
	—	—	—	5·5	5·0	1·75	25 mins.
	—	—	—	>5·0	2·25	1·25	35 ,,
	—	—	—	>5·5	2·5	1·0	0·5

Preparation of the trisulphuric acid by means of brucine.

3 g. of preparation 4 were electrodialysed in 170 ml. of water for 9 hours (at a current of 600 milliamps.), the temperature being kept below 25° (volume after dialysis 190 ml.). 150 ml. of this, corresponding to 2·4 g. of preparation 4, were neutralised with brucine and the excess of the base was removed with chloroform. The solution was kept in an ice-box and frozen repeatedly during the course of a week. The insoluble brucine salt was collected and washed with water in a centrifuge-tube. The weight of the air-dry substance was 1·8 g. The precipitate and the mother-liquor were treated separately with 20 ml. *N* NaOH and the brucine removed with chloroform. After neutralisation of the solution with HCl some magnesium chloride was added and the heparin precipitated with 1·5 vols. of acetone. On the next day the precipitate was dissolved in about 10 ml. of water and reprecipitated with about 100 ml. of acetone.

The yield of air-dry substance from the brucine precipitate was 580 mg. and from the mother-liquor 500 mg.

	Material recovered from brucine precipitate %	Material recovered from mother-liquor %
Moisture	14·82	17·70
Ash	33·30	37·27
CO_2 from uronic acid	4·99	5·5
Hexuronic acid equiv. to CO_2	22·02	24·25
Hexosamine found	17·5	—
Hexosamine calc.	19·6	—
Sulphur	10·67, 10·42 } 10·55	7·42, 7·29 } 7·36
S calc. for 3 atoms per mol. uronic acid	10·89	—
S calc. for 2 atoms per mol. uronic acid	—	8·0
Acetic acid	1·46	—

In another experiment performed in the same way 2 g. of preparation 4 were electrodialysed. The insoluble brucine salt which separated on freezing the solution was dissolved in 200 ml. of boiling water. After filtration through a hot funnel, the clear solution was left standing for 2 days in the cold and frozen. The same procedure was repeated once more with the precipitate which settled out. From the brucine salt, 475 mg. of air-dry heparin were recovered (moisture: 8·35 %; S: 11·40 and 11·58 %, mean 11·49 %; on dry substance 12·54 % S).

The previous experiment yielded a substance containing 12·34 % S calculated on dry substance with a recovery of 30 % of the uronic acids present in the initial material (preparation 4).

In spite of the twice repeated reprecipitation of the brucine salt from water, the yield in this experiment was about the same, and the sulphur content differed but slightly.

The heparin from this experiment was mixed with the material recovered from 3 g. of another heparin sample treated in exactly the same way. The mixture was submitted to analysis.

	%
Moisture	16·56
Ash	29·20
CO_2 from uronic acids	4·69
Hexuronic acid equiv. to CO_2	20·70
Hexosamine	17·10
Calc. from S content	19·8
S	10·63
Calc. for 3 atoms S per mol. hexuronic acid	10·23
Acetic acid	1·67

The Tollens-Lefèvre method of analysis. There are many modifications of the Tollens-Lefèvre method for the determination of uronic acids. At first the technique of Dickson *et al.* [1930] was employed; during the course of the work it was found necessary to change many of its details.

The inlet air was freed of CO_2 in an effective wash-bottle with concentrated potassium hydroxide solution. A Ω-shaped glass tube between the wash-bottle and the boiling flask rendered the escape of CO_2 backwards more difficult. Instead of a silver nitrate solution, solid silver sulphate suspended in water was kept in the trap for collecting the hydrochloric acid distilled over. The suspension was renewed after two analyses.

Seeing that the original technique of Dickson, Otterson and Link tended to give irregular figures 10–15 % too low in the analysis of known substances,

the influence of several factors was studied. More satisfactory results were only obtained after making the following changes.

The heating bath with Wood's alloy was kept at 145–150° during the hydrolysis, which was continued for 4 hours. Two microburners were used. The radiation of heat and the cooling by the refluxing liquid kept the temperature constant.

The strength of the hydrochloric acid was increased to 20%. One volume of concentrated hydrochloric acid was mixed with 1 vol. of water. Owing to these changes somewhat more hydrochloric acid distilled over, but with satisfactory cooling and an efficient trap this disadvantage did not impair the results. On the contrary the yield of CO_2 agreed in almost every analysis with the calculated figure.

