THE DETECTION OF EXTRACELLULAR DEOXYRIBONUCLEASE PRODUCED BY *CLOSTRIDIUM NOVYI* TYPE A, *CLOSTRIDIUM CAPITOVALE*, AND *CLOSTRIDIUM TETANI*

by

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INTRODUCTION

A. Statement of the problem

There is evidence to suggest a possible relationship between extracellular deoxyribonuclease (DNase) and pathogenicity (1,2,3). When massive tissue destruction occurs, deoxyribonucleic acid (DNA) is released as a highly viscous material. Apparently, if the infection is to spread, the organisms must destroy the DNA. Some bacteria accomplish this by producing DNase which breaks down highly viscous DNA into mononucleotides which are much less viscous.

Because of discoveries of an ever increasing number of bacterial species and of complex problems involving nomenclature of the known species, microbiologists are faced with the difficult task of classification. The production of extracellular deoxyribonuclease by some bacteria is of considerable value to the taxonomist in that it serves as a tool by which bacteria may be classified.

Many environmental factors can control the production of this enzyme in bacterial cells, and the influence of these may be different for each bacterial species. Temperature, pH, divalent cations and substances such as glucose and ethylenediamine tetraacetic acid (EDTA) have all been shown to have some effect on DNase production.

This study was undertaken for the purpose of determining

the degree, if any, of DNase activity in a selected group of Clostridia. The factors listed above were tested to determine what effect each had on the DNase activity of these species. In addition, this study was designed to determine if the present DNase test medium is sufficient to detect extracellular DNase produced by these organisms.

B. Review of the pertinent literature

Eisenberg in 1907 (4) discovered that culture filtrates of Cl. septicum and Cl. chauvoei contained thermolabile antigens which damaged washed leucocytes. Microscopic examination of hanging-drop preparations of the incubated mixtures revealed that the cytoplasmic membranes of the polymorphonuclear leucocytes had become rounded and that the cytoplasm had become agranular. The lobes of the nucleus exhibited a dramatic change in that they had fused together to form clear vesicles. Lymphocytes and macrophages were affected to a lesser degree. Human leucocytes were shown to be most sensitive. Rabbit leucocytes and guinea-pig leucocytes were relatively insensitive.

Butler (5,6) examined cervical smears from eighty-four post-partum patients. She observed that in clostridial infections, heavily encapsulated bacilli were present and that many of the leucocytes showed severe damage. These bacilli were identified as Cl. welchii. She also noted that damage to leucocytes occurred with both Cl. septicum and Cl. welchii, but that infection with any other anaerobic bacilli resulted in no damage to the leucocytes.

In 1945, Robb-Smith (7) showed that injection of culture filtrates of Cl. welchii into muscles of living rabbits caused karyolysis of the nuclei of polymorphonuclear leucocytes, muscle cells, and connective tissue cells. Further studies showed that similar nuclear changes could be observed in sections of muscle or liver by incubating them at 37 C with filtrates of Cl. welchii. Feulgen-positive material only was damaged by the culture filtrates. Based upon this fact, he concluded that the substance causing the damage was a deoxyribonuclease.

2. Elaboration of DNase by a variety of bacteria

a) Streptococci

It was discovered in 1948 (8) that deoxyribonucleoprotein and protein-free deoxyribonucleic acid (DNA) made up a significant part of the purulent and inflammatory exudates derived from patients with streptococcal infections. DNA was isolated in quantities ranging from 30 to 70 per cent of the total purulent sediment. Tillett, Sherry and Christensen (9) examined the effect of incubating this exudate with culture filtrates of streptococci. The destruction of feulgen positive material was attributed to production of DNase. Among the strains tested, Streptococcus pyogenes (Lancefield's Group A hemolytic streptococci [10]), which also possessed fibrinolytic properties, were uniformly potent in the production of DNase. On the other hand, they found that strains of Streptococcus viridans and Diplococcus pneumonia (pneumococci) produced no detectable level of DNase. Unless otherwise stated, DNase refers to extracellular DNase production.

All of the 36 strains of Group A hemolytic streptococci examined by McCarty (11) were found to produce DNase during growth in liquid cultures and that the enzyme was released into the medium. In conjunction with determining DNase activity, ribonuclease (RNase) activity was also measured. He noted that DNase was consistently produced in greater quantity than RNase and that the concentration of DNase in the culture increased logarithmically during active growth of the organism. For measurement of DNase, a viscosimetric method (12) was used. The potency of streptococcal nuclease was compared with nucleases from other sources, e.g. beef pancreas and the streptococcal DNase was shown to be approximately one-eighth as great when changes in viscosity were measured using equivalent amounts of the enzymes.

Further work by McCarty (13) showed that rabbit antisera against partially purified streptococcal DNase inhibited the action of the enzyme on its substrate. In addition, it was shown that the activity of pancreatic DNase was not affected by antisera to streptococcal DNase. Furthermore, antibodies against pancreatic DNase did not inhibit the streptococcal enzyme. Several patients recovering from streptococcal infections developed inhibitory antibody to streptococcal DNase. The level of antibody in these patients was occasionally very high although the number of patients developing this antibody was less than those developing antibodies against streptokinase and streptolysin O.

