JOURNAL OF CLINICAL MICROBIOLOGY, Jan. 1980, p. 30-34
0095-1137/80/01-0030/05$02.00/0

Vol. 11, No. 1

Antimicrobial Activity of Heparin

WALTER ROSETT[1] AND GLENN R. HODGES[1,2]*

Medical Service and Research Service, Veterans Administration Medical Center, Kansas City, Missouri 64128,[2] and the Department of Medicine, Univeristy of Kansas College of Health Sciences, Kansas City, Kansas 66103[1]

Single clinical isolates of eight species of microorganisms were incubated in solutions of heparin and brain heart infusion broth at various concentrations to determine the possible antibacterial effect of heparin. At heparin concentrations ranging from 12.5 to 400 U/ml, the effect on *Escherichia coli*, *Pseudomonas aeruginosa*, *Candida albicans*, *Staphylococcus aureus*, and *S. epidermidis* varied with brain heart infusion broth concentrations of 1.2 to 10% and inocula of 10^2 to 10^6 colony-forming units per ml; similar effects were not observed with *Klebsiella pneumoniae*, *Enterobacter aerogenes*, and *Citrobacter* spp. The minimal inhibitory concentrations of heparin for ten strains of each species were determined in 2.5% brain heart infusion broth with inocula of 10^4 colony-forming units per ml. All 10 isolates of *S. aureus* and all 10 of *S. epidermidis* were inhibited by heparin concentrations of 125 to 500 U/ml. Three *E. coli*, four *P. aeruginosa*, and nine *C. albicans* strains were inhibited by ≤500 U of heparin per ml. None of the *K. pneumoniae*, *E. aerogenes*, *Enterobacter cloacae*, and *Citrobacter* spp. was inhibited by heparin at ≤500 U/ml. Heparin inhibition of *S. aureus* in 2.5% brain heart infusion broth–500 U of heparin per ml could be quantitatively overcome by addition of magnesium, calcium, or magnesium and calcium. These data suggest that the growth of microorganisms from heparin-containing material may be suppressed.

In 1949, Stoker reported that heparin was bacteriostatic for *Staphylococcus aureus* in culture only when contaminated by "pus or trauma blood" (S. B. Stoker, J. Physiol. [London] **110:** 26P, 1949). The details or significance of this were not determined. A year later, Warren and Graham (6) reported that *S. aureus* and *Erwinia stewartii* were inhibited by heparin in a concentration of 150 U/ml when grown in protein-free medium. However, when brain heart infusion (BHI) broth was used as the culture medium, heparin in concentrations ranging from 15 to 7,500 U/ml did not inhibit the growth of these species. These investigators concluded that the bacteriostatic activity of heparin was neutralized by the protein in the medium. Christman and Doherty in 1956 studied this effect in different media and found several resistant strains (1). In spite of many detailed investigations of the other actions of heparin (3), further investigation of its bacteriostatic effect seem to have been neglected until our recent report (5), although Evans et al. (4) observed that heparin was more inhibitory to the growth of gram-positive cocci than was sodium polyanetholesulfonate in experimental blood cultures. We observed that the growth of single strains of *Escherichia coli*, *Pseudomonas aeruginosa*, *S. aureus*, and *Staphylococcus epidermidis* was inhibited in the presence of 1,000 U of heparin per ml in 2.0% brain heart infusion broth. In contrast, the growth of single strains of *Candida albicans*, *Citrobacter* spp., *Enterobacter aerogenes*, and *Klebsiella pneumoniae* was not inhibited under similar conditions. The present studies complement our initial observations.

MATERIALS AND METHODS

Recent isolates of microorganisms from the clinical laboratory were used for these studies. They were maintained in BHI broth (BBL Microbiology Systems, Cockeysville, Md.) at −70°C until needed. After removal from the freezer and inoculation into BHI broth, the organisms were incubated overnight at 37°C, washed three times in 0.9% saline, and diluted in normal saline or BHI broth for testing. Subcultures for quantitation were serially diluted 10-fold in 0.9% saline, plated on Trypticase soy agar (BBL), and counted after overnight incubation at 37°C.

Heparin sodium for injection prepared from porcine intestinal mucosa without preservative (Fellows Medical Manufacturing Co., Oak Park, Mich.), 1,000 U/ml in isotonic saline with pH adjusted to 7.0 with HCl, was used throughout the studies.

