# THE USE OF SODIUM AZIDE ($NaN_3$) AND CRYSTAL VIOLET IN A SELECTIVE MEDIUM FOR STREPTOCOCCI AND ERYSIPELOTHRIX RHUSIOPATHIAE[1]

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The isolation and study of certain microorganisms are often very difficult because of the presence of other organisms which overgrow the desired one on ordinary culture media. This is especially true when pathogens are to be isolated from contaminated material such as intestinal contents or from the upper respiratory tract where there is an abundance of saprophytic organisms. Contaminated milk samples mailed to the laboratory for mastitis diagnosis are usually overgrown to such an extent that pure cultures of the streptococci present can not be obtained for identification except with difficulty. It was in this connection that a study of selective media for streptococci was begun.

Edwards (1933, 1938) described two selective media which were of value. The first was a crystal-violet esculin blood agar and the other a crystal-violet sodium-azide broth. Hartmann (1936) investigated the value of several substances for the selective cultivation of mastitis streptococci from contaminated material. Of the substances tested, sodium azide appeared to be most efficient. Snyder and Lichstein (1940) used sodium azide as an inhibitor of gram-negative bacteria.

A combination medium employing sodium azide and crystal violet has been developed and found to have some advantages over the media referred to above. A description of this medium and its uses follows.

## METHODS

The base medium used in the investigation was either tryptose broth or tryptose agar. Broth medium was prepared by dissolving 3 grams of beef extract, 15 grams of bacto-tryptose, and 5 grams of NaCl in 1000 ml. of distilled water. The pH was determined by means of an electrometer and was adjusted to 0.2 of a pH unit above the final pH desired. The medium was filtered through absorbent cotton and dispensed in 150 ml. quantities into 200 ml. Erlenmeyer flasks. These were sterilized by autoclaving 20 minutes at 15 lbs. pressure. Tryptose agar base was prepared by the addition of 18 grams of agar per liter to the broth base.

A stock solution of crystal violet was made by adding 0.25 gram of crystal violet powder, dye content 94 per cent, to 100 ml. of distilled water. Sodium

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azide[2] stock solution was prepared by dissolving 1 gram of sodium azide powder in 25 ml. of distilled water. These stock solutions were sterilized by autoclaving 20 minutes at 15 lbs. pressure. When gentian violet was used, a stock solution was prepared in the same manner as for crystal violet. Gentian violet employed in ordinary staining methods was used. Various concentrations of the inhibiting substances were made by adding appropriate amounts of the stock solutions to the sterile base media with sterile pipettes. Glucose was added in some cases as an enrichment substance. This was added before sterilization in amounts to make 1 per cent concentration. When blood agar was used it was prepared by adding sterile, citrated horse blood to the melted agar base which was cooled to 48°C. The horse blood contained 0.5 per cent sodium citrate to prevent coagulation and was stored at 5°C. for 1 to 30 days before use. There was little or no hemolysis of the blood up to 30 days storage. After the various substances were added to the medium it was dispensed into sterile culture tubes in approximately 5 ml. amounts. Tubes containing agar were placed in a slanting position until hardened. The final pH of the medium was determined at the end of the pouring operation. The tubes of poured media were incubated overnight to determine sterility and were inoculated on the following day.

