VII. ON THE RELATIONSHIP BETWEEN THE SULPHUR CONTENT AND THE ANTICOAGULANT ACTIVITY OF HEPARIN PREPARATIONS

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ABOUT 20 years elapsed between the discovery of heparin by Howell in 1916 and its chemical identification. Howell [1928] reported on preparations which must have been reasonably pure and showed a positive naphthoresorcinol reaction, indicating the presence of a hexuronic acid. Fischer & Schmitz [1933], as well as Charles & Scott [1933], also worked with preparations of a high degree of purity, considered by them to be crystalline or semi-crystalline. Fischer & Schmitz [1933] claimed that they had succeeded in preparing a crystalline brucine salt of heparin. The elementary composition of this heparin indicated that it contained neither sulphur nor nitrogen. Their statement was unjustified, however, for on ignition the samples yielded a small amount of ash and were thus not homogeneous but a mixture of brucine and alkali salts.

Heparin was found by Jorpes [1935] to be a polysulphuric ester of chondroitin or some closely related substance. When the amino-sugar was identified as glucosamine [Jorpes & Bergström, 1936] heparin seemed to be a mucoitin polysulphuric acid. The occurrence of sulphur, accidentally observed by Howell already in 1928, was later denied [Fischer & Lipmann, 1935; Schmitz, 1935], but sulphur was soon afterwards recognized as an essential component of the active substance [Charles & Scott, 1936].

A new contribution to the discussion was made by these latter authors, Charles & Scott, in that they claimed that they had succeeded in isolating a crystalline barium salt of heparin. This salt would thus have to be regarded as the first chemically pure preparation of the anticoagulant. The free acid, derived from the barium salt as well as from an amorphous benzidine salt, was believed to have the composition $C_{25}H_{65}O_{50}N_2S_5$, with 11.5 % S. This statement has led to some confusion concerning the chemical nature of heparin, for all the evidence previously obtained indicated that heparin was not a definite chemical compound but a mixture of different polysulphuric esters, i.e. a polysaccharide (mucoitin) which could be esterified with sulphuric acid to a varying degree and might contain from 20 to 40 % of sulphuric acid. With increasing S content there was a steady increase in the anticoagulant activity. The different esters, having different S contents, could easily be separated as brucine salts by fractionation in water. The organic skeleton appeared to be the same in all of them, hexuronic acid, hexosamine (glucosamine) and acetic acid together making up at least 90 %. All this evidence was available in 1935.

It is quite natural that in the meantime those who have reviewed the subject have considered this alleged crystalline product to be pure heparin. They have not paid much attention to the fact that the amorphous preparations can contain 20 % more S (up to 13.5 % in the free acid) than the crystalline product, which has 11.5 %. The more recent discussions have rather neglected the previously

established facts. Thus Charles & Scott [1936] did not feel convinced that heparin contained hexuronic acid and hexosamine, in spite of the fact that, by means of most reliable reactions for detecting sugars the Tollens-Lefèvre procedure and the pyrrole reaction with Ehrlich's reagent for amino-sugars, they had been shown to make up 90 % of the ash-free preparations, if acetic acid also is assumed to occur in heparin. They found a considerable part of the nitrogen to occur in NH_2 groups. The amino group of heparin, however, is monoacetylated, $-\text{NH}.\text{OC}.\text{CH}_3$, as everywhere in the amino-sugars in nature. Of course part of it can become de-acetylated during the process of preparation, which includes heating with acids as well as with alkali, just as occurs in the preparation of chondrosine (mucosine) by acid hydrolysis. This negative aspect was most recently clearly expressed by Best [1938], who claims that "tests do not indicate the presence of glucuronic acid, which some previous observers had thought was present". The situation is somewhat perplexing when biochemists of to-day are unable to trace a substance which constitutes 25–30 % of the preparation and which was discovered by a physiologist 10 years ago. However, this matter need not be further discussed. The quantitative analysis according to Tollens-Lefèvre is quite reliable. The titration of the free acid obtained by electro-dialysis showed at an early stage that there was one carboxyl group to 2 or 3 sulphate groups. The titration curves also prove, as did the Van Slyke analysis, that the amino group is acetylated [Wilander, 1938].

In Charles & Scott's formula for the crystalline product there is one detail which immediately disproves its correctness. There are 2.5 atoms of S to each N atom. The same observation was made by Jorpes [1935] as the result of analysing the earliest samples of pure heparin available. It seemed improbable that heparin should contain 2.5 sulphate groups in each chondroitin (mucoitin) complex. A more plausible explanation was then obtained when it was found that the preparations could be divided by means of brucine into fractions having the composition of chondroitin (mucoitin) di- and tri-sulphuric acids and mixtures of these. With increasing S content there was, as mentioned, an increase in the anticoagulant activity. Additional analyses indicating the same thing were later published by Jorpes & Bergström [1937]. Heparin has therefore always been regarded by us as a mixture of mucoitin polysulphuric esters.