Standard microtiter techniques were used, with U-shaped-well microtiter plates and a 0.05-ml multiple hand diluter (Dynatech Laboratories Inc., Alexandria, Va.) (2). For testing two variables, plates were set up in a checkerboard pattern. Twofold dilutions of BHI broth (10 to 0.156%) in saline in the first seven rows

and twofold dilutions of heparin (400 to 0.31 U/ml) in the first 11 columns were used. The remaining row and column contained heparin and BHI broth controls, respectively. The minimal inhibitory concentration of heparin was defined as the lowest concentration of heparin for which there was no visible turbidity after overnight incubation at 37°C.

Cation determinations were made with an Automatic Clinical Analyzer (DuPont Co., Wilmington, Del.).

RESULTS

The relationships of BHI broth concentration, heparin concentration, and inoculum size were determined by inoculating 10^2 to 10^6 colony-forming units (CFU) of single isolates of *S. aureus*, *S. epidermidis*, *E. coli*, *P. aeruginosa*, and *C. albicans* per ml into different concentrations of BHI broth containing varying amounts of heparin. The results for *E. coli* are shown in Fig. 1A. At the lower inocula ($<10^5$ CFU/ml), the minimal inhibitory concentration increased with increasing concentrations of BHI broth. At the highest inocula, no inhibition was observed at heparin concentrations up to 400 U/ml. The results for *P. aeruginosa* were similar to those seen with *E. coli*. Data for *S. epidermidis* are shown in Fig. 1B. In contrast to the results for *E. coli* and *P. aeruginosa*, higher concentrations of BHI broth were required to overcome the inhibitory effect of heparin at all inocula used, and there was never visible growth in media containing less than 1.2% BHI broth even in the absence of heparin. The results for *S. aureus* were similar to those observed with *S. epidermidis*. *C. albicans* was inhibited by heparin at the low inocula ($<10^5$ CFU/ml) but was resistant to the effect of heparin when inocula of 10^5 and 10^6 were used (Fig. 1C). Visible growth was observed for all inocula (10^2 to 10^6 CFU/ml) of isolates of *K. pneumoniae*, *E. aerogenes*, and *Citrobacter* spp., independent of heparin and BHI broth concentrations.

Multiple recent isolates of nine different species of microorganisms were tested for their susceptibility to inhibition by heparin in 2.5% BHI broth at an inoculum of 10^3 to 10^4 CFU/ml. The gram-positive cocci (*S. aureus*, *S. epidermidis*, and *C. albicans*) were more susceptible to heparin than were the gram-negative rods (*Citrobacter* spp., *K. pneumoniae*, *E. aerogenes*, and *Enterobacter cloacae*) (Table 1). The gram-negative rods *E. coli* and *P. aeruginosa* showed an intermediate susceptibility to heparin.

The effect of heparin on organisms at various phases of growth was determined by inoculating 10^4 CFU of a heparin-susceptible *S. aureus* strain per ml from an overnight culture into BHI broth (5.0% final concentration). At 1, 2, 4, 8, and 24 h, samples were quantitated and subcultured into equal volumes of saline or heparin, incubated overnight at 37°C, and then quantitated. Subculture of *S. aureus* into medium containing BHI broth (final concentration, 2.5%) and heparin (final concentration, 500 U/ml) at any time during growth inhibited further growth (Fig. 2). In contrast, subculture into medium without heparin was not inhibitory.

The reversibility of heparin inhibition of *S. aureus* was tested by resuspending an overnight growth into 2.5% BHI broth with and without heparin, 500 U/ml. At intervals up to 8 h, samples were quantitated, diluted in saline to 10^3 to 10^4 CFU/ml, and subcultured in 2.5% BHI broth. Subsequently, samples of these subcultures were quantitated to determine 24-h growth curves. There were no differences in the subcultures from heparin-containing or heparin-free media.

