Microelectrophoresis of Heparin
(*A Modified Technique–But Unperfected*)

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ABSTRACT

Apparatus and technique for the microelectrophoresis of heparin on an agarose gel are described in detail. Four brands of commercial gut mucosa heparin were tested. All had a fast component and a slow component, the latter migrating 0.88 (0.86 to 0.91) relative to the fast band. The difference in the rate of migration of the commercial preparations (1.02 to 0.97 relative to the arbitrary standard of 1.0) is probably within the error found when the standard was run against itself (0.96-1.02). No mucopolysaccharide or protein impurities were found. There was no observed difference between the migration pattern of gut heparin and the one brand of lung heparin tested. Future attempts at further separation of the 2 fractions is also discussed.

Although heparin has been used clinically as an anticoagulant since 1935,[1,2] it still remains a "black box" in terms of its structure, metabolism, and locus of action. Heparin, or indications for its existence, was first noted in the 30 year period following 1880 when several workers studied a substance with anticoagulation ability that was found in tissue and blood of dogs after an antigenic protein had been injected.[1] In 1916, McLean, a sophomore medical student working in the laboratory of W. H. Howell at Johns Hopkins, stumbled onto a phosphatid with anticoagulant activity in tissue isolated from heart and liver of dogs.[3] Howell and Holt first theorized that heparin, so named because of its abundance in the liver, acted by preventing activation of prothrombin and causing the activation of pro-antithrombin.[4] In 1937, it was demonstrated that heparin was effective in the prevention of experimental thrombosis; it is now universally used in the treatment of disseminated intravascular consumptive coagulation, myocardial infarction, post-operative thrombosis, and pulmonary embolism.[1,5,6] Other uses have been indicated, but are not as uniformly accepted.

It is generally agreed that the structure of heparin is a long chain polysaccharide of alternating molecules of glucuronic acid and glucosamine linked by an 1,4 glycosidase bond.[6,7] Molecular weight estimates have varied between 8,000 and 18,000 with 16,000 being a generally accepted figure.[8,9] In addition, it is unique among mucopolysaccharides in having a high proportion of its amino groups combined with sulphate for form sulphamidoamino linkages (N-sulphate groups). These may form covalent linkages between the amino groups of adjacent molecules.[7] The fact that all mucopolysaccharides have some anticoagulant activity makes one consider the possibility that these N-sulphate groups may be connected with the higher degree of activity of this particular muco-polysaccharide.

The mechanism of action of heparin is a confused issue, and there are nearly as many theories as there are investigators to promote them. It is generally agreed that heparin inhibits thrombin, thromboplastin, factor V and factor IX.[6,1,2,11] In addition, there is now evidence that it inhibits activation of factor IX by factor XI .[10]

The state of standardization of heparin also leaves a great deal to be desired.[12] Originally, heparin was standardized on the basis of the amount of International Standard Heparin (ox lung) neutralized by 1 mg. protamine sulphate; generally speaking, this worked out to 86 units, or so, of heparin neutralized by 1 mg. of protamine. However, in the early 1960's, economic reasons forced a change from bovine lung to intestinal mucosa as the primary source of commercial heparin. Since then, there has been a fair amount of discrepancy in measurement of anticoagulant activity; figures now run around 125 IU/mg. where it used to be 100 IU/mg. with ox lung. Several new methods have

been introduced, but each is rather specific to the substrate at hand and values can vary by 40% from substrate to substrate.[5,13]

Much effort has been spent in investigating the various properties of heparin with regards to its specific activity, but with little results. Molecular weight has been shown not to be significant.[1,8,9,10,15] Heparin has also been broken down to its carbohydrate and saccharide components for various preparations. Jaques did a very detailed study along these lines and could only conclude that metachromatic activity increases with increased sulphur content.[16] Barlow *et al.* felt that the prerequisite for heparin activity might be the fixed distance between charges which permitted the polysaccharide to combine with positive charged sites on one of the proteins involved in the coagulation sequence. If this distance were rigidly fixed, any alteration of the charge arrangement of heparin would also perturb its biological activity.[9]

Since for the last 10 years, heparin has been obtained from hog, sheep, and ox intestinal mucosa, the International Standard is no longer valid. There has been no real attempt to set a new standard, but several studies have been done to investigate bioactivity of the new heparins. Bangham and Woodward, under the auspices of the WHO, investigated the above problems. Species dependent activity difference was found, but it was not consistent; nor was it as great as tissue difference within species, *i.e.* between gut and lung.[15] This finding has been confirmed by several other investigators.[1,7]

Microelectrophoresis has been shown by Jaques, van Arkel and others to be an effective method of examining certain properties of heparin.[7,8,17-24] They have examined the rate of migration and optical density of the various bands versus the specific for bovine lung heparin and other non-commercial preparations. In addition, they have been able to detect the presence of heparin in tissue, blood, and urine. Using the techniques to be described below, they have been able to demonstrate amounts of heparin as small as 0.01 μg.

