INTERACTION OF PHENOL WITH THE POLYSACCHARIDE OF BACTERIAL CELL WALL

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SUMMARY

Phenol forms an insoluble complex with the polysaccharide in the cell wall of M. lysodeicticus, and renders it resistant to the action of lysozyme. The formation of the stable complex in the wall accounts for the bactericidal action of phenol. The application of lysozyme in this study affords a unique means of measuring the rate and degree of reaction between phenol and the polysaccharide.

INTRODUCTION

The toxic action of phenol on the viability of bacteria has been investigated extensively ever since Joseph Lister discovered the antiseptic action of phenol in 1867. The mechanism of the antiseptic action still is unknown. The currently held view is that proposed by Reichen in 1909 (1), namely, that phenol penetrates the cell and poisons the protoplasm. As yet no information has been brought forward concerning the mode of action of phenol at the molecular level.

Strict reproducibility in tests with microorganisms is difficult to achieve because of difference between cultures, rates of growth and accumulation of metabolites. In the type of study to be presented, it was important to select a preparation that would be least liable to these effects, hence the choice of the lyophilized preparation of M. lysodeikticus. It is appreciated that lyophilization may influence the viability of the organism but this would not invalidate its use for the specific purpose of the study. This organism is a commonly used substrate for the assay of lysozyme, and it has been used in the study of the antiseptic action of phenol.

In the present study the use of phenol and lysozyme has afforded a novel means of investigating the disposition of the polysaccharide in the cell wall.

MATERIALS AND METHODS

The rate of disintegration of the M. lysodeikticus (lyophilized, Sigma Chemical Co.) by hen's egg-white lysozyme (Nutritional Biochemicals Corp.) was measured at 21° C by means of the turbidimetric method of Gorin, Wand and Papapavlou (2,3). A known quantity of the lyophilized specimen was suspended in dilute solutions of phenol of various concentrations. At intervals an aliquot (4.5 ml) of each was taken, and, to it a definite quantity of the lysozyme reagent (0.50 ml of 0.01% aqueous solution) added. The change in

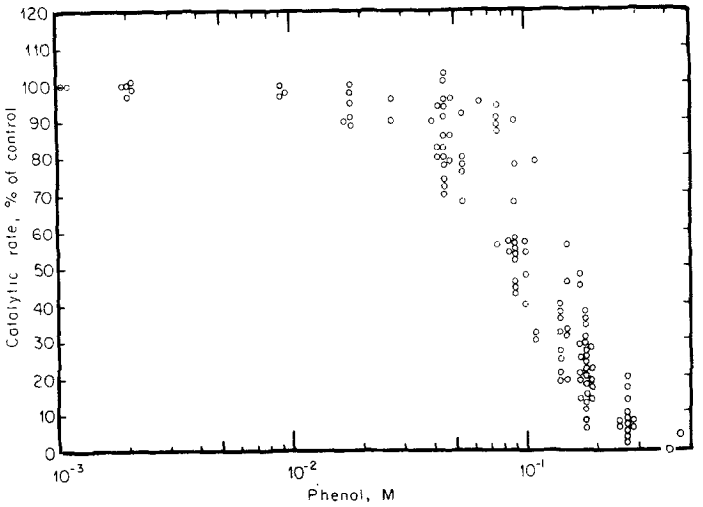

Fig. 1. Exposure of substrate, M. lysodeikticus, to phenol solution in 100 mM phosphate buffer at pH 6.0. Lysozyme then added (thus diluting the medium to 90 mM in phosphate) and the rate of the lysozyme action measured. Control assay made with 90 mM phosphate buffer at pH 6.0 without phenol.

the turbidity was followed at intervals with a Bausch and Lomb Spectrophotometer (Model Spectronic-20) and compared with that of a control without phenol.

