$$(-\log \gamma^*_{KCl} - (1 - g^*_{KCl})/2.303) + ((1 - g)/2.303 + \int_{m^{**}}^{m} (1 - g)\, d \log m) + \int_{m^*}^{m^{**}} (1 - g)\, d \log m$$

where γ^*_{KCl} is the activity of potassium chloride and g^*_{KCl} its osmotic coefficient, both at m^*. These values are readily obtained from the literature.[10] The quantity in the second parenthesis is independent of $m_g = 0.5$, and is given in Fig. 6 as a function of g. The last integral can be easily estimated with sufficient accuracy from the curve by which m^* was determined.

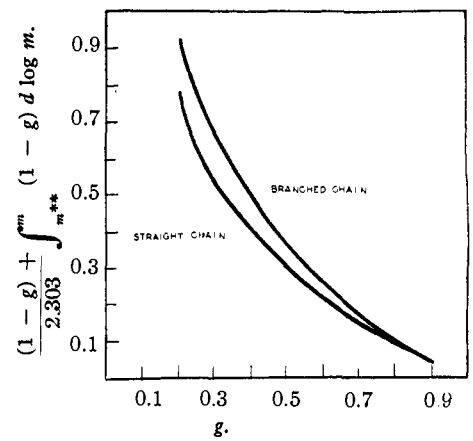


Fig. 6.—Graph for use in calculating activity coefficients of colloidal electrolytes from a single freezing point measurement.

Summary

1. It is shown that all data for osmotic coefficients of colloidal electrolytes are brought into coincidence on one of three curves, by merely changing the scale of concentration. There is one curve for straight chain compounds, one for branched and one for polycyclic colloidal electrolytes.

2. The osmotic and conductivity data indicate the presence of a small proportion of highly conducting micelles in very dilute solutions, a conclusion which is supported by previously published migration data.

3. The addition of potassium chloride or of potassium sulfate to very dilute solutions of potassium laurate promotes the formation of colloid, so that the result is distinctly less than additive; in higher concentrations, where the colloid is already fully formed, the effect on freezing point lowering is slightly more than additive. There is no marked indication of high ionic strength in solutions of colloidal electrolytes.

4. Owing to the remarkable regularity noted in item 1 of this summary it is possible to obtain the value of the activity coefficient with fair accuracy from a single freezing point determination.

STANFORD UNIVERSITY, CALIF. RECEIVED JUNE 7, 1943

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

Chemical Studies on Crystalline Barium Acid Heparinate[1]

BY M. L. WOLFROM, D. I. WEISBLAT,[2] J. V. KARABINOS,[3] W. H. MCNEELY[2] AND JAY MCLEAN

McLean,[4] working in Howell's laboratory, isolated a blood anticoagulant from dog liver and beef heart. This substance was further studied by Howell[5] and the name heparin was given to it. Jorpes[6] considers that the active material is a mucoitinsulfuric acid containing much more ester sulfate than the normal type of mucoitinsulfuric acid.

Charles and Scott[7] isolated from beef lung a crystalline barium salt possessing heparin activity and having the following recorded analysis: C, 18; H, 3.9; N, 1.68; S, 9.3; ash, 33.6; Ba, 19.8; amino-N (Van Slyke), 0.4. These analyses were apparently made on material that was not anhydrous.[8] Charles and Scott considered that glycuronic acid was absent in the substance and demonstrated that the biological activity was destroyed by nitrous acid or formaldehyde. A sample of the crystalline barium salt received from Dr. Charles was analyzed by Meyer and Smyth[9] with the following results: N, 2.36; amino sugar, 16.3; uronic acid, 20.1; acetyl, 2.2; sulfur, 9.8; ash, 33.3. Meyer stated that the substance was an acid salt and recorded an equivalent weight of 749. Reinert and Winterstein[10] reported an analysis of a neutral sodium salt regenerated from the crystalline barium salt and stated that this preparation contained 10% (dry basis) acetyl. Charles and Todd[8] considered that the crystalline barium salt prepared from beef liver was identical with that prepared from beef lung. They con-

(1) A preliminary notice of this work has appeared in *Science*, **97**, 450 (1943). The original version of this manuscript was received May 12, 1943, and then withdrawn by the authors for certain minor additions and alterations.—*The Editor.*

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

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

(4) J. McLean, *Am. J. Physiol.*, **41**, 250 (1916).

(5) W. H. Howell and E. Holt, *ibid.*, **47**, 328 (1918); W. H. Howell, *ibid.*, **63**, 434 (1923); **71**, 553 (1925).

(6a) E. Jorpes, "Heparin," Oxford University Press, London, 1939; (b) E. Jorpes, *Biochem. J.*, 203 (1942).

(7) A. F. Charles and D. A. Scott, *ibid.*, **30**, 1927 (1936).

(8) *Cf.* A. F. Charles and A. R. Todd, *Biochem. J.*, 112 (1940).

(9) K. Meyer and Elizabeth M. Smyth, *Cold Springs Harbor Symposia Quant. Biol.*, **6**, 97 (1938).

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

sidered that the crystals were hydrated (water content, 12.1) and reported the following analyses, calculated on the hydrated basis: C, 17.8; H, 3.1; N, 1.9; S, 9.6; Ba, 19.7; ash, 33.4; and calculated on the anhydrous basis: C, 20.2; H, 3.5; N, 2.2; S, 11.0; Ba, 22.5. The values on the "hydrated basis" are in agreement with those reported by Charles and Scott[7] and by Meyer and Smyth.[9] Charles and Todd reported N-acetyl as present and recorded values for this group varying from *ca.* 1.5 to 3%. Masamune, Suzuki and Kondoh[11] analyzed a crystalline barium salt prepared from beef lung and recorded the values (degree of dryness not stated): N, 2.1; hexosamine, 26.4; hexuronic acid, 28.7; acid-hydrolyzable S, 8.7; acetyl, 0.0; amino-N, 0.0; ash, 27.7. Jorpes and Bergström[12] cite an N-acetyl content in amorphous preparations of the sodium salt. Jaques, Waters and Charles[13] isolated the crystalline barium salt from dog, beef, pork and sheep tissue and noted a wide variation in biological activity, the anticoagulant potencies being in the order 10:5:2:1. No significant chemical differences were found in the material from these various sources.