The question as to the homogeneity of the product of Charles & Scott, supposed to be crystalline, is consequently of great interest. It will also be of importance when the question arises as to what is to be an international standard of heparin. In order to check our results we have repeated our earlier experiments on a larger scale and have subjected the different fractions of heparin to a very careful S analysis and a thorough biological standardization. No corrections of our earlier views have been found necessary.

*The fractionation of ordinary pure heparin by means of
brucine salts*

The heparin used was the commercial product supplied by Vitrum of Stockholm. It is a sodium salt prepared mainly in accordance with the Charles & Scott method and contains about 12 % moisture, 33 % ash, 2 % N and 9–10 % S. It is 8–10 times more active than the older commercial samples, its activity being fairly constant. This sodium salt is used in the intravenous treatment of thrombosis in man. The free acid was prepared by electro-dialysis against parchment in an apparatus constructed by E. Hammarsten [see Ågren, 1934]. Either 25 g. of heparin dissolved in 200 ml. were electro-dialysed for 48 hr. or smaller samples were treated for a shorter time. Electro-dialysis was continued until no

alkali was traceable with litmus in the cathode liquor. The acid was neutralized with brucine and the brucine salt fractionated in water. One part of it was soluble in water. In separating the insoluble fraction a clear mother liquor was obtained only after repeated freezing and thawing. This fraction was dissolved in boiling water and again frozen out. By repeating this procedure a series of mother liquors was obtained and a final less soluble brucine salt. It was also possible to separate a small fraction which was insoluble even in a large volume of boiling water. This last fraction showed the highest S content and the greatest anticoagulant activity.

The various brucine salts were treated with a small excess of NaOH and the brucine was removed with chloroform. The solution was then neutralized with HCl against litmus and the heparin precipitated with 1.5 vol. acetone. The precipitate was collected the following day, dissolved in a small amount of water and reprecipitated with alcohol.

For S analysis the substance was fused with carbonate-nitrate in a porcelain crucible. The precipitate of BaSO₄ was collected on a Neubauer-Gooch platinum-iridium filter, ignited, washed with HCl and dried. The error of the method is insignificant. On two occasions samples were sent to Dr Schoeller of Berlin for control analysis, the same figures then being obtained as by the ordinary method.

The biological standardization was performed on fresh ox blood as described by Jorpes [1935]. This method easily discloses differences of 20 % and usually differences as small as 10 % [see also Wilander, 1938]. At least 8 stands with 10 test tubes (5 standard and 5 unknown) were used for each standardization, for some of them 14 or 16 stands. The figures in Tables I-III ought to be correct to within 10-15 %.

Two preparations of 25 g. each were fractionated; the results are presented in Tables I and II. The experiment given in Table III is incomplete.

Table I. *Fractionation of 25 g. heparin as brucine salts. Yield of sodium salts after removal of the brucine*

Source of substance	Wt. air-dry substance g.	Anticoagulant activity % standard	S % of dry substance
1st mother liquor	3.37	60	7.42
2nd mother liquor	5.30	45-50	7.01
3rd mother liquor	5.25	100	10.97
Less soluble brucine salt	2.30	125	11.74
Insoluble brucine salt I	0.266	150-160	11.88
Insoluble brucine salt II	0.238	150	11.96
	16.72		

Table II. *Fractionation of 25 g. heparin of standard potency as brucine salts. Yield of sodium salts after removal of the brucine*

Source of substance	Wt. air-dry substance g.	Anticoagulant activity % standard	S % of dry substance
1st mother liquor	5.12	20-25	8.52
2nd mother liquor	6.52	75-80	9.58
3rd mother liquor	2.73	100-110	11.58
4th mother liquor	1.51	100	11.20
Less soluble brucine salt	0.61	140-150	12.13
Insoluble brucine salt I	0.32	130	11.72
Insoluble brucine salt II	0.09	150-160	12.26
	16.90		

Table III. *Fractionation of standard heparin as brucine salts. Yield of sodium salts from the most soluble fractions*

Source of substance	Anticoagulant activity % of standard	S % of dry substance
1st mother liquor	75	9.31
2nd mother liquor	80	10.41

Table IV. *Sulphur contents and anticoagulant activities of different heparin preparations*

S % of dry substance	Anticoagulant activity % of standard
7.01	45-50
7.42	60
8.52	20-25
9.31	80
9.58	75-80
10.41	80
10.97	100
11.20	100
11.58	100-110
11.74	125
11.72	130
11.88	150-160
11.96	150
12.13	140-150
12.26	150-160

It is evident from Table IV, where the samples are arranged in a series, that the anticoagulant activity increases with increasing S content. Only one sample (no. 3) out of 15 breaks the series, and that was obtained from the first mother liquor of this preparation. It is also apparent from the table that further additions of S cause a greater increase in the activity if introduced in the highly esterified samples, thus indicating that it is the abundance of ionic charges that makes the polysaccharide an anticoagulant. The bulk of evidence now available indicates that the activity is due to the ionic charge of heparin [Jorpes, 1938].