RESULTS

The resistance of the bacterial cell wall to the action of lysozyme increased with the duration of exposure of the preparation to phenol as indicated in Fig. 1. This is shown by the downward spread of the plots especially within the sigmoidal portion of the curve. It is evident that phenol in 50-200 mM concentration, caused about 50% inhibition of the enzyme reaction. In phenol concentrations up to 50 mM the phenol has no appreciable inhibitory action on the enzyme itself. At higher than 50 mM, however, phenol does produce immediate inhibition of the enzyme. The extreme right-hand portion of the curve indicates this inhibition. The inhibition of the enzyme that occurs with higher than 50 mM phenol, however, is completely reversible upon dilution of the preparation with water.

The bacterial cell wall becomes progressively and irreversibly resistant to the action of lysozyme as the reactive polysaccharide sites become complexed with phenol (Figs. 2 and 3). Some of the types of site are more reactive than others, but for any given type, the rate of the reaction increases with the phenol concentration while the degree of phenolation varies with the duration of exposure to the reagent. As long as any site remains uncomplexed it is liable to attack and rupture by the enzyme. As the cell fragmentation (4) proceeds the turbidity of the preparation decreases. Since the diameter

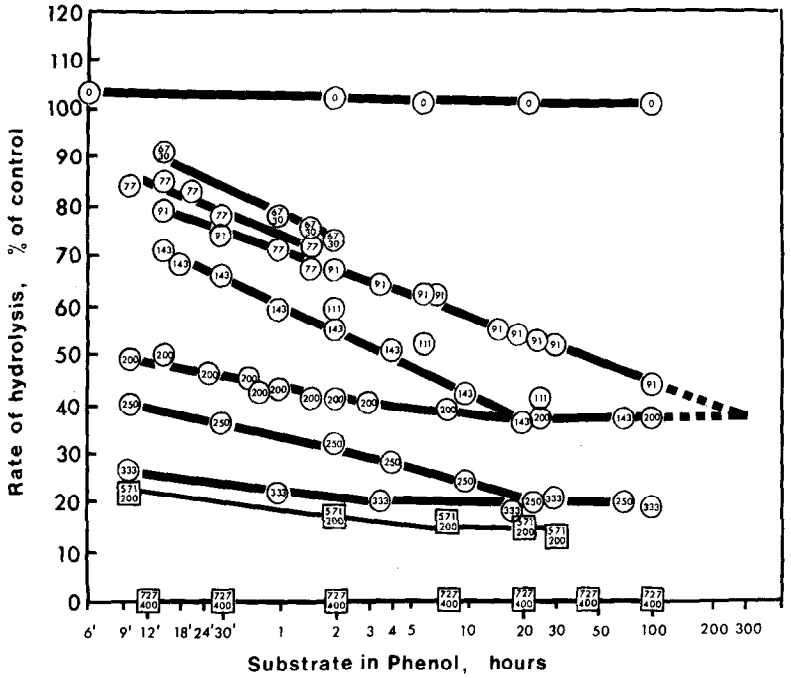

Fig. 2. Lysozyme susceptibility of the bacterial cell wall with two-step treatment with phenol in 50 mM phosphate buffer at pH 6.0. In the first step (10 minute treatment) the concentration (mM) of phenol is indicated by the numeral inside the circles. In the second step, buffer was added to reduce the concentration of phenol to less than 50 mM (unless as indicated in the squares) thus ensuring the full activity of the lysozyme. The upper values in the squares indicate the concentration of phenol in the initial treatment, and the lower values, that in the second treatment.

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of the individual cells approximated the wave length of light (4500 Å) used in the spectrophotometer the slightest degree of fragmentation renders the fragments incapable of scattering the light and contributing to the turbidity. The duration of exposure of the cells to phenol, required to produce complete saturation, represents a 'lag' or the 'threshold' period. Thus the lag varies inversely with the concentration of phenol. With 1 mM phenol the lag period was 20 hours; with 20 mM, 2 hours; with 50 mM, 0.1 hour, and with higher concentrations the lag was abolished. The relationship between the lag period and the phenol concentration is such that the product of the two at a given temperature is constant. For the preparation used, k =0.02 hour. mole.liter^{-1}.