However, little has been done in terms of commercial preparation examinations with the present gut heparins. This paper will present initial studies on such heparin using an electrophoretic technique modified from Jaques, van Arkel, and Wieme.[17,20,24]

METHODS AND MATERIALS

General principles. 1 μl of commercial heparin (1000 units/ml.) is applied to a cut in the agarose gel adjacent to the arbitrary reference solution (also 1 μl of 1000 units/ml). Electrophoresis is conducted for 40 to 60 minutes at 90 v and 30 mAmps per slide. Temperature is maintained at 10°C. The finished gel is fixed with cetavlon (cetyl trimethyl ammonium bromide), heat dried for two hours, stained and decolorized.

Comments: The several modifications made in these runs will be discussed as they become pertinent.

Microelectrophoresis Chamber: The chamber was modified from Jaques and Wieme as pictured in figure 1.[20,24] It is made of lucite. The electrodes, represented by black wavy lines are of platinum. Silver was tried first, but silver acetate formed as the wire dissolved and silver precipitated out into the agar. The area marked "b" is filled with buffer (see below), "a" is the agarose block, and "c" is the cooling agent, pentane. The agarose is poured to fill both "b" and "a" and is then cut out of the "b" section for the buffer. A recessed metal pan is designed to fit the lucite chamber; it is filled with ice during the run and acts as a heat sink. The dimensions of the chamber are not critical; this one was built to accommodate 2.5 x 7.5 cm. microscope slides 4 across.

Slide Cutter. Rather than use a two step procedure as outlined by Jaques, Whatman number 3 stiff filter paper was used in 5 mm. strips as a combination cutter-dryer.

Micropipettes. Originally, 2 lambda pipettes were used as suggested by Jaques. However, this was found to overflow the wells created in the prior step. 1 lambda pipettes were found to be quite suitable. It is suggested that practice with a dye be done several times to get used to the procedure as the colorless heparin solution is easy to spill out of the wells.

Reference solution. There were 4 different brands available in varying concentrations. The optimal banding pattern was discovered by trial and error to be in the concentration of 1000 units/ml. It was possible to dilute the solution out to Jaques solution of 0.1 to 0.5 μg in 2 μl, but this did not stain sufficiently dark to be considered the concentration of choice for this experiment. The 4 brands were Upjohn (gut and lung), Elkins-Sinn, Lilly and Organon. Lilly was chosen at random to be the standard.

Hence, each slide prepared had a μl of Lilly heparin (1000 μ/ml) and a μl of test solution. In addition, Lilly protamine sulfate was also used.

Solutions and Slides. 22.1 gm. of sodium barbital buffer is dissolved in 200 ml. of hot 0.5 M NaOH. The cooled solution is brought to pH 8.6 and is 0.12 M. It is used for coating the slides and the agarose block in the chamber proper. A second batch is mixed as above and brought to two liters in distilled water. It, too, is brought to pH 8.6 and is the buffer solution for the chamber.

For pre-coating the slides, 0.1 gm. of electrophoresis grade agarose is dissolved in 5 ml. 1% glycerol, 25 ml. barbital buffer and 70 ml. distilled water at 65°C. Agarose is the non-sulphated (actually contains 0.3% sulphate) polysaccharide of agar (agaropectin is the other) which makes up 70% of agar. It is used because it does not stain metachromatically as does agar.[25] A pure solution can be prepared after Hjerten,[26] but this is a tedious and time-consuming process; the commercial preparations are suitable for this experiment. The slides to be pre-coated (to help the final gel to adhere) are cleaned and stored in acetate. A thin layer of the hot solution is poured over them and allowed to set until dry. In the interim, dissolve 0.9 g. agarose in the 0.12 M buffer (100 ml.) at a temperature of 65-70°C. This is a long process and requires constant agitation. When the agarose is completely dissolved, 30 ml. are quickly squirted with a large syringe into a 15 cm. diameter petri dish and allowed to set for 10 to 15 minutes. This must be done rapidly as agarose tends to congeal more quickly, and less smoothly if not very hot, than the agar in bacteriology labs with which one is more familiar. The slides are now placed on the prepared bed and 20 ml. are applied over their surface in a like manner. This volume will coat the slides to a depth of 1 mm. which leaves adequate room for a well but is not so thick as to lead to a prohibitively high current flow through the slides. Once the slides have been poured, they may be used 24 hours afterwards (the same applies to the agarose block in the chamber) and be kept for weeks as long as they are not refrigerated. This would increase the ionic strength of the medium due to evaporation of water. In addition, electro-osmotic back-flow would thus increase. The chamber should be checked for evaporation of buffer and pentane as well as liquid from the agarose block; the latter presents as large crystals. The buffer solution may be used for up to two weeks as long as the terminals are reversed after each run.