Because heparin is highly anionic and known to bind calcium (3), the possible significance of this effect was determined. Solutions of calcium chloride and magnesium chloride were prepared in saline, and the pH was adjusted to 7.0 with sodium hydroxide. Growth of a heparin-susceptible *S. aureus* strain was determined in heparin (500 U/ml) with 2.5% BHI and the addition of serial twofold dilutions of these cations. Growth was quantitated after overnight incubation at 37°C and compared with growth of the following controls: 2.5% BHI broth, 2.5% BHI broth with heparin (500 U/ml), 10% BHI broth with heparin, and 25% BHI broth with heparin (Table 2). Maximal growth of *S. aureus* in 2.5% BHI broth was 4.19 $\log_{10}$. In contrast, it was 0.82 $\log_{10}$, 1.40 $\log_{10}$, and 4.00 $\log_{10}$ in media containing 2.5, 10, or 25% BHI broth, respectively, and heparin. The growth of *S. aureus* in 2.5% BHI broth with heparin was directly related to the concentration of calcium or magnesium added. However, the effect of heparin was more completely reversed by magnesium at a concentration similar to that found in 25% BHI broth than when calcium was added at a concentration similar to that found in 25% BHI broth. The growth of *S. aureus* in 2.5% BHI broth with heparin after the addition of various concentrations of calcium and magnesium was also dependent on the total concentration of cations present and was similar to the results observed when magnesium alone was added.

DISCUSSION

Heparin is a mucopolysaccharide composed of partially sulfated units of glucuronic acid and aminodeoxyglucose (3). The usual source is the mast cells in connective tissues of animal lungs and intestines. The properties of heparin are