The results shown in Fig. 4 indicate that the cell population in the preparation was comprised of at least four distinct categories or groups as indicated by the stepwise discontinuities in behavior towards phenol and lysozyme (shown in Fig. 4 by plot of susceptibility to lysozyme *vs*. log phenol concentration on abscissa). These groups may be designated A, B, C and D

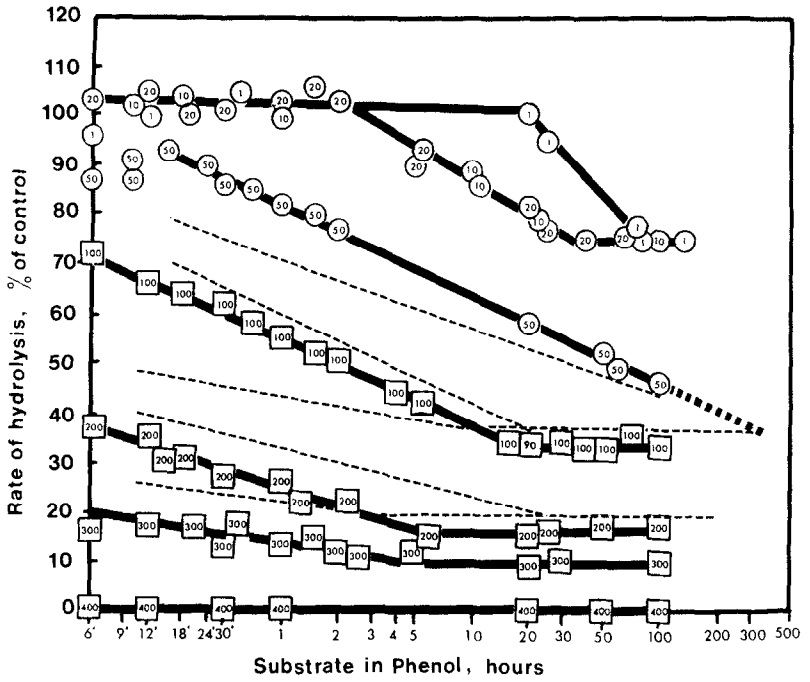

Fig. 3. Change in lysozyme susceptibility of bacterial cell wall treated with phenol in a single step where the phenol concentration remained constant (in 50 mM phosphate buffer, pH 6.0). The lines are broken from Fig. 2 and superimposed for comparison.

respectively. Group A was found to make up 25%; B, 38%; C, 17%; and D 20% of the population.

DISCUSSION

The results may best be explained as follows: The four groups of cells in the preparation (A, B, C and D) apparently differed sharply in the susceptibility to phenolation. The types of phenol-reactive polysaccharide site in the cell wall may be represented respectively as a, b, c and d, in descending order of reactivity with phenol. The group-A cells contain only type-a sites thus are the most readily phenolated. The group-B cells contain a and b sites; group-C, a, b and c; and group-D, a, b, c and d sites. The group-A reacts with phenol in concentrations up to 50 mM; group-B, with concentrations higher than 50 mM and up to 200 mM; group-C, with $>$200-400 mM; and group-D, with $>$400 mM phenol. With concentrations higher than 400 mM saturation occurs throughout the entire population almost immediately without lag. With all the groups and types of site the rate of phenolation increases with the phenol concentration, and, from the time the first cell or cells become saturated and lysozyme-resistant, the remaining cells, with like types of site, reach saturation at a linear rate.

