20. On the Chemistry of Heparin

By Erik Jorpes, *From the Chemistry Department of the Karolinska Institutet, Stockholm, Sweden*

(Received 21 January 1942)

The molecular structure of heparin

Howell's observation [1928] that heparin contains a uronic acid was confirmed by Jorpes [1935, 1, 2], who also found an amino-sugar and a high content of ester sulphates. On the basis of quantitative analyses, Jorpes concluded that heparin is a polysulphuric ester of chondroitin or of some closely related substance. When the amino-sugar was identified as glucosamine [Jorpes & Bergström, 1936], it became evident that the structural carbohydrate unit of heparin was mucoitin, if the uronic acid is assumed to be glucuronic acid. These findings were confirmed by Meyer [1938]. It is now generally agreed that heparin belongs to the same group of substances as chondroitin sulphuric acid [Reinert & Winterstein, 1939; Charles & Todd, 1940].

It is almost certain that the amino group is acetylated as is the case in all the other natural compounds, chitin, chondroitin sulphuric acid and the immune carbohydrates which contain an amino-sugar. The shape of the titration curve in the range pH 7-10 also clearly indicates that the amino group is not free [Wilander, 1938]. There was only a trace of buffering capacity in this range, whereas with chondrosin, where the amino group is free, there was a distinct rise of the curve. At least 50% of the theoretical amount of acetic acid has been isolated and identified as silver salt [Jorpes & Bergström, 1937].

The probable disaccharide unit of heparin.

If the assumption is made that the disaccharide unit has an internal structure similar to that of the ordinary disaccharides, there are four hydroxyl groups which could be esterified with sulphuric acid. Consequently the following compounds would have to be considered.

Mucoitinmonosulphuric acid	$C_{14}H_{21}SO_{14}N$	mol. wt. 459;	S 6.98%
Mucoitindisulphuric acid	$C_{14}H_{21}S_2O_{17}N$	mol. wt. 539;	S 11.86%
Mucoitintrisulphuric acid	$C_{14}H_{21}S_3O_{20}N$	mol. wt. 619;	S 15.5%
Mucoitintetrasulphuric acid	$C_{14}H_{21}S_4O_{23}N$	mol. wt. 699;	S 18.3%

The Na and Ba salts would have the following composition.

The Na salt of the:

Monosulphuric acid	$C_{14}H_{19}SO_{14}NNa_2$	mol. wt. 503;	S 6.36%
Disulphuric acid	$C_{14}H_{19}S_2O_{17}NNa_3$	mol. wt. 605;	S 10.58%
Trisulphuric acid	$C_{14}H_{17}S_3O_{20}NNa_4$	mol. wt. 707;	S 13.58%
Tetrasulphuric acid	$C_{14}H_{16}S_4O_{23}NNa_5$	mol. wt. 809;	S 15.81%

The Ba salt of the:

Disulphuric acid	$(C_{14}H_{18}S_2O_{17}N)_2Ba_3$	S 8.62%
Trisulphuric acid	$C_{14}H_{17}S_3O_{20}NBa_2$	S 10.80%

As early as 1935, fractions were isolated [Jorpes, 1935, 2] with a sulphur content corresponding very closely to that of a chondroitin or mucoitin trisulphuric acid. As the pure protein-free heparin had a lower sulphur content it was assumed to be a mixture of the different sulphuric esters, mainly di- and tri-sulphuric acids. It could not be proved, however, that a tetrasulphuric acid is also present in the natural heparin.

Crystalline heparin

In the meantime, the concept of a homogeneous crystalline heparin has been introduced. Crystalline heparin has been the subject of much discussion. Fischer & Schmitz [1933] thought that they had obtained a crystalline brucine salt of heparin. In the same year, Charles & Scott [1933] reported on a semi-crystalline preparation. Finally, in 1936, these authors [Charles & Scott, 1936] isolated a Ba salt of lung heparin, which they considered to be crystalline and which, since then, has been treated in the literature as the pure crystalline anticoagulant. Unfortunately, their preparation contained only 11.5% S in the free acid, or 20% less than the S content of the free acid of an amorphous liver heparin isolated from the insoluble brucine salt and first described by the present author in 1935. This 'crystalline' Ba salt of liver and lung heparin was studied by Charles & Todd [1940] and claimed by them to be a homogeneous compound, probably identical with the preparation described by Reinert & Winterstein [1939]. Both groups of authors were, in fact, still of the opinion that the compound contained $2\frac{1}{2}$ S atoms to each mucoitin unit.

