been condensed with a varied series of heterocyclic compounds.

2-Thiohydantoin, 1-benzoyl-2-thiohydantoin, 1-acetyl-2-thiohydantoin, rhodanine, pseudothiohydantoin, N^2-phenylpseudothiohydantoin, and N^2,-3-diphenylpseudothiohydantoin on condensation with the quinoline aldehydes gave products of the unsaturated "benzylidene" type in every instance. In the case of the 1-benzoyl and 1-acetyl-2-thiohydantoins, condensation was accompanied by simultaneous loss of the acyl group.

Hydantoin, 5-methylpseudothiohydantoin and 5 - methyl - N^2,3 - diphenylpseudothiohydantoin combined with the quinoline aldehydes producing aldol-like compounds in each case.

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[CONTRIBUTION FROM THE CHEMICAL LABORATORY OF THE OHIO STATE UNIVERSITY]

The Relation between the Structure of Heparin and its Anticoagulant Activity

BY M. L. WOLFROM AND W. H. MCNEELY[1]

In a previous publication[2] from this Laboratory, it was reported that the crystalline barium acid heparinate (from beef) of Charles and Scott[3] lost its biological activity on repeated crystallization from dilute acetic acid. Since this might indicate that the barium acid heparinate was merely a carrier for an active impurity, this inactivation was subjected to further investigation, the results of which are herewith reported.

The anticoagulant activities were determined in this Laboratory essentially according to the Foster[4] modification of the procedure of Reinert and Winterstein[5] and are expressed as Roche anticoagulant units per milligram (Roche ACU per mg.). It was found more convenient and more conducive to uniform results, to prepare a mixed sample of citrated beef plasma from a number of animals and to store the frozen plasma in small bottles for use as needed.

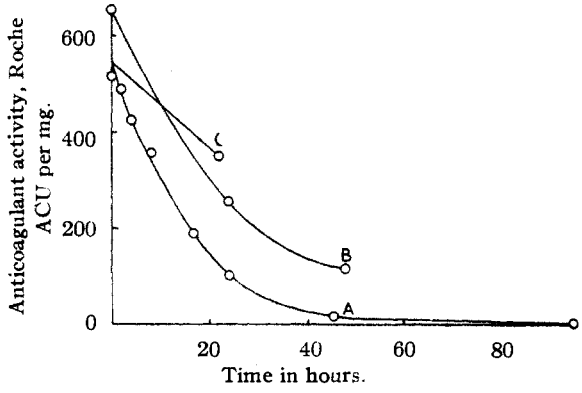

Fig. 1.—Inactivation of heparin salts (c 2, 68 ± 2°) by mild acidity. A, barium acid heparinate in 11% acetic acid (*cf.* Table I); B, sodium heparinate in 11% acetic acid; C, barium acid heparinate in water.

Unless otherwise noted, the barium acid heparinate used in the present work was the crystalline material prepared through the benzidine salt and the sodium heparinate used was the amorphous material prepared from this crystalline barium acid heparinate.

Curve A of Figs. 1 and 2 shows the rate of inactivation of a 2% solution of barium acid heparinate in 11% acetic acid solution at 68 ± 2°. The activities at each point were determined on the whole solution. Forty-eight hours of such treatment led to an essentially inactive solution from which there could be obtained with some difficulty, in 55% yield, a crystalline product similar in appearance to the original crystalline barium acid heparinate. These crystals were, however, considerably more soluble in dilute aqueous acetic acid (containing barium acetate) than the active crystals. In one experiment, crystallization was effected at the eight and twenty-two hour points of Curve A. A yield of 47 and 30%, respectively, of well-formed crystals was obtained. No significant differences in the activities, expressed as

TABLE I

INACTIVATION OF BARIUM ACID HEPARINATE (c 2, 68 ± 2°) IN 11% ACETIC ACID

Time, hr.	Solution activity, Roche ACU per mg.	Precipitate activity, Roche ACU per mg.	Precipitation yield, %
0		515	
2.1	490		
4.0	425		
8.0	355		
16.7	190		
24		95	86
24		115	94
24	100		
24	95		
45.5	15		
45.5		15	
95		3	
95	0		

(1) Hoffmann-La Roche Postdoctoral Fellow of The Ohio State University Research Foundation.