The stock cultures of the test organisms were grown on tryptose agar pH 7.4. Before inoculation into the test media, transfers were made to tryptose broth pH 7.4 and incubated 18–24 hours at 37°C. These 18–24 hour broth cultures were diluted in sterile saline immediately before inoculation into the test media. Due to great variation in the amount of growth of the different organisms at 18–24 hours incubation, it was necessary to dilute those organisms which grew abundantly considerably more than those which grew more slowly. Two drops of the 18–24 hour broth cultures of *Escherichia coli*, *Pseudomonas aeruginosa*, *Salmonella choleraesuis*, *Pasteurella multocida*, *Eberthella typhosa*, *Proteus vulgaris*, *Bacillus subtilis* and *Staphylococcus aureus* were diluted in 10 ml. of sterile saline. Streptococci and micrococci were diluted 5 drops in 10 ml. of saline. *Erysipelothrix rhusiopathiae*, *Corynebacterium pyogenes*, *Brucella abortus* and *Listerella monocytogenes* were diluted 10 drops in 10 ml. of saline. The test media containing the various dilutions of sodium azide and crystal violet were inoculated with 1 drop of the diluted saline suspensions delivered from a 1 ml. pipette. Plate counts of 1 drop of the saline suspensions showed that the numbers of organisms inoculated usually varied between 2,000 and 10,000 organisms. The inoculated cultures were incubated at 37°C. Observations were made at 24, and 48 hours, and final results recorded after 72 hours incubation.

## RESULTS

The presence of growth was usually determined macroscopically but in doubtful cases microscopic examinations were made. The amount of growth in each case was determined by comparing the growth with that of a control not containing inhibitory substances. Thus, the control is always designated as +++

[2] Obtained from Fairmount Chemical Company, Newark, New Jersey. Manufacturers analysis 95% sodium azide.

(see table 1) so that the comparison is a measure of the inhibitory action rather than a comparison of the amounts of growth of one organism contrasted to another. Growths designated +++ are comparable to that of the controls, ++ and + growths designate varying degrees of inhibition of growth, ± indicates faint growth, often 1 colony, 0 indicates no growth or complete inhibition. Many instances of ± growth occurred only after 72 hours incubation.

In table 1 the inhibitory effect of sodium azide blood agar upon the growth of the various organisms is shown. The streptococci, except *S. lactis*, are not

TABLE 1

*Inhibitory action of various concentrations of sodium azide in blood agar*

| | CONCENTRATION OF SODIUM AZIDE | | | | | |
|---|---|---|---|---|---|---|
| | 1-500 | 1-1,000 | 1-2,000 | 1-3,000 | 1-5,000 | Control |
| | Tryptose agar and 5 per cent horse blood. Final pH 6.8 | | | | | |
| *Streptococcus pyogenes* A | ++ | ++ | +++ | +++ | +++ | +++ |
| *S. pyogenes* C | ++ | +++ | +++ | +++ | +++ | +++ |
| *S. agalactiae* | ++ | ++ | +++ | +++ | +++ | +++ |
| *S. dysgalactiae* | ++ | ++ | +++ | +++ | +++ | +++ |
| *S. uberis* | +++ | +++ | +++ | +++ | +++ | +++ |
| *S. viridans* | ++ | +++ | +++ | +++ | +++ | +++ |
| *Diplococcus pneumoniae* | +++ | +++ | +++ | +++ | +++ | +++ |
| *S. lactis* | 0 | ± | ++ | ++ | +++ | +++ |
| *Micrococcus* Sp | + | ++ | +++ | +++ | +++ | +++ |
| *Staphylococcus aureus* | 0 | ± | +++ | +++ | +++ | +++ |
| *E. rhusiopathiae* | ++ | ++ | +++ | +++ | +++ | +++ |
| *P. aeruginosa* | 0 | 0 | ±* | ± | +++ | +++ |
| *S. choleraesuis* | 0 | 0 | 0 | ± | +++ | +++ |
| *Proteus vulgaris* | 0 | 0 | 0 | ± | +++ | +++ |
| *E. coli* | 0 | 0 | 0 | ± | ± | +++ |
| *E. typhosa* | 0 | 0 | 0 | ± | ± | +++ |
| *Aerobacter aerogenes* | 0 | 0 | 0 | ± | ± | +++ |
| *P. multocida* | 0 | 0 | 0 | + | + | +++ |
| *L. monocytogenes* | 0 | 0 | 0 | ± | + | +++ |
| *C. pyogenes* | 0 | 0 | 0 | ± | + | +++ |
| *B. subtilis* | 0 | 0 | 0 | ± | + | +++ |
| *Brucella abortus* | 0 | 0 | 0 | ± | + | +++ |