Furthermore, the excess of barium hydroxide was not titrated directly with 0·1 *N* hydrochloric acid, but only after removal of the barium carbonate. In the direct titration there is a possibility that, even if the flask is shaken vigorously, the hydrochloric acid may react with the barium carbonate. The glass beads were quickly washed with CO_2-free water in a suction funnel and the barium hydroxide was titrated in the suction flask. 0·1 *N* HCl was added in excess, the solution boiled and titrated with 0·1 *N* alkali against phenolphthalein.

For each analysis 250–300 mg. of the heparin preparations were taken. The reliability of the figures obtained was checked by analysing corresponding amounts of known substances before and after the analysis of the heparin. For this purpose a sample of chondroitinsulphuric acid prepared by the author's method [Jorpes, 1929] and an analytically pure crystalline sample of the glycuronogalactose isolated by Butler and Cretcher [1929] from gum arabic were used. The figure calculated for the CO_2 in the Tollens-Lefèvre analysis of the chondroitinsulphuric acid was 6·5%: found 6·50, 6·76, 6·57%. The corresponding figure calculated for the glucuronogalactose, $C_{12}H_{20}O_{12}.2H_2O$ is 11·22%: found 11·16%.

The colorimetric determinations of hexosamine were made by the method of Elson and Morgan, both on the mixed chondroitinsulphuric acids (preparation 1) and on the two samples of the supposed trisulphuric acid recovered from the insoluble brucine salt. The hydrolysate after the Tollens-Lefèvre analysis could be used for this purpose. Otherwise 50 mg. substance were hydrolysed in 7 ml. of 12% hydrochloric acid for 3 hours over a free flame. The hydrochloric acid was removed by distillation. After neutralisation the volume was made up to 10 ml. and 0·4–1·0 ml. of this solution was used for a test. Generally three different concentrations were analysed in duplicate. Each time four different concentrations of glucosamine between 0·3 and 1 mg. were used also in duplicate as standards. The readings were plotted on a semilogarithmic paper, the logarithmic ordinates corresponding to the scale readings of the Zeiss step photometer. In spite of the great variations of the single analyses a good approximation to the hexosamine content was generally obtained. The method is a real advance on the older one of Zuckerkandl and Messiner-Klebermass [1931].

The weak alkaline solution with the acetylacetone (volume 3 ml.) was heated at 90° in a water-bath for half an hour. After cooling 5·5 ml. of 95% alcohol were added. When the strongly acid Ehrlich reagent was added, air was blown through the solution and the volume was made up to 10 ml. (Filter S 53. Cups 0·5 cm.)

On the analysis of preparation 1 on three different days the figures 12·3, 12·9 and 14·4% calculated on air-dry substance were found. The content of hexuronic acid, 17·23%, would require 16% hexosamine.

In the supposed chondroitintrisulphuric acid recovered from the insoluble brucine salt of the first experiment the figure 17·5 % was found, for the acid recovered in the second experiment 16·9 and 17·3 %. The figures calculated from the sulphur content were 19·6 and 19·8 % respectively. For chondroitinsulphuric acid treated in exactly the same way, the figures 22·3 and 20·5 % were found on two different days; calculated 26·5 %.

From these analyses it is evident that all the heparin samples contained 1 mol. of hexosamine per mol. of hexuronic acid.

The content of acetic acid was first determined by the method of Kuhn and Roth [1933], hydrolysis with alkali in methyl alcohol being employed. The figures obtained agreed well with those calculated and were used in the preliminary report [Jorpes, 1935] as evidence of the chondroitin structure of the preparations. The method had unfortunately been checked only on penta-acetylglucose. When the preparations were later on submitted to acid hydrolysis either with 25 % sulphuric acid according to Levene and LaForge [1913] or with *p*-toluenesulphonic acid according to Friedrich and Rapoport [1932] a much lower acetyl content was found. All the analyses were therefore repeated with the last method. For chondroitinsulphuric acid figures which were only slightly (3 %) too high were obtained by this method[1].

For the sulphur determinations a sufficient amount of substance was taken to give a precipitate of barium sulphate weighing 20–40 mg., which was collected in a Neubauer-Gooch platinum-iridium microcrucible. Fusion was effected with sodium carbonate and potassium nitrate in porcelain crucibles.

The free sulphates were precipitated with barium chloride from 0·1 *N* hydrochloric acid solution. The precipitate could be filtered after one hour. In *N* hydrochloric acid 24 hours were necessary for a complete precipitation of the free sulphates.