The compound of Charles & Todd seemed to be crystalline. There was, however, no convincing evidence to justify the assumption of a crystalline structure. The crystals were 'too small and heavily twinned for the X-ray diagram'. Further information would also have been desirable regarding the S content and the optical rotation of their preparations. The S content of the Ba salt was 9.6% and of the NH_4 salt 12.2%. Both preparations were dried at room temperature and no information was given as to their water content. Compared with a Na salt described in this paper containing 13.8% S, the NH_4 salt ought to contain about 11% moisture. An analysis of the optical rotation would have been desirable. Thus, the preparation of Reinert & Winterstein, which was claimed by them to be derived from the 'crystalline' Ba salt, had a rotation of $+42^\circ$, a figure which is very much lower than the rotation $+63^\circ$ ($+66^\circ$?) of the strongest heparin preparations. Charles & Todd, in discussing their results, assumed that the pure heparin contains $2\frac{1}{2}$ sulphate groups to each mucoitin unit. Their conclusions were based on the S : N ratio. These acidic polysaccharides, however, are never completely free from traces of proteins or ammonium salts. The N content, therefore, does not correspond to the content of hexosamine.

As to the product described in 1939 as pure 'crystalline' heparin by Reinert & Winterstein of the Hoffman-La Roche laboratory, it is evident that it was no more than a mixture of the different polysulphuric esters described above. The Na salt, recovered from the 'crystalline' Ba salt, contained 11.1% S in the air-dry substance (11.9% of the dry substance), and showed an optical activity of $+42^\circ$. Consequently it corresponds to the purified heparin which was used by the present author as starting material for the fractionation of the brucine salts of heparin. From this material the mucoitin di- and tri-sulphuric acids were isolated. Although the present author found that the material contained at least one fraction with 12.5% S in the Na salt, and a composition very similar to that of a mucoitin trisulphuric acid, Reinert & Winterstein assumed, in agreement with Charles & Todd, that there were $2\frac{1}{2}$ S atoms to each hexosamine residue in their preparation. Further, the findings of Jorpes & Bergström [1937], that these strong heparin preparations with more than 12% S may have a specific rotation of $+66^\circ$, seemed also to have escaped the attention of these authors. The low S content and the low specific

rotation of the heparin samples of Reinert & Winterstein prove that these samples consisted of pure heparin but were by no means crystalline homogeneous compounds. The anticoagulant activity also corresponds to this. A sample of the commercial Liquaemin Roche, which was claimed to be prepared from 'crystalline salts', and which was analysed by the author in September 1940, was found to have the same strength (100 %) as our standard powder, whereas the trisulphuric acid has an activity of 140–150 %. All these facts clearly indicate that the product of Reinert & Winterstein was not a crystalline compound but a mixture of the different fractions isolated and separated from each other as far back as 1935. It must be admitted therefore that the discussion of crystalline heparin so far has rested on an insecure foundation.

In two later reports, crystalline heparin has been discussed. Thus, Jaques & Waters [1940] reported on a crystalline Ba salt isolated from the blood of dogs in anaphylactic shock which appeared to be homogeneous. Furthermore, Jaques [1940, 1] reported on crystalline Ba salts of heparin, isolated from the lungs of different animals, all of which had a similar composition. The water-free Ba salts of these heparins contained the following amounts of S, viz. dog heparin 10.8, that from the cow 10.8, from the pig 10.4, and from the sheep 11.6 %. No further chemical details were given. Evidently, the S content of these preparations corresponds exactly with that of the Ba salt of the mucoitin trisulphuric acid discussed above (calculated at 10.8 % S). Furthermore, if the figure given for the S content of sheep heparin is correct (11.6 %), it would indicate the presence of a certain amount of the tetrasulphuric acid of mucoitin.

In this last paper the very interesting observation was made that heparins from different animals, in spite of their similarity in chemical composition, showed individual biological activities as tested in a coagulation system, the dog heparin being 2.4 times stronger than the ox heparin, and the pig heparin having only 44 % of the strength of the latter. The sheep heparin was still weaker.

Is heparin activity due to one definite homogeneous chemical compound only?

There is one discrepancy which is very remarkable between the findings of the present author and the statements made in the literature. The strongest heparin fractions, the chemical composition of which corresponds to that of 'crystalline' heparin, make up only a small part of the heparin preparations, e.g. usually 15–20 % in ox-liver heparin and a still smaller percentage in heparin from lungs and spleen. If the heparin is very little influenced by the procedure of preparation, the main part of the natural heparin must have a lower S content and a weaker anticoagulant activity than the strongest fractions separated from it as Ba or brucine salts. Consequently, these fractions alone, even if they were crystalline, cannot be claimed to be the natural heparin, because the main part thereof consists of a polysaccharide of a much lower degree of esterification. The question arises whether these weaker fractions occur in the body or if they are solely decomposition products.