* One colony after 72 hours incubation.

inhibited in any of the concentrations of sodium azide in blood agar. *Diplococcus pneumoniae* likewise is not inhibited. *E. rhusiopathiae* is only slightly inhibited. The micrococci, *Staphylococcus aureus* and *Streptococcus lactis* are definitely inhibited by the concentrations above 1-2,000. In the case of *S. aureus*, a marked inhibition was noted in the 1-500 and 1-1,000 concentrations whereas no inhibition was observed in the 1-2,000 concentration. The other species of organisms are definitely inhibited in concentrations greater than 1-5,000. *P. aeruginosa* proves to be more resistant than any other of the gram-negative organisms.

A series of tests were made using the same concentrations of sodium azide as in table 1, except that tryptose broth was used instead of tryptose blood agar. Results of these tests are similar to those obtained on blood agar. Several of the gram-negative rods, *P. aeruginosa*, *E. coli*, *S. choleraesuis*, and *E. typhosa*, are not inhibited at 1–2,000 concentration as is the case in the blood agar media containing 1–2,000 sodium azide. More inhibition of the streptococci was noted in the broth of 1–500 concentration than on blood agar of the same concentration of sodium azide.

The action of sodium azide in broth at pH 7.4 instead of pH 6.8 was compared. It was noted that there was considerably less inhibition at pH 7.4 than at pH 6.8. This is particularly true in the 1–500 concentration among the streptococci. For example, *S. lactis* is completely inhibited in the 1–500 and 1–1,000 concentrations at pH 6.8, while the same concentrations at pH 7.4 yield abundant growth. *S. aureus* is completely inhibited in the 1–500 concentration but abundant growth is obtained at pH 7.4 in all concentrations of sodium azide. *Brucella abortus* is completely inhibited by all concentrations of sodium azide at a pH of 6.8, while fair growth is obtained in all concentrations except 1–500 at a pH of 7.4.

TABLE 2

*Inhibitory action of crystal violet alone and in combination with sodium azide*

| | TRYPTOSE AGAR pH 6.8 | | | | |
|---|---|---|---|---|---|
| | Crystal violet 1–200,000 | Crystal violet 1–200,000, 5 per cent blood | Crystal violet 1–500,000, 5 per cent blood | Crystal violet 1–500,000, $NaN_3$ 1–2000, 5 per cent blood | Crystal violet 1–100,000, $NaN_3$ 1–1000, 5 per cent blood |
| *S. pyogenes* A | 0 | + | +++ | +++ | 0 |
| *S. pyogenes* C | | | | | |
| *S. agalactiae* | | | | | |
| *S. dysgalactiae* | | | | | |
| *S. uberis* | + | ++ | +++ | +++ | 0 |
| *S. viridans* | | | | | |
| *Micrococcus* sp | | | | | |
| *S. lactis* | 0 | 0 | ± | ± | 0 |
| *S. aureus* | 0 | 0 | 0 | 0 | 0 |
| *E. rhusiopathiae* | +++ | +++ | +++ | +++ | +++ |
| Gram-negative rods | +++ | +++ | +++ | 0 | 0 |
| *B. subtilis* | 0 | 0 | 0 | 0 | 0 |
| *C. pyogenes* | | | | | |
| *L. monocytogenes* | | | | | |

Table 2 shows the inhibitory action of crystal violet on the various groups of organisms. The effect of the addition of 5 per cent blood is shown and the inhibitory action of various concentrations of crystal violet alone and in combination with sodium azide are compared. When the results were the same for different organisms they are grouped in order to make the material more concise.

It will be noted in table 2 that a concentration of 1–200,000 of crystal violet is inhibitory to the streptococci, staphylococci, micrococci, and the gram-positive

rods except *E. rhusiopathiae*. The gram-negative organisms grow abundantly in that concentration. The addition of 5 per cent blood to the medium decreases the effect of crystal violet and allows the growth of all streptococci except *S. lactis*. A concentration of 1–500,000 crystal violet allows the growth of all pathogenic streptococci, but inhibits *S. aureus* completely and permits only a few colonies of *S. lactis* to develop on the medium.

