CCLXX. STUDIES ON HEPARIN.
IV. OBSERVATIONS ON THE CHEMISTRY OF HEPARIN.

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SINCE Howell & Holt [1918] demonstrated that dog liver contained a substance, heparin, that retards the coagulation of blood *in vitro*, much work has been done to devise methods for isolating very active preparations of this material. Chemical analyses of such products, however, have differed greatly, owing to the fact that the substance has not yet been isolated in pure form. Howell [1928] obtained a product which was N- and P-free and concluded that heparin was a derivative of glycuronic acid. Schmitz & Fischer [1933] believed heparin to be a trisaccharide $C_{18}H_{32}O_{17}$ containing one carboxylic group. The product isolated by Scott & Charles [1933] gave a positive α-naphthol reaction but the test for glycuronic acids with naphthoresorcinol was negative. Their preparation contained approximately 2% N and was readily inactivated by nitrous acid. These facts suggested the presence of an amino-group essential for the physiological action of heparin. The more recent work of Jorpes [1935] has contributed much to the chemistry of heparin. His results led him to advance the theory that heparin is a chondroitin polysulphuric acid which would explain the difficulties encountered in attempting to remove inorganic impurities from heparin preparations. He also found that his product contained approximately 2% N and gave no test for glycuronic acid. Schmitz [1935] more recently has isolated a very active preparation which was not a sulphuric acid derivative and which contained only 4–5% ash.

Because of the divergence of opinion as to the chemical nature of heparin, much work has been done in these laboratories in an attempt to isolate this substance in pure form. The active substance is completely soluble in formamide and in butylamine but addition of other organic liquids to solutions of heparin in these solvents produces only amorphous precipitates. It was thought that perhaps the failure to crystallize heparin was due to its high ash content. The heparin was therefore prepared as the calcium salt and, after removal of calcium, reprecipitated with glacial acetic acid, yielding a product usually with about 5% ash. Further attempts to reduce the ash content were unsuccessful until it was found that precipitation of heparin from aqueous solutions by means of benzidine reduced the ash content to 0·7%. Attempts to crystallize the resultant preparation by means of various solvents or as an alkaloidal salt only resulted in amorphous products. Finally barium acetate was used and a crystalline barium salt was obtained. The details of the procedure used for isolating the crystals as well as their analyses and other chemical findings are recorded below.

EXPERIMENTAL.

To prepare heparin containing about 60 units per mg. we have used as our initial material crude heparin obtained from ox lung according to the method previously described [Charles & Scott, 1934]. Following our usual procedure

[Scott & Charles, 1933] the crude anticoagulant was then purified by Lloyd's reagent and acetic acid. The product contained 15 units per mg. Using this material highly active preparations of uniform activity were obtained in the following manner.

20 g. of heparin (15 units per mg.) are dissolved in 2 l. of water. The reaction is adjusted to *p*H 5·0 with glacial acetic acid and 20 g. of Lloyd's reagent are added; after 12–15 hours, the mixture is filtered by suction. The reaction of the filtrate is adjusted with acetic acid to *p*H 4·0 and 20 g. of Lloyd's reagent are added. After filtration, NaCl is added to 0·85 % followed by acetone (approx. 25 % final concentration) to produce a flocculent precipitate. The precipitate is removed by centrifuging and discarded and the heparin precipitated from the clear centrifugate by the addition of acetone to a final concentration of 66 %. After 12–15 hours, the clear supernatant liquid is removed by filtration and the precipitate washed thoroughly with alcohol and dried. 6 g. of this material are then dissolved in 300 ml. of water and 10 % $CdCl_2$ is added until no further precipitation occurs; after 4–5 hours the mixture is centrifuged and the precipitate rejected. NaCl is then added to the clear liquid to 0·85 % and the heparin precipitated by adding 2 vol. of acetone. The purified heparin is separated by filtration, washed with alcohol and dried. The activity is 60–65 units per mg.