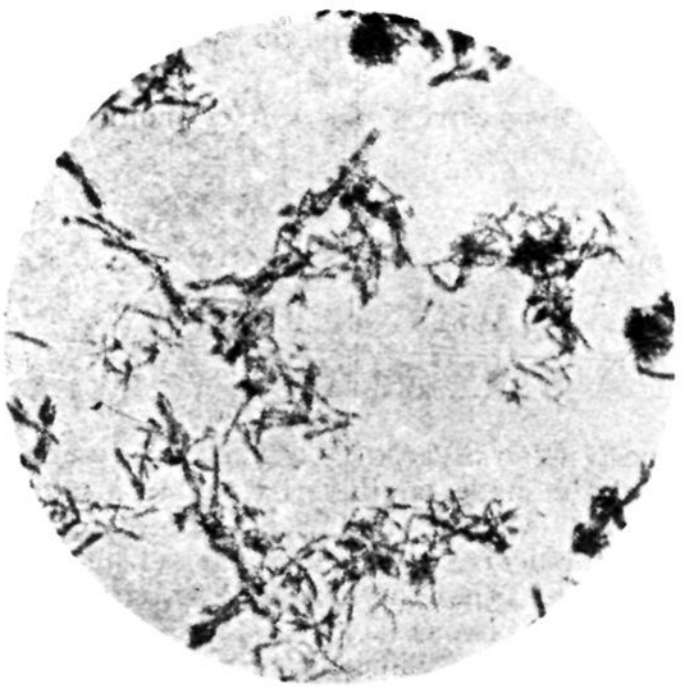


Fig. 1.—Barium acid heparinate, first crystallization (I), × 600. Photomicrograph by Professor W. M. MacNevin of this Laboratory.

In the present work we have prepared the crystalline barium salt (Fig. 1) from "Roche heparin" and have subjected it to a thorough analytical investigation. We also prepared samples of the neutral sodium salts of chondroitinsulfuric acid and mucoitinsulfuric acid and from these neutral salts we prepared acid salts by repeated precipitation from a high concentration of acetic acid. The preparation of these mucoitinsulfates and chondroitinsulfates was effected in order to obtain material suitable for the establishment of the accuracy of the analytical procedures used. The data obtained are collected in Table I, wherein we have designated the crystalline barium salt as barium acid heparinate. The data of this table are recorded on thoroughly dried samples. All of these materials hold water tenaciously and when dried are extremely hygroscopic.

The elementary (C, H, N, S and Ba) analyses for the crystalline barium acid heparinate shown in Table I agree well with those recorded by Charles and Todd.[8] The purification through the benzidine salt led to crystalline material with essentially the same analysis as that not so treated. This purification procedure is nevertheless considered to lead to a slightly better product, as can be noted in the higher rotation value. The acid-hydrolyzable sulfur was 11.3% and that calculated from the ash (as barium sulfate) was 5.6%. All ashing was done in platinum with concentrated sulfuric acid. The acid-hydrolyzable barium content was 23.8% and that calculated from the ash (as barium sulfate) was 23.8%. Therefore the Ba:S ratio was 1.0:2.0; all sulfur was present as sulfate sulfur; and all barium can be considered as attached to sulfate. This leaves the free acidity (neutral equivalent 800 ± 50) as carboxyl acidity. The titer curve shown in Fig. 9 indicates a simple monobasic acid type of compound and thus the carboxyl groups, if more than one are present, are not close enough in space to influence this titration. The nature of the curve of Fig. 9 indicates the absence of a lactone of any stability. The influence of any basic nitrogen present does not appear in the titration.

In a product isolated from a natural source, a contaminant ash may be expected. The concordance shown above between the ash (40.5%) and the barium and sulfur values, shows the absence of significant amounts of such an ash, as does also the ash value (40.4%) obtained (Table I) on converting the entirely ash-free benzidine salt into crystalline barium acid heparinate.

N-Methyl and O-methyl were absent in the barium acid heparinate. The nitrogen content was not present as free amino nitrogen and the observation of Charles and Todd[8] regarding the slow evolution of nitrogen in the Van Slyke apparatus (acetic and nitrous acid only present) was confirmed (*cf.* Fig. 10). Contrary to the findings of these workers, however, the nitrogen group is not acetylated. No acidity was obtained from properly purified material when distilled at constant volume with *p*-toluenesulfonic acid. The Kuhn-Roth[14] CH_3-C analysis (chromic acid oxida-

(11) H. Masamune, M. Suzuki and Y. Kondoh, *J. Biochem.* (Japan), **31**, 343 (1940).

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

(13) L. B. Jaques and E. T. Waters, *J. Physiol.*, **99**, 454 (1941); L. B. Jaques, *Science*, **92**, 488 (1941); L. B. Jaques, E. T. Waters and A. F. Charles, *J. Biol. Chem.*, **144**, 229 (1942).

(14) R. Kuhn and H. Roth, *Ber.*, **66**, 1274 (1933).

TABLE I

ANALYTICAL DATA[a] (DRY BASIS[23])

Constituent	"Roche Heparin"	Crystalline BaH heparinate (I)	Crystalline BaH heparinate (through benzidine salt[b])	Mucoitinsulfuric acid Na salt	Mucoitinsulfuric acid NaH salt	Chondroitinsulfuric acid Na salt	Chondroitinsulfuric acid BaH salt
C		20.12	20.19	31.80	31.58	33.87	31.55
H		2.61	2.85	5.05	5.00	3.99	4.58
N (Dumas)	2.60	2.03	1.94	2.51	2.58	2.74	2.70
Amino-N (Van Slyke) as N	0.10[c]	0.0[d]		0.15		0.04	
S (Parr bomb)	11.66			6.06	6.64	5.85	
S (acid-hydrolyzable)		11.44	11.31				5.78
N-Acetyl as CH_3CO (described method)	0.30	0.0		7.80		8.37	
N-Acetyl (Kuhn-Roth[h])	0.0			5.70		8.82	
O-Acetyl				0.0		0.0	
OCH_3	0.0						
NCH_3	0.0			0.0		0.0	
Ash (H_2SO_4)	37.3	40.50	40.41	27.74	19.5	30.5	23.78
Anhydrohexuronic acid	22.2	17.8	17.9	29.0		31.6	
Ba		23.35[e]	23.78[e]				14.12[f]
Anhydrohexosamine	22.2 ± 1.5	18.7[g] ± 1.3	17.3[g] ± 1.2	29 ± 2		31 ± 2	
Biuret test	(−)	(−)	(−)	(−)	(−)	(−)	(−)
Neut. equiv., electrometric		800 ± 50	750		500		490
Neut. equiv., periodic consumption			1900[g]				
Rel. visc., *c* 0.5, 25°, H_2O	1.24	1.10	1.09	1.33		1.37	
$[\alpha]^{25}D$ (H_2O, *c* 2)	+42°	+44°	+47.5°	−15°	−7.4°	−24°	−11.5°

[a] Most of these data represent averages of a large number of closely agreeing results. [b] Ash-free. [c,d] Extrapolated initial value; increases slowly with time in the presence of the acetic and nitrous acid reagents as per Fig. 10. [e] Ba as calcd. from ash (H_2SO_4), 23.8. [f] Ba as calcd. from ash (H_2SO_4), 14.0. [g] Determined on sodium salt regenerated from the crystalline barium acid salt and recalculated to the barium acid salt basis. [h] Ref. 14 (chromic acid oxidation).

tion) was negative. These two methods showed the presence of acetyl in the salts of mucoitinsulfuric and chondroitinsulfuric acids. Thus the amino group of the aminohexose of barium acid heparinate is neither free nor acetylated but is in some other type of combination. This is in agreement with the Japanese workers[11] but their postulation of a C-O-SO_2-NH-C linkage is based upon faulty stoichiometry and on the fact that they did not recognize the substance as being an acid salt.