Microelectrophoresis Technique. The agar-layered slides are cut out by following the edge of the slide with a scalpel and lifting the slide from the underlying layer. Before making the cuts for the wells, it is imperative that the chamber be precooled to the proper temperature. Pentane fills the central chamber so that it will cover the slides when they are added. Petroleum ether is recommended by some, but was not used as it will soften the lucite and is highly flammable. Ice should be added to the pan overlaying the chamber and kept at a filled level throughout the run as it acts as a heat sink.

The cuts for the wells are to be made immediately prior to use. A 5 mm. wide strip of Whatman #3 MM paper is inserted to make a cut 2 cm. from the end of the slide, 5 mm. from the long edge, and perpendicular to the long axis. Let the paper sit 10 seconds or so to dry the slit, withdraw it, and pipette 1 lambda of the test solution into the well. In order to reduce confusion, be sure that the slit end of the slide is away from you, and the reference solution goes on the left. The runs were made with 2 sets of 2 identical slides; one pair was marked by cutting out a small square of agar between the wells and the near end of the slide. The slides are placed face down in the pentane with the agarose making contact at each end with the chamber blocks. The slits are placed at the negative electrode end of the chamber. Current is passed through the slides to promote about 30 mm. migration of the fast heparin band. In the current experiments, this was about 40 minutes at 80-100 V, and 120 mA for 4 standard microscope slides. These figures will vary from time to time and apparatus to apparatus.

Fixation and Staining. Immediately after the run, slides are placed in 0.1% cetavlon for one hour to fix the mucopolysaccharides. The cetavlon is made to a 1.0% solution and diluted 1:10 before use. If proteins are to be stained, it is necessary to fix the slide in a mixture of 1 volume formol in 4 volumes methanol for 15 minutes prior to fixing with cetavlon. After the slides have been fixed, they are lifted carefully out and put in another petri dish covered with filter paper moistened in 0.1% cetavlon and heat dried at 37°C for 2-4 hours. If protein staining is to be done, 300 mg. of Lissamine green are dissolved in 100 ml. 1% acetic acid. The slide sits in this for 15 minutes and is