For the elucidation of this question I have analysed heparin from different organs and from different species of animals qualitatively and quantitatively. The fate of heparin during the course of its preparation has been followed. Furthermore, the fractionation with brucine has been once more studied in detail. The decreasing solubility of the brucine salts which parallels the increasing S content, offers an excellent means of studying the crude polysaccharide of heparin. The only way of obtaining the brucine salts is, of course, by means of electrodialysis.

Heparin from ox spleen

200 kg. of ox spleen were worked up in the usual way. The protein-free polysaccharide fraction, 4.6 g., was dialysed in a cellophane sac against distilled water. The substance dialysing out, had only 4 % of the standard heparin activity. The heparin inside the sac

was precipitated with alcohol in the presence of sodium chloride. The yield was 3.3 g. air-dry substance with 9.12 % moisture and 45–50 % heparin activity. The water-free substance analysed as follows: ash 29.8; N 2.83; S 9.35; hexosamine 22 %. $[\alpha]_D^{20} = +29^\circ$.

Fractionation by means of the brucine salt. 2.69 g. of the dry substance were electro-dialysed for 2½ hr. The substance removed with the neutralized anodic fluid, 0.54 g., showed a S content of 2.7 % and only 7 % of the standard heparin activity. The inner cell contained, as found after neutralization of a small sample, 2.4 g. substance with 9.17 % S in the Na salt and a heparin activity of 50–55 %. The different Na salts obtained after fractionating the brucine salts in water, analysed as shown in Table 1.

Table 1. *Na salts of heparin from ox spleen as recovered from the different fractions of brucine salts*

Substance recovered from	Air-dry substance g.	Moisture %	S % of dry substance	$[\alpha]_D^{20}$ (dry substance)	Heparin activity of air-dry substance, % of standard
Mother liquor: 1st	1.000	17.3	8.3	+26°	40
2nd	0.255	12.6	10.3	+33°	60
3rd	0.028	13.4	12.6	+39.4°	100 (?)
Insoluble brucine salt	0.038	13.2	13.9	+50°	100

Heparin from lungs of cattle

500 kg. of ox lungs yielded 20.8 g. of air-dry substance after dialysis of the Na salt initially obtained (23 g.). Moisture 7.98 %. Anticoagulant activity of the air-dry substance, 50 %. The dry substance analysed as follows: ash 38.7; N 2.9; S 10.62; hexosamine 24.9 %. $[\alpha]_D^{20} = +38.7^\circ$.

Fractionation by means of the brucine salt. 9.2 g. dry substance were electro-dialysed in 350 ml. of water for 8 hr. The anode liquor contained 2 g. of dry substance with 8.7 % S and a heparin activity of 35 %. The inner cell contained 7.8 g. of heparin calculated as dry Na salt with 9.75 % S and 60 % heparin activity. The original strength of the air-dry substance which was electro-dialysed was 50 %. $[\alpha]_D^{20} = +41^\circ$. The results of the analysis of the Na salts obtained after fractionation of the brucine salts are given in Table 2.

Table 2. *Na salts of heparin from the lungs, recovered from the different fractions of brucine salts*

Substance recovered from	Air-dry substance g.	Moisture %	S % of dry substance	$[\alpha]_D^{20}$ (dry substance)	Heparin activity of air-dry substance, % of standard
Mother liquor: 1st	1.26	16.7	5.48	—	7
2nd	1.375	19.6	10.8	+39.5°	50
3rd	1.209	14	12.2	—	80
Insoluble brucine salt	0.3467	12.6	13.2	+56.7°	110

Heparin from ox liver

Fractionation by means of brucine. A Na salt of commercial heparin was dialysed for 24 hr. in a cellophane sac against distilled water in order to remove free sulphates. 4.1 g. dry substance containing 11.1 % S and having a heparin activity of 110 % of the standard were dissolved in 150 ml. of water and electro-dialysed. After 6½ hr. no alkali was present in the water passing the cathode chamber.

The heparin activity still present in the inner cell after electro-dialysis was 75–80 % of the original. Anticoagulant activity, corresponding to the loss, was found in the anode fluid. The water (10 l.) which had washed the anode during electro-dialysis was collected, neutralized and concentrated *in vacuo* to 101 ml. It contained 0.723 g. of dry substance with the heparin activity unimpaired (110 %) and with 11.3 % S. Thus, about 18 % of the

heparin originally present had passed the anodic parchment membrane without any loss of heparin activity or of sulphur.

