THE EFFECT OF HEPARIN ON THE GROWTH OF BACTERIA AND YEASTS

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Comparatively recent investigations by Heilbrunn and Wilson (1949*a*) have directed attention to the fact that, in eggs of the worm *Chaetopterus*, there occurs a marked increase in the viscosity of the protoplasm immediately prior to the formation of the mitotic spindle that precedes cell division. In their studies, they have been able to prevent gelation of the protoplasm and, hence, cell division by placing the cells in an environment containing an anticoagulant, such as heparin or dicumarol. They have also reported (1949*b*) that the effect of heparin on cell division is not a killing one and is, to some extent, reversible.

Following publication of the papers mentioned above, an attempt was made, under the direction of Dr. L. E. Anderson, of the Botany Department of Duke University, to determine whether heparin would have a similar effect on the division of cells in the root tips of pea seedlings. Concurrently with this experiment we undertook to determine the effect of heparin on bacterial reproduction, despite a report by Stoker (1949) that, except in the presence of trauma blood or pus, heparin did not prevent bacterial reproduction. In our initial experiments we used *Micrococcus pyogenes* var. *aureus* and *Bacterium stewartii* as test organisms. The medium used in these tests was Difco brain heart infusion, and the heparin was in the form of the sodium salt, as prepared by the Connaught Medical Research Laboratories, Toronto, Canada. Reproduction of the bacteria was determined by the usual plating methods.

In these tests a stock solution of heparin, at a concentration of 10,000 ppm, was prepared and sterilized by passage through a Seitz filter. The final pH of this stock solution was 6.4. With this base solution, a dilution series was prepared in duplicate. The nutrient concentration was kept constant, but the concentration of heparin ranged from 10 ppm to 5,000 ppm. After 24 hours, although there were no apparent differences in growth, plate counts were made; and the number of colonies developing showed no significant differences at any of the tested concentrations of heparin.

At this time it was noted that, in the nutrient solutions used by Anderson in his study of the effect of heparin on pea seedling root development, there was a considerable bacterial growth in the controls that contained no heparin, whereas those solutions containing heparin and into which the pea seedling roots had grown contained no visible contamination.

Following the suggestion of Stoker that a cofactor was required if heparin was to be bacteriostatic, extracts of pea seedling roots were prepared and used in a new series of heparin dilutions. Again the results were uniformly negative in that the heparin had no effect on bacterial reproduction.

Inasmuch as the nutrient solutions used by Anderson were inorganic, and since it has been suggested by Anderson and Wilbur (1950) that heparin reacted with the basic proteins of the nuclei of rat liver cells, presumably by displacing nucleic acids of the nucleus and mitochondria, we prepared the following protein-free medium:

Sodium chloride, 5 g; dipotassium phosphate, 2.72 g; ammonium sulfate, 4.72 g; glucose, 2 g; L-asparagine, 2 g; and 1 ml of a solution containing 1 gram each of ferric chloride, magnesium chloride, and calcium chloride per liter.

This medium was prepared in such concentrations that the nutrient levels could be kept constant while the concentration of heparin was varied.

Duplicate tubes containing dilutions of 50, 100, 200, 1,000, and 30,000 ppm of heparin were inoculated with two loopfuls of a 24-hour-old culture of *B. stewartii*. After 48 hours' incubation at room temperature, no growth was apparent in heparin concentrations of greater strength than 100 ppm. Plate counts were made, and, though there was no significant difference between the number of colonies developing on plates prepared from the controls and those containing 50 ppm, there was a significant reduction in plates prepared from the 100 ppm concentrations. The number of colonies developing on plates prepared from concentrations higher than 100 ppm was practically zero.

A limited series of similar tests with *M. pyogenes* var. *aureus* indicated a similar response to heparin by this representative of the gram-positive group of bacteria.

To determine whether heparin had a bactericidal as well as a bacteriostatic effect, 100 ml of a 72-hour-old culture of *B. stewartii* was concentrated by centrifuging and resuspending until a final volume of 4 ml of cell suspension was obtained. To two 1-ml portions of this concentrated suspension there was added 0.1 gram of powdered heparin. After 24 hours' incubation at room temperature, plate counts were made. There were no significant differences in the number of colonies developing on plates prepared from the control tubes and those growing in plates prepared from the tubes containing heparin. Presumably, there was little increase in the number of cells in the control tubes because of the large number of bacteria concentrated in a small amount of medium.

Perhaps the most interesting result of this test was that, in the plate containing the organisms that had been in contact with heparin, approximately 10 per cent of the colonies were of an unusual, rough type. In none of our previous cultures had we observed this type of variation. After repeated transfers on an organic, heparin-free medium, the variant has failed to revert to the original type.

In determining the effects of heparin on yeast reproduction, no attempts were made to measure growth rates by plating because of the difficulty of separating the cell clumps sufficiently to assure the development of colonies from single cells. The method used was one of direct observation, in which suspensions of actively growing yeasts were placed on vaseline-ringed slides and covered with a cover glass. The slides were then examined, and the locations on the slides of individual cells or small groups of cells were determined. Several hours later

the increase in the number of the cells could be determined by re-examining the slides.

As in the experiments on bacterial growth, a protein-free medium was used. The heparin concentrations used were 5,000 ppm and 10,000 ppm. Two slides for each of these concentrations of heparin and two control slides containing no heparin were prepared. Nutrient concentration and pH were kept constant.

On each slide, 15 fields containing 2 to 5 cells were located by the use of microscopes with calibrated, movable stages. Eight hours later the same microscope fields were relocated, and the number of cells was again determined. These counts of 90 fields and approximately 360 cells at the beginning of the test period showed that heparin, in the concentrations used, had no effect on yeast reproduction.

The results of these experiments confirm those previously reported by Stoker (1949), that heparin is not bacteriostatic in an organic medium, but they demonstrate that it has a marked inhibiting effect on the growth of bacteria when the latter are cultivated in a protein-free medium. The explanation for these differences would seem to be that the heparin or some bacteriostatic component of heparin reacts with and is rendered inactive by the proteins of the medium. That this explanation is justified is evidenced by the above-mentioned report of Anderson and Wilbur (1950) that heparin unites with certain basic proteins.

The fact that heparin causes bacteriostasis in an organic medium if a cofactor, in the form of trauma blood or pus, is added to the medium indicates that heparin has more than one bacteriostatically active component—one component not requiring a cofactor, but possibly combining with basic proteins and thus being rendered inactive; and the other component requiring a cofactor, but being unaffected by the presence of extraneous proteins.

This conception of heparin as a complex of active fractions facilitates the explanation of some of the apparently contradictory effects ascribed to it, such as the increased viscosity and ultimate gelation of rat liver *brei* and of nuclear suspensions resulting from low concentrations of heparin and its effect in preventing increases in cellular viscosity.

The activity of heparin as a mutagenic agent is to be expected, since it has been demonstrated that it displaces desoxyribonucleic acid and ribonucleic acid from cells (Anderson and Wilbur, 1950).

The failure of heparin to inhibit budding by yeast cells may be due to the impermeability of the yeast cytoplasmic membrane, but it is at least equally probable that the ratio of cellular protein to heparin was so great that the amount of protein reacting with heparin in any one cell was insufficient to inhibit reproduction.

SUMMARY

The results of the experiments reported here indicate that heparin in a protein-free medium was bacteriostatic at concentrations of 100 ppm or greater. Heparin had no bactericidal effect at a concentration of 100,000 ppm; but, at this concentration, it did induce the development of a high percentage of mutants. Heparin failed to inhibit budding in yeasts at a concentration of 10,000 ppm.

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