The neutral equivalent (800 ± 50) of the barium acid heparinate is in agreement with the value of 749 (degree of dryness not stated) obtained by Meyer and Smyth.[9] That the fundamental unit, within the limits of experimental error, is twice this however, is indicated by the rapid initial periodic acid (sodium metaperiodate) consumption[15] (Fig. 11, equivalent weight 1900 on the barium acid salt basis). The equivalent weight calculated from the total nitrogen content is 700. The nitrogen value of 1.1 listed in Table III can be interpreted as indicating a unit of 1400. Previous investigators[8] have reported that heparin is stable toward periodate and although the initial periodate uptake which we have observed is a small effect, we believe that it is real. The absence of furanoside linkages unsubstituted on carbons 5 and 6 was indicated by the absence of formaldehyde in the periodic acid titers. The neutral equivalent of 800 obtained for barium acid heparinate compares with the value of 500 found for both sodium acid mucoitinsulfate and barium acid chondroitinsulfate.

The specific rotation of +47.5° (25°, *c* 2, H_2O, D line) found for barium acid heparinate is intermediate between the value of +36° recorded by Masamune, Suzuki and Kondoh[11] and the values +53 to +56° reported by Jaques, Waters and Charles.[13] The rotation changes observed between the rotations of the neutral (carboxylic ion) and acid (largely undissociated carboxyl) salts of mucoitinsulfuric and chondroitinsulfuric acids are in the direction predictable from Levene's[16] acid rule if a hexuronic acid of the D-series is present in these substances. This rotation effect is reversed for heparinic acid (specific rotation +55° for sodium heparinate regenerated from the crystalline barium acid salt of specific rotation +47.5°) but the complexity of the molecule does not warrant emphasis upon this finding.

Uronic acid was definitely present in barium acid heparinate and was detected both qualitatively and quantitatively. The nature of the uronic acid is undetermined but the data obtained are expressed in anhydrohexuronic acid units. The uronic acid is considered to be the source of the acidity of the barium acid salt. Amino sugar was present and was identified as D-glucosamine by isolation as the hydrochloride, in agreement

(15) *Cf.* E. L. Jackson and C. S. Hudson, THIS JOURNAL, **59**, 994, 2049 (1937); **60**, 989 (1938); H. A. Rutherford, F. W. Minor, A. R. Martin and M. Harris, *J. Research Natl. Bur. Standards*, **29**, 131 (1942).

(16) P. A. Levene, *J. Biol. Chem.*, **63**, 95 (1925).

TABLE II

ANALYTICAL SUMMATIONS

Constituent	Barium acid heparinate[a] % [b]	Molar ratio	Sodium heparinate[a] % [a]	Molar ratio	Sodium mucoitinsulfate % [b]	Molar ratio	Sodium chondroitinsulfate % [b]	Molar ratio
Anhydrohexosamine	18.7[c] ± 1.3	1.0	21.4[c] ± 1.5	1.0	29 ± 2	1.0	31 ± 2	1.1
Anhydrohexuronic acid	17.9	0.9	20.5	1.0	29.0	1.1	31.6	1.0
N-Acetyl (as CH_3CO)	0.0		0.0		7.8	1.0	8.4	1.1
Ester sulfate (as SO_3)	28.2	3.0	32.3	3.0	14.7	1.0	14.6	1.0
Sodium			12.4[d]	4.0	9.3	2.4	9.9	2.4
Barium	23.6	1.5						
	88.4		86.6		89.8		95.5	

[a] Calculated to this basis from data obtained on the crystalline barium acid salt. [b] Data of Table I. [c] Determined on the sodium salt regenerated from the crystalline barium acid salt. [d] Calculated from the sulfuric acid ash (37.90) on the sodium salt regenerated from the crystalline barium acid salt, 12.2. [e] Average values based upon a large number of analyses of preparations of the crystalline barium acid salt, some of which were purified through the benzidine salt.

with the work of Jorpes and Bergström[17] on amorphous sodium heparinate. The amino sugar values are expressed as anhydrohexosamine units.

We now had at hand sufficient analytical data to allow of a summation as shown in Table II. The sodium chondroitinsulfate exhibits a summation of 96% and molar ratios well in accord with accepted values[18] save for the reflection, in the sodium figure, of the ash content of this amorphous material. The same holds for the undoubtedly less pure sodium mucoitinsulfate except that the summation was lower. This summation is based upon a polysaccharide chain (anhydrohexose units). If the molecular size is that of a tetrasaccharide, then 99% would be the theoretical summation expressed in anhydrohexose units.

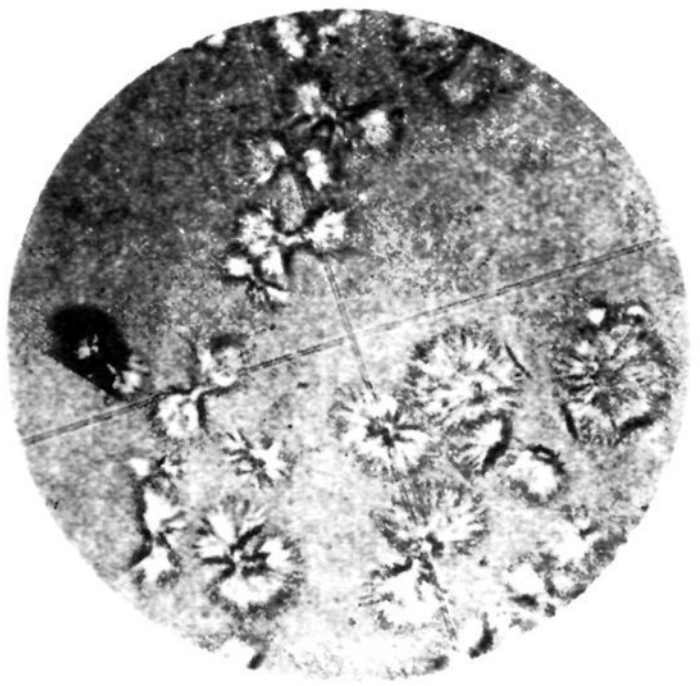


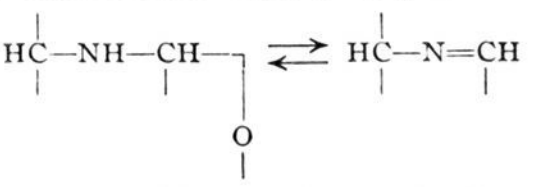
Fig. 2.—Product obtained from barium acid heparinate after five crystallizations, × 500. Photomicrograph by Professor W. M. MacNevin of this Laboratory.