About 70 % of the original potency is recovered in the highly purified form by this method. The preparations, however, contain a larger proportion of inorganic material, the greater part of which can be removed in the following manner. 1 g. of the purified heparin (60 units per mg.) is dissolved in 30 ml. of water and acidified to about *p*H 5 with acetic acid. To this is added 1 ml. of a saturated solution of ammonium oxalate. The mixture is warmed to 50° and centrifuged. The process of adding oxalate solution, heating and centrifuging is repeated until the addition of ammonium oxalate to the supernatant liquid no longer gives a precipitate. The clear brown supernatant liquid is decanted and evaporated to dryness *in vacuo*. The residue is dissolved in 5 ml. of water and the heparin precipitated by the addition of 45 ml. of acetic acid. After centrifuging the precipitate is redissolved in 5 ml. of water and reprecipitated with 45 ml. of glacial acetic acid. The precipitate is washed with alcohol and ether and then dried, yielding a white powder containing 70–80 units per mg. and about 5 % ash.

Many attempts were made to reduce further the ash of this purified heparin. It seemed likely that the inorganic material was held in combination with the acid groups of heparin. Neuberg & Schuchardt [1935] have shown that benzidine combines with the phosphoric acid group of phosphoglyceric acid and it seemed probable that benzidine might combine with the acid groups of heparin. Furthermore, the benzidine could subsequently be removed from such a complex by treatment with aqueous solutions of sodium or ammonium hydroxide. Using this process, it has been found that the ash content of heparin can be reduced to 0·7 % by the following treatment. 100 mg. of heparin, after the ammonium oxalate treatment, are dissolved in 2 ml. of water and 5 % benzidine hydrochloride in dilute HCl is added, giving a brown tarry precipitate. The addition of the benzidine solution is continued until, after centrifuging, no further precipitate is obtained. The precipitate is washed thoroughly with methyl alcohol and dried. This material is then suspended in water (5 ml. per 100 mg.) and made alkaline with sodium or ammonium hydroxide. The mixture is heated to 75° until a clear brown solution is obtained. When the solution is cooled slowly to 6° the benzidine precipitates and is removed by centrifuging. The supernatant

liquid is brown and contains a few small crystals of benzidine which are dissolved by adding 2 vol. of methyl alcohol. From this solution the heparin is precipitated by 2 vol. of ether and after washing with alcohol and ether is dried. This material is then dissolved in 5 ml. of water and the heparin precipitated by the addition of 45 ml. of glacial acetic acid. The precipitate is washed twice with 90% acetic acid, then with alcohol and ether. The dried material contains only 0·7% ash.

Barium salt of heparin. 50 mg. of the heparin of low ash content (0·7%) are dissolved in 4 ml. of water and 1 ml. of 5% barium acetate is added. This causes a slight turbidity. After 12–15 hours at 20° a brown tarry inactive material separates. The clear centrifuged supernatant liquid is decanted and to it 1 ml. of glacial acetic acid is added. A white precipitate forms which is dissolved by heating to 70–75°. When cooled slowly to room temperature a crystalline precipitate forms. A photomicrograph of the crystals is shown in Plate IV. The mixture is cooled to 6° for several hours and then centrifuged. The crystals are washed twice with 90% acetic acid, methyl alcohol and finally with ether; the final traces of ether are removed *in vacuo*. Two samples of crystals were prepared, each obtained from different lots of lung. The results of the chemical analyses of both crystalline preparations are shown in Table I. Samples of the crystals were ashed in the presence of oxygen. To other samples, one or two drops of H_2SO_4 were added before ashing. Since the weights of ash determined by each procedure were practically identical, the inorganic material must have been present as a sulphate. In the preparation of sample I the benzidine was removed by treatment with ammonia, whereas in sample II NaOH was used. The ash contents however, were essentially the same. Since the use of NaOH did not increase the ash content, the inorganic substance forming the ash must be $BaSO_4$. The total S was found by first estimating the S present as volatile SO_3 by the method of Pregl [1930] which was found to be 4·78%. The residue was then ashed and from the inorganic material ($BaSO_4$) it was found that the non-volatile S was 4·58%. Hence the total S in the crystals is 9·36%. These S values indicate that Ba is present in the crystalline salt in the form RSO_3—Ba—SO_3R. From such a substance, one-half of the S content would be determinable as an oxide of S, whilst the other half would be estimated as non-combustible material in the form of $BaSO_4$.