Investigation into DNase production continued and in 1950, Brown (14) conducted a survey of nuclease production by streptococci. A total of 267 cultures, representing 24 groups and species of the genus Streptococcus was examined for the production of DNase and RNase. All of 42 cultures of Group A streptococci tested produced both nucleases. About one-fourth of the Group B strains produced both nucleases, one strain produced RNase only, and the remaining Group B strains produced neither nuclease. Most strains of Group C and some strains of Groups F, G, and L produced DNase but not RNase.

Bernheimer and Ruffier (15) studied 34 strains of streptococci for their ability to produce DNase in broth culture. They were able to demonstrate that cells in the resting state elaborated DNase into the surrounding culture medium. The largest quantity of enzyme was found to be produced by strains belonging to Group A and by certain strains belonging to Groups C and D. When Group A streptococci were fractionated, a substance was found which partially inhibited the activity of DNase produced by these strains. At the same time, this substance failed to inhibit the DNase formed by strains of Groups B and C or by yeast cells. This inhibitor was identified as ribonucleic acid. Yeast ribonucleic acid differed from streptococcal ribonucleic acid in that it failed to inhibit the DNase of the Group A streptococci (16).

DNase activity in Group A streptococci was also studied by Wannamaker (17). He noted a paradox previously mentioned

by McCarty (13). In the immunologic response following natural infections with Group A streptococci, only a moderate number of patients developed neutralizing antibodies against DNase, even though all strains of Group A streptococci produce DNase. He presented evidence that Group A streptococci can produce three distinct enzymes, each independently capable of depolymerizing intact DNA. The three streptococcal DNase's were shown to migrate separately on starch zone electrophoresis and to be serologically distinct. It is possible that the three enzymes may have slightly different chemical activity and the end products of digestion may be different for each of the three components. In addition, they showed differences in the degree of inhibition by citrate and versene and in optimal pH for activity. Further investigation revealed that within Group A streptococci, strains differ markedly in their distribution of the three components (DNase's). Some strains have a high level of component A and a low level of B and C, while other strains have a high level of B and a low level of A and C, etc. Within Group A streptococci, DNase B appears to be much more commonly encountered than either DNase A or C. Antibody studies in rabbits have shown that antibodies to each of these components are highly specific and strongest against component A. Based on these findings, a possible explanation of the above paradox was presented suggesting that those patients who developed neutralizing antibodies were those infected with strains having a high level of component A.

b) Staphylococci

Cunningham, Catlin and Privat De Garilhe (18) discovered extracellular DNase activity in Staphylococcus aureus. All of the strains of S. aureus tested in their experiment produced DNase. The enzyme was purified by boiling and was shown to be an exonuclease, breaking DNA into mononucleotides. The optimum pH was 8.6 and the DNase was shown to be unusually stable.

Investigations of the relationship between coagulase and DNase have been described by several workers. Weckman and Catlin (1) studied the production of DNase in staphylococci from clinical sources. Eighty-seven isolates were studied and isolates which produced coagulase also produced DNase. DiSalvo in 1958 (19) investigated DNase production by S. aureus and S. albus. The organisms he used were all obtained from various clinical sources. A total of 304 isolates were studied for DNase production. All of the 204 isolates of S. aureus produced DNase while the 100 isolates of S. albus produced no DNase.

The problem of biochemical differentiation between virulent and avirulent staphylococci was investigated by Burns and Holtman in 1960 (2). Two-hundred strains of S. aureus were tested biochemically for coagulase production, pigment production, gelatin liquifaction, egg yolk reaction, production of acid from mannitol, growth on tellurite agar, reduction of tetrazolium agar medium and DNase activity. The purpose of the study was to test the correlation between coagulase production and the other seven biochemical tests. The final analysis revealed that

96 per cent of the coagulase-positive organisms also produced DNase, substantially greater than the correlation between coagulase and any of the other six biochemical tests. A similar study was performed by Kilmer in 1962 (20) using fluorescine amine agar, phenophthalein diphosphate agar, mannitol salt agar, egg yolk agar and DNase test agar. The study was designed to determine how closely the biochemical tests correlated. The results of the investigation showed that DNase production and mannitol fermentation most closely correlated with coagulase production. Further studies on the relationship between coagulase and DNase production were carried out by several other investigators (21, 22, 23, 24). Each of these investigators reported that at least 95 per cent of those isolates which produced coagulase also produced DNase.

The effect of vitamins on DNase activity was investigated by Fusillo and Weiss (25). Thiamine or nicotinamide was added to a basal medium containing DNA and the effect of added vitamins was observed. The addition of either vitamin alone or in conjunction resulted in no increase or decrease in DNase activity.

A method for detecting staphylococcal contamination of foodstuffs was presented as a practical use for DNase determination by Chesbro and Auborn (26) in 1967. Food contaminated with enterotoxin A-producing S. aureus was homogenized, and the DNase was extracted using protamine sulfate. Calf thymus DNA was used as a substrate, and the level of DNase was determined using a spectrophotometric procedure (27).