No sulphuric acid had been split off from the heparin inside the cell, because two 5 ml. samples of the concentrated anode fluid did not contain any free sulphates precipitable with BaCl_2 in 0.5 *N* HCl.

As to the chemical structure and the anticoagulant activity of the heparin, the electro-dialysis of this experiment proved to be quite harmless. Apparently the free acid is stable at room temperature in aqueous solution, at least for a certain time. A similar finding was made in determining the amount of free sulphates in the heparin preparations. In 0.5–0.75 *N* HCl no sulphuric acid was split off from the heparin during 24–48 hr. at room temperature.

In another experiment, 9.2 g. of the same heparin were electro-dialysed in 150 ml. of water for 7 hr. The heparin activity inside the cell was 80 % of the original. The anode fluid, about 10 l., was neutralized and concentrated *in vacuo* to 200 ml. It contained 1.143 g. of dry substance (12.5 %) with 8.5 % S and an anticoagulant activity of 70 %. In 10 ml. of this solution there was only a trace of sulphate precipitable with BaCl_2 in acid solution; consequently only a trace of sulphate had been set free from the heparin during the electro-dialysis.

In a further experiment with 10 g. of heparin, 1.8 % of the S was split off during electro-dialysis and recovered from the anode liquor as free sulphates.

6 and 10 g. dry Na salt of heparin (dialysed) were electro-dialysed in two runs of 8 and 11 hr. duration. The combined anode fluids were collected, neutralized and concentrated to a small volume. They contained free sulphates corresponding to 3.5 % and ester sulphates corresponding to 16 % of the total S present in the heparin which had been submitted to electro-dialysis. The combined inner fluids were neutralized with brucine and the brucine salts fractionated in water as usual. The brucine was this time removed with a saturated solution of calcium hydroxide at room temperature. The Ca salts were twice reprecipitated with only 1 vol. of alcohol from an almost neutral or faintly alkaline solution; the results are given in Table 3.

Table 3. *The Ca salts recovered on removal of brucine from the different fractions of brucine salts*

Substance recovered from	Air-dry substance g.	Moisture 100° <i>in vacuo</i> over P_2O_5 %	% of dry substance			$[\alpha]_D^{20}$ calc. per dry substance	Heparin activity of air-dry substance, % of standard
			Ash	S	Ca		
Mother liquor: 1st	7.20	10.4	29.8	8.80	10.4	+45.7°	65
2nd	0.75	6.54	29.6	10.80	10.3	+45.4°	100
3rd	1.60	10.2	30.3	11.20	—	+57.2°	100
4th	0.60	9.9	36.3	12.00	12.0	+61.8°	125
Insoluble brucine salt	2.00	10.6	33.9	12.56	12.3	+63.5° ?	135–140

The Ca salts recovered (10.92 g.) contained about 80 % of the dry substance and about 70 % of the heparin activity calculated to be found in the inner cell after removal of the heparin which had passed the anode membrane. Evidently no considerable destruction with loss of heparin activity had taken place during electro-dialysis, even in this experiment, which revealed a certain loss of ester sulphates (3.5 %).

In two other experiments samples of the inner fluid were neutralized following electro-dialysis for 8 and 10 hr. The Na salts of the inner fluids were carefully assayed against the Na salt of the starting material.

There had been no detectable loss of anticoagulant activity during electro-dialysis; the potency of the samples did not deviate more than 5 % from the strength of the heparin used as starting material.

As is evident from these experiments, very little sulphuric acid, if any at all, is split off from the heparin during electrodialysis. The various fractions of heparin of different sulphur content, separated from each other as brucine salts, must therefore be assumed to be performed in the protein-free purified heparin. They must occur together before electrodialysis because a considerable part of the heparin recovered from the brucine salts has a 10–20 % higher S content and a 30–50 % stronger anticoagulant activity than the material submitted to electrodialysis. This could not be if the starting material had been a uniform product, which suffered partial destruction during electrodialysis, thus giving rise to new, less active fractions with lower S content.

The composition of the strongest heparin samples

The composition of the strongest heparin samples obtained after removal of the brucine from the insoluble fractions of the brucine salt is of particular interest in relation to the crystalline heparin problem. In this way, Na salts of heparin from ox liver can be obtained with steadily increasing S content; the compositions and anticoagulant activities of a series of such samples are given in Table 4.