Table I. *Analysis of heparin.*

Ba salt of heparin	C %	H %	N %	S %	Ash ($BaSO_4$) %	Ba %
Sample I	17·87	3·89	1·63	9·24	33·8	19·9
Sample II	18·00	3·88	1·73	9·36	33·3	19·6
Ba-free heparin (calc.)						
Sample I	22·22	4·84	2·01	11·49	—	—
Sample II	22·39	4·82	2·14	11·64	—	—

The amino-N content of the crystals (Van Slyke) was found to be 0·40%.

On heating in a platinum boat at 110° in a current of dry air the crystals attained constant weight in 2 hours, the loss in weight being 5%.

An attempt was next made to remove the Ba and analyse the ash-free material. Again the use of benzidine was found to be the most satisfactory means of accomplishing this.

Benzidine salt of heparin. 200 mg. of crystals are dissolved in 10 ml. of water. Benzidine hydrochloride in dilute HCl is added until no further precipitate forms. This precipitate is washed, first with methyl alcohol, then with

ether and dried. The material is suspended in 10 ml. of water and *N* NaOH added until alkaline. The suspension is heated to 70–75° and the resulting clear solution cooled slowly to 0°. The benzidine is removed by centrifuging, and the heparin precipitated by the addition of 9 vol. of acetic acid. After washing thoroughly with methyl alcohol and ether, the material is dried, redissolved, precipitated again as the benzidine compound and the benzidine removed as described above. The resultant product is dissolved in 7 ml. of water and the benzidine compound again formed. The precipitate is washed thoroughly with alcohol and ether and dried. Analyses of this compound are shown in Table II. Since the S content of Ba-free heparin is 11·56%, and the benzidine-heparin compound contains 8·46% S, it can readily be calculated that heparin constitutes 73·3% of the benzidine-heparin compound. From this relationship the composition of benzidine-free heparin is estimated and is also shown in Table II.

Table II. *Analysis of heparin.*

Material	C %	H %	N %	S %	Ash %
Benzidine-heparin compound	38·05	5·40	5·60	8·46	1·09
Benzidine-free heparin (calc.)	23·09	4·99	2·10	11·54	—

Miscellaneous experiments.

Action of nitrous acid on heparin. The crystalline material when treated with $NaNO_2$ in the presence of acetic acid (*p*H 4·0) was almost completely inactivated, whilst no inactivation occurred in solutions having *p*H 7 or higher. Solutions of heparin at *p*H 4 without $NaNO_2$ showed no loss of potency under similar conditions.

Action of formaldehyde on heparin. The heparin crystals, on treatment with about 100 parts of formalin for 1 hour at 45° at *p*H 8 and *p*H 4 lost about 50% of their activity. Controls run under the above conditions in the absence of formaldehyde showed no loss.

Colour tests. The Molisch test for carbohydrate compounds was positive. The naphthoresorcinol test for glycuronic acid was negative as was Tollens's phloroglucinol reaction for pentoses or glycuronic acid. Control tests showed that heparin did not interfere with these reactions.

Action of acid alcohol on heparin. The benzidine was removed from the benzidine-heparin compound by treatment with ammonia as described above. 10 mg. of this material were dissolved in 1 ml. of 0·1 *N* HCl in 95% methyl alcohol. This solution was kept at 10° for 20 hours. The heparin was precipitated by the addition of 9 ml. of glacial acetic acid, washed with ether and dried. A weighed sample of this material was assayed and found to have lost approximately 80% of its potency. The acetic acid supernatant liquid from the inactivated heparin was evaporated to dryness and the residue redissolved in 1 ml. of water. When tested with barium acetate this solution gave a precipitate indicating the presence of $SO_4^=$. A control experiment in which the heparin was precipitated immediately from acid alcohol without loss of potency did not show the presence of $SO_4^=$ in the filtrate. When acid water was used instead of methyl alcohol no inactivation of the heparin occurred over 20 hours.

