SELECTIVE ACTION OF THALLIUM ACETATE AND CRYSTAL VIOLET FOR
PLEUROPNEUMONIALIKE ORGANISMS OF HUMAN ORIGIN

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The prevalence of pleuropneumonialike organisms (PPLO) has been summarized by Morton et al. (1952). Their incidence and significance in infections of the genitourinary tract have been discussed by Ruiter and Wentholt (1953a) and Edward (1952). Subsequent reports of the finding of these organisms in infections of man (Slingerland and Morgan, 1952; Ruiter and Wentholt, 1953b) and of poultry (Markham and Wong, 1952; Grumbles et al., 1953) increase the importance of searching for these organisms in suspected infectious diseases. The culture medium for the isolation and propagation of pleuropneumonialike organisms has to be highly nutritive, and an incubation period of from three to five days is required. Under these conditions it is often difficult and sometimes impossible to exclude bacteria. It is highly desirable, therefore, to be able to inhibit a wide variety of bacterial species but, at the same time, permit growth of pleuropneumonialike organisms. That the metabolism of pleuropneumonialike organisms isolated from man may be different from that of bacteria in general is suggested by the work of Smith et al. (1950). These authors observed that pleuropneumonialike organisms behaved neither like gram positive nor gram negative organisms. They were not inhibited by 1:50,000 concentration of potassium tellurite which suppresses growth of most gram negative microorganisms nor by crystal violet in a concentration of 1:100,000 which suppresses growth of most gram positive microorganisms. The crystal violet can be added to the culture medium before sterilization, but the potassium tellurite does not withstand autoclaving and has to be added aseptically to the sterile medium.

In the work of Smith et al. (1950) ascitic fluid was added to the medium in a final concentration of 25 per cent for the necessary enriching substance. It was found later by Smith and Morton (1951) that a particular fraction from serum in a final concentration of one per cent served satisfactorily as the enriching substance. However, it was discovered that crystal violet usually inhibited growth of pleuropneumonialike organisms in medium enriched with one per cent of the serum fraction whereas the strains grew in the presence of crystal violet if the medium was enriched with ascitic fluid.

It was decided to investigate this discrepancy in selective inhibitory action in the presence of 25 per cent ascitic fluid and one per cent fraction A, and also to investigate the desirability of using thallium acetate which Edward (1947) employed along with penicillin as a selective inhibitor with the L5 strain and a strain from mouse catarrh.

MATERIALS AND METHODS

There was a difference in species protein in the enriching substances, human ascitic fluid and bovine serum fraction. Serum fraction A was prepared therefore from human, bovine, and horse sera by the method of Smith and Morton (1951).

Hemoglobin (Difco), known to reduce the toxicity of crystal violet for bacteria, was added to the pleuropneumonialike organism-enrichment broth to a concentration of one or 1.5 per cent.

A difference in the protein content of the serum fraction and ascitic fluid might have a bearing on the toxicity of crystal violet for pleuropneumonialike organisms. The serum fraction and ascitic fluid were assayed for their total protein content. The protein content of the culture medium was varied by adding different amounts of serum fractions or by the use of a
sterile solution of egg albumin (Merck, soluble). Egg albumin, by itself, does not support good growth of pleuropneumonia-like organisms; therefore, the standard amount of serum fraction also was added with the egg albumin.

A 10 per cent aqueous solution of thallium acetate was prepared, sterilized in the autoclave at 121 C for 20 minutes, and added to the sterile basal media to give the desired concentrations. The thallium acetate also may be added directly to the basal media before autoclaving. The basal medium was enriched with either 25 per cent ascitic fluid or one per cent serum fraction.

The solid medium was "bacto-PPLO agar", pH 7.8, enriched with one per cent bacto-pleuropneumonia-like organism serum fraction. This has been used for maintaining the strains of pleuropneumonia-like organisms in the laboratory for the past few years. The liquid medium was "bacto-PPLO broth", pH 7.8. This has the same composition as the "PPLO agar" medium except that the agar is omitted.

Six strains of pleuropneumonia-like organisms from human male and female genitourinary tracts were used. Twelve species of bacteria were included among the test organisms.