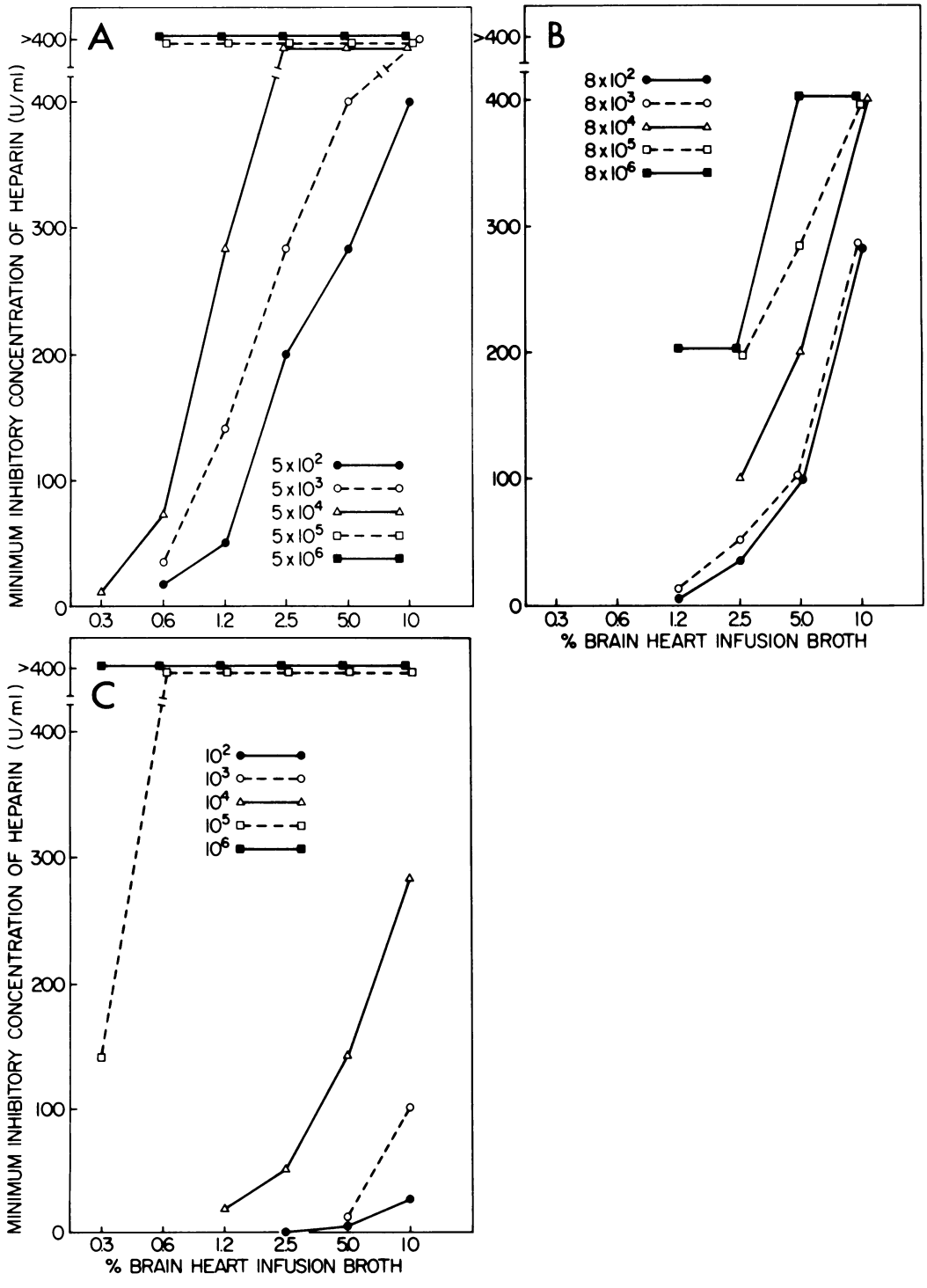

FIG. 1. *Relation of inoculum and concentration of BHI broth to geometric mean minimal inhibitory concentration of triplicate tests of heparin against E. coli (A), S. epidermidis (B), and C. albicans (C).*

TABLE 1. *Cumulative inhibition of nine different bacterial species by heparin*[a]

Species	No. of isolates tested	Cumulative no. of isolates inhibited at a heparin concn of:					
		31 U/ml	62 U/ml	125 U/ml	250 U/ml	500 U/ml	>500 U/ml
S. aureus	10	0	0	4	10	10	10
S. epidermidis	10	0	0	0	7	10	10
C. albicans	10	0	3	6	7	9	10
P. aeruginosa	10	0	0	0	1	4	10
E. coli	10	0	0	0	2	3	10
Citrobacter spp.	10	0	0	0	0	0	10
K. pneumoniae	10	0	0	0	0	0	10
E. cloacae	10	0	0	0	0	0	10
E. aerogenes	8	0	0	0	0	0	8

[a] Using 2.5% BHI broth, an inoculum of 10^3 to 10^4 CFU/ml, and overnight incubation at 37°C.

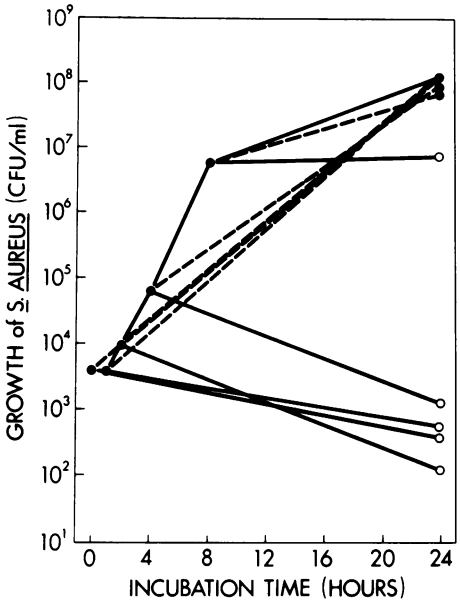

FIG. 2. *Relation of inhibition of S. aureus by heparin and phase of growth from which inoculum was obtained. Growth was measured in 5% BHI broth without heparin (●——●), after subculture into 2.5% BHI broth (●- - -●), and after subculture into 2.5% BHI broth with heparin, 500 U/ml (●——○).*

related to its highly anionic nature and molecular weight of 12,900. Primarily known for its anticoagulant activity, the result of activation of antithrombin III and interference with factor IXa activity, heparin's other activities include stimulation of the release of diacylglycerol lipase, binding and inhibition of multiple hormones and enzymes, and the binding of calcium. Further, heparin inhibits transcription of deoxyribonucleic acid, polynucleotide kinase activity, incorporation of labeled thymidine into the deoxyribonucleic acid of rat thymocytes, and *E. coli* deoxyribonucleic acid-dependent ribonucleic acid nucleotidyltransferase activity.