(17) E. Jorpes and S. Bergström, *Z. physiol. Chem.*, **244**, 253 (1936).

(18) P. A. Levene, "Hexosamines and Mucoproteins," Longmans, Green and Co., London, 1925.

The viscosity (Table I) of the crystalline barium acid heparinate is low and a high polymer (400 anhydro units or greater) structure is not indicated for this substance. A slightly higher viscosity is shown by solutions of the sodium salts of mucoitinsulfuric and chondroitinsulfuric acids. The summation (88.4%) for the barium acid salt (and of 86.6% when calculated to the sodium salt basis) is low in comparison with that (95.5%) obtained for chondroitinsulfuric acid and thus the possible presence of a still unascertained constituent is not excluded.

The nature of the nitrogen linkage in the heparinic acid molecule is different from that in mucoi tinsulfuric and chondroitinsulfuric acids, wherein the acetamido group, R-NHCOCH$_3$, is present. In the crystalline barium acid heparinate, the amino group is not acetylated and is not free, since N-acetyl is absent and the initial amino-nitrogen (Van Slyke determination and ninhydrin color) is negative. The sulfur present is acid-hydrolyzable sulfate and is equivalent to the barium content. The data of Table II clearly show that all of the carboxyl of the uronic acid content is free. Thus neither sulfate nor carboxyl is available for combination with the nitrogen and, of the known constituents, there remains only the sugar aldehyde group. Such a type of combination, R-CH(NHR′)(OR″), is known, as, for example, in tetramethylglucose anilide,[19] and is in possible tautomerism with an acyclic structure.



The data of Table II call for a N:S:Ba ratio of 2:6:3 or 1:3:1.5. This ratio of nitrogen to sulfur (1:3) is that postulated by Jorpes[12] for his most active amorphous preparation of the sodium salt. Employing a tetrasaccharide unit consisting of two moles each of glucosamine and hexuronic acid, upon which are distributed six ester sulfate groups, we arrive at the formula

(19) J. C. Irvine and R. Gilmour, *J. Chem. Soc.*, **93**, 1429 (1908).

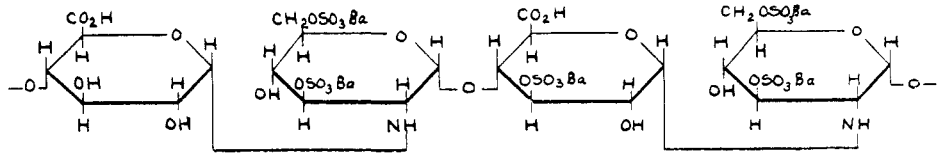


Fig. 3.

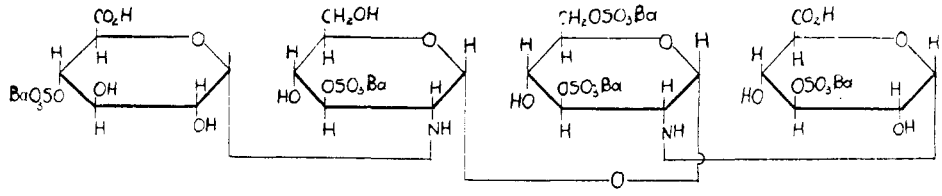


Fig. 4.

$C_{24}H_{34}O_{39}N_2S_6Ba_3$ requiring: C, 18.24; H, 2.17; N, 1.77; S, 12.18; Ba, 26.10; equiv. wt., 790. The required sulfur and barium contents are clearly too high for those determined (S, 11.3; Ba, 23.6) and this type of structure can be eliminated for the crystalline barium acid heparinate.

Charles and Todd[8] interpret their analytical data in accordance with the ratio N:S = 1:2.5. Calculating this to the tetrasaccharide basis and eliminating the acetyl group contained in the formula of Charles and Todd, we arrive at $C_{24}H_{35}O_{36}N_2S_5Ba_{2.5}$ requiring: C, 20.14; H, 2.47; N, 1.96; S, 11.20; Ba, 24.00; anhydrohexosamine, 22.5; anhydrohexuronic acid, 24.5; equiv. wt., 716. The elementary analyses required are in precise agreement with the data of Table I and with those of Charles and Todd. The equivalent weight is in fair agreement with that cited in Table I but the calculated values for anhydrohexosamine and anhydrohexuronic acid, particularly the latter, are definitely higher than found. It may be stated that these values are group analyses and are not as precise as are analyses for the elements. This type of structure is therefore not eliminated and can be developed approximately as shown in Fig. 3.

The heparinic acid salts show no strong reduction until after hydrolysis. The formula of Fig. 3 is represented as a repeating unit. Lack of reducing power[19a] might also be represented by cyclization of the formula or by the introduction of one trehalose type of linkage, the latter being shown in Fig. 4.

The sulfate distribution in the formulas of Fig. 3 and Fig. 4 is such that one mole of periodic acid would be consumed per unit but is not unique. The finer structural details are arbitrarily represented.

Another possible interpretation of the analytical data recorded in Tables I and II would be to maintain the N:S ratio of 1:3 therein shown and introduce a hexose as a fifth component, whereupon we arrive at the formula $C_{30}H_{42}O_{43}N_2S_6Ba_3$ requiring: C, 20.90; H, 2.46; N, 1.63; S, 11.17; Ba, 23.92; anhydrohexosamine, 18.7; anhydrohexuronic acid, 20.3; equiv. wt., 862. The calculated anhydrohexuronic acid value is in fair agreement with that recorded in Tables I and II. Otherwise, the agreement with the determined values is excellent. The establishment of this formula will require a search for the possible missing component and work along these lines is now in progress in this Laboratory. A cyclic representation of such a structure is shown in Fig. 5. The cyclization would require that, in the event one of the nitrogen linkages were hydrolyzed, the molecule would remain unaltered except for the appearance of one amino group and the analytically negligible addition of one mole of water. The same experimental situation could arise, however, in a long-chain structure with the added difference that the molecular size would decrease.

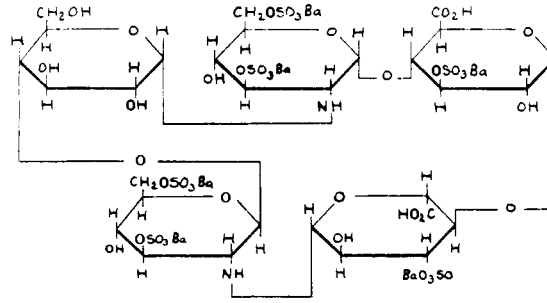


Fig. 5.