The method was used to compare staphylococcal growth with nuclease production in foods under varying conditions of temperature, aerobiosis, and competition from other microorganisms. It was concluded that the DNase was produced under any condition that permitted growth of the S. aureus, and that no interference with the test was encountered. The sensitivity of the enzymatic detection of DNase was comparable to the sensitivity of serological detection of enterotoxin A. The test is particularly useful because the entire procedure takes less than three hours to complete.

c) Corynebacteria

The discovery of RNase (28) in the broth cultures of Corynebacterium diphtheriae was followed by a report by Messinova, Yusupova and Shamautdinov (29) of extracellular DNase activity in the culture fluid of C. diphtheriae. A total of 156 strains of various corynebacteria species were studied. Of 134 strains of C. diphtheriae, 104 strains were positive for DNase activity. None of the other species produced DNase.

d) Gram-negative bacteria

Streitfeld, Hoffmann and Janklow (30) investigated the production of DNase by Pseudomonas species. A collection of 25 strains of Ps. aeruginosa and Ps. fluorescens were tested for DNase activity. DNase production was demonstrated in both strains of Ps. fluorescens and 22 of 23 strains of Ps. aeruginosa.

Another study of DNase activity in Pseudomonas species was performed by Sadouski and Levin (31). The production of DNase

was studied in 23 marine and 3 dairy strains of Ps. putrefaciens, 15 strains of fish-pathogenic fluorescent pseudomonads, 38 strains of fluorescent pseudomonads isolated from haddock and 34 related organisms. All strains of Ps. putrefaciens produced both DNase and RNase. Of the remaining 87 organisms examined, 26.5 per cent produced RNase only, while 14.5 per cent produced DNase and RNase.

Valu (32) in 1965 discussed the usefulness of the DNase test as a method of differentiating Enterobacter hafniae and Serratia marcescens. A total of 24 strains of E. hafniae and three strains of S. marcescens were examined for their ability to produce DNase. All of the E. hafniae strains tested were unable to produce a detectable level of DNase, while all cultures of S. marcescens produced DNase. All cultures in this study were tested at 25 C, 30 C and 37 C, although nothing was reported concerning the quantity produced at each temperature.

Elston and Elston (33) conducted a similar study of DNase production by Serratia and E. hafniae and also included in their study other members of the genus Enterobacter. Ninety-three isolates were used, including 51 Serratia marcescens, 33 E. hafniae, 6 E. cloacae, and 3 E. liquifaciens. All 51 isolates of Serratia were positive for DNase production while all the Enterobacter species were negative.

A third investigation into the production of DNase by Serratia and related organisms was carried out by Schreier (34). One-hundred isolates of Klebsiella, 29 E. cloacae, 16 E. aerogenes, 75 Serratia and 15 intermediate coliforms were tested for DNase

production. All of the Serratia strains exhibited DNase activity while 100 per cent of the remaining organisms produced no detectable level of DNase.

An examination of DNase activity in gram-negative bacilli was described by Rothberg and Swartz (35). The examinations included: 182 isolates of Escherichia coli, 117 Proteus, 77 Klebsiella, 58 Serratia, 26 Enterobacter, 10 Citrobacter, 8 Herellea, 7 Alcaligenes, 6 Providence, 7 Salmonella, and 4 Mima. Nine strains of Proteus, 58 Serratia and 1 Alcaligenes were found to be positive for DNase activity. All of the remaining strains produced no DNase. Unfortunately, the authors did not mention the various species used in their study other than in the case of E. coli.

A similar survey of DNase production by gram-negative bacilli was described by Martin and Ewing (36). Sixty of 62 isolates of E. liquifaciens, 194 of 194 Serratia, 6 of 10 P. vulgaris, 100 of 100 V. cholerae, 12 of 12 Aeromonas hydrophilia, 14 of 19 A. salmonicida, 1 Flavobacterium, and 1 Alcaligenes were positive for DNase activity while 22 strains of E. coli, 36 Shigella, 236 Salmonella species, 10 P. mirabilis, 10 P. morgani, 12 P. rettgeri, 20 Providence species, and 10 Aeromonas shigelloides produced no detectable level of DNase.

Gilardi (37) investigated DNase production in a collection of nonfermenting gram-negative bacteria. Four of 35 isolates of Ps. aeruginosa, 38 of 39 Ps. maltophilia, and 1 of 8 isolates of Ps. stutzeri produced DNase while 3 Ps. fluorescens, 19 Ps. putida,

4 Ps. pseudomaliei, 5 Ps. alcaligenes, 60 Acinetobacter anitratum, 27 A. lwoffi, 12 Moraxella duplex, 3 Alcaligenes faecalis and 16 A. odorans var. viridans produced no DNase. A study of extracellular DNase production by isolates of Aeromonas and Serratia was performed by Bottone and Allerhand (38). A total of 7 A. hydrophilia, 1 A. salmonicida and 2 A. hydrophilia var. formicans were studied and all exhibited DNase activity. In addition, all of the 175 isolates of S. marcescens produced DNase.

e) Bacillus subtilis

The presence of an extracellular DNase in culture filtrates of Bacillus subtilis has been studied and described (39, 40, 41). The DNase described appeared to be Ca^{++} dependent and degraded single-stranded DNA from the 5'-terminus and double-stranded DNA from the 3'-terminus.

3. DNase production by clostridial species

Since the reports of Eisenberg (4), Butler (5, 6) and Robb-Smith (7), very little work has been published on the DNase of Clostridia. The fact that DNase destroys nuclei has been demonstrated (7). It has also been shown that the culture filtrates of certain clostridial species also damage nuclei (5, 6). It is logical to then ask the question: Do these culture filtrates contain DNase? It seemed worthwhile to Warrack, Bidwell and Oakley to examine culture filtrates of Cl. welchii and Cl. septicum for their ability to attack DNA (3). Because of difficulties, not discussed, in the dealing with Cl. welchii filtrates, they discussed mainly the DNase of Cl. septicum. All cultures of Cl. septicum and Cl. welchii produced DNase.

