# PHENOL AS THE PRESERVATIVE IN INSULIN INJECTIONS

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The purpose of this paper is to describe a laboratory investigation into the use of phenol as the added bacteriostatic in insulin injections, an investigation which arose partly because other accepted agents, such as chlorocresol and phenylmercuric nitrate, are considered unsuitable either for physiological or for chemical reasons, and partly because The United States Pharmacopœia XIV recommends phenol or cresol in the different injections in concentrations ranging between 0·1 and 0·28 per cent. Insulin preparations of American origin have been found to contain phenol as the bacteriostatic, whereas certain commercial insulins of non-American origin have contained either cresol or *o*-cresol. In view of these facts it was considered desirable to study in detail the merits of phenol as the preservative, especially as there is also published evidence[1,2,3] showing that the rate of loss of phenol through rubber closures on storage is smaller than with other bacteriostatics. The particular preparations to be considered are the B.P. injections of insulin, globin insulin and protamine zinc insulin. The first two are in acid solution, *p*H about 3, and the last is neutral, *pH* 6·9 to 7·3.

The British Pharmacopœia, 1953, requires all injections dispensed in multi-dose containers to contain "sufficient of a bacteriostatic to prevent the growth of micro-organisms," but it further states "A bacteriostatic need not be added if the medicament itself has sufficient bacteriostatic power." The classical example of a suitable bacteriostatic is 0·5 per cent. of phenol; but no indication is given how activity is to be measured, or how comparisons with other substances are to be made. Although described as bacteriostatic, a solution containing 0·5 per cent. of phenol possesses considerable bactericidal properties which vary according to the nature of other constituents of the solutions, its temperature and the type of infecting organism, and these must be taken into account in assessing the comparative antibacterial properties of any other solution.

It is well known that acid solutions themselves are inhibitory to bacterial growth and at sufficiently low *p*H values are lethal; the activities of bacteriostatics are also affected by *p*H value. It seems proper, therefore, in any comparative work to use 0·5 per cent. of phenol in neutral solution as the standard, and to attribute any advantage gained by acidity or from any constituent of the solution to the natural bacteriostatic properties of the preparation. Anti-fungal properties would need separate consideration since moulds are much less sensitive to acid conditions than are bacteria. We have evidence, for example, of the growth of *Cladosporium* in insulin solution in the presence of 0·17 per cent. of cresol after several months in cool storage.

### Bacteriostatic and Bactericidal Tests with Phenol

To test the influence of acidity on the bacteriostatic properties of phenol solution, serial dilutions were made in 0·5 per cent. peptone water and adjusted to the required *p*H values with 0·02M phosphate-citrate buffer. These solutions were inoculated with (*a*) mixed 24-hour cultures of *Staphylococcus aureus* (including the F.D.A. strain), (*b*) mixed 24-hour cultures of *Bacterium coli* (N.C.T.C. 86 and 5934), and (*c*) a spore suspension of *Bacillus subtilis*, and the concentrations just preventing growth in 5 to 7 days at 37° C. and at 22° C. recorded. Similar solutions were also inoculated with mixed mould cultures, including *Penicillium*, *Aspergillus* and *Cladosporium* species, and tested at 22° C. only. Numerous replicates were made at different times and Table I gives the maximum concentrations in the whole series which just prevented growth under the conditions quoted. At *p*H 4 or less, the acidity alone was sufficient to prevent bacterial growth. There were no significant differences between tests at 37° C. and 22° C., and addition of 2 per cent. of glycerol did not affect the results.

TABLE I

BACTERIOSTATIC CONCENTRATIONS OF PHENOL IN PEPTONE SOLUTION

| Test organism | Percentage concentration just preventing growth at | | | |
|---|---|---|---|---|
| | *p*H 3 | *p*H 4 | *p*H 5 | *p*H 7·5 |
| *Staph. aureus* | | | 0·1 | 0·2 |
| *Bact. coli* | | | 0·15 | 0·2 |
| *B. subtilis* (spores) | | | 0·2 | 0·25 |
| Mould spores | 0·25 | 0·25 | 0·2 | 0·2 |