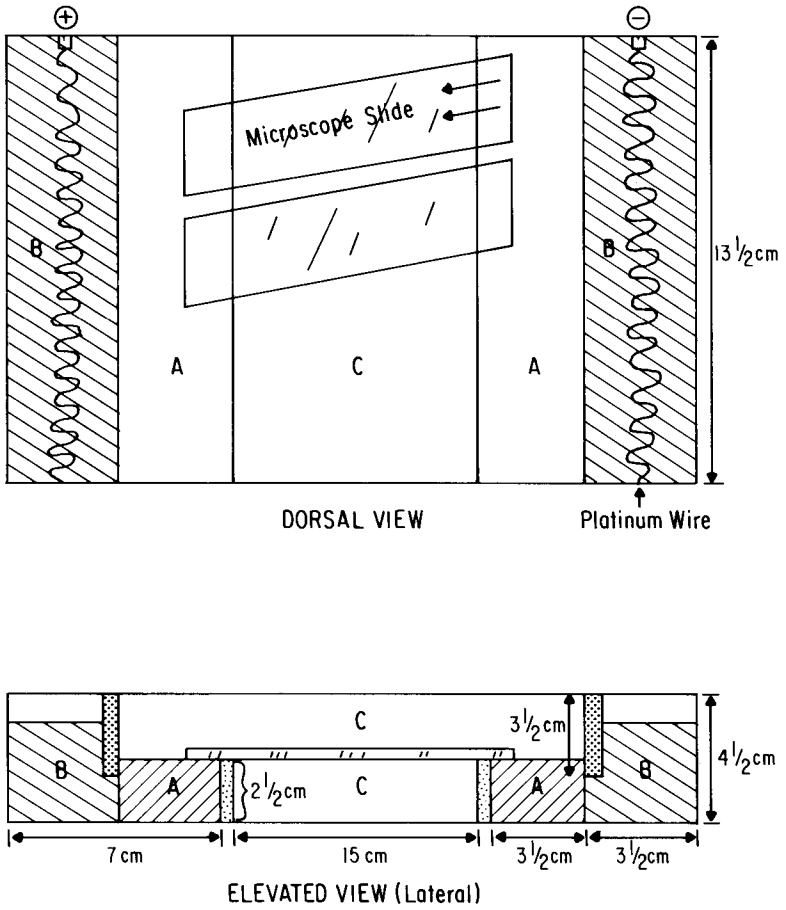

Figure 1
ELECTROPHORESIS CHAMBER

then decolorized for 30 to 45 minutes in 1% acetic acid. The toluidine blue solution consists of 40 mg. toluidine blue in 20 ml. distilled water and 80 ml. dry acetone. The references cite 10-15 minutes as sufficient, though it was found that 30-45 minutes was really necessary for an effective stain. The slide is then rinsed colorless (for the background) in 1% acetic acid for 2-8 hours prn. The slide is then placed on wet filter paper in a covered petrie dish to prevent evaporation and drying. Some authors suggest drying under a 250 W infrared lamp for 2 hours with a circulating fan. This may sufficiently dry the slide before staining to eliminate this step.

PLAN

It was intended to compare the 4 commercial preparations for impurities and migration rates. In addition, a few other heparins were run. During the course of the term, 8 runs were made with 2 slides each, and 12 runs of 4 slides each. Of these 20 runs, 12 yielded adequately reliable data to be discussed. Statistical conclusions are not possible with such small sample sizes.

RESULTS

Group 1: A comparison of 4 commercial gut heparins with equal concentrations of heparin (1000 units/ml). See table 1.

It should be noted that no protein peaks were picked up in any of the runs. In order to test the efficacy of the protein stain and fixing technique used, a few drops of

human blood were pipetted into two different slide wells for one run. The results are in table 2.

In addition to the above, two other trials were made. One compared pattern of two identical drugs at a given concentration and 20 times that concentration. After duplicate trials, the only difference was that the more concentrated band of heparin was darker, larger in all dimensions (edge by 1 mm.) but both had the same center.

The last trials involved comparison of standard Lilly gut heparin with two non-heparins: Upjohn lung heparin and Lilly protamine sulphate. See table 3.

Slides 46 and 47 had the standard heparin on one side and the other side consisted of 1 lambda protamine sulphate and 1 lambda heparin applied to the same well.

DISCUSSION

An established technique has been used, with few changes, to look at commercial preparations of heparin. The bulk of the trials were directed at comparing four commercial brands. No impurities or extra fractions were picked up at the level of sensitivity at which the trial was run. In two slides, a very fast fraction, moving about twice as fast as heparin fast component, was picked up. However, since this was on two different drugs, but not duplicated within that run on identical slides, this is probably artifact. The trials were able to duplicate the findings that gut heparin has a fast and slow band.[7, 20] Resolution was not as good as could be expected; this will be discussed below. The Lilly sample arbitrarily picked as the standard solution was assigned a migration value of 1.00 for the fast peak. The fast peaks of the other three commercial preparations ranged from values (relative to the standard) of 0.98 to 1.02. On a sample size this small it is difficult to obtain a valid statistical analysis. However, looking at the other figures involved, it seems likely that any discrepancy from the standard value are random errors. First, one may look at the slow values divided by the fast peak values for the standard. This ratio should be constant, but is varied for each set of trials. If the test substance is examined in the same fashion, the slow/fast ratio is identical to that for the standard sample in each set of trials; this value varies between 0.86 and 0.91. However, this does indicate a sameness of relative migrations of fast and slow peaks for all samples. Second, if the last set of figures in table 1 is examined, it can be seen that the discrepancy for standard runs on the same slide when the standard is run against itself are equal to those between the standard and test substances. Thus, it can be tentatively stated that within the limits of the procedure, no differences have been found between the four commercial brands of gut heparin tested. In addition, a fast and slow peak are seen with the latter having a migration distance of 0.86 to 0.91 that of the former.

It was felt that perhaps the technique used for protein staining was not being done correctly and that protein peaks in the solutions were being missed. However, the runs made with human blood and heparin showed that the lissamine green procedure does pick up the presence of proteins quite satisfactorily. Proteins fractions were not seen in the heparin samples on these runs either.

In the runs using gut and lung heparin, no more difference was seen between the two types than is found between different preparations of gut heparin. In view of the striking difference in potency of the heparin derived from these two tissue sources, this does not seem an adequate technique for helping to shed light on the reasons, structurally or otherwise, for this difference.