Addition of sodium azide to the medium containing crystal violet inhibits the growth of all of the species in the gram-negative-rod group and does not alter the effects of crystal violet.

It is possible to inhibit all organisms except *E. rhusiopathiae* completely by increasing the concentrations of crystal violet and sodium azide to 1–100,000 and 1–1,000 respectively.

It is further noted that colonies of *S. agalactiae* and *S. uberis* absorb enough crystal violet to give them a distinctly violet color. *S. viridans* and *S. dysgalactiae* absorb only a moderate amount and *S. pyogenes* none at all.

Gentian violet was used in a similar experiment and compared with crystal violet. The inhibitory action is practically the same as crystal violet except in minor instances.

During the course of this study it was noted that differences in hemolysis of blood agar varied to some extent with the streptococci, micrococci and pneumococci. *S. dysgalactiae* produced beta hemolysis in sodium azide blood agar as compared to gamma hemolysis in blood agar. A wide greenish zone of hemolysis around colonies of organisms commonly designated as alpha hemolytic was also observed. The zone of hemolysis of beta hemolytic streptococci, such as *S. pyogenes*, was definitely extended.

It is universally recognized that the addition of a fermentable carbohydrate to blood agar commonly reduces and often inhibits the production of hemolysin by streptococci. Since hemolysis is more marked in the presence of sodium azide, a series of tests were made in a medium to which one per cent glucose was added. It is noted that in the presence of glucose, species of hemolytic streptococci are unable to produce hemolysis while on media not containing glucose definite hemolysis is produced. In this study it was observed that alpha hemolytic streptococci produced a yellowish-green color which spread over the entire plate in the presence of glucose while that characteristic was not found in media not containing this carbohydrate.

Microscopic examination of stained smears from the inoculated media containing higher concentrations of sodium azide, in some cases revealed many unusual morphological characteristics. Several species, particularly the gram-negative rods, were so changed as to be unrecognizable. For example, *Aerobacter aerogenes* was observed to form large ovoid cells several times the length and width of those seen from ordinary media. Transfers of these atypical cells into media containing no sodium azide produced cells typical of the species in size and shape. The other species of gram-negative rods and *Bacillus subtilis* exhibited similar morphologic changes. The streptococci in general were not affected morphologically by sodium azide.

## DISCUSSION

A selective medium using sodium azide and crystal violet as inhibiting agents is able to eliminate much of the difficulty in culturing streptococci and *E. rhusiopathiae* from contaminated materials. A concentration of 1–2,000 sodium azide in blood agar having a pH of 6.8 did not affect the growth of these organisms and is very effective in inhibiting gram-negative organisms. The pH of the medium is an important factor in obtaining the maximum effect of the sodium azide. Edwards (1938) found that *E. coli* was inhibited by a 1–10,000 concentration of sodium azide if the pH of the medium was 6.8 but was only partially inhibited when the pH was 7.4. The results of this study not only confirm Edward's observation on *E. coli*, but show a similar effect on all organisms which are inhibited by sodium azide.

The mechanism by which sodium azide is able to inhibit growth of certain bacteria was not studied. Since this substance is an inhibitor of the enzyme catalase, it is possible that its inhibitory effect may be due to the inhibition of certain enzyme systems within the bacterial cell. Schattenfroh (1896) who was the first to study the effects of azide compounds on bacteria found that both sodium and ammonium azides inhibited bacterial growth.