(2) M. L. Wolfrom, D. I. Weisblat, J. V. Karabinos, W. H. McNeely and J. McLean, THIS JOURNAL, **65**, 2077 (1943); *Science*, **97**, 450 (1943).

(3) A. F. Charles and D. A. Scott, *Biochem. J.*, **30**, 1927 (1936).

(4) R. H. K. Foster, *J. Lab. Clin. Med.*, **27**, 820 (1942).

(5) M. Reinert and A. Winterstein, *Arch. intern. pharmacodynamie*, **62**, 47 (1939).

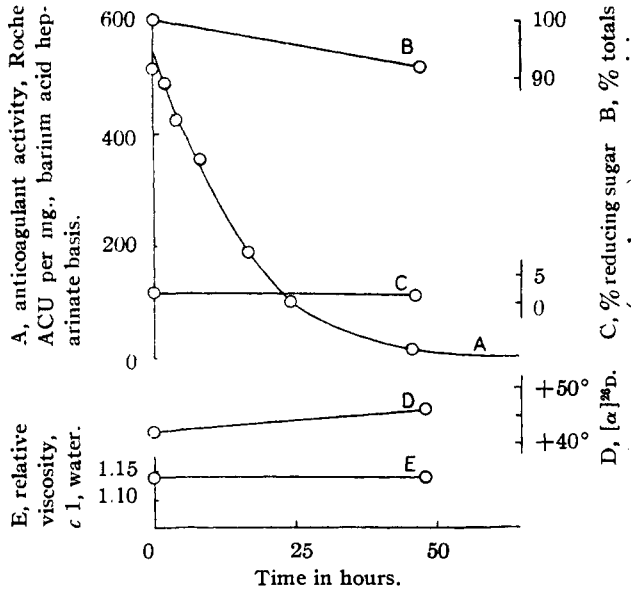

Fig. 2.—Inactivation of barium acid heparinate (c 2, 68 ± 2) in 11% acetic acid. D taken on sodium heparinate; B on both sodium and barium acid heparinate (average value).

Roche ACU per mg., were found between the crystalline and mother liquor material of each point. Table I demonstrates that activities determined on the whole neutralized solution are in agreement with those obtained by precipitating the carbohydrate material and assaying it separately. No error was thus introduced by the salts formed in the neutralization of the acetic acid. These experiments also demonstrate that there was no simple elimination of an active impurity.

The heparin inactivation is not due to the barium ion since the loss in activity of the sodium salt (Curve B of Fig. 1) paralleled that of the barium acid salt. The sodium salt has a higher heparin content and thus a higher initial activity. Slightly differing acidities are also involved, since the sodium heparinate, which should exist in the solution as sodium acid heparinate, will be buffered with sodium acetate at a higher pH than the solution of the barium acid salt in the dilute acetic acid.

The acetic acid is not necessary for the inactivation, since a solution of the barium acid salt (pH 3.6 at 25°) was slowly inactivated at 68° under its own acidity. That the inactivation is due solely to the effect of pH at the temperature employed, is indicated by the inactivation of an aqueous solution of sodium heparinate buffered with sodium diacid phosphate to a pH of 2.6 at 25°, this pH value being identical with that of the dilute acetic acid used. The activity dropped from an initial value of 550 Roche ACU per mg. to 50 Roche ACU per mg. at forty-eight hours, thus closely paralleling Curve B of Fig. 1.

No significant change in the very low reducing value (*ca.* 2% expressed as D-glucose) of the barium acid heparinate occurred during the inactivation (Curve C of Fig. 2). This finding does not eliminate the possibility of a sugar aldehydic group being bound to an amino nitrogen radical as postulated in our previous communication,[2] since it is known that many such types of linkages are hydrolyzed by alkali and would thus yield a reducing value in the analysis. However, the low reducing value found would appear to eliminate any appreciable quantity of this kind of bond, such as would seem to be demanded by the concomitant amino nitrogen release to be described later.

The optical rotation (Curve D of Fig. 2) changed very little during the inactivation. This indicates the absence of any extensive degradation of the heparin molecule and shows that the change did not involve gross changes in the optically active centers of the molecule.