Table 4. *Compositions and anticoagulant activities of heparin fractions with high S content*

No.	g.	Moisture %	% of dry substance			Anticoagulant activity of the dry substance in % of standard (dry substance)
			S	Ca	Na	
1. Na salt	0.260	13.0	11.73	—	—	125
2. Na salt	0.600	9.9	12.00	—	—	125–130
3. Na salt	0.240	12.5	12.10	—	—	125–130
4. Na salt	0.770	17.4	12.5	—	—	135–145
5. Ca salt	2.00	10.6	12.56	12.60	—	135–140
6. Na salt	0.775	6.7	12.90	—	—	140–145
7. Ca salt	0.294	12.85	13.2	12.60	—	153.
8. Na salt	0.650	17.5	13.8	—	10.43	160

The brucine salt of the liver heparin with the lowest solubility obtained in one experiment (sample 8) showed an exceptionally high content of S, i.e. about 14 % of the dry Na salt. In fact, it had been obtained from a preparation of the crude heparin which showed an unusually strong activity, 125 % of the standard powder. This salt, 0.650 g., was analysed on four different occasions. The air-dry substance yielded 11.54 (11.48, 11.57) % S corresponding to 14.0 % of the dry substance (17.5 % moisture). On another occasion 13.7 % (13.8, 13.55) were obtained on analysing the dry substance (100° *in vacuo* over P₂O₅). The ash content of the dry substance was 38.1 %; Na, 10.43 %.

This sample also showed a somewhat stronger biological activity than the other samples, about 160 (160, 160, 150)%. The figures given were found in assays on three different days.

The S content of this sample equals or is slightly higher than the theoretical figure calculated for a mucoitin trisulphuric acid. Taking into account the fact that the substance is amorphous and certainly contains traces of foreign substances including salts, this figure might indicate admixture with a trace of a tetrasulphuric acid. So far, however, no conclusive proof could be obtained for the occurrence of such an acid in heparin. The analysis of this sample in any case disproves the earlier claims that pure heparin contains 2½ sulphate groups to each mucoitin unit, as do also the figures given by Jaques for the S content of the Ba salts of lung heparins from different animals.

The polysaccharide fractions with lower S content

As to the nature of the remainder of the heparin polysaccharide after removal of the most active fractions, it would be of interest to know whether they have a similar organic skeleton. Particularly in lung heparin, one might expect the occurrence of a considerable

amount of chondroitin sulphuric acid originating from the cartilage of the bronchi. A sample of lung heparin was therefore fractionated as Ba salt. One fraction separated when BaCl_2 was added at neutral reaction (I), another after the addition of $\text{Ba}(\text{OH})_2$ (II). The mother liquor was precipitated with alcohol and the precipitate dialysed in a cellophane sac against water. An insoluble fraction (III) was separated. The dialysed solution was concentrated to a small volume and $\text{Ba}(\text{OH})_2$ added until no further precipitate formed (IV). To the mother liquor, alcohol was added (precipitate V). The Ba salts were transformed into Na salts and then analysed (Table 5).

Table 5

g.	Moisture %	% of dry substance					Heparin activity of air-dry substance %
		Ash	Nitrogen	Sulphur	Uronic acid	Amino- sugar	
I 6.5	10.0	41	2.45	11.0	19.5	14	100
II 1.3	11.0	—	2.9	6.2	—	—	15
III 2.7	10.5	39	3.3	7.2	21	14.5	15
IV 1.15	7.1	—	2.95	4.8	—	—	—
V 4.35	10.9	37	2.55	0	22	17	Nil

As is evident from Table 5, all fractions have a similar composition. They are all built up of an acetylated hexosamine and a hexuronic acid. A considerable part of the polysaccharide from the lungs is sulphur-free, and lacks heparin activity.

The stability of heparin during autolysis, tryptic digestion and alkaline extraction

The first stage at which destruction of heparin could take place is during the autolysis of the organs. In fact, Charles & Scott [1933] found autolysis to be favourable for the preparation of heparin. The recent findings of Jaques [1940, 2], that liver contains a heparinase capable of splitting heparin, indicate possible partial destruction of heparin during autolysis. The experience hitherto gained, however, indicates that no such destruction occurs. I have made repeated experiments, using both fresh and autolysed liver and lungs of cattle, and dog liver, and have found the same activity and the same S content of the heparin from one and the same source irrespective of whether it was extracted before or after autolysis for 36 hr.