The last run consisted of two sets of slides. The first contained heparin in one well and protamine sulphate in the other. The second set contained heparin in one well and equal volumes of heparin and protamine sulphate in the other. The first set of slides showed a normal run for heparin, though technical difficulty with crystallization of the agarose in the chamber prevented adequate migration in the time allotted for resolution of the two bands. No protamine sulphate band was seen. The reason for this is not known. Its charge character, being very basic, could have caused reverse migration of the end of the slide. The second set of slides showed the same heparin peak. The other well had a white precipitate which could be seen even before fixation; upon staining, the heparin in that well took the MPS stain. It is assumed that the strongly basic protamine sulphate and strongly acidic heparin formed an insoluble complex which precipitated in the well, thus preventing migration. However, another weaker heparin band was seen on that side of the slide having travelled the same distance as

TABLE 1

Slide #	Lilly fast mm.	Lilly slow MPS mig.	Test fast mm.	Test slow MPS mig.	Lilly MPS Bands	Test MPS Bands
(Elkins-Sinn)						
1	22	—	23	—	1	1
2	23	—	23	—	1	1
7	31	27	31	27	2	2
8	30	26	30	26	2	2
11	18(38)	—	17(39)	—	1?	1?
12	17	—	19	—	1	1
17	23	—	23	—	1	1
18	23	—	23	—	1	1
34	25	22	26	23	2	2
35	26	23	27	24	2	2
ave.	28.0*	24.5	28.5*	25.0		
%Std.	1.00	0.86	1.02	0.88		
%fast		0.86		0.86		
(Upjohn)						
3	17	—	13	—	1	1
4	23	—	23	—	1	1
5	29	27	32	29	2	2
6	31	27	31	27	2	2
13	27	25	28	26	2	2
15	30	26	30	27	2	2
27	23	21	23	21	2	2
28	25	22	25	22(?)	2	2(?)
34	25	22	26	23	2	2
35	26	23	27	24	2	2
ave.	27.7*	25.0	28.5	25.5		
%Std.	1.00	0.90	1.01	0.92		
%fast		0.90		0.91		
(Organon)						
9	18(39)	—	18(39)	—	1(?)	1(?)
10	19	—	19	—	1	1
25	26	22	25	22	2	2
26	23	21	23	21	2	2
29	25	22	24	—	2	1
30	25	22	25	22	2	2
32	23	21	23	21	2	2
33	23	21	23	21	2	2
ave.	24.4*	21.6	24.0*	21.5		
%Std.	1.00	0.89	0.98	0.88		
%fast		0.89		0.89		
(Lilly)						
36	24	21	24	21	2	2
37**	24	21	23	20	2	2
37a**	23	20	24	21	2	2
39	24	21	23	20	2	2

*Fast averages calculated only for those runs where two fractions were clearly delineated.

TABLE 2

Slide content	Protein band	MPS band	Prot. mig.	MPS mig.
21. Heparin	0	2	—	21,17
Blood	2	1	9,5	18
22. Heparin	0	2	—	20,18
Blood	2	1	10,4	19

the lone heparin sample on the other side. Thus, not all of the heparin was complexed with the protamine.

The most perplexing question remains as to why certain runs did show two heparin fractions and certain runs did not. Looking back through the particulars of the runs the factors of time and distance seem to play the most important parts in determining the number of bands seen. With one exception, all runs of 1 band were less than 30 minutes. Of course, the shorter the distance travelled the poorer the resolution, but current and time seem more important than simple distance. This is shown when one looks at current vs. time. Better resolution is seen with lower current (30 mA/slide) and longer time than vice-versa even though distance travelled is the same.

Resolution must be improved, especially within the two peaks. For future work, two proposals should be considered for modifying the present system and two possible suggestions for other techniques.

First, a longer slide must be tried to gain better resolution. This could be accomplished by enlarging the chamber and laying two or three slides end to end in a support before coating them with agarose. The agarose would form a continuous path for the electrophoresis and give about a 10-fold increase in migration distance. Another suggestion is to cut apart the two fractions, reapply them to fresh slides, and run electrophoresis a second time on them. This would give a clear border between the two and allow inspection within each fraction.

If the slide agarose gel does not prove to be successful in separating these two fractions, disc gel electrophoresis after Davis has been shown to be a more sensitive technique.[27] Departing completely from electrophoresis, it must be considered that impurities or other fractions may not manifest themselves except by different molecular weight than the standard heparin molecule. Since this is entirely possible, a sephadex gel column merits consideration as the next technique employed.

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