The literature contains no record of the recrystallization of barium acid heparinate. This has now been accomplished, the recrystallization (material not purified through the benzidine salt was used) being effected from dilute acetic acid solution as in the first crystallization. The analytical data after repeated crystallization (five times) are recorded in Table III and therein compared with the original material. The ash decreases slightly but otherwise no significant differences appear except for the nitrogen present as amino nitrogen (Van Slyke determination and

(19a) The crystalline barium acid heparinate is now known to show a slight reduction (see experimental portion). Appropriate alterations can be made for this property in the figures shown without altering any of the fundamental conclusions herein reported.

ninhydrin color), which is appreciable in the repeatedly crystallized material. A photomicrograph of this five times crystallized material is shown in Fig. 2.

The variation of the anticoagulant activity of the crystalline barium acid heparinate is recorded in Table IV. The five times crystallized material is substantially biologically inactive. The interesting further observation is made that prolonged drying of the original crystalline material likewise deactivates it. Since the dilute acetic acid recrystallization is a very mild procedure but is accompanied by the release of a glucosamino group from its previous combination, the conclusion may be drawn that the biological deactivation is produced by the hydrolysis of the group thus combined. It is probable that the apparent hydrolytic action resulting in crystalline material does not lead to the production of two entities but is of an intramolecular nature. This would favor the cyclic (Fig. 5) or long-chain (Fig. 3) type of structure and would eliminate that depicted in Fig. 4. The data in Tables III and IV show that the toluidine blue test[20] is no criterion of anticoagulant potency and that anticoagulant potency may not vary with sulfur content (which parallels ash content).

TABLE III

RECRYSTALLIZATION OF BARIUM ACID HEPARINATE; ANALYTICAL DATA

Number of crystallizations		1	3	5
$(\alpha)^{25}D$, H_2O, c 2		+44	+43	+42
Anal., %	Carbon	20.12	...	20.07
	Hydrogen	2.60	...	2.94
	Nitrogen	2.03	...	2.40
	Ash (H_2SO_4)	40.50	39.72	39.29
	Init. amino-N (Van Slyke) as N[a]	0.0	...	1.1
Equiv. wt. by titration		800 ± 50	...	745
Toluidine blue test		+	+	+
Ninhydrin reaction[b]		−	...	+

[a] Extrapolated initial values. [b] Intense blue to violet color on heating the substance (1 mg.) with ninhydrin (5 mg.) for 90 sec. at 100° in 1 cc. of 50% aqueous pyridine solution; strong positive test with D-glucosamine hydrochloride and chondrosin (glucosamino-glucuronic acid[21]).

TABLE IV

ANTICOAGULANT ACTIVITY

No. of crystallizations	1 (I)	3	5
ACU/mg.[a]	595	118	7

[a] Performed on material containing *ca.* 10% of moisture and corrected to the dry basis by moisture determination in a separate sample. Drying to constant weight at 100° under reduced pressure and over phosphorus pentoxide lowered the activity of I from 595 to 74 ACU/mg.

Experimental

Preparation of Crystalline Barium Acid Heparinate.—Unless otherwise noted, the crystalline barium acid heparinate used in this work was prepared from "Roche heparin" (neutral sodium salt; P, 0.1; inorganic sulfate absent; other analytical data given in Table I; 550 ACU/mg.) essentially according to the procedure of Charles and Scott[7] except that the preparation was made directly from the commercial sodium salt instead of through the intermediate amorphous benzidine salt. In one experiment, the amorphous benzidine salt was washed thoroughly with hot water and obtained completely ash-free.[22] It was then transformed into the crystalline barium acid salt. The analytical data obtained on this preparation (see Table I) did not differ essentially from those obtained on the same compound prepared directly from "Roche heparin" by the following procedure. It is believed, however, that the benzidine procedure effects some purification. "Roche heparin" (3.0 g.)[23] was dissolved in 120 cc. of water. Barium acetate (40 cc., 5%) was added and the whole allowed to stand for several days at 15–20° until clear. The insoluble material (A, 0.5–0.6 g.) was removed by centrifugation. The clear, yellow centrifugate was filtered through a hardened filter paper and the filtrate was heated to 65°. Glacial acetic acid (30 cc.) was added and the solution allowed to cool slowly to room temperature. The crystalline material (I, Fig. 1) was removed by centrifugation and was washed successively with 80% acetic acid, glacial acetic, 95% ethanol, and finally with ether; yield 1.9–2.1 g. The mother liquors were cooled to 0–5° and yielded a second crop of crystals which were separated as above; yield 0.3–0.4 g. From the filtrate, an amorphous fraction (B, 0.7–0.9 g.) was obtained by precipitation with excess acetic acid. Further amounts (0.5–0.7 g.) of crystalline material (kept separate from fraction I) could be obtained from fractions A and B.

Fraction I (Fig. 1) was examined[24] under a polarizing microscope. Low birefringence was shown as indicated by a first order gray interference color of all crystals. Crystals were in rosets which showed positive birefringence on using a retardation plate. Application of the Becke line test showed the crystals to have positive birefringence with the index of the long direction of the crystal being 1.544 ± 0.002 and the short direction, perpendicular to the above, being 1.537 ± 0.002. Crossed Nicols with a roset in the center of the field showed an extinction cross which indicated parallel extinction for the orientation of the crystals as they radiated from the center of the roset. No diffraction lines could be obtained by the X-ray powder method. The preparation showed a positive reduction toward Tollens reagent and gave a reducing value by the Hagedorn and Jensen method that was 2.5% of D-glucose.

Conversion of the Crystalline Barium Acid Heparinate into Sodium Heparinate.—The crystalline barium acid heparinate (550 mg.) was dissolved in 6 cc. of hot water and made alkaline to litmus with 10% sodium hydroxide solution. To this solution was added an excess of sodium carbonate (2.0 cc., *N*). The barium carbonate was removed by centrifugation. Upon addition of 3–4 volumes of absolute ethanol, the sodium heparinate was precipitated as a sirupy mass. The sirup was redissolved and the above treatment repeated. The sirup was dissolved in 5 cc. of water and precipitated by the addition of four volumes of glacial acetic acid. The precipitated salt was removed by centrifugation and washed successively with glacial acetic acid, 95% ethanol, absolute ethanol and finally with ether. This material (sodium acid heparinate) was redissolved in water and made alkaline to phenolphthalein with 10% sodium hydroxide (carbonate free). Addition of 8–9 volumes of alcohol precipitated the neutral sodium heparinate. The salt was dissolved and reprecipitated by the addition of several volumes of ethanol and ether; yield

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

(21) P. A. Levene, *J. Biol. Chem.*, **140**, 267 (1941).

(22) All ash determinations were made in platinum with concentrated sulfuric acid (see section below on analytical methods).