The rather low initial viscosity of the barium acid heparinate (relative viscosity 1.14, c 1.0, 25°, water) was recovered unaltered after inactivation. Likewise, the inactivated material still gave the toluidine blue test,[6] supposedly indicative of a sulfated polysaccharide, and was precipitable from aqueous solution by an excess of acetic acid or of ethanol. Thus the molecular size has undergone no marked change.

The nitrogen bond in heparin is not acetylated,[2] as it is in the related mucoitin and chondroitin hydrogen sulfates. In order to determine if this nitrogen bond was involved in the loss of activity, the inactivation was followed by the Van Slyke analysis for amino nitrogen. These data are plotted in Fig. 3. The inverse relationship between activity and initial (nitrogen evolution in the first five minutes) Van Slyke amino nitrogen is striking and is evidence that the nitrogen bond is concerned in the inactivation. The relationship is nearly linear but is probably best represented by the curved line shown in Fig. 3. Upon extrapolation of this function to zero initial amino nitrogen, the corresponding activity value for the barium acid

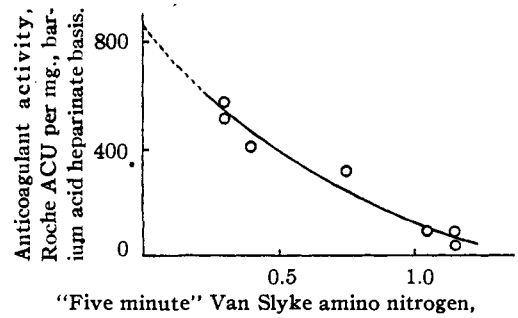

Fig. 3.—Relation between anticoagulant activity and Van Slyke amino nitrogen content.

(6) L. Lison, *Compt. rend. soc. biol.*, **118**, 821 (1935); E. Jorpes, *Acta Med. Scand.*, **88**, 427 (1936).

salt is roughly 850 Roche ACU per mg. or *ca.* 950 Roche ACU per mg. on the basis of the neutral sodium salt. This value represents a measure of the maximum activity attainable, at least for beef heparin. Since the isolation and purification of heparin is lengthy and involves some treatment with weak acids, partial degradation of the nitrogen bond in the product is unavoidable. The degree of definition of the curve makes the extrapolation very approximate; the initial value so obtained may be high. The nitrogen is not lost during the inactivation, the total content as measured by the Dumas method being unchanged at 2 ± 0.2%. Since the curve of Fig. 3 shows that the activity is nearly lost when one-half (1%) of the nitrogen content appears as the free amino group, one is tempted to speculate that one-half of the nitrogen bonds may differ from the other half of the bound nitrogen. This is, however, very improbable and would require more experimental justification than is at present available.

In Fig. 10 and in Tables I and III of our previous publication,[2] an extrapolation to zero amino nitrogen at zero time was made. This is an incorrect extrapolation, as the active barium acid heparinate has a definite (*ca.* 0.3%) though small initial amino nitrogen content.

The bare trace of reducing value exhibited by the active and inactivated heparin eliminates the chance that the nitrogen liberated in the Van Slyke assay was derived from the reduction of the nitrous acid, a possibility with strongly reducing substances, such as polyhydric phenols.[7]

The amino nitrogen results provide additional information on the possibility that the activity of heparin might be due to an active impurity. Stacey and Youd[8] have stated that the immunological properties of many polysaccharides were due to the presence of traces of protein impurities. If the active blood anticoagulant were a protein impurity, attached as required by Fig. 3 to at least every other nitrogen in heparin, the molecular weight of heparin would be very large and its protein content would be high indeed. The analytical data on heparin[2] are not indicative of such a situation.

