19. OBSERVATIONS ON THE STRUCTURE OF THE BARIUM SALT OF HEPARIN

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The question of the nature of heparin, an inhibitor of blood coagulation in mammals, has engaged the attention of various workers since its discovery in the liver of dogs by Howell & Holt [1918]. The early preparations of heparin were very impure but Howell [1928] examined a fairly pure material and considered it to be a glycuronic acid derivative. Charles & Scott [1933] were able to confirm the carbohydrate nature of heparin although they were unable to obtain a positive test for glycuronic acid; they noted further that their most active preparations contained some 2% of nitrogen. A valuable series of investigations by Jorpes [1935] and Jorpes & Bergström [1936] clarified the position considerably as their evidence indicated that heparin might be regarded as containing polysulphuric esters of a mucoitin. Jorpes & Bergström were able to isolate glucosamine on hydrolysis of heparin, while the amount of CO_2 evolved on heating with strong HCl indicated the presence of a large proportion of uronic acid residues. It should, however, be borne in mind that although the uronic acid concerned is probably glycuronic acid its identification as such is not rigid, and actual isolation of the acid in question from heparin would be desirable. The isolation of a crystalline barium salt of heparin by Charles & Scott [1936] represented a marked advance, since a great deal of the confusion existing in the literature on heparin is doubtless due to the varying degrees of purity of the amorphous preparations used by different workers.

The work described in this paper has been undertaken with the object of confirming the homogeneity of the crystalline barium salt and of obtaining further insight into its chemical constitution.

Experimental

Barium salt of heparin

Several methods for the purification of heparin have been developed in the Connaught Laboratories, and these have been used to prepare material both from beef liver and beef lung suitable for conversion into the barium salt. The crude heparin was prepared according to the method of Charles & Scott [1934] and purified by either of the following methods.

I. Crude heparin (*ca.* 150 g.) is dissolved in alkaline water (5 l.), the acidity adjusted to *p*H 5 with acetic acid and an aqueous solution of $CdCl_2$ run in until no further precipitation occurs. The mixture is warmed to 60° and filtered. To the filtrate NaCl is added to 0·85% and the heparin precipitated by the addition of 95% alcohol (2 vol.).

II. Crude heparin (*ca.* 150 g.) dissolved as above in alkaline water is treated with a solution of $(NH_4)_2CO_3$ (1200 ml. of 20%), warmed to 60° and allowed to

stand several hours. After removal of the dark brown precipitate by centrifuging, the filtrate is made just acid to litmus and animal charcoal (60 g.) added. The mixture is warmed to 60°, stirred 1 hr. and allowed to stand 4–5 hr. before filtering. The filtrate, to which 1 % of acetic acid is added, is again treated with charcoal, and the process repeated until the filtrate is practically colourless. To the final solution NaCl is added to 0·85 % and the heparin precipitated as above with alcohol.

In preparing the barium salt it has been found most satisfactory first to prepare the ammonium salt, which can readily be obtained with an ash content < 1 %. Its preparation may be carried out in two ways.

A. Heparin (10 g.) purified by either of the above methods is dissolved in ammoniacal water (125 ml.) and $(NH_4)_2CO_3$ solution (15 ml. of 20 %) is added. After warming to 60° the mixture is allowed to cool and filtered. From the clear filtrate the heparin salt is precipitated by the addition of acetic acid (9 vol.), collected, washed with alcohol and ether and dried.

B. Benzidine method. This has already been described [Charles & Scott, 1936].

Conversion of the ammonium salt into the crystalline barium salt is carried out as already described by Charles & Scott [1936]. In the present work the barium salt was prepared from heparin isolated from lung and liver by the above methods and was analysed for potency, ash content and nitrogen. The results are shown in Table 1, the potency being determined by the method of Charles & Scott [1936].

Table 1

Starting material	Methods	% ash	Potency u./mg.	% N
Beef lung	$CdCl_2$-$(NH_4)_2CO_3$	33·1	98	2·13
,,	$CdCl_2$-benzidine	33·8	98	2·04
,,	Charcoal-benzidine	33·7	104	1·89
,,	Charcoal-$(NH_4)_2CO_3$	33·1	97	1·90
Beef liver	$CdCl_2$-$(NH_4)_2CO_3$	33·8	100	2·20
,,	$CdCl_2$-benzidine	33·2	98	2·01
,,	Charcoal-$(NH_4)_2CO_3$	33·3	98	1·90

Mr L. B. Jaques of the Connaught Laboratories has prepared in similar fashion from beef intestine a crystalline barium salt apparently identical with the products from lung and liver; it had an ash content of 33·4 %, contained 1·89 % N and had a potency of 100 u./mg.