(23) Unless otherwise noted, preparative weights of substances are of material containing *ca.* 10% moisture. All analytical data, concentrations and specific rotation values are expressed on the dry basis. Unless otherwise noted, drying was effected by heating to constant weight over phosphorus pentoxide at 105–110° at water pump vacuum or at 75° at 0.1 mm.

(24) Crystallographic work by Mr. A. Pace of this Laboratory.

300–350 mg. of amorphous material, spec. rot. +55° (23°, *c* 2, H_2O, D line).

Preparation of the Neutral and Acid Sodium Mucoitinsulfates.—Sodium mucoitinsulfate (neutral salt) was prepared from commercial pig gastric mucin[25] (40 g.) according to the procedure of Meyer, Smyth and Palmer.[26] In this procedure, treatment with Lloyd's reagent was limited to three minutes and was followed by a filtration through a prepared bed of activated charcoal and Super-Cel (Johns-Manville); yield 2.3 g. This material was further purified by solution in 25 cc. of water, filtration through a bed of activated charcoal and Super-Cel and reprecipitation with five volumes of ethanol (95%). Saturated sodium chloride aqueous solution was added in the ratio of 0.1 cc. per 500 cc. of total solution in order to coagulate the colloid formed. The white precipitate was removed by centrifugation and washed with absolute alcohol–ether (1:1); yield 1.6 g. of the neutral amorphous sodium salt. This purified preparation differs in rotation and analysis (Table I) from that recorded by Meyer and co-workers[26] who cite higher nitrogen and lower sulfur values.

The above neutral sodium salt (2 g.) was converted to the sodium acid salt by solution in 10 cc. of water containing 0.2 cc. of glacial acetic acid and a small amount of insoluble material was removed by centrifugation. The product was precipitated from the centrifugate by the addition of 10 volumes of glacial acetic acid and was washed successively with 80% acetic acid, 95% ethanol, absolute ethanol, acetone and ether; yield 1.8 g., equiv. wt. by titration 530. The above procedure was repeated twice; equiv. wt. by titration, 500. This final material (amorphous) was used for the analytical data recorded in Table I for sodium acid mucoitinsulfate.

Preparation of Sodium Chondroitinsulfate and Barium Acid Chondroitinsulfate.—Crude sodium chondroitinsulfate[27] (50 g.) was dissolved in 500 cc. of water and filtered through Super-Cel to give a clear yellow solution. Six volumes of glacial acetic acid added to the filtrate precipitated the sodium acid salt which was removed by filtration and washed with glacial acetic acid. The damp product was dissolved in 400 cc. of water and precipitated by pouring into 600 cc. of 95% ethanol. The filtered product was washed with 95% ethanol, dissolved in 200 cc. of water and 10% sodium hydroxide (*ca.* 160 cc.) was added slowly and with cooling to neutralization. This solution was poured into three volumes of 95% ethanol and the resultant precipitate of the amorphous neutral sodium chondroitinsulfate was removed by filtration and washed with 95% ethanol; yield 30 g., inorganic sulfate absent. The analytical data for this preparation are recorded in Table I.

In order to prepare the amorphous barium acid chondroitinsulfate, the partially neutralized barium salt of Levene and La Forge[27] (2 g.), prepared from crude sodium chondroitinsulfate, was treated as described above for the transformation of sodium mucoitinsulfate into sodium acid mucoitinsulfate and the product (1.8 g., equiv. wt. by titration 560) was purified by two further precipitations effected in the above-described manner; equiv. wt. by titration 490. The analytical data for this preparation are recorded in Table I.

Analytical Methods (Miscellaneous).—Comparative analytical data were obtained on the above-described salts of heparinic, mucoitinsulfuric and chondroitinsulfuric acids and these data are recorded in Table I. All analyses are recorded on the dry basis and drying was effected by heating to constant weight over phosphorus pentoxide at 105–110° and water pump vacuum or at 75° and 0.1 mm. pressure.

The samples for carbon and hydrogen were admixed with chromic acid or lead chromate. The sulfur values on the barium salts were determined as acid-hydrolyzable sulfate and it was found for barium acid heparinate that sulfide and sulfite sulfur were absent. The sulfur in the sodium salts was determined by the Parr bomb (sodium peroxide fusion) method and concordant results were obtained when both procedures were used. Ash determinations were made in platinum with concentrated sulfuric acid. Dry ashing gave very appreciably lower (10–15%) results.

Hexosamine Determination.—The hexosamine analyses were performed on the sodium salts according to the method of Elson and Morgan[28] as modified by Palmer, Smyth and Meyer[29] except that the time of hydrolysis at 100° with 4 *N* hydrochloric acid was lengthened to twelve hours. That these were the optimum hydrolytic conditions for heparinic acid, is clearly shown by Fig. 6. Jorpes[6b] recommended thirty to sixty minutes at 100° with 12 *N* hydrochloric acid for this hydrolysis. That these conditions are too vigorous is shown by Fig. 7 on data obtained with "Roche heparin." The anhydrohexosamine value obtained by us for sodium heparinate, regenerated from the crystalline barium acid salt, was 21.4 ± 1.5%. When hydrolysis was effected for twelve hours at 100° with 6 *N* hydrochloric acid the same anhydrohexosamine value was

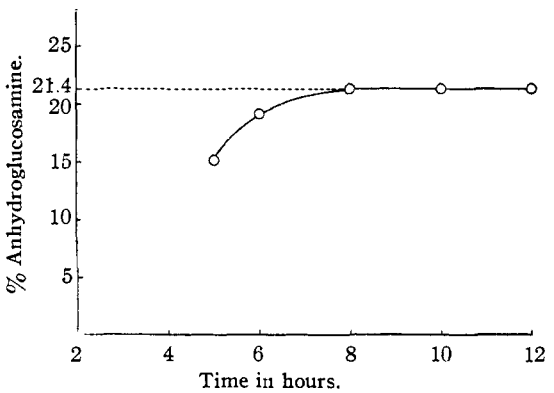


Fig. 6.—Variation of the anhydrohexosamine content of sodium heparinate (*c* 0.8), regenerated from the crystalline barium acid salt, with time of hydrolysis at 100° with 4 *N* hydrochloric acid.

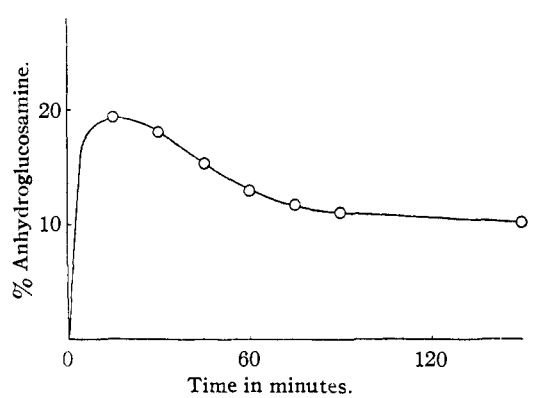


Fig. 7.—Variation of the anhydrohexosamine content of sodium heparinate (*c* 0.8), regenerated from the crystalline barium acid salt, with time of hydrolysis at 100° with 12 *N* hydrochloric acid.