The DNase produced by Cl. septicum was shown to be antigenically distinct from the DNase of Cl. welchii.

The study of DNase production in Clostridial species was continued by Mukhin, Firsova and Messinova (42). They studied 5 strains of Cl. septicum and 45 strains of Cl. perfringens. The test strains were inoculated at 37 C for 24 hr anaerobically. All the strains of Cl. septicum tested exhibited DNase activity, but only 28 of the 45 strains of Cl. perfringens did. It was also demonstrated that citrate and EDTA caused a complete inhibition of DNase activity when added to the substrate.

4. Factors influencing DNase production by bacterial species

Glucose and dextran are two substances which have an effect on the level of DNase production. Mukhin and his co-workers were able to demonstrate a marked increase in DNase production by enriching their basal medium with 0.5 per cent glucose and an even greater increase with the addition of dextran (42).

Mukhin et al. (42) were able to show that an increase or decrease in pH beyond an optimum of 7.0 would cause a decrease in DNase production by Cl. welchii and Cl. septicum. Jarvis and Lawrence (43) determined the levels of DNase activity in S. aureus over a pH range of 6.0 to 10.0 and demonstrated a pH optimum of 8.7. The production of DNase over a pH range of 4.0 to 9.0 was investigated by Porter and Laskowski (44) who noted a pH optimum of 7.0 for streptococci. Streitfeld et al. (29) studied the effect of pH on 25 Strains of pseudomonas species over a range of 6.0 to 8.0 and showed that a pH of 7.0 was optimal. The effect of pH on the rate of depolymerization

of DNA by staphylococcal DNase was observed by Cunningham et al. (18), who showed that a pH of 8.6 was optimal. Wannamaker (17) also studied the effect of pH on each of 3 distinct DNase's called components A, B, and C, which were found to be produced by Group A hemolytic streptococci. Component A was shown to have been optimally produced at pH 8.5, component B at 7.5, and component C at 5.5. Jeffries et al. (45) studied the effect of pH over a range of 6.0 to 8.0 and concluded that the optimum pH for Serratia strains was 7.0. In a similar study, Nestle and Roberts (46) studied the effect of pH on DNase production by S. marcescens and noted an optimum pH of 8.6.

Temperature also influences the production of DNase. Jeffries et al. (45) studied the effect of temperature on the production of DNase for strains of Serratia and showed that 30 C was the optimum temperature for DNase production. In the same study, they were able to show that the optimum temperature for DNase activity by Bacillus megaterium was 25 C while 37 C was optimal for Streptomyces rimosus. Valu (32) studied the effect of temperature on DNase production by Serratia and was able to demonstrate an optimum temperature of 30 C.

Divalent cations have also been shown to effect DNase production. Tillett et al. (9) working with Group A streptococci, noted that Mg^{++} markedly accelerated the activity of DNase produced by streptococci but not streptokinase. Brown (14) used 267 isolates of streptococci and confirmed the work of Tillett et al. (9). Lamanna and Mallette (47) worked with

cultures of streptococci and found that Na^{+} stimulated RNase activity and that Mg^{++} stimulated DNase activity whereas Mg^{++} inhibited RNase activity. RNase was inhibited by concentrations as low as 0.0005 M and inhibition increased as the concentration of Mg^{++} increased. Potter and Laskowski (44) were able to demonstrate that Mg^{++} has a definite inhibitory effect beyond its optimum concentration of 0.02 M.

Cunningham et al. (18) studied the effect of Mg^{++} and Ca^{++} on DNase activity in staphylococci and discovered that Mg^{++} had no effect, and that, unlike streptococci, Ca^{++} was necessary for DNase activity. A concentration of 0.01 M Ca^{++} was optimal for DNase activity. Fusillo and Weiss (25) studied the effect of Ca^{++} on cultures of S. aureus and demonstrated that Ca^{++} was not necessary for DNase activity. Streitfeld et al. (30) examined the effect of Mg^{++} and Ca^{++} on the activity of DNase produced by pseudomonas strains. They found that there was optimal activity when Ca^{++} was added to the test medium.

Mukhin et al. (42) worked out the effect of divalent cations on DNase activity for cultures of Cl. welchii and Cl. septicum, and they were able to show that Mn^{++} and Co^{++} activated DNase while Mg^{++} and Cu^{++} inhibited it. It was reported by Messinova and Yusupova (48), that several divalent cations, namely Mg^{++}, Mn^{++}, Co^{++}, and Fe^{++} functioned as activators of DNase of Corynebacterium diphtheriae. On the other hand, Cu^{++} was shown to have an inhibitory effect.

C. Methods used for determination of DNase Production

1. Viscosimetric methods

Laskowski and Seidel (49) developed a viscosimetric method for measurement of DNase production. Thymonucleic acid in borate buffer was placed into an Ostwald viscosimeter immersed in a water bath at 37 C. The culture filtrate supernatant was placed into the viscosimeter, thoroughly mixed and the viscosity was read at regular intervals for 30 minutes. This test procedure is based on the fact that as the viscosity of the DNA decreases, the flow rate increases. The viscosity constant of this reaction was calculated from the formula: $K = (1/t) \log (n_o/n_t)$, where n_o = relative viscosity at time zero, and n_t = relative viscosity at time t. One unit of DNase activity was defined as that amount of enzyme which under specified conditions would give the value $K = 1.0 \times 10^{-3}$.