There is not much reason, either, to assume a destruction of heparin during the digestion with the pancreatic extract, already recommended by Howell and applied by Charles & Scott in their routine method. However, this phase in the process of preparation was also controlled. It was found to be completely harmless.

To the trypsin solution used in the preparation of heparin a Na salt of heparin was added, 7.6 mg./ml. in one experiment and 0.75 mg. in another. The reaction was made slightly alkaline to litmus, toluene was added and the samples digested at 37° for 4 days. In both samples the activity after digestion, as compared with the original strength, was still 100 %. The addition of 20 ml. of N HCl and 2 ml. of a 10 % BaCl_2 solution to 20 ml. of the former sample did not cause any detectable precipitation of BaSO_4 , a fact which indicates that no sulphuric acid had been set free during the digestion.

If there is any possibility at all of destroying the heparin, it might be during the first stage of its preparation, i.e. during the alkaline extraction or the acidification with H_2SO_4 . If the amounts of NaOH and of $(\text{NH}_4)_2\text{SO}_4$ are properly balanced, as recommended by Charles and Scott, to give a pH of 9.4–9.1 in the mixture, no destruction seems to take place, as is evident from the following experiment.

1 g. of heparin of standard potency (10.96 % S) was dissolved in a mixture of 30 ml. 10*N* NaOH and 80 ml. 50 % ammonium sulphate (by volume) (pH 9.4). The solution was

kept at 40° for 1 hr., and at 85–90° for 1 hr. more. The solution was then diluted with water and the heparin precipitated with alcohol. Free sulphates were removed by dialysis. S content of the heparin recovered, 11.01 %. Heparin activity, 100 (possibly 95) % of the standard. No destruction whatsoever had occurred.

Attention was also paid to the question of a possible destruction of heparin by the H_2SO_4 added to the first extract. Not even at this stage of the preparation could any decrease in heparin activity be traced.

METHODS

Amino-sugar. The quantitative determination of the amino-sugar content of the heparin preparations presents several difficulties. At first, the amino-sugar is only slowly liberated, boiling with conc. HCl for several hours being necessary for hydrolysis of the disaccharide [Jorpes & Bergström, 1937]; furthermore, the free amino-sugar, glucosamine, is partly destroyed during this hydrolysis. Thus, it was found that of the glucosamine 10 % was destroyed in 1 hr., 15 % in 3 hr., and about 30 % in 6 hr., if boiled in a water bath with conc. HCl.

Glucosamine and galactosamine are known to give the same colour intensity in the Elson-Morgan reaction [Smyth *et al.* 1937], but in disaccharide units the linkage influences the colour formation. An accurate figure for the amino-sugar content therefore cannot be obtained. It was also observed that lower figures were consistently obtained, if the hydrolysis lasted for some hours. The heparin preparations were therefore hydrolysed for only 30 min., or 1 hr.

If material was available, 50 mg. were hydrolysed in a water bath in conc. HCl for 30 min. The HCl was distilled off, the solution cautiously neutralized and the volume adjusted to 10 ml.; 0.25 ml. of this solution and of its dilutions 1:1.5 and 1:2 were taken for analysis. The different modifications of the Elson-Morgan method [Nilsson, 1936; Boyer & Fürth, 1935; Smyth *et al.* 1937; Sørensen, 1938; Hewitt, 1938] were tried; the following technique was preferred.

A series of 10 ml. pyrex test-tubes marked at 5 ml. are used. To 0.25 ml. of the test solution, containing 0.4–1 mg. of the hydrochloride of the amino-sugar per ml., is added 0.2 ml. of $N \text{ Na}_2\text{CO}_3$ containing 1 ml. acetylacetone in 25 ml. The test tubes are boiled in a water bath for 15 min. After cooling at room temperature for 1 hr., 2.5 ml. of glacial acetic acid are added to each tube of the series and then 0.25 ml. of the Ehrlich reagent. The tubes are boiled in a water bath for 10 min. Glacial acetic acid is filled up to 5 ml. After mixing with a glass rod and keeping for 20 min. the colour intensity is read through filter S53 of the step photometer using 1 cm. cells.

Usually three different concentrations of an unknown solution are analysed simultaneously, all of them in duplicate. The sugar concentration is read off from a curve on semi-logarithmic paper obtained by analysing in duplicate four different concentrations of a known glucosamine solution. Such a curve is determined every time a series is boiled. Three different unknown samples can be analysed simultaneously, making a total of 26 test tubes in the series.