(25) Obtained through the courtesy of Dr. David Klein of The Wilson Laboratories, Chicago, Illinois.

(26) K. Meyer, Elizabeth M. Smyth and J. W. Palmer, *J. Biol. Chem.*, **119**, 73 (1937).

(27) P. A. Levene and F. B. La Forge, *ibid.*, **15**, 69 (1913).

(28) L. A. Elson and W. T. J. Morgan, *Biochem. J.*, **27**, 1824 (1933).

(29) J. W. Palmer, Elizabeth M. Smyth and K. Meyer, *J. Biol. Chem.*, **119**, 491 (1937)

found. A standard glucosamine hydrochloride solution treated in the same manner with 6 *N* hydrochloric acid underwent no detectable destruction. A sample of chondrosin (amorphous) hydrolyzed for twelve hours at 100° with 4 *N* hydrochloric acid gave an apparent hexosamine content of 49.7% (calcd. 50.0%). Without hydrolysis the value was about one-half of this. N-Acetylglucosamine on preliminary hydrolysis for twelve hours at 100° with 4 *N* hydrochloric acid, gave an apparent hexosamine content of 81 ± 5 (calcd. 81%). Without hydrolysis the values were erratic and very low. Our reproducibility with this colorimetric procedure was about 7%.

The hexosamine present in the crystalline barium acid heparinate was identified as D-glucosamine. An amount of 1.000 g. (dry basis) of the crystalline salt was refluxed with 4 *N* sulfuric acid for ten hours. The barium sulfate was removed by centrifugation and the centrifugate was neutralized hot with barium carbonate. The precipitate formed was removed by centrifugation and the centrifugate was decolorized with activated charcoal (50 mg.), concentrated under reduced pressure to 5 cc. and absolute ethanol added slowly to precipitate a small amount of flocculent material that was removed by filtration. The filtrate was concentrated under reduced pressure to a sirup that crystallized on the addition of a few drops of concentrated hydrochloric acid. Further crystallization was induced by the slow addition of acetone to a volume of 20 cc. followed by overnight standing at icebox temperature; yield 210 mg. (83%), spec. rot. +65° (H_2O, D line), hexosamine value 100–103% of theoretical using D-glucosamine hydrochloride standard. The material was recrystallized from water by the addition of methanol and acetone; recrystallization yield 80%, spec. rot. +72° (final, 25°, *c* 1.8, H_2O, D line), comparing with the generally accepted value of +72.5°.

Uronic Acid Determination.—The general procedure of Lefèvre and Tollens[30] was used. The method of operation was that of Whistler, Martin and Harris[31] and the apparatus employed was that of Burkhart, Baur and Link.[32] A weight of sample sufficient to yield 10–15 mg. of carbon dioxide was used. Figure 8 shows a typical extrapolation curve for crystalline barium acid heparinate. Using this procedure, mucic acid and D-glucono-δ-lactone were found to give negligible extrapolated values. Chondrosin (amorphous) gave a value of 51.4% hexuronic acid (calcd. 54.5).

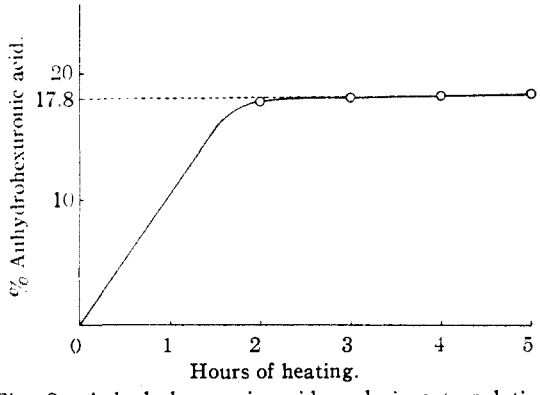


Fig. 8.—Anhydrohexuronic acid analysis extrapolation, crystalline barium acid heparinate.

Crystalline barium acid heparinate gave a positive qualitative Tollens[33] naphthoresorcinol test on following exactly the directions of Neuberg and Kobel,[34] the final benzene extract in this test showing an absorption band at 5700–5800 Å. with a maximum at 5700 Å.

N-Acetyl Determination.—For this analysis, the sample (200 mg.) was heated at 100° for one hour with 20% aqueous *p*-toluenesulfonic acid and was then distilled at constant volume. The acetic acid was determined in the distillate by titration. In the apparatus used, a constant blank of 0.06 cc. of 0.1 *N* sodium hydroxide per 10 cc. of distillate was found and this was subtracted from all recorded data. This blank was not altered when neutralized D-glucosamine hydrochloride (100 mg.) and D-galacturonic acid (100 mg.) was added. Acetanilide was analyzed by this method: calcd. CH_3CO, 31.8; found, 31.6. In order to remove any adsorbed volatile acidity from the amorphous neutral sodium salts analyzed, these were dissolved in water, the *p*H adjusted to phenolphthalein alkalinity by the addition of dilute sodium hydroxide and the product precipitated by the addition of alcohol and ether. This process was repeated (generally three times) until the initial aqueous solution was neutral or just alkaline to phenolphthalein. The crystalline barium acid salt was washed thoroughly with alcohol and ether and dried at 110° under reduced pressure and over powdered soda lime and phosphorus pentoxide. O-Acetyl[35] was absent in sodium mucoitinsulfate and sodium chondroitinsulfate on application of the Kunz[36] acetyl assay. Final verification of the presence or absence of N-acetyl was made by application of the Kuhn-Roth[14] CH_3-C assay.

Neutral Equivalent Determination.—The neutral equivalent values were determined on the acid salts by means of titration, using a glass electrode. The data are recorded in Table I. A typical titration curve for barium acid heparinate is shown in Fig. 9.

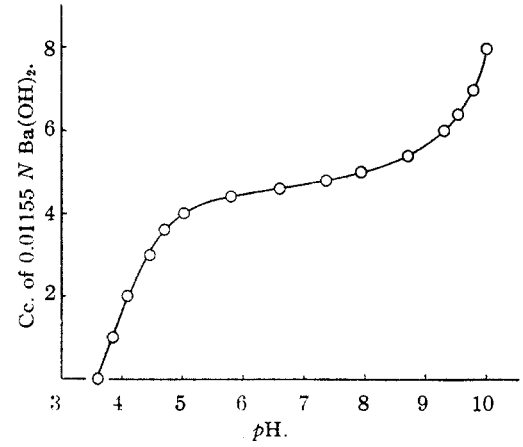


Fig. 9.—Titer curve of crystalline barium acid heparinate (*c* 0.0009143) at 25°.