These findings indicate that the barium salts prepared by various routes from different beef tissues are identical and support our view that the salt is a chemical individual.

From the barium salt, by treatment with $(NH_4)_2CO_3$ followed by filtration from $BaCO_3$ and precipitation with acetic acid, the amorphous ammonium salt may be prepared; this material contains 0·5 % or less of ash and biological tests indicate a 90 % recovery of activity; the balance is possibly adsorbed on the $BaCO_3$. The results of a series of analyses on these salts indicate that heparin contains 5 S atoms for every 2 N atoms, a result in agreement with earlier observations [Charles & Scott, 1936] and with the findings of Reinert & Winterstein [1939]; the analytical values are discussed later in this paper.

The ammonium salt of heparin was reconverted into the barium salt in the usual way. After collecting the first crop of crystals a second fraction was obtained by cooling in the ice-chest; the residual material in the mother liquor was isolated by evaporation *in vacuo*, and washing free of barium acetate with 80 % acetic acid. The sulphur content of the various products was determined.

The results collected in Table 2 show that the ammonium salt can be converted into a barium salt of uniform sulphur content, differing only in physical appearance due to mode of separation.

Table 2

Substance	Weight (g.)	S %	S content (mg.)
Ammonium salt*	2·00	12·2	244
Ba salt, 1st crop	1·77	9·6	170
Ba salt, 2nd crop	0·17	9·8	17
Ba salt from mother liquor	0·22	9·6	21

* The ammonium salt used was dried at room temperature.

The claim of Jorpes & Bergström [1939] that heparin is a mixture of sulphuric esters which can be separated by fractionation with brucine into several fractions with varying sulphur content prompted us to apply the process to the crystalline barium salt. Using either brucine or brucine sulphate the results were negative; both brucine precipitates and supernatants on working up gave material of the same sulphur content and all could be reconverted into a barium salt apparently identical with the starting material.

Condition of nitrogen in heparin. Heparin yields glucosamine on hydrolysis and it is virtually certain that all the nitrogen in the molecule is accounted for by amino-sugar residues. While it would be expected that the amino-groups would be acetylated as in, for example, chondroitin sulphuric acid, small amounts (up to *ca.* 0·5 %) of nitrogen are liberated from heparin in normal Van Slyke estimations. In view of the observation of Hynd & Macfarlane [1926], who found that quantitative evolution of nitrogen from glucosamine under the usual Van Slyke conditions was only attained after some 16 hr. shaking, we felt it was desirable to apply the same technique to heparin. Dr C. P. Stewart of Edinburgh University, to whom we are deeply indebted, carried out for us parallel determinations on *N*-acetylglucosamine (kindly supplied by Dr W. T. J. Morgan) and on the barium salt of heparin. The results showed that the amino-N value for heparin increased gradually with the time of reaction until after 16 hr. the value reached (1·84 %) represented virtually all the N present. Under the same conditions *N*-acetylglucosamine gave no measurable amount of nitrogen even in 16 hr.

A series of *N*-acetyl determinations were made by three different analysts but the results obtained even with the same sample were not concordant (Found: *N*-acetyl 1·26, 1·56; 1·76, 2·9; 2·2). It is difficult to lay much stress on these values although they indicate that part at least of the N in heparin is acetylated. The curious result of the Van Slyke determinations is doubtless due to slow hydrolysis of the *N*-acetyl grouping.

Inactivation of heparin with acids

That heparin loses its potency when treated with alcohol containing small amounts of HCl has already been reported [Charles & Scott, 1936]. This inactivation is attended by loss of sulphur-containing groups, and by varying the acid and alcohol concentration and the temperature and duration of reaction a correlation between sulphur content and activity has been obtained. The results are collected in Table 3. In each case the products were precipitated as amorphous barium salts and the ash content taken as a measure of sulphur content. Potency is expressed relative to the barium salt of heparin.

Table 3

Product	*N* HCl conc.	% MeOH	Temp.	Time	% ash	Potency
Heparin Ba Salt	—	—	—	—	33·5	100
1	0·01	25	5°	1 hr.	31·2	72
2	0·01	50	5°	1 hr.	30·5	57
3	0·025	50	5°	1 hr.	28·6	43
4	0·05	50	5°	1 hr.	27·9	40
5	0·1	99	5°	10 min.	26·0	22
6	0·1	99	5°	20 min.	24·6	20
7	0·028	99	5°	17 hr.	24·5	20
8	0·1	99	5°	1 hr.	22·4	13
9	0·05	99	5°	17 hr.	21·0	9
10	0·1	99	5°	2 hr.	20·1	8
11	0·1	99	5°	17 hr.	18·0	4
12	0·1	99	20°	1 hr.	15·2	2·7
13	0·1	99	20°	2 hr.	12·7	1·5
14	0·1	99	20°	7 hr.	8·5	1·0
15	0·1	99	20°	17 hr.	7·4	0·6

These results indicate that the potency varies with the sulphur content of the products.