The nature of this active nitrogen bond in the heparin molecule is at present unknown The nitrogen is unquestionably that of the D-glucosamine present. The slight loss of sulfate during the inactivation (Curve B of Fig. 2) suggests the possibility that the nitrogen might be joined in a sulfonic acid (R—NH—SO_2—OH) type of structure. The extreme stability of N-alkylsulfonic acids toward acid hydrolysis[9] would seem to rule out such a possibility, although it is true that this stability might be altered by the hydroxyl substituted radical present in heparin. The liberation of 1.1% N as amino nitrogen from such a linkage in barium acid heparinate would require the release of 2.5% S. In the experiments herein described, a 1.0% S loss (barium acid salt basis) was found. The barium acid salt, however, showed an initial amino nitrogen content of *ca.* 0.3%, the experimentally found sulfur equivalent (0.3%) of which should be added to the above 1.0% to give a corrected sulfur loss of 1.3%. This value is approximately one-half of that expected should there have been one sulfate group combined with the nitrogen. Sulfation experiments yet to be described, failed to restore the activity of inactivated heparin, a result not in harmony with the postulation of a sulfated nitrogen atom, although the sulfation experiments might have caused some hydrolysis of glycosidic bonds with resultant activity loss. Furthermore, the sulfation conditions might have been too acidic for the introduction of such an acid-sensitive sulfate radical. The present evidence therefore neither strongly favors nor completely eliminates the presence of a sulfated nitrogen atom in the heparin molecule. We do know that the sensitive nitrogen bond in heparin is hydrolyzed by weak acids at moderate temperature to free the amino group. Thus, heparin gives a slow evolution of nitrogen in the Van Slyke apparatus, over long periods of time.[2,10] A similar behavior of some proteins has been reported.[11] In our previous publication,[2] an analytical summation of 88% for the crystalline barium acid heparinate was cited as evidence for the possible presence of an unknown substituent. Such a substituent might well be that attached to the nitrogen atom. If such a constituent were combined with one-half of the nitrogen, a molecular weight for this of less than 200 would raise the analytical summation to 100%. Our previously reported[2] analytical data demonstrate the absence of volatile acids in the hydrolytic products of heparin, as well as the absence of any substance containing the CH_3—C group.

Since a considerable amount of work has been published in an attempt to show that the anticoagulant activity of heparin is proportional to the sulfate content[12] and that practically any sulfated polysaccharide will have some anticoagulant action,[13] a careful investigation of any sulfate loss during the inactivation was made. It was found (Curve B of Fig. 2) that the inactiva-

(7) N. W. Stuart, *N. H. Agr. Expt. Sta., Tech. Bull.* **50**, 6 (1932); *Plant Physiol.*, **10**, 135 (1935); H. E. Carter and S. R. Dickman, *J. Biol. Chem.*, **149**, 571 (1943).

(8) M. Stacey and F. R. Youd, *Biochem. J.*, **32**, 1943 (1938).

(9) L. F. Audrieth, M. Sveda, H. H. Sisler and M. Josetta Butler, *Chem. Rev.*, **26**, 49 (1940).

(10) A. F. Charles and A. R. Todd, *Biochem. J.*, **34**, 112 (1940).

(11) D. G. Doherty and C. L. Ogg, *Ind. Eng. Chem., Anal. Ed.*, **15**, 751 (1943).

(12) E. Jorpes, *Biochem. J.*, **29**, 1817 (1935); E. Jorpes and S. Bergström, *J. Biol. Chem.*, **118**, 447 (1937); E. Jorpes, *Biochem. J.*, **36**, 203 (1942).

(13) S. Bergström, *Naturwissenschaften*, **23**, 706 (1935); *Z. physiol. Chem.*, **238**, 163 (1936); E. Chargaff, F. W. Bancroft and Margaret Stanley-Brown, *J. Biol. Chem.*, **115**, 155 (1936); H. Elsner, W. Broser and E. Bürgel, *Z. physiol. Chem.*, **246**, 244 (1937); P. Karrer, H. Koenig and E. Usteri, *Helv. Chim. Acta*, **26**, 1296 (1943).

tion was accompanied by a slight sulfate loss but that this was less than 10% of the total sulfate ester initially present. As noted previously, the inactivated product still gave the toluidine blue test and, as will be discussed below, did not recover activity on sulfation. It is not excluded that a sulfated polysaccharide may be the essential carrier of heparin activity, but our results do indicate that such activity is concerned primarily with the nitrogen bond and not with the sulfate ester groups. Since the active material does contain ester sulfate, there will be a rough correlation between activity and sulfur content of very crude heparin preparations. The early (1936) observations of Charles and Scott[3] that heparin is inactivated by nitrous acid in acetic acid at a *p*H of 4 and by formalin at 45°, are more in support of a unique nitrogen linkage as the center of activity than of a sulfate ester. Charles and Todd,[10] on the other hand, at a later date (1940), desulfated heparin with very dilute methanolic hydrochloric acid at room temperature, or below, and reported a correlation between sulfur content and anticoagulant activity. Herein, they failed to note or to comment on the fact that their data indicated that the activity decreased very much faster than the sulfate content. The demonstration by Jaques[14] of an enzyme which inactivated heparin without the liberation of sulfate, suggests an attack on a non-sulfated nitrogen bond. The possibility of glycosidic bond hydrolysis was, however, not eliminated in the work of Jaques.