TABLE 2. *Effect of added calcium and magnesium*[a] *on growth of S. aureus in heparin*

Composition of medium							Growth[b]
BHI broth (%)	Heparin (U/ml)	Cations (mM × 10^2)					
		Added		Total			
		Ca^{2+}	Mg^{2+}	Ca^{2+}	Mg^{2+}	$Ca^{2+} + Mg^{2+}$	
2.5	0	0	0	1.88	4.93	6.81	4.19
2.5	500	0	0	1.88	4.93	6.81	0.82
10.0	500	0	0	7.50	19.8	27.3	1.40
25.0	500	0	0	18.8	49.3	68.1	4.00
2.5	500	1.48	0	3.36	4.93	8.29	0.76
2.5	500	2.95	0	4.83	4.93	9.76	0.18
2.5	500	5.90	0	7.78	4.93	12.7	0.67
2.5	500	11.8	0	13.7	4.93	18.6	1.03
2.5	500	23.6	0	25.5	4.93	30.4	3.34
2.5	500	0	5.8	1.88	10.7	12.6	1.39
2.5	500	0	11.6	1.88	16.5	18.4	1.40
2.5	500	0	23.2	1.88	28.1	30.0	2.36
2.5	500	0	46.4	1.88	51.3	53.2	3.75
2.5	500	0	92.8	1.88	97.7	99.6	3.26
2.5	500	1.48	5.8	3.36	10.7	14.1	1.25
2.5	500	2.95	11.6	4.83	16.5	21.3	1.74
2.5	500	5.90	23.2	7.78	28.1	35.9	2.52
2.5	500	11.8	46.4	13.7	51.3	65.0	3.86
2.5	500	23.6	92.8	25.5	97.7	123.2	3.78

[a] As chloride salts, pH adjusted to 7.0.

[b] Growth = $\log_{10}$ final CFU per milliliter − $\log_{10}$ initial CFU per milliliter.

Heparin inhibits the growth of microorganisms. Gram-positive organisms are relatively susceptible, and, to this effect, gram-negative organisms are relatively resistant. Heparin inhibition is dependent on the presence of minimal nutrients (BHI broth) and is overcome by addition of higher concentrations of nutrient (BHI broth). Alternatively, heparin inhibition can be quantitatively overcome by concentrations of divalent cations comparable to those contained in concentrations of BHI broth which allow equivalent growth. The concentration of magnesium or combination of calcium and magnesium required to overcome heparin inhibition was similar to that of 25% BHI broth. This suggests that heparin inhibition is a result of the reduction of effective divalent cations, magnesium, calcium, or both, from the growth media. These observations are consistent with and com-

plement the results of earlier studies (1, 4, 6).

The actual mechanism by which heparin acts has not been defined. Since it has been established that heparin binds calcium, it seems likely that heparin acts by chelation of cations essential for bacterial growth. Other possibilities consistent with the data are inhibition of cell wall permeability or transport of divalent cations, or intracellular inhibition of their utilization.

The growth of microorganisms is inhibited when material for culture is contaminated by heparin solution containing a preservative, usually benzyl alcohol (5). Our present studies emphasize the possibility that the growth of microorganisms may also be inhibited when material for culture is contaminated by heparin solution not containing a preservative. Caution should be used when interpreting the results of such cultures if they are negative.

ACKNOWLEDGMENT

We thank Sue Larkin, Renee Russell, and Virginia Hartley for assistance in preparing this manuscript.

LITERATURE CITED

1. **Christman, J. F., and D. G. Doherty.** 1956. The antimicrobial action of heparin. J. Bacteriol. **72**:433–435.
2. **Conrath, T. B.** 1972. Handbook of Microtiter® procedures. Dynatech Corp., Cambridge, Mass.
3. **Ehrlich, J., and S. S. Stivala.** 1973. Chemistry and pharmacology of heparin. J. Pharm. Sci. **62**:517–544.
4. **Evans, G. L., T. Cekoric, Jr., and R. L. Searcy.** 1968. Comparative effects of anticoagulants on bacterial growth in experimental blood cultures. Am. J. Med. Technol. **34**:103–112.
5. **Ferguson, R. L., W. Rosett, G. R. Hodges, and W. G. Barnes.** 1976. Complications with heparin-lock needles. A prospective evaluation. Ann. Intern. Med. **85**:583–586.
6. **Warren, J. R., and F. Graham.** 1950. The effect of heparin on the growth of bacteria and yeasts. J. Bacteriol. **60**:171–174.