Physiological assay.

In the present investigation the two crystalline samples of heparin were compared physiologically with two standardized preparations. The first standard was prepared in these laboratories and was three times as active as the com-

mercial product of Hynson, Westcott and Dunning. This ratio has been checked many times. The second was the commercial material of Hynson, Westcott and Dunning. This was used so that the unitage of the crystalline heparin could be directly correlated with the more active preparation of Jorpes.

The method of assay was as follows. 3·520 mg. of sample I were dissolved in 3·50 ml. of isotonic saline, and 5·282 mg. of sample II in 5·30 ml. of isotonic saline. Each dilution was diluted further 1:40, 1:45 and 1:50. In order to compare the potency of our material with that of Jorpes, a solution of commercial heparin (Hynson, Westcott and Dunning) was made containing 3·643 mg. in 7·30 ml. This latter solution was used without further dilution. Into a series of tubes 0·1, 0·2 and 0·3 ml. of each solution was measured. Isotonic saline was added where necessary to bring the volume in each tube to 0·3 ml. By means of a cannula inserted in the carotid artery of a cat anaesthetized with sodium amytal, 0·7 ml. of blood was added to each tube. The tubes were kept at 20° for 2 hours and then examined. It was found that sample I or sample II in a concentration of 1 mg. in 45 ml. showed the same potency as the control solution containing 1 mg. in 2·0 ml. Hence the ratio of the activity of the crystals to that of the commercial product of Hynson, Westcott and Dunning is approximately 22:1. Thus the crystals are about twice as active as the purest preparation isolated by Jorpes. When the assay was performed by the method formerly described by us [Scott & Charles, 1933] the crystals had a potency of approximately 500 units per mg.

Discussion.

A method of obtaining very active heparin preparations has been described. Using this procedure a quantity of heparin was purified from different lots of ox lung. After reducing the ash content of these preparations to 0·7% by means of ammonium oxalate and by benzidine, the crystalline barium salt was formed. Analyses of each lot of crystals are in agreement (Table I) and when calculated on a Ba-free basis, the results indicate that the empirical formula of heparin can be expressed as $C_{25}H_{65}O_{50}N_2S_5$. From the analytical and qualitative results it appears that S is present in heparin in the form $—SO_3H$. The positive Molisch reaction indicates that a carbohydrate grouping is present in crystalline heparin. Tests with naphthoresorcinol and with phloroglucinol were negative, indicating the absence of glycuronic acid and pentoses. The fact that negative tests were obtained substantiates our former findings and agrees with the more recent results of Jorpes. The possibility that the carbohydrate is combined with the $—SO_3H$ groups as in chondroitin sulphuric acid has been suggested by Jorpes. Indirect evidence that such may be the case is indicated by the work of Bergstrom [1936] who showed that certain polysaccharides, when sulphonated, had a definite anticoagulant action on blood. Further, our work shows that when ash-free heparin was treated with methyl alcohol containing a small amount of HCl, (0·1 *N*), at 10° the potency was destroyed and that this inactivation was accompanied by the liberation of $SO_4^=$. Gebauer-Fuelnegg & Dingler [1930] have shown that $—SO_3H$ groups can readily be removed from sulphonated cellulose by treatment with acid methyl alcohol. The fact that the potency of the heparin inactivated by methyl alcohol could not be recovered with aqueous or alcoholic NaOH indicates that the loss in potency was not due to esterification. Finally, it should be noted that the analyses are not in agreement with Jorpes' view that heparin is a chondroitin polysulphuric acid, the carbon content of heparin being much too low. However, it is quite possible that heparin is very similar in structure to chondroitin sulphuric acid.