In the present work, we have performed some sulfation experiments, employing the procedure highly recommended by Karrer, Koenig and Usteri.[13] For this purpose, the active heparin preparations were converted to their more soluble ammonium salts. The data are tabulated in Table II and demonstrate that a sharp drop in activity occurred on sulfation of the active or partially inactivated heparin preparations. This is predictable, since the chlorosulfonic acid used, with an excess of pyridine, in the sulfation is a strong acid and the resultant salt with the weak base pyridine would hydrolyze the acid-sensitive

TABLE II

ANTICOAGULANT ACTIVITIES, BEFORE AND AFTER SULFATION,[a] OF HEPARIN AND RELATED PRODUCTS

Substance	Anticoagulant activity, Roche ACU per mg. Before sulfation	After sulfation
Ammonium heparinate	630	75
Ammonium acid heparinate	570	70
Partially inactivated ammonium heparinate	300	40
Inactivated sodium heparinate	50	7
Sodium acid mucoitinsulfate	12	50
Sodium chondroitinsulfate	...	1
Chondrosin	...	0

[a] Sulfation according to procedure of Karrer, Koenig and Usteri, ref. 13.

(14) L. B. Jaques, *J. Biol. Chem.*, **133**, 445 (1940).

nitrogen linkage, the seat of the anticoagulant activity of the heparin molecule. These results are also in agreement with the failure of Charles and Todd[10] to obtain any activity upon sulfation of a nearly completely desulfated and inactive heparin sample.

It cannot be overlooked that sulfation of various polysaccharides does yield products with some blood anticoagulant activity.[13] In this connection, our sulfation of sodium acid mucoitinsulfate is of interest (Table II). This polysaccharide, the most closely related of any to heparin, had apparently not been previously sulfated. It is interesting that the sodium acid mucoitinsulfate possessed inherently a small but measurable activity that was enhanced slightly on sulfation. It seems very probable that this type of activity is not that of heparin. The relatively rapid disappearance of heparin activity *in vivo* compared to the slow disappearance *in vivo* of the activity of the synthetic polysaccharide poly (hydrogen sulfates), as reported by Karrer and co-workers,[13] suggests a difference in the mode of action. The blood coagulation mechanism is very complicated,[15] is influenced by many factors, and the synthetic sulfated anticoagulants may well enter the system at a point different from that of heparin.

As previously mentioned, we have reported[2] the essentially complete inactivation of the crystalline barium acid heparinate on five crystallizations from dilute acetic acid. Since this indicated the elimination of an active impurity and was a much faster inactivation than could be explained by the expected contact with hot, dilute acetic acid (*cf.* Fig. 1), this work was repeated. It was found that if the recrystallization operations were performed expeditiously, only a slight drop in anticoagulant activity was noted, this drop being that predictable from the data of Fig. 1. Long standing, or aging, at room temperature, over periods of days, of the crystals in contact with the acetic acid mother liquor, produced a decrease in activity, a 15% drop on three days of standing being observed in one case. These original experiments were carried out when the influence of acidity and drying temperature (for the acid salt) on activity was not yet known. The drop in activity was due probably to long standing in the acetic acid solutions or to drying of the samples at elevated temperatures, which also is known to lead to practically complete inactivation[2] for the barium acid salt. Our previous observation[2] that the loss in activity is accompanied by the liberation of a little more than one-half of the nitrogen as a free amino group, has been adequately confirmed. In our previous publication,[2] we reported a practically quantitative yield on recrystallization. This is in error. The yield is approximately 70%. Quantitative recovery of material is possible by

(15) J. H. Ferguson, *Science*, **97**, 319 (1943); J. H. Ferguson in "Colloid Chemistry, Vol. V, Biology and Medicine" (J. Alexander, editor), Reinhold Pub. Corp., New York, N. Y., 1944, p. 951.

precipitation of the mother liquors with an excess of such solvents as glacial acetic acid, ethanol, or ethanol and ether.