The inoculum of pleuropneumonia-like organisms was prepared by grinding an agar block about one cm square of a 3 day old culture (the area selected contained abundant growth) in 5 ml of broth. After the agar particles sedimented, 0.05 ml of the supernatant was inoculated into the liquid medium, or a standard loopful was streaked across one-sixth of an agar plate. The broth and agar media contained various concentrations of thallium acetate. The agar plate cultures were incubated at 37 C for 5 days with readings being made on the third and fifth days with the aid of the microscope employing a magnification of 100 X. The broth cultures were incubated at 37 C for 3 days. A loopful of the cultures was streaked on one-sixth of a PPLO-agar plate and incubated for 3 days in order to detect growth in the tubes of broth.

The bacterial inoculum was prepared by diluting an 18 hour old broth culture 1:1,000 with 0.9 per cent saline and then inoculating 0.05 ml of this dilution into 5 ml of the broth medium or streaking a loopful on one-sixth of an agar plate. The inoculated broth and agar cultures were incubated at 37 C for 5 days. Readings were made each day to determine turbidity in the broth or colony formation on the solid medium. These plates were examined also with the microscope employing a magnification of 100 X in order to detect possible production of "L" forms.

RESULTS

The serum fractions prepared from human, bovine, and horse sera were equally ineffective in detoxifying crystal violet for pleuropneumonia-like organisms. This ruled out the species of protein as being the important factor.

Hemoglobin in concentrations of one and 1.5 per cent in pleuropneumonia-like organism-enrichment broth reduced the toxicity of the crystal violet to some extent but not as completely as desired for the pleuropneumonia-like organisms.

It was estimated that the culture medium was enriched with ascitic fluid in a final concentration of 25 per cent, 3.1 mg of protein per ml of medium were provided. When 3 per cent serum fraction A was added to the medium in a final concentration of one per cent, only one mg of protein per ml of medium was provided. When 3 per cent serum fraction A was added to provide about the same amount of total protein in the medium, the toxicity of crystal violet was partially overcome. If the amount of the serum fraction A was increased to 6 or 10 per cent, the toxicity was decreased further. If one per cent serum fraction A and sufficient egg albumin were added to make the total protein content of the medium comparable to that provided by 25 per cent ascitic fluid, the toxicity of crystal violet was practically overcome. An enrichment of one per cent serum fraction A and sufficient egg albumin to supply the same amount of total protein provided by 10 per cent serum fraction were the most effective in overcoming the toxicity of crystal violet for pleuropneumonia-like organisms.

The protein content of the liquid medium was increased to 10 mg per ml either by the addition of egg albumin or by increasing the content of serum fraction A. It was decreased to as little as 0.1 mg per ml by employing the more purified serum fraction B. Throughout this 100-fold range in protein content of the medium, the pleuropneumonia-like organisms grew in the
Escherichia coli, three discrete Escherichia coli, inhibited except that the colonies of S. swarming growth. No exceedingly were observed on plates inoculated with bacteria. Salmonella schottmuelleri, Micrococcus pyogenes, Corynebacterium xerose, Bacillus albus, and Staphylococcus albus were not inhibited by thallium acetate, 1:1,000 concentration, but the strains of Corynebacterium xerose and Micrococcus pyogenes var albus and aureus used in these studies were inhibited. Because of the possible production of "v" forms it is desirable not to use penicillin in the culture media to suppress the growth of bacteria when looking for pleuropneumonia-like organisms. The medium as employed by Morton et al. (1951) containing crystal violet and potassium tellurite exerts its selective action when

Thallium acetate. In liquid medium the growth of Proteus was delayed 2 to 4 days, and the growth of all the other bacteria tested, except the streptococci, was prevented. On solid medium S. faecalis and S. pyogenes grew practically uninhibited except that the colonies of S. pyogenes were exceedingly small. Proteus grew as two or three discrete colonies instead of the characteristic swarming growth. No "v" type colonies were observed on any of the thallium acetate plates inoculated with bacteria.