Sulphur. For the S analyses, 25–40 mg. of the material were fused with carbonate-nitrate. The precipitate of BaSO_4 was collected in a Neubauer-Gooch platinum iridium crucible, washed, ignited and washed again with dil. HCl, ignited and weighed. Each analysis was made in duplicate or triplicate.

Anticoagulant activity. The anticoagulant activity was determined by the method described by Jorpes [1935, 2] and by Wilander [1938, p. 58]. Fresh ox blood taken direct from the vessel at the slaughterhouse was quickly decanted into a series of small test tubes, each containing 0.2 ml. of a dilute heparin solution. Five tubes in a stand contain standard heparin, 0.2 ml. each of a solution with 10, 5, 2.5, 1.25 and 0.63 mg. air-dry standard heparin in 32 ml. of physiological saline. The remaining five tubes of the same

stand contain the unknown sample in the corresponding dilutions. Readings are made at room temperature after 1½, 3, 5, 8 and 12 hr., possibly also after 24 hr. Care must be taken that no air enters the test tubes and that the two parts of the stand, the cover and the bed, are tightly clamped together.

A difference in strength of 10 % can be distinguished by this method. The figures of the anticoagulant activity of the heparin preparations given in Table 4 are therefore correct within 5–10 %. They were first determined in one or two series, each comprising 9 stands. Five of these samples, nos. 1, 2, 4, 5 and 6, were assayed once more as air-dry powder against the air-dry standard heparin. The potency of four of the samples did not deviate more than possibly 5 % from the strength found during the preliminary assaying. One of them only (no. 4) showed a 5–10 % greater potency than that found on the first day.

Heparin standard

The principles of expression of the anticoagulant activity of heparin, which have been applied by different workers, are almost as many as the authors themselves. Charles & Scott [1933] elaborated in detail the method of determining the cat unit, originally suggested by Howell. One unit was the amount of anticoagulant which prevented coagulation of 1 ml. of cat blood kept for 24 hr. at 0°; 1 mg. of pure heparin contained about 500 units.

A few years later, Best & Murray [1938] suggested that this unit should be replaced by another which corresponded to 0.01 mg. of pure heparin, assumed to be crystalline.

Other units have been recommended by Schmitz [1935], Chargaff *et al.* [1936], de Puoz [1938], Reinert & Winterstein [1939], Astrup [1938], and by Jores of Promonta in Hamburg.

Schmitz suggested as a unit the amount of heparin, of which $\frac{1}{30}$ doubled the logarithm of the coagulation time in 0.1 ml. of plasma. Chargaff *et al.* used hen's plasma and a muscle kinase of standard potency as suggested by Fischer & Schmitz [1932]. They defined an inhibitor unit which was very small, $\frac{1}{15}$ of the original Howell unit.

De Puoz, and Reinert & Winterstein of the Hoffman-La Roche laboratory expressed the potency of the heparin in anticoagulant units (A.C.U.). One A.C.U. keeps 1 ml. of recalcified citrated plasma of cattle uncoagulated at 37° for 4 hr. The necessary amount of Ca was to be titrated on each plasma specimen separately; 1 mg. of their pure product contained 500 A.C.U.

Realizing the complete impossibility of defining with accuracy an anticoagulant unit in terms of its action in some coagulation system, Bergström *et al.* [1937] tried to avoid all kinds of units by expressing the anticoagulant activity in mg. of a standard heparin; for this purpose, the protein-free heparin polysaccharide of ox liver was selected.

This principle has been found very convenient. The samples of ox liver heparin have an activity which is constant within about 10 %. The strength of the standard powder is about 65 % of that of the strongest heparin available, the mucoitin trisulphuric acid from ox liver, presumably corresponding to the 'crystalline' heparin of Best and Murray which, according to them, contains 100 units per mg. In the Swedish clinical records, therefore, the amount of anticoagulant given has been expressed in mg. of the commercial heparin.

Because of differences in their mode of action [Astrup & Fischer, 1938; Dam & Glavind, 1939], no synthetic anticoagulants can replace the heparin as standard substance.

DISCUSSION AND SUMMARY

The protein-free polysaccharide carrying heparin activity is quite different if prepared from different organs. The organic skeleton seems to be the same, namely, an acetylated amino-sugar combined with a hexuronic acid. The preparations differ in S content and anticoagulant activity, the liver heparins being the most active. The preparations are all

inhomogeneous. From all of them, fractions with a higher S content can be isolated either as Ba or as brucine salts.

It is of particular interest to know whether these fractions with varying S content really occur in the living animal or if they are secondary degradation products arising during the process of preparation. The former alternative is the most probable, because the heparin with the highest S content is very resistant. It is stable enough to resist both autolysis of the organs and tryptic digestion, and also alkaline extraction with subsequent acidification; it will therefore hardly give rise to any considerable amount of fractions with lower S content.