The bactericidal properties of phenol solutions at different *p*H values were measured in comparison with 0·5 per cent. of phenol in neutral solution (*p*H 7·5). Serial dilutions in either saline solution or nutrient broth were adjusted to the required *p*H values with 0.02M phosphate-citrate buffer and inoculated heavily with mixed cultures of *Staph. aureus* and *Bact. coli* as used in the bacteriostatic tests to give initial counts of the order of $20 \times 10^6$ per ml. After short intervals, standard samples were removed and the surviving bacteria counted (by plating 0·1 ml. amounts in nutrient agar). The results from the several groups of tests made were somewhat variable, and typical ones are shown in the composite Table II. They show that phenol solutions have a substantial and fairly rapid bactericidal activity, and that a 0·2 per cent. solution at *p*H 3 is at least equivalent in activity to a standard 0·5 per cent. solution at *p*H 7·5. At *p*H 3·5, a 0·2 per cent. solution is less effective and is somewhat inferior to the standard.

Tests were also made to ascertain the survival of *B. subtilis* spores under the same conditions. Whereas they remained viable in 0·5 per cent. phenol at *p*H 7·5 for at least two weeks, there was an appreciable death rate with as little as 0·1 per cent. of phenol at *p*H 3 and 4 within 8 days.

### Tests with Insulin Injections

*Insulin.* Insulin solution is adjusted to *p*H approximately 3, and contains only crystalline insulin and added glycerol. The total solids

## TABLE II

BACTERICIDAL PROPERTIES OF PHENOL SOLUTIONS

| Test Organism | Tests in | after (hr.) | Surviving organisms in phenol solutions (per cent.) at *p*H 3 | | | | at *p*H 3·5 | | | | at *p*H 4 | | | at *p*H 7·5 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | 0·4 | 0·3 | 0·2 | 0·1 | 0·4 | 0·3 | 0·2 | 0·1 | 0·4 | 0·3 | 0·2 | 0·5 |
| *Bact. coli* | Saline solution | ½ | | | | | 0: + | 0: + | ++: +++ | +++ | + | +: +++ | +++ | +++ |
| | | 1 | | | | | 0 | 0: + | +: ++ | +++ | 0: + | +: +++ | : +++ | 0: + |
| | | 2 | | | | | 0 | 0 | 0: + | +: +++ | 0 | 0: + | ++: +++ | 0 |
| | Nutrient broth | ½ | + | +: ++ | ++ | +++ | 0: + | 0: + | +: +++ | +++ | | | | ++ + |
| | | 1 | 0 | 0 | 0 | +: ++ | 0 | 0 | + | +++ | | | | 0 |
| | | 2 | 0 | 0 | 0 | 0 | 0 | 0 | 0: + | +: ++ | | | | 0 |
| *Staph. aureus* | Saline solution | ½ | | | | | 0: ++ | +: ++ | ++ | +++ | ++ | ++: ++ | +++ | +++ |
| | | 1 | | | | | 0 | 0: ++ | 0: ++ | +++ | 0: + | + | ++: +++ | ++: +++ |
| | | 2 | | | | | 0 | 0 | 0: ++ | +: +++ | 0 | 0 | 0: ++ | 0: + |
| | Nutrient broth | ½ | + | + | ++ | ++ | 0: + | 0: + | 0: ++ | +++ | • | | | +: +++ |
| | | 1 | 0 | 0: + | 0 | +: ++ | 0 | 0 | 0: + | +: ++ | | | | 0: ++ |
| | | 2 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | +: ++ | | | | 0 |

Initial inoculum equivalent to approx. $2 \times 10^6/0{\cdot}1$ ml.

O = no survivors in 0·1 ml.

\+ = few survivors only, less than 100.

++ and +++ = increasingly large numbers of survivors.

Two values indicate responses in different tests.

and organic matter present is very small, hence the bactericidal and bacteriostatic levels of phenol under these conditions will not be different from those found in the foregoing tests with phenol in broth or in buffer solution.

*Globin Zinc Insulin.* This injection has a *p*H value of about 3. To confirm that phenol exerts the same activity in it as in other solutions at similar *p*H values, a standard preparation of globin zinc insulin was made at *p*H 3·15 without any added bacteriostatic. To portions of this were added different amounts of phenol and these were inoculated with mixed 24-hour cultures of *Staph. aureus*, *Bact. coli*, *Pseudomonas pyocyanea* and *Proteus vulgaris*, and the survivors counted after short intervals. Results are given in Table III. They confirm clearly the earlier findings at low *p*H values and show that 0·2 per cent. of phenol is satisfactory for globin zinc insulin.