The question arises whether the preparations with lower S content consist of highly esterified heparin and impurities. It has already been shown that their organic skeleton is the same, i.e. mucoitin. The content of uronic acid is considerably higher in these samples than in those with high S and ash contents. The amino-sugar is also demonstrated to be the same, glucosamine. The original assumption that ordinary heparin is a mixture of different poly- (di- and tri-) sulphuric esters thus seems to be justified.

The properties of the heparin preparations are strongly influenced by the introduction of additional sulphuric acid groups. This makes their barium and brucine salts less soluble or insoluble in water. The salts of the samples with 7.0-7.42% S behave like salts of chondroitin sulphuric acid, and they are not precipitated with barium chloride or barium hydroxide. On the other hand, the fractions with a content of S greater than 10.41% give less soluble or insoluble barium and brucine salts. This excludes the possibility that the former fractions contain considerable amounts of the highly esterified stronger heparin. In fact, an attempt was made to fractionate one of these samples (7.01% S) once more with brucine. After electrodialysis the solution was neutralized with

brucine. No insoluble brucine salt was precipitated. After cooling the concentrated solution, part of the brucine salt separated out and was removed in the centrifuge. From 5 g. containing 7.01 % S there were obtained 0.97 g. of a fraction containing 5.17 % S and 2.15 g. of a fraction containing 7.80 % S. The anticoagulant activities were 15 and 50 % of standard respectively. As could be expected, at least the first fractions shown in Table IV are not homogeneous, but they do not contain any considerable amount of highly esterified heparin.

Standard heparin

It will become necessary in the future to have a reference substance, a standard of heparin, as is already the case with other biological products used in medicine. A homogeneous crystalline product, if available, would be valuable for that purpose. However, the amorphous preparations of the pure liver heparin already have a very constant strength. For this reason Bergström *et al.* [1937] suggested that these should be used as standard. The same constant potency was found by Murray & Best [1938]. The potency of these liver preparations is sufficiently constant for practical use, varying by not more than 10–15 % in different preparations. This strength has been used by us as standard. We express the anticoagulant activity in mg. of this standard, considering this to be the most practical expression for that activity.

Recently at least six different units have been suggested by various workers, a tendency which would appear undesirable. Any method of assay may of course be applied in order to check the potency of a preparation against a standard, but the strength can hardly be expressed in a simpler way than in milligrammes of this standard. There is no reason for the use of smaller units, for in blood analysis as well as in therapy quantities of the order of milligrammes will be dealt with.

CONCLUSIONS

It is evident from Tables I–III that the anticoagulant activities of different heparin preparations obtained in a similar manner from the liver, vary with their S contents. Preparations of liver heparin containing from 7 to 12.26 % S show potencies varying from 45 to 160 % of the activity of the standard heparin from which they are derived. Pure liver heparin is a mixture of mucoitin polysulphuric esters which can be separated from each other as brucine salts. The starting material has a fairly constant strength and is used as heparin standard, the anticoagulant activity being expressed in milligrammes of this standard.

These preparations are sodium salts of heparin. The most potent samples, which contain about 10 % Na, contain about 13.5 % S in the free acid. For this and other reasons the alleged crystalline barium salt of heparin isolated by Charles & Scott in 1936, which has a content of S corresponding to about 11.5 % in the free acid, cannot be considered as the anticoagulant substance itself. Its homogeneous nature must be doubted. In any case, when this substance is discussed it must be kept in mind that it is merely a fraction taken out from the series of different polysulphuric esters of mucoitin which constitute heparin.

REFERENCES

- Ågren (1934). *Skand. Arch. Physiol.* **65**, 1.
Best (1938). *Brit. Med. J.* 12 Nov. p. 977.
Bergström, Jorpes & Wilander (1937). *Skand. Arch. Physiol.* **76**, 175.
Charles & Scott (1933). *J. biol. Chem.* **102**, 437.
—— — (1936). *Biochem. J.* **30**, 1927.
Fischer & Lipmann (1935). *Hoppe-Seyl. Z.* **237**, 273.
—— & Schmitz (1933). *Hoppe-Seyl. Z.* **216**, 264.
Howell (1928). *Johns Hopk. Hosp. Bull.* **42**, 199.
Jorpes (1935). *Biochem. J.* **29**, 1817.
—— (1938). *Skand. Arch. Physiol.* **80**, 202.
—— & Bergström (1936). *Hoppe-Seyl. Z.* **244**, 253.
—— — (1937). *J. biol. Chem.* **118**, 447.
Murray & Best (1938). *J. Amer. Med. Ass.* **110**, 118.
Schmitz (1935). *Hoppe-Seyl. Z.* **236**, 1.
Wilander (1938). *Skand. Arch. Physiol.* **81**, suppl. 15.