These polysaccharide fractions with lower S content also possess a certain anticoagulant activity. The biological activity is probably not due to admixture with the more active mucicetin trisulphuric acid, because the fractions are obtained from the mother liquors after removal of the less soluble brucine or Ba salts of the trisulphuric acid. The possibility, however, that the more soluble salts influence the solubility in water of the less soluble salts of the trisulphuric acid is not excluded. It seems as if all these different fractions carry an anticoagulant activity of their own, rapidly decreasing with decreasing S content.

The recent findings of Jaques, that there is a species specificity, also add further evidence on this point. Heparin from dog liver is 2.5 times more active than ox liver heparin with the same S content. Taking into account also the fact that heparins with about the same S content originating from different organs show different anticoagulating effects, the liver heparins being the strongest, heparin cannot be considered to be a homogeneous compound in the same sense as the other crystalline hormones, such as insulin.

In order to explain the exceptionally high biological activity of dog heparin, an attempt was made at isolating a tetrasulphuric acid as brucine salt from dog liver heparin. No fraction with a higher S content than that of the trisulphuric acid could, however, be obtained.

Whilst the fact still remains, that the exceptionally strong electric charge and the molecular configuration are the most outstanding properties of the heparin polysaccharide, it must be admitted that we actually know very little about the ultimate underlying principle causing the anticoagulating activity.

This investigation has been aided by grants from the Rockefeller Foundation and from the Therese and Johan Andersson Memorial Foundation.

REFERENCES

- Astrup, T. [1938]. *Enzymologia*, 5, 12.
 Astrup, T. & Fischer, A. [1938]. *Proc. Soc. exp. Biol.*, N.Y., 38, 494.
 Bergström, S., Jorpes, E. & Wilander, O. [1937]. *Skand. Arch. Physiol.* 76, 175.
 Best, C. H. & Murray, D. N. G. [1938]. *J. Amer. med. Ass.* 110, 118.
 Boyer, R. & Fürth, O. [1935]. *Biochem. Z.* 282, 242.
 Chargaff, E., Bancroft, F. W. & Stanley-Brown, M. [1936]. *J. biol. Chem.* 115, 149, 155.
 Charles, A. F. & Scott, D. A. [1933]. *J. biol. Chem.* 102, 425, 437.
 ——— [1936]. *Biochem. J.* 30, 1927.
 Charles, A. F. & Todd, A. R. [1940]. *Biochem. J.* 34, 112.
 Dam, H. & Glavind, J. [1939]. *Skand. Arch. Physiol.* 82, 221.
 Fischer, A. & Schmitz, A. [1932]. *Hoppe-Seyl Z.* 210, 129.
 ——— [1933]. *Hoppe-Seyl Z.* 216, 264, 274.
 Hewitt, L. F. [1938]. *Biochem. J.* 32, 1554.
 Howell, W. H. [1928]. *Bull. Johns Hopk. Hosp.* 42, 199.
 Jaques, L. B. [1940, 1]. *Science*, 92, 488.
 ——— [1940, 2]. *J. biol. Chem.* 133, 445.

- Jaques, L. B. & Waters, E. T. [1940]. *Amer. J. Physiol.* **129**, P 389.
- Jorpes, E. [1935, 1]. *Naturwissenschaften*, **23**, 196.
- [1935, 2]. *Biochem. J.* **29**, 1817.
- Jorpes, E. & Bergström, S. [1936]. *Hoppe-Seyl Z.* **244**, 253.
- [1937]. *J. biol. Chem.* **118**, 447.
- Meyer, K. [1938]. *Cold Spr. Harb. Symp. Quant. Biol.* **6**, 97.
- Nilsson, I. [1936]. *Biochem. Z.* **285**, 386.
- de Puoz, J. [1938]. *Schweiz. med. Wschr.* **68**, 524.
- Reinert, M. & Winterstein, A. [1939]. *Arch. int. Pharmacodyn.* **62**, 47.
- Schmitz, A. [1935]. *Hoppe-Seyl Z.* **234**, 216.
- Smyth, E. M., Palmer, J. W. & Meyer, K. [1937]. *J. biol. Chem.* **119**, 491.
- Sörensen, M. [1938]. *C.R. Lab. Carlsberg*, **22**, 487.
- Wilander, O. [1938]. *Skand. Arch. Physiol.* **81**, Suppl. 15.