TABLE III

BACTERICIDAL PROPERTIES OF PHENOL IN GLOBIN ZINC INSULIN

| Test organism | Period of Test (hr.) | Surviving organisms from Globin insulin + phenol (per cent.) 0·3 | 0·25 | 0·2 | 0·15 | Nil | 0·5 per cent. of phenol in saline solution |
|---|---|---|---|---|---|---|---|
| *Staph. aureus* | 1 | 0 | ++ | +++ | +++ | +++ | ++ |
| | 2 | 0 | 0 | + | + | +++ | 0 |
| | 6 | 0 | 0 | 0 | 0 | +++ | 0 |
| | 24 | 0 | 0 | 0 | 0 | 0 | 0 |
| *Bact. coli* .. | 1 | 0 | 0 | +++ | +++ | +++ | + |
| | 2 | 0 | 0 | 0 | 0 | +++ | 0 |
| | 6 | 0 | 0 | 0 | 0 | +++ | 0 |
| | 24 | 0 | 0 | 0 | 0 | 0 | 0 |
| *Ps. pyocyanea*.. | 1 | 0 | 0 | 0 | ++ | +++ | 0 |
| | 2 | 0 | 0 | 0 | + | ++ | 0 |
| | 6 | 0 | 0 | 0 | 0 | + | 0 |
| | 24 | 0 | 0 | 0 | 0 | 0 | 0 |
| *Proteus vulgaris* | 1 | + | + | ++ | +++ | +++ | + |
| | 2 | 0 | 0 | + | ++ | +++ | 0 |
| | 6 | 0 | 0 | 0 | + | +++ | 0 |
| | 24 | 0 | 0 | 0 | 0 | +++ | 0 |

Initial inocula equivalent to 3–4·5 × $10^6$ orgs./0·1 ml.
O = no surviving organisms in 0·1 ml.
+ = few survivors only, less than 100.
++ and +++ = increasingly large numbers of survivors.

*Protamine Zinc Insulin.* This injection is prepared at *p*H about 7. It is evident, therefore, that if a concentration of less than 0·5 per cent. of phenol is to be of any value, one or more of the constituents must contribute to the antibacterial properties of the final preparation. There is already published evidence[4,5] that protamine (salmine) is itself antimicrobial. To check this, a series of tests with phenol and protamine were made, using protamine at a concentration of 0·5 mg./ml., the normal amount used in the injection. Graded amounts of phenol were added to the protamine solution in 0·5 per cent. peptone water buffered to *p*H 7, and these were inoculated with 24-hour cultures of the test organism as before. For comparison, a similar series of dilutions of phenol without protamine were included. Surviving organisms were counted at intervals up to 24-hours with results as recorded in Table IV. The

TABLE IV

BACTERICIDAL PROPERTIES OF PHENOL AND PROTAMINE IN PEPTONE SOLUTIONS (*p*H 7)

| Test Organism | Period of test (hr.) | Surviving organisms from | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | Protamine (0·5 mg./ml.) with phenol (per cent.) | | | | | | Phenol alone (per cent.) | | | | | | |
| | | 0·4 | 0·3 | 0·25 | 0·2 | 0·15 | 0 | 0·5 | 0·4 | 0·3 | 0·25 | 0·2 | 0·15 | 0 |
| *Staph. aureus* | 1 | ++ | +++ | +++ | ++ | ++ | +++ | ++ | ++ | + | + | +++ | +++ | +++ |
| | 2 | ++ | ++ | ++ | ++ | ++ | +++ | | ++ | ++++ | ++++ | ++++ | ++++ | ++++ |
| | 6 | 0 | 0 | 0 | 0 | 0 | ++0 | 0 | ++0 | ++++ | ++ | +++ | +++ | +++ |
| | 24 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | +++ | G | G | G | G |
| *Bact. coli* | 1 | 0 | 0 | + | ++ | ++ | +++ | ++ | ++ | + | + | + | +++ | +++ |
| | 2 | 0 | 0 | 0 | ++ | 0 | ++0 | | ++ | ++++ | ++++ | ++++ | ++++ | ++++ |
| | 6 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | +++ | ++ | ++ | +++ | +++ |
| | 24 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | ++++ | ++++ | +++ | G | G |
| *Ps. pyocyanea* | 1 | 0 | 0 | 0 | + | + | ++ | ++ | + | + | + | + | | |
| | 2 | 0 | 0 | 0 | 0 | 0 | ++ | + | ++ | ++++ | ++++ | ++++ | | |
| | 6 | 0 | 0 | 0 | 0 | 0 | 0 | + | ++ | ++++ | ++++ | ++++ | | |
| | 24 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | ++++ | ++++ | ++++ | | |

