THE EFFECT OF PENICILLIN ON EGGS OF THE SEA URCHIN, ARBACIA PUNCTULATA*

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It has been shown (1, 2) that the general cell inhibitors urethane, chloral hydrate, and sulfanilamide inhibit cell division of fertilized *Arbacia* eggs, and that this inhibition results from an inhibition of the specific fraction of the cell respiration upon which cell division depends. It was considered to be of interest to study the effects of the chemotherapeutic agent, penicillin, on these cells.

Materials and Methods

Eggs and sperm of the sea urchin, *Arbacia punctulata*, were obtained essentially as described by Just (3). Handling of the eggs and data, and the respiratory experiments were carried out as in previous reports (1, 2). The penicillin[1] solutions and the sea water used in the sea urchin egg experiments were adjusted to pH 7.5. This pH was selected because of the instability of penicillin at pH greater than 7.9 (4) (sea water has a pH of about 8.2), and because this increase in hydrogen ion concentration does not materially affect cell division of fertilized sea urchin eggs. The per cent of cells in a suspension was determined by high speed centrifugation of a 1 ml. sample in a hematocrit tube.

Effect on Cell Division

Penicillin (P) inhibits cell division of the fertilized sea urchin egg. This inhibition is directly related to P concentration (Fig. 1), the inhibition at 250 units (U)/ml. being slight, inhibition at 3000 U/ml. being complete (for reasons explained later, the dotted line in Fig. 1 represents approximately the corrected relation between cell division and penicillin concentration in that range of concentrations). In this respect, the action of P on fertilized sea urchin eggs is identical with that of numerous other cell inhibitors such as urethane, chloral hydrate, and sulfanilamide. One very important difference between the action of P and these other inhibitors on fertilized sea urchin eggs is its much greater tendency to kill the cell (vacuolated cytoplasm, pigment clumps). As the con-

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[1] The penicillin used was the therapeutic preparation manufactured by Chas. Pfizer & Co., Inc. and put up in 100,000 unit sterile lots.

centration of P is increased in the range employed, more and more cells are found dead for any particular period of contact with the drug. Similarly with any one concentration of P, the number of cells killed increases with time. This is in distinct contrast to the other inhibitors mentioned, *e.g.* concentrations of sulfanilamide which completely inhibit division will kill very few cells even after several hours contact.

Tests were made of the reversibility of this inhibition. At a concentration of P of 7500 U/ml. reversal could be obtained by washing the cells in sea water after one-half hour's contact with the drug, but not after 1 hour's contact. This is not in reference to those cells which are obviously dead (vacuolated cytoplasm, pigment clumps, cytolysis, etc.), but in reference to cells which as

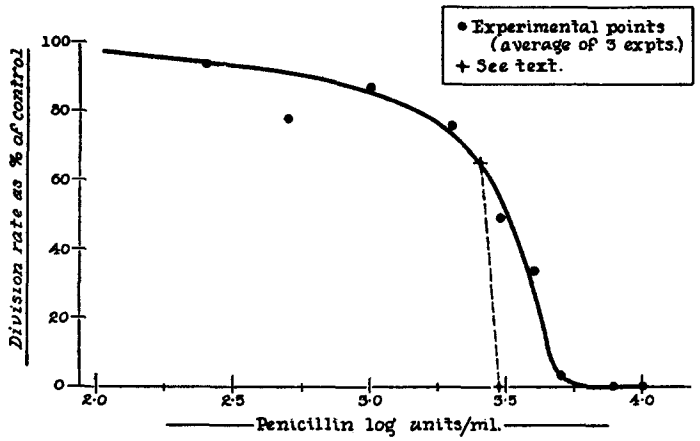

FIG. 1. Inhibition of cell division by penicillin. About 1 per cent cell suspension by volume.

far as appearance is concerned are normal. This also is in distinct contrast to the other inhibitors, *e.g.* an exposure longer than 70 minutes to a 0.34 M (3,029 mg. per cent) solution of urethane is required to cause irreversible damage, this concentration being almost three times that necessary to inhibit division completely (1). With 0.04 M (690 mg. per cent) sulfanilamide, which just completely inhibits division, complete reversal can be obtained even after 3 hours' contact (2).

It has been repeatedly stated (5–7) that P acts as a bacteriostatic on bacteria, assuming that it retards the rate of division. Actually, however, it is extremely difficult with cells which separate after division, to establish whether the decreased number of cells resulting from the action of inhibitors is due to a decreased rate of division of each cell or whether it is due to the killing or complete inhibition of some cells with others unaffected. With fertilized sea urchin. eggs, however, the divided cells are still an intact entity and, therefore, it is

easy to determine in this case at least which of the two possibilities obtains. As seen in Fig. 2 penicillin decreases the division rate of each cell.