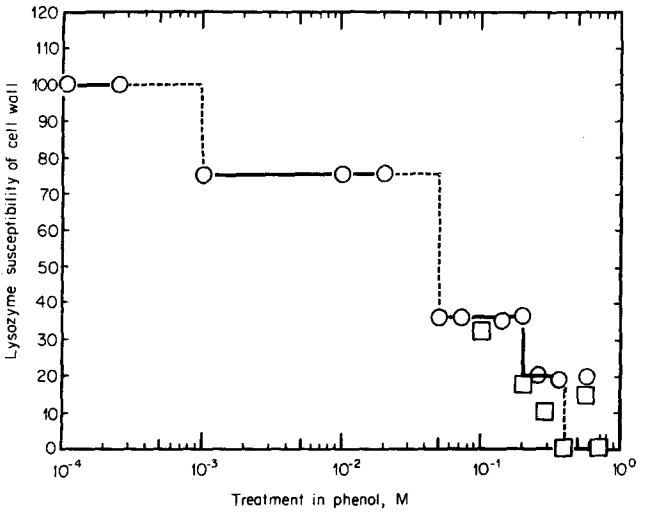

Fig. 4. Phase diagram of the formation of irreversible phenol-bacterial-cell-wall complexes. The open circles indicate assay values with less than 50 mM phenol solution, and the squares, those with greater than 50 mM.

The presence of the four groups may suggest that they may be artifacts since it is known that lyophilization may cause damage to some of the organisms, possibly to the older or less hardy individuals in the population (1). However, the group phenomenon may exist even in a normal population since individual bacteria are known to display periodic abrupt changes in growth pattern (5) and thus gives rise to certain differences which could account for group behavior.

The effectiveness of phenol at concentrations as low as 1 mM, along with the prolonged lag period with such concentrations, and the irreversibility of the phenol complex, indicate that the primary target of the phenol action is polysaccharide. The fact that the cell wall of M. lysodeikticus is completely digestible by lysozyme indicates that the wall polysaccharide is completely accessible to the enzyme. It is conceivable furthermore that the cell wall may have regional differences in sensitivity to phenol. It is reasonable to suppose that the region on the cell wall most sensitive to lysozyme and to phenol or other antiseptics may be the central 'ring' around the organism where cell division occurs. Thompson (6) has postulated that penicillin exerts its bacteriostatic and bactericidal action at this region. Alteration of the polysaccharide in this area doubtless could inhibit mitosis and thus be fatal to the cell.

As a rule the product of the concentration of the antiseptic and the lag period in build-up to the final threshold event, is constant for a given temperature. It is of interest that this rule has analogy also with refer-

ence to nerve excitation by means of an electrical stimulus: the threshold current multiplied by the lag interval is a constant, 1×10^{-3} ampere.second or 1×10^{-3} coulomb (7).

The kinetic pattern of phenolation of the cell wall and that of the antiseptic action of phenol, are similar in that they are characterized by a lag period and by linearity of the subsequent reaction. The rates of the two processes also are comparable thus indicating that both are due to phenolation of the wall material. The lag in the antiseptic action of phenol on M. lysodeikticus (8) is shorter than that with the same phenol concentration in the production of complete resistance to lysozyme.

The bacterial cell wall would appear to contain numerous pores, the size of which varies with the hydrogen-ion concentration and the ionic strength of the medium (9). Furthermore, the pores appear to be lined with anionic groups since the adsorption of phenol is impeded at hydrogen-ion concentrations below pH 4 and also in the presence of high concentrations of salts. The continuation of the phenolation of the lyophilized specimen, even after the dilution of the phenol of the medium with water, suggests that an excess of phenol is retained in the lyophilized bacterium. On the other hand, the antiseptic action of phenol on the living bacterium is arrested when the phenol is diluted to an ineffective level (1,8).

For antiseptic action, Withell (8) has estimated that 10^9 phenol molecules per bacterium are necessary. The phenol-acetylglucosamine ratio, with M. lysodeikticus, according to Withell's data, would be unity. At the saturation level the acetylglucosamine unit can hydrogen-bond up to 3 molecules of phenol. The packing of phenol in tight meshes of the polysaccharide chains (10) appears to form a stable complex since the phenol is unextractable. In this state the antiseptic action is slow and irreversible. In this process phenol presumably displaces hydrogen bonds in the wall (11). It is clear therefore that the phenol is not simply adsorbed on the cell surface, or occluded among the cell clumps.

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