Oxidation of Sodium Heparinate with Periodic Acid.—Sodium heparinate (0.3616 g. per 100 cc. of soln.) regenerated from the crystalline barium acid salt, which in turn had been prepared from the benzidine salt, was oxidized at room temperature (28°) with very dilute sodium metaperiodate (0.0391 molar) and the results are shown in Fig. 11. It is seen that an initial rapid consumption of periodate occurred in an otherwise slow and continuous absorption of the oxidizing agent. Extrapolation (Fig. 11) to zero time from the point at which the initial reaction was completed, gives the equivalent weight of 1700 for the sodium salt or 1900 for the barium acid salt. This value is higher than that (1200) previously reported[1] but is considered to be a better figure since it was obtained on what is believed to be material of higher purity. Formaldehyde (dimedon method) and formic acid (a significant

(30) K. U. Lefèvre and B. Tollens, *Ber.*, **40**, 4513 (1907).

(31) R. L. Whistler, A. R. Martin and M. Harris, *J. Research Natl. Bur. Standards*, **24**, 13 (1940).

(32) B. Burkhart, L. Baur and K. P. Link, *J. Biol. Chem.*, **104**, 171 (1934).

(33) B. Tollens, *Ber.*, **41**, 1788 (1908).

(34) C. Neuberg and Maria Kobel, *Biochem. Z.*, **243**, 435 (1931).

(35) M. L. Wolfrom, M. Konigsberg and S. Soltzberg, THIS JOURNAL, **58**, 490 (1936).

(36) A. Kunz and C. S. Hudson, *ibid.*, **48**, 1982 (1926).

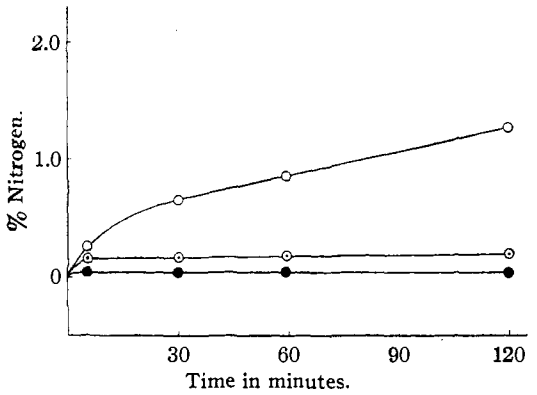


Fig. 10.—Variation of amino-N (Van Slyke) with time: O, crystalline barium acid heparinate; ●, sodium chondroitinsulfate; ⊙, sodium mucoitinsulfate.

titratable acidity with methyl red indicator) were absent. The figure of 1900 compares with the neutral value of 800 ± 50 found for barium acid heparinate.

Recrystallization of Barium Acid Heparinate.—The crystalline salt (Fraction I, 2.00 g.) was dissolved in water (80 cc.) and heated to 65°. Barium acetate (5 cc. of 5% aqueous solution) was added, followed by glacial acetic acid (10 cc.), and the whole was cooled slowly to room temperature. The crystals formed were removed by centrifugation and washed as described previously; yield 1.32 g. This process was repeated three times and the results are recorded in Table III. Figure 2 is a photomicrograph of the five times (yield practically quantitative) crystallized salt.

Anticoagulant Activity.—The bioassays were performed by Dr. R. H. K. Foster at the Laboratory of Hoffmann-La Roche, Inc., Nutley, New Jersey, according to the procedure described by him[37] and are expressed as Roche anticoagulant units (ACU) per mg. The marked lowering of the anticoagulant activity of the crystalline barium acid salt on repeated recrystallization is shown by data recorded in Table IV.

"Roche heparin" (550 ACU/mg.) was essentially deactivated (85 ACU/mg.) in 1% solution on treatment with 3% hydrogen peroxide in 0.02 *N* ammonium hydroxide either by heating for fifteen minutes at water-bath temperature or on standing for one day at room temperature. Sulfate ion was liberated in this process in appreciable amount.

We are indebted to the Hoffmann-La Roche Company for a research grant. Dr. H. M. Wuest, director of research of this corporation, gave his counsel throughout this work. Dr. R. H. K. Foster of the Hoffmann-La Roche Laboratory carried out the bioassays. We are indebted to the Hoffmann-La Roche Laboratory for some of the analytical data, a part of which were obtained by Mr. J. E. Varner of the Ohio State Laboratory. We also acknowledge the assistance of Messrs. R. J. Morris, C. D. DeWalt, Jr., and Robert L. Brown of this Laboratory.

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

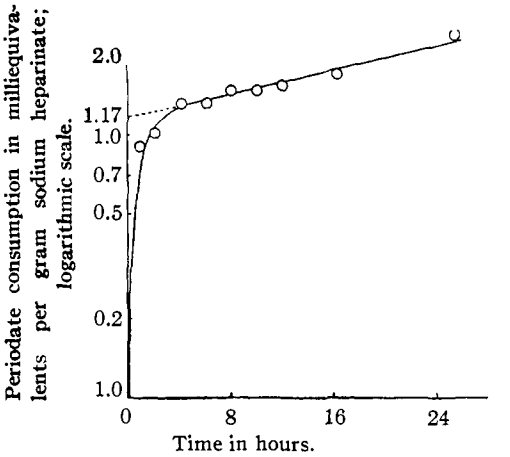


Fig. 11.—Rate of consumption at 28° of periodate (0.039 molar sodium metaperiodate) by purified sodium heparinate (0.362 g. per 100 cc. soln.).

Summary

1. Analytical data are cited for the crystalline barium acid salt of heparin (barium acid heparinate) in comparison with those obtained for the neutral and acid salts of mucoitinsulfuric acid and chondroitinsulfuric acid.

2. The following ratio was obtained for barium acid heparinate: anhydrohexosamine:anhydrohexuronic acid:SO_3:Ba = 2.0:1.8:6.0:3.0.

3. Summation of the above data indicates the possible presence of another constituent.

4. D-Glucosamine was identified (as D-glucosamine hydrochloride) in the hydrolyzate of the crystalline barium acid heparinate.

5. Sodium heparinate, purified through the crystalline barium acid salt, consumed initially 1 mole (per 1900 equivalent weight) of periodic acid.

6. The amino group of the D-glucosamine component of barium acid heparinate is not acetylated and is not free.

7. Barium acid heparinate loses its anticoagulant potency on repeated crystallization from warm, dilute acetic acid. This change is accompanied by the appearance of free amino nitrogen in the molecule.

8. Crystalline barium acid heparinate is biologically inactivated by prolonged drying at 100°.

9. Sodium heparinate is inactivated by weakly ammoniacal hydrogen peroxide.

10. Neither sulfate content nor toluidine blue staining power are true criteria of heparin activity.

11. Two alternative structures for the general architecture of the heparinic acid molecule are proposed.

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