A similar viscosimetric procedure was developed by McCarty (12). A 0.1 per cent solution of calf thymus DNA in veronal buffer (pH 7.5) was placed together with the enzyme extract to be tested into an Ostwald viscosimeter. Further modifications of the viscosimetric technique have been described, (49, 50, 51, 52).

2. Colorimetric method

A colorimetric procedure for determining DNase production was devised by Allfrey and Mirsky (27). This method was based on a colorimetric determination of acid-soluble desoxypentose-purine compounds released by enzyme degradation, reacting with diphenylamine reagent. The optical density was read at 600 mμ and the optical densities obtained were converted to desoxy-pentose-phosphorus equivalents by comparison with a standard.

3. Agar plate techniques

a) HCl precipitation method

Jeffries et al. (45) devised a rapid agar plate method for determining DNase production. Their medium contained NaCl, glucose, $MgSO_4$, $FeSO_4$, agar and DNA in distilled water. The organisms to be tested were incubated on the surface of the gelled medium for 24 hr. The enzymatic activity was then assayed by flooding the plates with a small volume of 1.0 N HCl. DNase production was indicated by a clear zone around the colony. Clear zones represented areas of the acid-soluble hydrolytic products of enzyme degradation, whereas cloudy areas represented acid-insoluble DNA. The diameter of the clear zone was related to the amount of extracellular enzyme produced.

A micromethod for determining DNase production was introduced by Jarvis and Lawrence (43). A hot solution containing agar and calf thymus DNA was mixed with hot tris-HCl buffer (pH 8.7) and $CaCl_2$. One ml of this hot mixture was spread on a microscope slide and outlined with cellulose tape. A hole was bored in the agar and a few drops of a broth culture of test organisms were added to the well. The slide and test organisms were incubated for 20 hr, removed and dipped into 1.0 N HCl. The diameter of the clear zone was then measured using a vernier caliper.

b) Toluidine blue method

A modified test for detection of DNase activity was developed by Streitfeld et al. (30) while working with pseudomonas strains. The agar plate method of Jeffries et al. (45)

was unsuccessful in this study because of pyocyanin production. Pyocyanin, when acidified, becomes red and it becomes impossible to detect clear zones indicative of DNase activity. Toluidine blue, because of its metachromatic properties, stains intact DNA a royal blue, while the lower molecular weight products of hydrolysis are stained a bright pink color. For this reason, toluidine blue (0.1%) was used as the assay reagent for DNase production. Assay plates were flooded with toluidine blue and observed ten minutes later for bright pink zones which indicated DNase production.

Schreier (34), while working with strains of Serratia marcescens, proposed a modification of the agar plate method for detecting DNase production. The modified test medium was prepared as follows: One hundred mg of toluidine blue O were added to 1 liter of DNase test medium (Baltimore Biological Laboratories, Baltimore BBL). Strains to be tested were inoculated onto agar plates and incubated for 24 hr. After incubation, the plates were examined for bright pink zones around the test colonies which indicated the presence of lower molecular weight hydrolytic products of DNA degradation.

c) Methyl green method

Smith, Hancock and Rhoden (53) developed another agar plate technique for testing DNase production. Methyl green was added to DNase test agar (Difco) such that a final dye concentration was 0.003 to 0.008 per cent. The organisms to be tested were spotted onto the agar plates, incubated for 24 hr and observed for DNase production. DNase-producing colonies were surrounded

by a clear zone in an otherwise green agar. The mechanism of the reaction is explained as follows: Methyl green combines with polymerized DNA to form a stable, colored complex at pH 7.5. If the DNA is hydrolyzed (depolymerized), the methyl green is freed to a colorless compound (54). This principle has been known for some time and methyl green has been used as a nuclear stain in histochemical preparations (55).

d) Acridine orange method

An overlay technique using acridine orange has been developed by Lachica and Deibel (56). Test organisms were streaked onto plate-count agar (Difco) or tellurite glycine agar and incubated for 24 hr at 37 C. The plates were then overlaid with an acridine orange-calf thymus DNA medium and incubated for 3 hr. To detect fluorescence, the plates were examined in the dark using an ultraviolet lamp. The intact DNA-acridine orange complex produced a light green fluorescence while clear halos developed around colonies that produced DNase.

4. Indigogenic reaction

Wolf, Horwitz, Mandeville, Vazquez and Von Der Muehll (57) have described a new method for detection of DNase production using a specific substrate: 5-bromo-4-chloro-3-indolyl-thymidine-3'-phosphate. A positive reaction for DNase activity is indicated by the production of a blue-green indigo color formed by the cleavage of the substrate at the site of attachment of the indolyl ring to the thymidine-3'-phosphate. Working with cultures of staphylococci, they were able to show that only S. aureus

produced a blue-green indigo color. The color developed within 4 hr and intensified after overnight incubation.

MATERIALS AND METHODS

A. Solutions and media

1. Thioglycollate broth without indicator

Thioglycollate broth without indicator,[1] was used to store stock cultures of the organisms tested. Using a sterile loop, organisms from a thioglycollate broth culture or blood agar plate were inoculated into a tube of thioglycollate without indicator. Following incubation for 3 hr at 37 C, the tubes were removed and stored at room temperature in the dark. Uninoculated tubes of thioglycollate were also kept at room temperature in the dark.