Experimental

Anticoagulant Activity Assay.—The Foster[4] modification of the procedure of Reinert and Winterstein[5] was employed. In this method the activity is determined by comparing the weight of unknown necessary to give a 50% clot with recalcified beef plasma, with the weight of "standard heparin" required to give the same percentage clot. The "standard heparin" is a reference sample kept by the Hoffmann-La Roche Company and has a value of 570 "Roche Anticoagulant Units" per mg. In the present study where most of the samples had activities of less than 350 Roche ACU per mg., it was first necessary to carry out assays on a series of three dilutions of each sample. More assays were then generally run at the proper dilution and the arithmetical mean taken as the final value. The results are expressed as Roche ACU per mg. (dry basis).[16]

It was found that best results were obtained with a uniform sample of frozen beef plasma prepared in our Laboratory by mixing and freezing the citrated plasma from a number (five were used) of different animals. It was essential to carry out the preparation of the plasma in the minimum possible time. The deterioration in the clotting quality of the blood was most rapid while the blood cells were in contact with the citrated plasma. If sufficient centrifuging capacity is available to prepare all of the plasma in a short time, it is preferable to mix the plasma after the removal of the blood cells. Otherwise, the whole blood may be mixed before centrifuging.

Amounts of 130 cc. of plasma were placed in 250-cc. rubber-stoppered narrow-mouthed bottles and frozen by rotating on their sides in a 2.5-cm. depth of Dry Ice–ethanol freezing mixture. In this manner, the plasma was frozen in a shell around the sides of the bottle. The frozen plasma supply was stored in a cold room at −10°. Before an assay, a bottle of frozen plasma was melted by immersion in a 37° water-bath and the bottle contents filtered through a cotton plug. For each lot of frozen plasma, only one standardization for calcium chloride addition was required.

Inactivation of Heparin by Mild Activity.—A 2% solution of crystalline barium acid heparinate, purified through the benzidine salt, in 11% acetic acid solution (*p*H 2.6 at 25°) was heated at 68 ± 2° (vapor of *n*-propyl bromide). At various time intervals, aliquots of this solution (containing 5 mg. of the heparin salt) were removed, made slightly alkaline with a 5% excess of 0.25 *M* sodium carbonate and the whole solution assayed for anticoagulant activity. Samples were held in a refrigerator if the assay could not be run immediately. The results are diagrammed in Curve A of Figs. 1 and 2 and are tabulated in Table I. In order to determine the effect of the introduced salts on the activity values, the heparin content of several aliquots (containing 10 mg. of heparin) was precipitated with glacial acetic acid. The precipitate was washed with 90% acetic acid, absolute ethanol, and ether and dried under reduced pressure over phosphorus pentoxide at room temperature. The recovery of material was 85–95% and no significant differences in activity were noted (Table I).

Similar results were obtained when sodium heparinate, purified through the benzidine salt, was substituted for the crystalline barium acid heparinate (Curve B of Fig. 1).

A corresponding inactivation of the sodium salt[17] was obtained by substituting for the dilute acetic acid a like volume of phosphoric acid, sodium diacid phosphate buffer solution (0.1 *M* phosphoric acid + 0.04 *M* sodium hydroxide) of the same *p*H (2.6 at 25°). The activity of the material dropped from an initial value of 550 Roche ACU per mg. to 50 Roche ACU per mg. (inactivated product precipitated with glacial acetic acid) after forty-eight hours at 70°.

Barium acid heparinate was inactivated slowly (Curve C of Fig. 1) under its own acidity (*p*H 3.6 at 25°) at 68 ± 1°.

Crystals similar in appearance to those of the barium acid heparinate could be recovered with some difficulty, in 55% yield, from the inactivated (forty-eight hour point of Curve A of Figs. 1 and 2) material. These were considerably more soluble in dilute aqueous acetic acid (containing barium acetate) than the original material.

No significant change in the low reducing value of the crystalline barium acid heparinate occurred during the inactivation (Curve C of Fig. 2). An initial reducing value of 1.7% (expressed as D-glucose; Hagedorn-Jensen method) changed to 1.0%.