With a view to obtaining an insight into the nature of the above inactivation the ammonium salt of heparin (1 g.) was treated with 0·1 *N* HCl in 99 % MeOH at 20° during 24 hr. The reaction products were then precipitated by addition of acetic acid (9 vol.), collected, washed with ether and dried. Analysis of the material obtained showed that approximately 80 % of the S had been removed, although the remainder appeared still to be present in an ammonium salt the NH_4-N/S ratio being 1/1. The methoxyl content was 7 % which is probably significant since heparin dissolved in MeOH and reprecipitated showed no methoxyl content. The inactivated product was not obtained in crystalline form and is certainly not a single substance; unlike heparin, it had definite though weak reducing properties, and was at least partially dialysable. Further work is in progress on the nature of this material but its properties indicate that some breakdown of the carbohydrate molecule takes place in the treatment with acidic alcohol. This may explain why attempts to re-introduce sulphate groups into the inactivated material failed to yield any product with significant physiological action even although materials containing as much as 8 % S were obtained.

Experiments were also carried out in which heparin was hydrolysed by heating with *N* H_2SO_4, hydrolysis being continued until the solutions had a reducing value corresponding to half that expected for complete hydrolysis to monosaccharide derivatives. So far it has not been possible to obtain any crystalline identifiable product from these experiments, possibly because of the small amounts of material employed.

Oxidation experiments

The use of periodic acid as a specific oxidizing agent for α-glycols introduced by Malaprade [1928] has recently been applied by Hudson and his collaborators [cf. Jackson & Hudson, 1937] in an elegant series of investigations on sugar derivatives. Application of this reagent offers the possibility of learning something of the distribution of sulphuric acid groupings in heparin and we have begun a series of investigations to this end. Preliminary experiments show that heparin itself is stable to periodic acid while heparin inactivated by various processes shows varying degrees of reducing power towards this reagent. The results of the investigations will be reported later.

Discussion

It has been shown that the crystalline barium salt of heparin prepared by various methods from different beef tissues has uniform composition and physiological activity. It can be converted into an ammonium salt and the latter reconverted almost quantitatively into a barium salt with the same composition as before. Moreover, the heparin from the barium salt showed no evidence of separating into distinct fractions with brucine. The ratio S/N in heparin appears to be 5/2 on the basis of analyses of the barium and ammonium salts. The same ratio has recently been found by Reinert & Winterstein [1939] for the sodium salt.

In a recent publication Jorpes & Bergström [1939] cast doubts both on the crystalline nature and on the homogeneity of the barium salt without apparently having themselves examined it. Of its crystalline nature there can be no doubt; we had hoped to obtain some insight into the detailed structure of the salt by X-ray examination but Dr W. H. Taylor of the Manchester College of Technology found that the crystals were too small and heavily twinned for the X-ray diagram to be of much assistance; we are indebted to Dr Taylor for examining the material. As regards homogeneity we consider that all the available evidence suggests that the barium salt is a chemical individual; the possibility that we are dealing with mixed crystals seems unlikely in view of the various interconversions carried out without change in composition. In the communication of Jorpes & Bergström [1939] there appears the remarkable statement that the ratio S/N = 2·5/1 in the formula suggested by Charles & Scott [1936] for the barium salt "immediately disproves its correctness". Since the ratio might equally well be written 5/2 and there is no evidence to show that heparin is built of a recurring fundamental unit of 1 mol. glucosamine and 1 mol. uronic acid, each unit containing the same number of sulphate residues, we are unable to see the force of the argument presented. As regards sulphur content it should be noted that heparin preparations contain varying amounts of water according to the drying procedure adopted; this may explain the varying sulphur content quoted by different workers although it should not affect the more significant S/N ratio.

The brucine fractionation experiments of Jorpes & Bergström [1939] carried out on impure amorphous heparin preparations are interesting, although some separation was to be expected. One striking feature emerges on analysis of the figures published by these authors; the recovered material accounts for 70 % of the weight and 70 % of the S of the heparin used, while the unrecovered material accounts for some 30 % of the weight and about 50 % of the original activity. Thus, if no degradation of the active heparin has occurred in the experiment, the material lost in the brucine fractionation has an activity per unit weight equal to that of the most active material isolated. Equally striking is the low sulphur content of the material which has apparently been lost. It is doubtless true that crude heparin may consist of a mixture of compounds of varying sulphur content, but this does not in any way reflect on the homogeneity of the barium salt isolated from it.