2. Enriched thioglycollate broth

Tubes containing 10.0 ml of thioglycollate broth without indicator were enriched by adding 0.5 ml of horse serum.[2] The horse serum had been inactivated by incubation for 30 min at 56 C. This enriched thioglycollate medium was used for preparing 24 hr cultures of the organisms to be studied.

3. DNase test agar

DNase test agar was prepared from the commercial formulation of Difco Laboratories and was used as the basic substrate in most of the experiments described in this paper. This medium, which contains 0.2 per cent calf-thymus DNA, was prepared according to the manufacturer's directions. Various

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[1] Difco Laboratories, Detroit, Michigan.

[2] Microbiological Associates, Albany, California.

test substances i.e., $CaCl_2$,[1] $MgCl_2$,[1] glucose[1] and dextran[2] (type 100-C) were added to the DNase test agar in the following concentrations: 0.01 per cent, 0.10 per cent, 0.50 per cent and 1.00 per cent. After autoclaving for 15 min at 250 C, the agar was cooled to 45 C and poured into 15 X 60-mm plastic petri dishes. Toluidine blue agar plates were prepared exactly as described above except that before autoclaving, 100 mg of toluidine blue[3] were added to each liter of DNase test agar.

4. Defined media

Several defined media were used in this research. The basal medium was prepared as follows:

Component	Amount
Calf-thymus DNA[4]	2 g
Bacto-tryptone[5]	15 g
Bacto-agar[5]	20 g
NaCl[1]	5 g
Distilled water	1000 ml

The resulting pH of this medium was 7.3. Three more defined media were prepared exactly as described above except that the pH of the media was adjusted to 6.0, 7.0 or 8.0. The pH was adjusted using either 0.25 N NaOH or 0.25 N HCl. The pH

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1 Mallinckrodt, New York, New York.

2 Sigma Chemical Company, St. Louis, Missouri.

3 Allied Chemicals, New York, New York.

4 Mann Research Laboratories, New York, New York.

5 Difco Laboratories, Detroit, Michigan.

adjustment was made just prior to autoclaving. The final pH after autoclaving was shown to have varied by no more than 0.1 pH units. Additional defined media were prepared exactly as described above except that 100 mg of toluidine blue were added to each 1000 ml of DNase test agar solution before autoclaving.

B. Equipment

1. Anaerobic system

GasPak anaerobic jars, GasPak envelopes and anaerobic indicators were purchased from BBL. This anaerobic system, which provided an atmosphere of CO_2 and H_2, was used according to the manufacturer's directions. The disposable anaerobic indicators were stored at 4 C until used.

2. Petri dishes

The sterile 15 X 60-mm plastic petri dishes used in this research were obtained from Falcon Plastics.

3. Syringes

The syringes used were plastic disposable 1-ml syringes, equipped with a 25-gauge needle. These syringes were purchased from the American Hospital Supply Company.

4. Pipets

All pipets used were plastic and were obtained from Falcon Plastics.

C. Clostridial species studied

The organisms used in this research were all stock cultures obtained from the National Communicable Disease Center, United States Public Health Service, Department of Health Education

and Welfare, Atlanta, Georgia (NCDC). Those organisms used were Clostridium novyi Type A, Cl. capitovale and Cl. tetani. Biochemical tests performed on the above stock cultures confirmed their identity. Each test culture was examined at regular intervals for evidence of contamination.

D. Inoculation and incubation procedures

Before inoculation, the agar plates were placed into a 37 C incubator for twenty minutes with the covers ajar. This allowed excess moisture to be removed, thus preventing the spread of organisms in the surface liquid. A 24-hr growth of the organism to be tested was mixed well on a vortex mixer to insure an equal distribution of cells. To each plate, exactly 3 drops of liquid from a 1-ml tuberculin syringe equipped with a 25-gauge needle were dropped onto the center of the agar plate. Care was taken not to touch the tip of the needle to the liquid on the agar surface. Each of the agar plates was then incubated anaerobically at 37 C for 48 hr.

E. Inoculum size and colony counts

Colony counts were performed to demonstrate the fact that a varying inoculum size would produce no significant difference in the number of viable cells at the end of a 24-hr incubation period. Tubes of thioglycollate without indicator were inoculated with 1, 2, 3, or 5 drops of a 24-hr culture of Cl. novyi Type A. These tubes were then incubated for 24-hr at 37 C. Serial 10-fold dilutions to 10^{-10} were performed using tubes containing thioglycollate without indicator. Brain Heart

Infusion agar[1] pour-plates were prepared in triplicate using a 1-ml inoculum from dilution tubes of 10^{-3} thru 10^{-10}. The pour-plates were incubated for 24 hr at 37 C, and colony counts were then performed.

F. Assay methods for DNase activity

1. HCl precipitation

After incubation, the agar plates which did not contain toluidine blue were assayed for DNase activity according to the method described by Jeffries et al. (45). The agar plates were flooded with 1.0 N HCl and allowed to stand for 5 minutes. At the end of this time, the plates were examined for clear zones around the colonies.

2. Toluidine blue agar

After incubation, the agar plates containing toluidine blue were observed for the formation of bright pink zones around the colonies as described by Schreier (34).

3. Measurement of zone size

To estimate DNase activity in both the HCl precipitation method and the toluidine blue agar method, zone diameters were measured with a vernier caliper.