A very slight rotation change occurred during the inactivation (Curve D of Fig. 2). As measured on the sodium salt in the 11% acetic acid solution, the initial specific rotation of +42 ± 2° (26°, *c* 2, D line) changed to +46 ± 2°.

The relative viscosity (Curve E of Fig. 2) in aqueous solution (*c* 1.0, 25°) of the barium acid heparinate was 1.14. The relative viscosity of the inactivated product (forty-eight hour point of Curve A of Figs. 1 and 2) recovered by precipitation with glacial acetic acid was likewise 1.14 (*c* 1.0, 25°, water).

Essentially completely inactivated (3 Roche ACU per mg., ninety-five hour point of Curve A, Figs. 1 and 2) barium acid heparinate, recovered by precipitation with glacial acetic acid, still gave the toluidine blue test. All partially inactivated preparations gave a positive test. The test was performed by dissolving one drop of a 1% aqueous solution (or *ca.* 0.5 mg. of solid) of the substance to be tested, in 2 cc. of water. To this solution was added one to five drops of aqueous toluidine blue solution (1:2000) and the resultant solution observed under artificial light. A color change from blue to reddish-violet indicated a positive test.

The Sulfate Loss During Inactivation.—(Curve B, Fig. 2). The acetic acid solution of the sodium heparinate remained clear throughout the inactivation. The sulfate ion was determined in the inactivated solution and was found to be 1.1% (as S) of the original sodium heparinate (12.9% S content) or 8.5% of the total sulfur.

During the inactivation of the barium acid heparinate, a brown, oily precipitate separated. This was removed by centrifugation and ashed. Considered as barium sulfate, the ashed residue was found to represent 0.95% (as S) of the original barium acid heparinate (11.3% S content) or 8.4% of the total sulfur in good agreement with the loss from the sodium salt. That the residue was barium sulfate was indicated by the fact that it dissolved in concentrated sulfuric acid to give a clear solution and was reprecipitated on dilution with water.

Variation of Van Slyke Amino Nitrogen with Activity.—The Van Slyke amino nitrogen assays were performed in a Fisher improved micro Van Slyke deamination apparatus. In each case the deamination was carried out for exactly five minutes. The results are diagrammed in Fig. 3. A definite relationship between these values and the anticoagulant activity is demonstrated. Some of the assays were carried out on the sodium salt, but all have been calculated to the barium acid salt basis. The points of highest activity represent the initial material before inactivation.

The total nitrogen (Dumas) on an inactivated sample (95 Roche ACU per mg.) was found to be 2.17%.

In Fig. 10 and in Tables I and III of our previous publication,[2] an extrapolation to zero amino nitrogen at zero time was made. This is an incorrect extrapolation, as the barium acid heparinate has a definite (*ca.* 0.3%) though small initial amino nitrogen content.

Inactivation of Barium Acid Heparinate on Recrystallization.—Active barium acid heparinate was recrystallized

(16) All activity and analytical data are expressed on the dry basis. Drying was performed by heating to constant weight over phosphorus pentoxide at 75° and 1 mm. pressure. For the activity determinations, this was done on a separate sample and correction applied.

(17) In this one case, this sodium salt was not purified through the crystalline barium acid salt.

as described in the preceding communication[2] and the activity determined after several recrystallizations. If the operations were performed expeditiously, no great drop in anticoagulant activity was noted. Long standing (periods of days) of the crystals in the acid (acetic acid) mother liquor produced a decrease in activity, a 15% drop on three days standing being observed in one case. The practically complete inactivation previously reported[2] on five recrystallizations was not confirmed. These earlier experiments, performed by Dr. D. I. Weisblat, were carried out when the influence of acidity and drying temperature (of the acid salt) on activity were not yet known. The drop in activity was due probably to long standing in the acetic acid solutions or to drying of the samples at elevated temperatures, which also is known to lead to practically complete inactivation[2] for the barium acid salt.

In our previous publication,[2] we reported a practically quantitative yield on recrystallization. This is in error. The yield is approximately 70%. Quantitative recovery of material is possible by precipitation of the mother liquors with an excess of such solvents as glacial acetic acid, ethanol, or ethanol and ether.