After boiling for 1 or 2 hours with 10 % hydrochloric acid the ester sulphates were split off. In preparation 2 of Table III 10·87 and 10·41 % S were thus obtained after acid hydrolysis. The content of total S found on ignition was 11·17 %.

Dialysis and electrodialysis were employed to remove the free sulphates and the positive ions from the heparin preparation. By electrodialysis the free acid and its alkaloid salts could easily be prepared.

The electrodialysis was performed with an apparatus constructed by E. Hammarsten [see Ågren, 1934]. 150–200 ml. of solution can be dialysed at one time. The solution is cooled by circulating acetone from a store flask with solid carbon dioxide.

200 mg. of preparation 2, dissolved in 10 ml. of water, were dialysed against water in a collodion sac. The outer fluid was changed daily during 3 days. Its volume was 1500 ml. The free sulphates found in it corresponded to 3·6 % S in the sample, whereas the calculated figure was 3·46 %. It did not contain any ester sulphates.

In two experiments with electrodialysis the heparin activity and the partition of the sulphates were observed. In the first experiment 200 mg. of preparation 2 were dissolved in 20 ml. of water and electrodialysed in a smaller apparatus for 2·5 hours. The current through the solution decreased from 500 to 380 milliamps. in half an hour and then remained constant. The sulphur of the free sulphates found in the anode liquor corresponded to 3·45 % of the heparin sample (calculated 3·46 %). In the inner cell no definite decrease of the heparin activity could be shown. In the anode liquor about 1 % of the heparin activity could be traced, in the cathode liquor none.

[1] All these analyses were made by Sune Bergström, medical student.

When this experiment was repeated, the content of ester sulphates found in the cell after dialysis corresponded to 7·3 % of the sample (calculated 7·71 %). The sulphate-S found in the anode liquor corresponded to 3·2 %. On the liquor of the cell a thorough biological assay was made, showing no decrease in the anticoagulating activity (see Table V).

Thus neither the ester sulphates nor any heparin activity are lost during electrodialysis.

Table V. *Heparin activity of the cell liquor before and after electrodialysis.*

200 mg. of preparation 2 in 20 ml.; 500–380 milliamps. half an hour and 380 milliamps. 2 hours. *b* before and *a* after electrodialysis.

mg. heparin per 100 ml. of blood...	1·25		0·63		0·31		0·16	
	b	*a*	*b*	*a*	*b*	*a*	*b*	*a*
	Time of coagulation in hours.							
Stand 1	>24	>24	10	10	3·5	3·5	0·75	0·75
2	>24	>24	10	4	2·75	2·75	0·5	0·5
3	>24	>24	8	8	3·75	3·75	1·0	1·25
4	>24	>24	10	10	1·5	2·75	1·5	1·5
5	—	—	3	3	1·25	1·25	0·75	0·75

Table VI. *The heparin activity of the supposed chondroitintrisulphuric acid and of the acid recovered from the brucine mother-liquor, as compared with the activity of the initial material (preparation 2).*

1 = preparation 2.
2 = the trisulphuric acid (580 mg.) recovered in the first experiment.
3 = the substance (500 mg.) recovered from the mother-liquor.
4 = trisulphuric acid (475 mg.) recovered in the second experiment.

Sample ...	2	1	2	1	2	1	2	1
mg. heparin per 100 ml. of blood...	1·25		0·63		0·31		0·16	
	Time of coagulation in hours.							
Stand 1	—	—	3·5	3·5	2·5	2·5	—	—
2	>11	11	3·5	2·5	2·5	2·5	—	—
3	>11	11	3·5	2·5	2·5	1·5	—	—
4	>11	11	3·5	?	2·5	2·5	—	—
Sample ...	4	1	4	1	4	1	4	1
Stand 1	—	—	>11	2·5	1·25	0·5	0·5	0·5
2	—	—	>11	1·5?	2·5	0·5	—	—
3	>11	11	11	3·75	2·5	1·25	0·5	0·5
4	24	11	11	3·5	2·5	1·5	—	—
5	>24	24	24	11	2·5	1·5	—	—
Sample ...	3	1	3	1	3	1	3	1
Stand 1	2·5	11	1·5	6·5	—	2·5	—	—
2	2·5	—	1·5	6·5	—	2·5	—	—
3	2·5	24	1·5	3·5	—	2·5	—	—
4	2·5	—	1·5	6·5	—	2·5	—	—
5	2·5	11	1·5	6·5	—	2·5	—	—

The biological assay.