Penicillin on Oxygen Consumption

Penicillin in concentration sufficiently high to inhibit cell division completely has no effect on the oxygen consumption of either unfertilized or fertilized eggs (11 determinations). Failure to inhibit oxygen consumption of bacteria has also been reported (8).

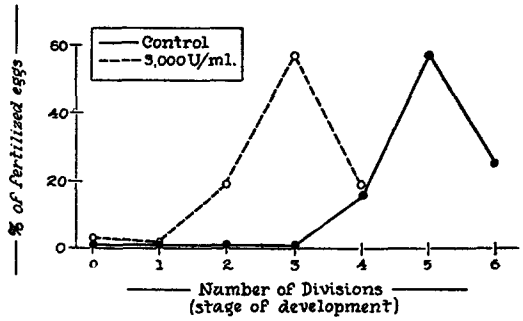

FIG. 2. Distribution of development of control fertilized eggs as compared with inhibited fertilized eggs. Average of three 3 hour experiments. 1 per cent egg suspensions.

Effect of Varying the Number of Cells per Unit Volume

As seen in Fig. 3 there is an inverse relation between percentage of cell suspension and inhibition of division. A concentration of penicillin of 3000 U/ml. was chosen to test this relationship because in this immediate region any particular change in P concentration will result in the largest change in inhibition by P; *i.e.*, it is the region most sensitive to changes in P concentration (see Fig. 1). As seen in Fig. 3 there is considerable spread of points taken from different experiments. This would be expected for the same reason that this concentration was selected—its great sensitiveness.

On the assumption that the P concentration did not change upon the addition of the cells, and assuming that P inhibition is a result of adsorption of P onto some cellular component such as an enzyme, this inverse relationship between percentage of cell suspension and inhibition of division should not have been found. This statement deserves clarification. Let us place a single cell in a solution of P. Presumably the P is free to diffuse through the cell wall and into the protoplasm and there may adsorb onto one or more cell components; if this is the mode of inhibition by P, then the amount adsorbed is directly related to the inhibition produced. The cell wall, however, prevents the outward diffusion of the cell components. According to the principle of

mass action, the amount of P adsorbed onto or combined with one molecule of any one component (whether inside of or outside of a cell) is directly related to the concentration of P in solution and inversely related to the concentration of the component. Adding one more or a thousand more cells to this suspension of one cell we already have in no way alters the concentration of the component in each of the individual cells; the cells are separate entities. As already stated, it is assumed that the concentration of this component in the solution of P surrounding the cells is zero. Thus, as long as the concentration of P is kept constant (and not decreased appreciably by significant quantities of P being adsorbed from solution by the component in question), the inhibition produced must be independent of the number of cells in the suspension. The inverse relation is apparent, however, for which there are at least two possible explanations:

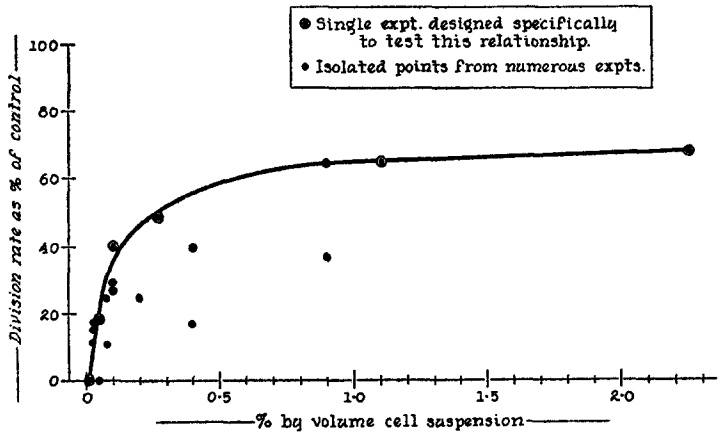

FIG. 3. Effect of percentage cell suspension on inhibition of division by 3000 U/ml. penicillin.*

1. The cells are capable of destroying P, thus decreasing its concentration. This is made unlikely by the nature of Fig. 3; the curve should not approach a maximum.

2. Some cellular component (egg jelly? protein? enzyme?) is adsorbing (or dissolving) sufficient P from solution to decrease P concentration and thus cause the effect seen.