Distribution of Activity between Crystals and Mother Liquor on Crystallization of Partially Inactivated Barium Acid Heparinate.—Crystalline barium acid heparinate was inactivated for eight and twenty-two hours (Curve A of Figs. 1 and 2) with dilute acetic acid as described previously. The inactivated product was crystallized and its activity was determined. The activity of the mother liquor material was then assayed in solution. The concentration of the latter was determined by subtraction of the crystalline material and correcting for an estimated 5% loss on washing. The yield of well-formed crystals was 47% at the eight hour point and 30% at the twenty-two hour point. No significant differences in the activities expressed as Roche ACU per mg., were found between the crystalline and mother liquor material of each point.

Conversion of Barium Acid Heparinate to the Neutral and Acid Ammonium Salts.—For purposes of sulfation, the ammonium salt was desired since it was more soluble in the sulfating mixture than either the barium acid or sodium salts. To obtain the ammonium salt, the general procedure of Charles and Scott[3] was followed, with some modification. An amount of 200 mg. of a partially deactivated barium acid heparinate (320 Roche ACU per mg.) was dissolved in 4 cc. of water and the benzidine salt precipitated as an oil by the slow addition, with stirring, of 4 cc. of a freshly prepared, saturated, aqueous solution of pure benzidine dihydrochloride. The separated oil was removed by centrifugation and hardened to a granular solid by trituration with several portions of methanol; yield 155 mg.

An amount of 120 mg. of the above benzidine salt was suspended in 6 cc. of water and treated under stirring with 0.4 cc. of 3 *N* ammonium hydroxide. The oily benzidine salt dissolved and a white slurry of benzidine formed. The mixture was heated at 70° for ten minutes and was then kept in the ice box for two hours to complete the benzidine crystallization. After benzidine removal by centrifugation, the centrifugate was diluted with 12 cc. of methanol. Upon the slow addition, with stirring, of 12 cc. of anhydrous ether, a milky sol formed. This sol was allowed to age overnight and was then flocculated by the addition of 10 mg. of ammonium chloride. The flocculated, neutral ammonium salt of the partially inactivated heparin was then removed by centrifugation, washed by centrifugation with absolute ethanol and ether, and then dried under reduced pressure over phosphorus pentoxide at room temperature; yield 84 mg., activity 300 Roche ACU per mg.

Such a neutral ammonium salt could be converted to the ammonium acid salt by the following general procedure. The clear sol resulting from the dispersion of 500 mg. of a neutral ammonium salt in 25 cc. of water was diluted very slowly, under stirring, with 9 volumes of glacial acetic acid. The flocculated ammonium acid salt was separated by centrifugation and washed with 90% acetic acid, absolute ethanol, and ether and was then dried as above; yield 445 mg.

Sulfation Experiments.—Sulfation was carried out with pyridine and chlorosulfonic acid as described by Karrer, Koenig and Usteri.[13] The sulfated products were isolated by pouring the reaction mixture into an excess of ice and water and precipitating the products by the slow addition of ethanol, followed by centrifugation and washing with ethanol and ether. Solids were obtained by this treatment.

The solubility of all of the heparin samples in the sulfating mixture appeared to decrease with increasing temperature. Upon warming to 60° the ammonium and ammonium acid heparinates precipitated as a gel which redissolved on cooling. This phenomenon was reversible.

The anticoagulant activities of the various products before and after sulfation are recorded in Table II.

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Summary

1. Very mild acidity causes the heparin molecule to lose its blood anticoagulant activity.

2. The rate of loss in activity is proportional to the rate of appearance of a free amino group in the molecule, substantially complete inactivation occurring when somewhat more than one-half of the nitrogen is released as free amino (Van Slyke) nitrogen.

3. Inactivation of the heparin molecule by mild acidity is accompanied also by a very slight change in optical rotation and by the loss of only 8% of the total sulfur content.

4. No other significant changes in the heparin molecule than those listed in 2 and 3 above have been detected during the inactivation.

5. A sharp drop in anticoagulant activity results when heparin is subjected to sulfation.

6. Mucoitin hydrogen sulfate possesses a low anticoagulant activity which is slightly enhanced by sulfation.

7. The seat of biological activity in the heparin molecule is its nitrogen linkage.

COLUMBUS, OHIO RECEIVED JANUARY 12, 1945