Several attempts to use recalcified oxalate blood only showed the complete unreliability of this technique. As cats could not be used because of the expense involved, an attempt was made to use horse blood. The blood was filled directly from the vein into small test-tubes containing a certain amount of heparin

solution. The next step was to collect ox blood from the newly opened vessels at the slaughter house. This source of blood would evidently be the cheapest. Fortunately it proved to be quite satisfactory.

The blood was quickly decanted from paraffined vessels into series of small test-tubes (70 × 8 mm.) accommodated in a stand (30 × 4 × 2 cm.). The tubes had a capacity of 2·5 ml. and contained 0·2 ml. of heparin solution and a glass bead. Each stand contained 10 tubes fixed in holes. All the tubes could be simultaneously tightly closed by lowering the strongly attached cover, which was faced underneath with rubber. After the tubes were filled with the blood, the cover was quickly lowered and fixed, and the stand turned over several times. The time that elapsed before the glass bead no longer moved freely was taken as the time of coagulation. The tubes were left standing at room temperature. Readings were made at shorter intervals at the beginning, the last ones after 10, 12 or 24 hours. Between 10 and 20 stands were used each day.

The strength of an unknown sample was checked against a known in two ways. Either 5–6 stands were used for each sample of heparin, as in Table IV, or each stand contained 4 or 5 different concentrations both of the unknown and of the known sample, as is shown in the other tables.

The second method is to be preferred. The individual variations of the blood from different animals are hereby eliminated.

Differences smaller than 20–30 % in the activity can hardly be expected to be distinguished with this technique.

In order to compare the purified heparin preparations with those of Charles and Scott, some experiments were performed on cats. Two of the animals gave a unitage of 450 and 540 per mg. of preparation 3. These experiments were performed by O. Wilander.

Table VII. *Heparin activity of the commercial preparations, as compared with the activity of the supposed chondroitintrisulphuric acid.*

1 = the heparin recovered from the insoluble brucine salt in the second experiment.
2 = heparin (Kahlbaum).
3 = heparin (Hynson, Westcott and Dunning). Lot No. 126.

mg. heparin per 100 ml. of blood...	6·3	0·63	3·2	0·32	1·6	0·16
	Time of coagulation in hours.					
Preparation ...	2	1	2	1	2	1
Stand 1	3	5	1	>1	1	1
2	8	>8	3	3	1	1
3	—	—	3	5	1	2
4	8	>8	2	3	1	1
Preparation ...	3	1	3	1	3	1
Stand 1	7	>8	3	5	1·5	2
2	8	>8	2	3	1·25	2
3	5	8	3	3	2	2
4	5	>8	3	3	2	2

Discussion.

The first question to be answered is whether the substances analysed were identical with those of the previous investigators or not. At first it seemed unlikely that some of them should have overlooked the nitrogen content [Schmitz and Fischer, 1933]; and it is still more surprising that no mention is made in the literature of the sulphur. Only in one place is it mentioned that

heparin contains calcium and sulphuric acid, namely in the list added to the heparin (Kahlbaum). Here the routine analyst apparently, and not the research chemist, made the note.

There are several considerations which almost make the presence of these acidic groups in heparin necessary. First the ash amounting to 25–35 % can hardly be explained without them. 1·5 vols. of acetone do not precipitate any sodium chloride added to the solution in order to facilitate the flocculation. Furthermore there is a striking similarity to the properties of chondroitinsulphuric acid. The most convincing evidence however is that the supposed chondroitintrisulphuric acid prepared from the liver shows such a strong heparin activity. It might be mentioned that preparations 1–5 of Tables I and II have a nitrogen content 1·63–1·93 % and an ash content 38·4–41·5 % of about the same order of magnitude as those of Charles and Scott. The general properties are quite identical. An attempt to test the heparin with cats gave also the same results, about 500 units per mg. substance.

Schmitz and Fischer describe a brucine salt stated to be crystalline, which they prepared from brucine sulphate and the barium salt of heparin. Their purified product was 32 times as active as the crude material obtained from Kahlbaum, which contained 8 % of phosphorus, partly in nucleic acids. That the chondroitintrisulphuric acid forms an insoluble brucine salt and is more than 10 times as active as the commercial products of to-day, is also an indication of the identity of the different heparin samples.