The second suggestion can be checked as follows; there are two possibilities:

(*a*) The relation might depend upon solubility coefficients. This is not compatible, however, with Fig. 3. If P is more soluble in the egg than in the medium, then the maximum reached in the graph should not be. Conversely,

* It is unfortunate that only one complete experiment designed to test this relationship was made. The isolated points from other experiments, however, definitely follow the same trend.

if the P is less soluble in the egg, then practically no P (less than 1 per cent) would be adsorbed by a 1 per cent suspension.

(*b*) The P is being adsorbed by some cellular component. As already explained, increasing the percentage of cell suspension increases proportionately the amount of adsorbing surface (A) with which the P in solution comes into contact, but the concentration of A within each individual cell is not increased; this is a heterogeneous system.

When a cell suspension comes into contact with a solution of P, AP_a is formed until equilibrium is established according to Equations 1 and 2.

(1) $$A + aP \rightleftharpoons AP_a$$

where A = adsorbent
P = penicillin
a = number of molecules of P combining with one molecule of A.

According to the law of mass action,

(2) $$\frac{(A)(P)^a}{(AP_a)} = K.$$

The initial concentrations of A and P are thus decreased by amounts relatively equal to the amount of AP_a formed. If more cells are added, the A in these cells is all in the free, uncombined state. If

(3) $$\frac{(A)(P)^a}{(AP_a)} > K$$

pertains, more AP_a is formed until the system again reaches equilibrium. Every time this happens the concentration of P is decreased, and as (P) decreases the tendency for P to adsorb onto A decreases because its pressure (concentration) is decreasing. Finally, P reaches a concentration below which it will not combine significantly with A, and once this point is reached, increasing the percentage cell suspension *ad infinitum* leads to no further decrease in the concentration of P. This is the condition seen in Fig. 3.

It is to be noted from Fig. 3 that as percentage suspension approaches 0 (as (P) approaches 3000), cell division rate approaches 0, thus cell division is completely inhibited at 3000 P (marked as + in Fig. 1). At suspensions greater than 1 per cent the true P concentration at 3000 U/ml. is at the critical concentration of P below which no P is bound by the cells (see Fig. 3). The percentage of control division rate at this critical concentration is about 65 per cent. From Fig. 1 it is seen that the concentration of P causing this amount of inhibition in a 1 per cent suspension is at log 3.42 P (about 2650 U/ml.); (this is both true and apparent concentration of P at this point). Points on the curve of Fig. 1 below this concentration are therefore true points. Points above this concentration are false because the concentration of P is

less than the critical concentration. The true curve in this region must approximate the dotted line.

Experiments (four in number) were set up as follows to determine experimentally whether P was being bound by the cells. The plan was to place an egg suspension in a concentration of P of 3000 U/ml. and then remove these eggs and determine the P concentration by growth assay. A 1.75 per cent suspension of fertilized eggs was permitted contact with a P concentration of 3000 U/ml. (solution 1) for one-half hour, at the end of which time the cells were removed by centrifugation. Now a 0.075 per cent cell suspension of fertilized eggs was allowed contact with the P solution to be assayed (solution 1), the inhibition to be compared with that produced on a similar suspension by a concentration of P of 3000 U/ml. (solution 2). A typical result is seen in Table I. This indicates definitely that the cells are binding P.[2] One other frequent observation which was made agrees with this interpretation. Growth experiments were run in Syracuse dishes with slightly concave bottoms. In the more concave dishes the cells would tend to aggregate near the center.

TABLE I

	Cell division rate as per cent of control
Solution 1 (unknown)	38
Solution 2 (3000 U/ml.)	11

In such instances the cells near the center were invariably inhibited to a considerably less degree than those scattered towards the periphery. The latter ones were usually all dead.

An important question is whether the combination of P with the cell which apparently accounts for the phenomenon just discussed is the actual point of attack directly responsible for the inhibition of cell division produced by P. Because of the fact that this adsorption takes place in the range of concentration where the inhibition curve is steepest (Fig. 1) this might at first seem likely. Results shown in Fig. 3, however, are incompatible with this interpretation. At cell suspensions greater than 0.9 per cent little or no more P is removed from solution by the cells beyond a maximum amount when an initial P concentration of 3000 U/ml. is used. Yet, it must be that as more cells are added the amount of P bound per cell decreases proportionately, since it is difficult to conceive in a presumably homogeneous cell suspension that some

[2] A 1 per cent suspension of *Arbacia* eggs has about 47,500 eggs/ml. (9). The density of these eggs is about 1.09; each milliliter of suspension has about 11 mg. of eggs. The manufacturer's assay of the penicillin sodium used in these experiments was approximately 755 U/mg. A concentration of 3000 U/ml. is, therefore, equivalent to 3.97 mg./ml. The standard of penicillin sodium, however, is 1650 U/mg.