Initial inocula—*Staph. aureus* $20 \times 10^8$/0·1 ml.
*Bact. coli* $30 \times 10^8$/0·1 ml.
*Ps. pyocyanea* $100 \times 10^8$/0·1 ml.

O = no survivors in 0·1 ml.
+ = few survivors only, less than 100.
+ + and + + + + = increasingly larger numbers of survivors.
G = visible growth.

strong lethal action of the protamine solution is clearly shown, which action is enhanced by adding even 0·15 per cent. of phenol. Two tests with *Ps. pyocyanea* showed the protamine solution to be still lethal at half the concentration normally used, but at a lower level; it was without action against bacterial spores.

To confirm these observations, protamine zinc insulin preparations, made to the normal formula but containing either phenol or cresol as preservative, were examined. They were inoculated with the same test organisms, and the survivors counted. Table V gives the composite results of 2, or in some cases 3, such tests, and shows complete killing of the test organisms in 6 hours and often in 2 hours. The killing rate with 0·2 per cent. of phenol was almost equal to that with 0·2 per cent. of cresol; this rate can be considered satisfactory for the purpose required.

TABLE V

BACTERICIDAL PROPERTIES OF PROTAMINE ZINC INSULIN

| Test organism | Period of test (hr.) | Surviving organisms from protamine zinc insulin with | | | | |
|---|---|---|---|---|---|---|
| | | Phenol per cent. | | | Cresol 0·2 per cent. | Nil (control) |
| | | 0·3 | 0·25 | 0·2 | | |
| *Staph. aureus* .. | 1 | +:++ | +:+++ | ++:++ | +:++ | +++ |
| | 2 | + | + | +:+ | + | +++ |
| | 6 | 0 | 0 | 0 | 0 | +++ |
| | 24 | 0 | 0 | 0 | 0 | ++:+++ |
| *Bact. coli* .. | 1 | + | + | +:++ | + | +++ |
| | 2 | 0 | 0 | 0 | 0:+ | ++ |
| | 6 | 0 | 0 | 0 | 0 | ++ |
| | 24 | 0 | 0 | 0 | 0 | ++ |
| *Ps. pyocyanea* .. | 1 | | + | + | 0:+ | +++ |
| | 2 | | 0 | 0 | 0 | ++ |
| | 6 | | 0 | 0 | 0 | ++ |
| | 24 | | 0 | 0 | 0 | ++ |
| *Proteus vulgaris* | 1 | | +:++ | ++:+++ | 0 | +++ |
| | 2 | | 0:+ | + | 0 | +++ |
| | 6 | | 0 | 0 | 0 | +++ |
| | 24 | | 0 | 0 | 0 | +++ |

Initial inocula—*Staph. aureus* 0·9–3 × $10^6$/0·1 ml.
*Bact. coli* 2–4 × $10^6$/0·1 ml.
*Ps. pyocyanea* 5 × $10^6$/0·1 ml.
*Proteus vulgaris* 1·6 × $10^6$/0·1 ml.

O = no survivors in 0·1 ml.
\+ = few survivors only, less than 100.
++ and +++ = increasingly larger numbers of survivors.

## SUMMARY

1. Acid conditions enhance the antibacterial properties of phenol such that a 0·2 per cent. solution at *p*H 3 behaves similarly to a 0·5 per cent. solution at neutral *p*H values; it does not behave in this way to moulds.

2. 0·2 per cent. of phenol is, therefore, a suitable bacteriostatic for insulin and globin insulin injections at *p*H 3.

3. Because of the antibacterial properties of protamine, 0·2 per cent. of phenol is also a suitable bacteriostatic to add to protamine zinc insulin injection.

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