The heparin preparations of Tables I and II have a uniform composition. Even if there is uncertainty as regards the uronic acid analysis, the assumption that the preparations consist of esters of sulphuric acid and chondroitin seems justified, and it is strongly supported by the results of the treatment with alkaloids. After the regeneration of the heparin from the alkaloid salts, preparations were again obtained with a similar composition except for the sulphur content, which now agreed exactly with the calculated figure for a chondroitintrisulphuric acid. The fractionation very strongly supports the view that the heparin activity belongs to this acid and not to any impurity. As there is not much hope of obtaining these acidic polysaccharides in a real crystalline state, the formation of a brucine salt, which can be reprecipitated after solution in boiling water, with the heparin activity not only preserved but increased as compared with the original material, seems at present to be satisfactory evidence of such a conclusion.

It is furthermore interesting to note that this supposed chondroitintrisulphuric acid, which apparently will prove to be the strongest acid of the animal body, bears a resemblance to all the synthetic anticoagulants, which are sulphonic or polysulphonic acids. Liquoid Roche is a salt of polyanetholesulphonic acid. Congo red, germanin, Chicago blue and chlorazole fast pink are all of them polysulphonic acids [*v.* Huggett and Rowe, 1933–34].

Even if the presence of the three sulphuric acid radicals makes it probable that the anticoagulating activity is located in them it is too early to make definite statements without further proof. In any case the acetamido-group seems to be of no importance for the heparin activity. In Table II the content of acetic acid varies between 2·24 and 4·10 %, and in the trisulphuric acid it is only about 1·5 %. Seeing that the content of uronic acid and hexosamine is almost constant in all the samples and slightly higher in the regenerated ones, it is evident that the acetyl group is split off during the preparation. At an early stage of the preparation, the material is heated to 70° at a slightly alkaline reaction, and during the regeneration from the brucine salt it is kept in *N*

sodium hydroxide solution for an hour or more. The heparin activity is not unfavourably influenced by this.

There is, however, one detail, which is in disagreement with the previous discussion, namely the negative naphthoresorcinol reaction. As stated by Charles and Scott it is negative even in concentrated solutions. If the reaction is performed simultaneously under quite similar conditions with heparin and chondroitinsulphuric acid, and the coloured substance is taken up in benzene, a marked difference will be seen. The test with heparin is almost or quite negative. With Howell's technique it was found positive. Furthermore the evolution of carbon dioxide on acid hydrolysis cannot be explained without the presence of a uronic acid. The similarity with the chondroitinsulphuric acid is also very striking. The only possibility, which could explain this discrepancy, is, that the ester sulphates may be united with the uronic acid, thereby retarding or preventing the colour formation.

Summary.

Heparin, the anticoagulant from the liver, was purified by the method recommended by Howell and by Charles and Scott. After a certain degree of purity was reached, no further purification seemed possible. The samples were then analysed and found to contain about 17–19 % of hexuronic acid and the amount of hexosamine calculated for 1 mol. of hexosamine per mol. of uronic acid. Acetic acid was found to be present.

The organic material of the heparin samples therefore seemed to be chondroitin.

The ash, amounting to 40 % of the air-dry substance, consisted of magnesium sulphate. Only a small part of the sulphate in heparin could be precipitated with barium chloride, the remainder occurring as ester sulphate. For each mol. of chondroitin about 2·5 mols. of sulphur were found as ester sulphates.

These components together made up more than 90 % of the heparin samples. The analysis thus indicated that the heparin was a chondroitinpolysulphuric acid.

On dialysing and electrodialysing both the ester sulphates and the heparin activity remained in the collodion sac or in the inner cell. On neutralising an electrodialysed solution of heparin with brucine and cooling the solution, an insoluble brucine salt slowly settled out. The substance recovered after removal of the brucine showed a stronger heparin activity than the previous samples and a content of uronic acid, hexosamine and ester sulphate corresponding to the composition of a chondroitintrisulphuric acid.

As regards the nature of the active group, the presence of the sulphuric acid radicals is the most prominent feature in the structure of heparin. All the synthetic anticoagulants, *e.g.* Liquoid Roche, germanin and Chicago blue are also strongly acidic, all of them being polysulphonic acids.

The author's thanks are due to Stiftelsen Therese och Johan Anderssons minne for several grants during the past years.

Grateful acknowledgment is made for the valuable assistance of Olof Wilander and Sven Linde during the performance of this work.

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