From the crystalline salt of heparin an amorphous benzidine-heparin compound was prepared. The composition of heparin calculated from the analysis of this compound (Table II) is practically identical with that estimated for ash-free heparin from the Ba salt (Table I).

The crystalline Ba salt of heparin contains N, part of which is associated with the anticoagulant action, as shown in experiments with nitrous acid or formaldehyde. This confirms our earlier findings [Scott & Charles, 1933]. The inactivation does not appear to be due to acidity since the control solutions showed no loss in potency. Neither can the inactivation be ascribed to oxidation or reduction since heparin is comparatively stable to such reagents. An estimation of amino-N (Van Slyke) showed that only about 25% of the N was present in this form.

The physiological activities of heparin preparations obtained by different workers are difficult to correlate. This is largely due to the fact that different methods of assay have been used. In these laboratories known amounts of heparin were mixed with cat blood and the samples kept for a definite time before being examined. Jorpes, on the other hand, used ox blood and inverted the tubes at certain intervals during the test. Fischer & Schmitz [1935] have defined the unit in terms of the clotting time of a system of hen plasma and muscle extract. These considerations, together with the fact that no common standard preparation has been used, have contributed to a lack of uniformity in expressing the unitage of heparin preparations. It seems logical that the unit should be expressed in terms of a highly active material which can readily be prepared and which is of uniform composition and activity. Further, definite conditions for the assay of heparin should be established with the object of obtaining the most reliable method for the comparison of different preparations. The crystals, when assayed physiologically by the method formerly described [Scott & Charles, 1933], showed a potency of about 500 units per mg. In order to obtain a relation between the potency of Jorpes's most active preparation and the crystalline material, the activity of the latter was compared with the commercial product of Hynson, Westcott and Dunning. It was found that the crystals were 22 times as active as the commercial product. This is about twice the potency of Jorpes's preparation.

Summary.

A method has been described for obtaining very active preparations of heparin. Benzidine was found to be most satisfactory for removing inorganic materials. The crystalline barium salt was formed and analyses showed that the empirical formula of heparin could be expressed as $C_{25}H_{65}O_{50}N_2S_5$. An amorphous benzidine-heparin compound was formed, analysis of which gave the same empirical formula for heparin. Evidence has also been presented confirming the finding of Jorpes that the sulphur is present in heparin as $—SO_3H$ groups. The crystalline product contained nitrogen, part of which was present as $—NH_2$ groups. Experiments with nitrous acid and formaldehyde indicated that the amino-nitrogen was associated with the physiological activity of heparin. Certain colour tests indicated the presence of a carbohydrate complex and the absence of pentoses and glycuronic acid. Potency assays showed that the crystalline preparation was 22 times as active as the commercial standard used by Jorpes.

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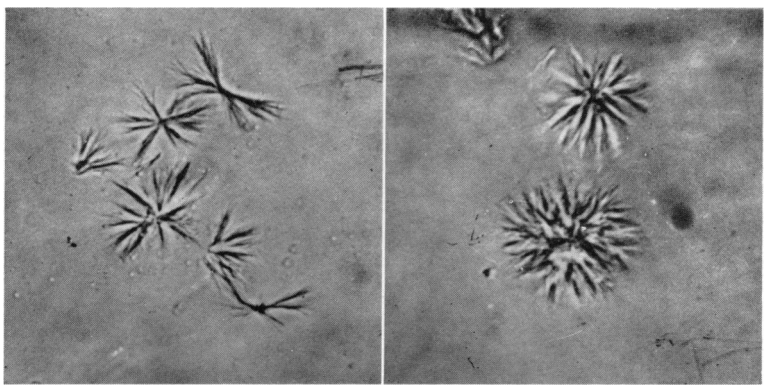

A photomicrograph of the crystalline barium salt of heparin.
Magnification $\times 1000$.

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