cells bind P while others do not. The amount bound per cell in a 2 per cent cell suspension would be approximately one-half that bound per cell in a 1 per cent cell suspension. If the amount bound per cell decreases then the inhibition should decrease. The curve in Fig. 3, however, is asymptotic to a level of division rate inhibition of approximately 25 per cent. Another point against such an interpretation is seen in Fig. 1. It was calculated that at concentrations of P below approximately 2650 U/ml. little or no binding of P occurred, yet it is seen that concentrations of P as low as 250 U/ml. do inhibit cell division.

Hence, it is concluded that P is bound by the fertilized egg at least at two loci. One of these at concentrations of about 2650 U/ml. P and above, binds P in sufficient quantities to reduce significantly the concentration of P in solution and thus to account for the observed inverse relation between inhibition and the number of cells per unit volume; this binding is dissociated from the inhibitory effect of P. Undoubtedly then, the inhibitory effect results from a binding or adsorption of P on some other cellular component(s) but in this case the amount of P bound is so small that the P concentration is practically unchanged.

Antagonism by Peptone

Because it had been demonstrated that P could be bound in significant quantities by some cellular component, and because peptone antagonizes sulfonamide action in bacteria, it was decided to see whether P activity would be changed by peptone. It has been reported that it has no effect on P activity on bacteria (8, 10, 11).

There were no consistent indications that 1 per cent peptone antagonized P action (concentration 3000 U/ml.) but occasionally it had a slight antagonistic action, evidenced principally by the fact that in its presence fewer cells were killed. The peptone itself inhibited division slightly. 1 per cent egg albumin and 5 per cent sea urchin egg cytolysate were both inhibitory *per se* and synergized with P inhibition.

DISCUSSION

It seems fairly certain that penicillin achieves its inhibitory action on fertilized sea urchin eggs by a different mechanism than the inhibitors sulfanilamide, urethane, and chloral hydrate, previously studied. Cell division undoubtedly results from a chain of events, any one of which if blocked will interfere with division. One of these is the respiratory processes which provide the energy for the process, and apparently sulfanilamide, urethane, and chloral hydrate attack at this point. While it is conceivable that penicillin might inhibit this fraction of the total respiration and cause simultaneously an equivalent increase in the other respiratory fraction which is unconcerned with

division so that the over-all oxygen consumption is unchanged, it is probably unlikely. Penicillin then must attack some other vital link in the process of division. From these experiments it is impossible to determine the mechanism of this inhibition. The fact that it has a great tendency to kill the cell is probably of some significance. Charcoal experiments (12) indicate that penicillin is a surface-active substance.

In view of the facts that penicillin is surface-active, it is bound in relatively large amounts by some component of the sea urchin egg, and peptone has a slight antagonistic action in these experiments, it is quite probable that penicillin antagonists will be found in bacterial experiments which act by binding penicillin. In fact, there is evidence that penicillin is bound by some component of whole blood. In the experiments on decreasing penicillin excretion in the dog by simultaneous administration of *p*-amino hippurate (13), in some instances the renal clearance was less than the glomerular filtration rate.

The question can be raised regarding the high concentrations of penicillin required for inhibition of cell division as compared to those required for a similar effect on bacteria. Actually penicillin is a powerful inhibitor for the sea urchin egg. Penicillin sodium of a potency equivalent to the standard of 1650 U/mg. in a concentration of 180 mg. per cent would inhibit completely division of the sea urchin egg, whereas a concentration of 690 mg. per cent of sulfanilamide is required. Thus penicillin is four times as powerful an inhibitor for the sea urchin egg on a weight basis as sulfanilamide. Whether or not penicillin inhibits the sea urchin egg division by the same mechanism as it inhibits bacterial division is a question which cannot be definitely answered. The difference in concentrations required for inhibition in the two cases cannot be used as a criterion. From the observations made there is nothing to suggest that the mechanisms in the two cases differ.

SUMMARY

1. Penicillin in the range of concentration from 250 U/ml. to approximately 2650 U/ml. inhibits the rate of cell division of the fertilized sea urchin egg from 0 to 100 per cent.

2. Penicillin in the same range of concentrations has no effect on the oxygen consumption of the unfertilized or the fertilized eggs.

3. Penicillin is bound by some component of the sea urchin egg in amounts sufficiently large to lower the initial concentration, this binding apparently not being related to the inhibitory action.

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