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[1] Difco Laboratories, Detroit, Michigan.

RESULTS

A. Varying inoculum size

In order to perform multiple experiments designed to estimate the quantity of DNase produced by Clostridia, a standard inoculum size was required. Therefore, tubes of thioglycollate medium were inoculated with 1, 2, 3, or 5 drops of a 24-hr thioglycollate culture of Cl. novyi, Type A. These tubes were incubated for 24 hr at 37 C. Serial tenfold dilutions of each culture were made in thioglycollate without indicator and 1-ml of the 10^{-3} thru 10^{-10} dilutions were used to inoculate pour plates. Three pour plates were made for each dilution used. After 24 hr incubation at 37 C, colony counts were performed. Results are shown in Table 1. From Table 1, it can be seen that each of the varying inoculum sizes resulted in the same colony count. Because of this data, an inoculum size of 3 drops was arbitrarily chosen.

B. DNase activity of the stock cultures

Evidence of DNase production by the organisms used is presented in Table 2. DNase activity was assayed using the HCl method of Jeffries et al. (45). The experiment was repeated five times under similar conditions.

C. Comparison of the HCl and toluidine blue methods

Streitfeld et al. (30) showed that with cultures of Pseudomonas species, identical results were obtained when

TABLE 1

Growth of 24-hr Cultures of Cl. novyi A in Thioglycollate as Determined by Pour Plate Technique

Dilution	Colony Count Obtained When The Inoculum of The 24-hr Culture Had Been:[1]			
	1 drop	2 drops	3 drops	5 drops
10^{-3}	TNTC[2]	TNTC	TNTC	TNTC
10^{-4}	TNTC	TNTC	TNTC	TNTC
10^{-5}	TNTC	TNTC	TNTC	TNTC
10^{-6}	51.0	47.3	47.3	50.7
10^{-7}	6.0	5.0	6.0	5.0
10^{-8}	0	0	0	0
10^{-9}	0	0	0	0
10^{-10}	0	0	0	0

1 Values are the average of three determinations. In no case was the variation greater than 2 colonies from the average.

2 TNTC: Too numerous to count.

TABLE 2

Deoxyribonuclease Activity of Three Clostridial Species[a]

Expt. No.	Zone Diameters of DNase Activity[b]		
	Clostridium novyi A	Clostridium capitovale	Clostridium tetani
1	16.0	14.5	16.0
2	16.0	14.0	16.0
3	16.0	14.0	16.0
4	16.5	14.0	15.5
5	16.0	14.0	16.0
Average	16.1	14.1	15.9

[a] Using the HCl method.

[b] Zone diameters are expressed in mm to the nearest 0.5 mm.

DNase production was measured using the HCl and toluidine blue overlay procedures. In the research reported here, toluidine blue was present in the medium during the production of DNase and therefore, it was necessary to determine whether toluidine blue affected detectable DNase. The data obtained from measurement of zones of DNase using the HCl and toluidine blue methods are shown in Table 3. Color photographs of the DNase test by both methods are shown in Plates 1 thru 4.

TABLE 3

Deoxyribonuclease Activity as Detected by The Toluidine Blue and HCl Methods

Expt. No.	Assay Method	Zone Diameters of DNase Activity[a]		
		Clostridium novyi A	Clostridium capitovale	Clostridium tetani
1	HCl	16.0	14.0	16.0
	Tol-Blue[b]	16.0	14.0	16.0
2	HCl	16.5	14.0	16.0
	Tol-Blue	16.0	14.0	16.0
3	HCl	16.0	14.0	16.0
	Tol-Blue	15.5	13.5	16.0
4	HCl	16.0	13.5	16.0
	Tol-Blue	16.0	14.0	16.0
5	HCl	16.0	14.0	16.0
	Tol-Blue	16.0	14.0	15.5

[a] Zone diameters are expressed in mm to the nearest 0.5 mm.

[b] Tol-Blue: Toluidine blue.

Plate 1

Uninoculated DNase test agar plate flooded with 1.0 N HCl.