As a result of the investigations so far carried out on heparin one may consider it as a mucoitin polysulphuric acid. Accepting as a basis the mucoitin formula of Levene [1925] and regarding as a fundamental unit a tetrasaccharide structure made up of 2 mol. *N*-acetylglucosamine and 2 mol. glycuronic acid it is clear that each unit must contain 5 sulphuric acid residues, i.e. heparin should have the formula

$$C_{22}H_{29}O_{12}\begin{cases}\text{-}(OSO_3H)_5\\ \text{-}(COOH)_2\\ \text{-}(NHCOCH_3)_2\end{cases}$$

This formula has also been proposed by Reinert & Winterstein [1939] who have shown that the analytical values of the sodium salt are in accordance with it. The crystalline barium salt has been analysed on numerous occasions, and contains 5 Ba atoms to every 10 S atoms; since the sodium salt of Reinert & Winterstein [1939] contains 7 Na atoms to every 5 S atoms one must conclude that the carboxyl groups in the barium salt are free or that they are lactonized, i.e. that the glycuronic residues are in the form of glycuronolactone. We have been unable to demonstrate the presence of lactone groupings although the mode of preparation of the barium salt would not conflict with their presence; the presence of free carboxyls is supported by the fact that a 1% aqueous solution of the salt has *p*H *ca.* 3·3 (calomel electrode) and that it readily takes up approximately 2 equivalents of NaOH in the cold. On this view the formula of the barium salt should be $(C_{28}H_{39}O_{38}N_2S_5)_2Ba_5$. Allowing for $24H_2O$ in the hydrated crystals the analytical values are in fairly good agreement with the calculated values. (Found: Ba, 19·7; N, 1·9; S, 9·6; H_2O (loss at 60° in high vac.), 12·1 $(C_{28}H_{39}O_{38}N_2S_5)_2Ba_5$ $24H_2O$ requires Ba, 19·8; N, 1·6; S, 9·3; H_2O, 12·5. Found in anhydrous salt C, 20·2; H, 3·5. Required C, 22·5; H, 2·6. Charles & Scott [1936] found in hydrated salt C, 18·0; H, 3·9. Required C, 19·3; H, 3·6.

Treatment of the barium salt with $(NH_4)_2CO_3$ gives an amorphous ammonium salt whose analysis is also in agreement with the heparin formula under discussion. It would appear to contain either 5 or 6 (NH_4) radicles to every 5 S atoms. (Found (anhydrous): NH_4-N, 6·1; bound N, 2·3; S, 12·0, $C_{28}H_{39}O_{38}N_2S_5(NH_4)_5$ requires NH_4-N, 5·6; bound N, 2·2; S, 12·7. $C_{28}H_{38}O_{38}N_2S_5(NH_4)_2$ requires NH_4-N, 6·6; bound-N, 2·2; S, 12·5.)

Assuming that there are two free carboxyl groups in the barium salt the formula containing 6 (NH_4) radicles could be understood on the grounds that the second carboxyl might be too feeble to form an (NH_4) salt under the conditions employed. It can, of course, be readily understood that the sodium salt of heparin formed by means of NaOH will contain 7 Na atoms to every 5 S atoms.

It should be realized that although the analytical values obtained are in agreement with the formula proposed, they do not constitute proof of its correctness. It is wellnigh impossible to deduce an accurate empirical formula of this magnitude on the basis of analysis of salts containing some 33% of ash. The formula is however in accordance with the known facts about heparin and the analytical values do not conflict with it. Whether or not the molecular formula of heparin corresponds to the simple tetrasaccharide structure, or whether it is a complex formed by fusion of a number of such units cannot be stated definitely at present.

The question of a standard heparin preparation is discussed by Jorpes & Bergström [1939]. It seems reasonable to suppose that the most satisfactory standard would be the stable crystalline Ba salt rather than amorphous preparations from liver whose activity admittedly varies.

Summary

1. Various methods are described for the preparation of a crystalline barium salt of heparin.

2. Evidence is presented for the view that the barium salt, isolated from various tissues, is identical in properties and is a chemical individual.

3. By treatment with acidified methyl alcohol under varying conditions heparin yields a series of products of lower physiological activity, decrease in activity being accompanied by a decrease in sulphur content. Material completely

inactivated in this way appears to have undergone structural alteration in addition to removal of sulphate groupings.

4. The structure of heparin is discussed. The results of investigations to date are best explained on the basis that it is a mucoitin sulphuric acid in which the basic tetrasaccharide unit contains 5 sulphuric ester groupings. Analyses of the barium salt and of the ammonium salt prepared from it are in agreement with this view. In the barium salt the carboxyl groups appear to be unsubstituted.

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