### TABLE 1

The effect of thallium acetate in "bacto-PPLO broth" with one per cent serum fraction in suppressing growth of bacteria and pleuropneumonia-like organisms of human origin

<table>
<thead>
<tr>
<th>MICROORGANISM</th>
<th>CONCENTRATION OF THALLIUM ACETATE</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0</td>
</tr>
<tr>
<td>Pleuropneumonia-like organism, Campo strain</td>
<td>++++</td>
</tr>
<tr>
<td>Pleuropneumonia-like organism, 607 strain</td>
<td>++++</td>
</tr>
<tr>
<td>Pleuropneumonia-like organism, 39 strain</td>
<td>++++</td>
</tr>
<tr>
<td>Pleuropneumonia-like organism, 48 strain</td>
<td>+</td>
</tr>
<tr>
<td>Pleuropneumonia-like organism, 60 strain</td>
<td>++++</td>
</tr>
<tr>
<td>Pleuropneumonia-like organism, 110 strain</td>
<td>++++</td>
</tr>
<tr>
<td>Aerobacter aerogenes</td>
<td>++++</td>
</tr>
<tr>
<td>Bacillus subtilis</td>
<td>++++</td>
</tr>
<tr>
<td>Corynebacterium xerose</td>
<td>++++</td>
</tr>
<tr>
<td>Escherichia coli, strain FS</td>
<td>++++</td>
</tr>
<tr>
<td>Escherichia coli, strain B</td>
<td>++++</td>
</tr>
<tr>
<td>Micrococcus pyogenes var albus</td>
<td>++++</td>
</tr>
<tr>
<td>Micrococcus pyogenes var aureus</td>
<td>++++</td>
</tr>
<tr>
<td>Proteus mirabilis</td>
<td>++++</td>
</tr>
<tr>
<td>Proteus vulgaris</td>
<td>++++</td>
</tr>
<tr>
<td>Pseudomonas aeruginosa</td>
<td>++++</td>
</tr>
<tr>
<td>Salmonella schottmuelleri</td>
<td>++++</td>
</tr>
<tr>
<td>Streptococcus faecalis</td>
<td>++++</td>
</tr>
<tr>
<td>Streptococcus pyogenes</td>
<td>++++</td>
</tr>
</tbody>
</table>

+++ = maximum amount of growth for that strain.
+ = very few colonies.
(+) = growth delayed 2 to 4 days.

### DISCUSSION

Whereas Edward (1947) observed that two strains of pleuropneumonia-like organisms (L5 and mouse catarrh) were inhibited by a 1:500 concentration, we have not found this to be the case with the 6 strains of pleuropneumonia-like organisms of human origin employed in these studies. Edward also observed that a diphtheroid and Staphylococcus albus and Staphylococcus
25 per cent ascitic fluid is employed but not when one per cent serum fraction is employed as the enriching substance. The presence of as much as 10 mg or as little as 0.1 mg of protein per ml of broth has caused no apparent change in the selective action of thallium acetate. Since only the streptococcal species of the bacteria tested were not inhibited by the thallium acetate, it is interesting to recall that McKenzie (1941) recommended its use in the diagnosis of streptococcal mastitis.

It is not the intent here to evaluate thallium acetate for the isolation of pleuropneumonialike organisms from clinical material. For clinical work the concentration may have to be less than that employed here since primary isolates may be more sensitive. Thallium acetate has proven very useful in working with cultures of pleuropneumonialike organisms and is practically a necessary adjunct to the medium in certain metabolic studies.

The insusceptibility of pleuropneumonialike organisms to the action of thallium acetate, a substance toxic to animals as well as bacteria, indicates another dissimilarity between pleuropneumonialike organisms and bacteria in general.

ACKNOWLEDGMENT

We wish to express our appreciation to Dr. C. W. Christensen, Difco Laboratories, for his helpful suggestions and interest in the work.

SUMMARY

The noninhibitory action of crystal violet for pleuropneumonialike organisms is influenced by the total protein content of the culture medium.

The noninhibitory action of thallium acetate for pleuropneumonialike organisms does not appear to be influenced by the total protein content of the culture medium.

The antibacterial effect of thallium acetate appears to be similar to the combined effects of crystal violet and potassium tellurite.

The presence of thallium acetate in culture media as an inhibitor for bacteria has the advantages that (a) it has not been observed to produce "t" forms from bacteria, (b) it has a wide bacterial spectrum, (c) only one substance needs to be added to the culture medium to inhibit both gram positive and gram negative organisms, (d) it may be added to the culture medium prior to sterilization, and (e) its selective action is independent of the total protein content of the culture medium.

REFERENCES


Smith, P. F., and Morton, H. E. 1951 The separation and characterization of the growth factor in serum and ascitic fluid which is required by certain pleuropneumonia-like organisms. J. Bact., 61, 395-405.