Micrococci, *S. aureus*, *D. pneumoniae*, and *E. rhusiopathiae* were not inhibited by sodium azide. The addition of 1–500,000 crystal violet to the medium increased the selectivity of the medium by inhibiting *S. aureus*. It was not possible to change the concentrations of the inhibitory substances to eliminate the other three species without also inhibiting the streptococci. However, the colonies of micrococci can usually be distinguished from streptococci by their larger size and greenish zone of hemolysis around them when grown on sodium azide blood agar. In cases when *E. rhusiopathiae* or *D. pneumoniae* may occur with streptococci, they must be differentiated by other recognized procedures.

It is noted that *E. rhusiopathiae* is the most tolerant of all species studied being able to grow in sodium azide 1–1,000 and crystal violet 1–100,000 concentrations. This fact makes it possible to increase the concentrations to a point where only this organism and no other will grow. A crystal-violet sodium-azide blood agar containing 1–1,000 sodium azide and 1–100,000 crystal violet was prepared. Saline suspensions of all the species of organism used in this study were mixed and cultured on this medium. Pure cultures of *E. rhusiopathiae* were obtained from the mixture on this medium.

The effect of sodium azide on hemolysis of red blood cells is of interest. All hemolytic action appears to be enhanced. Possibly the methemoglobin formed by the combination of sodium azide and hemoglobin is more easily altered than oxyhemoglobin. Certain organisms may be capable of changing methemoglobin and not oxyhemoglobin because some streptococci were able to produce hemolysis on sodium azide blood agar but were non-hemolytic on ordinary blood agar. The marked discoloration of sodium azide blood agar by beta hemolytic strains of streptococci described by Snyder and Lichstein (1940) was not confirmed. In fact, beta hemolytic strains of streptococci used in this study produced a wide,

clear zone of hemolysis on sodium azide blood agar which was wider than that seen on ordinary blood agar.

## CONCLUSIONS

1. A concentration of 1–2,000 sodium azide in 5 per cent blood agar having a pH of 6.8 was found to be most effective in inhibiting organisms other than the streptococci.

2. Sodium azide in a medium at pH 6.8 is a more effective inhibiting agent than when used in a medium at pH 7.4.

3. A concentration of 1–500,000 crystal violet appears to be most effective in inhibiting organisms not inhibited by sodium azide.

4. Pathogenic streptococci grew well in 1–500,000 crystal violet while *Staphylococcus aureus* did not grow in this concentration of the dye.

5. A combination of 1–2,000 sodium azide and 1–500,000 crystal violet in 5 per cent blood agar having a pH of 6.8 was found to inhibit almost all organisms except the streptococci.

6. *Diplococcus pneumoniae*, *Erysipelothrix rhusiopathiae* and the micrococci were not effectively inhibited on the combination medium.

7. Hemolytic properties of the streptococci grown on sodium azide blood agar are somewhat different from those observed on ordinary blood agar.

8. *E. rhusiopathiae* is not inhibited by concentrations of crystal violet and sodium azide which inhibit all other organisms studied including the streptococci.

9. A blood agar medium having a pH of 6.8 and containing 1–1,000 sodium azide, 1–100,000 crystal violet and 5 per cent blood is recommended for the selective isolation of *E. rhusiopathiae* from contaminated material.

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### *Literature Cited*

EDWARDS, S. J. 1933 Studies on bovine mastitis. IX. A selective medium for the diagnosis of streptococcus mastitis. J. Comp. Path. Therap., **46**, 211–217.

EDWARDS, S. J. 1938 The diagnosis of streptococcus mastitis by cultural methods. J. Comp. Path. Therap., **51**, 250–263.

HARTMANN, G. 1936 Ein Beitrag zur Reinzüchtung von Mastitisstreptokokken aus verunreinigtem Material. Milchw. Forsch., **18**, 116–122.

SCHATTENFROH, A. 1896 Ueber die Wirkung der Stickstoffwasserstoffsauren Salze auf pflanzliche Mikroorganismen. Arch. Hyg., **27**, 231–233.

SNYDER, M. L., AND LICHSTEIN, H. C. 1940 Sodium Azide as an Inhibiting Substance for Gram-negative Bacteria. J. Infectious Diseases, **67**, 113–115.