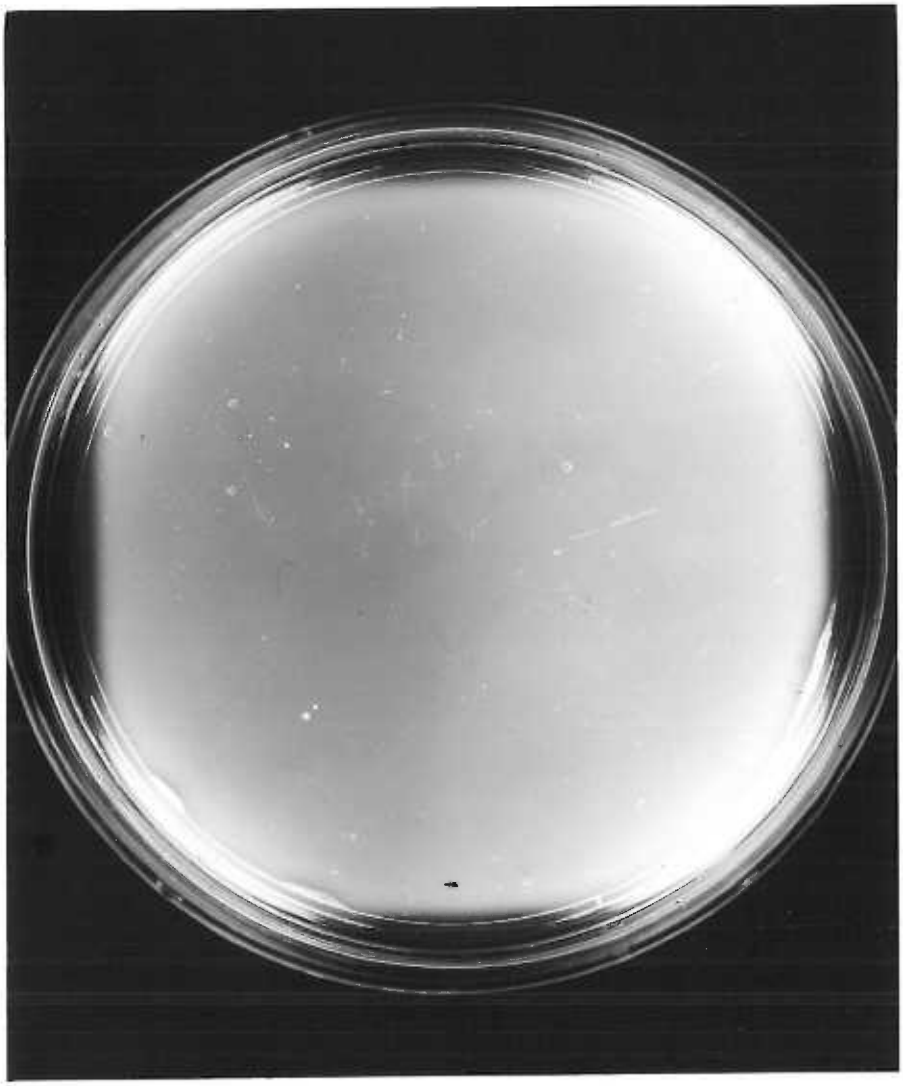


Plate 2

DNase test agar plate inoculated with Cl. novyi Type A, incubated for 48 hr, and flooded with 1.0 N HCl. Dark area shows the action of DNase. The photograph does not show the film of clostridial growth.

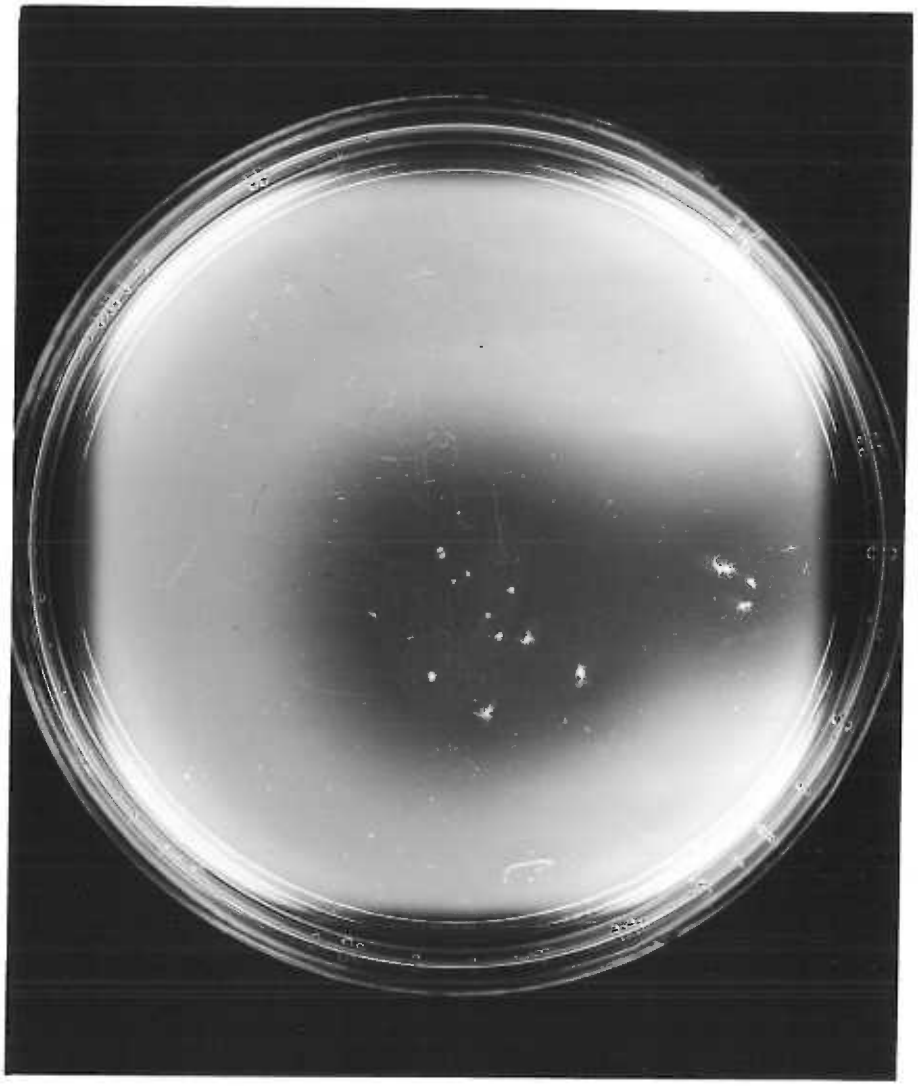


Plate 3

Uninoculated toluidine blue-DNase test agar plate.

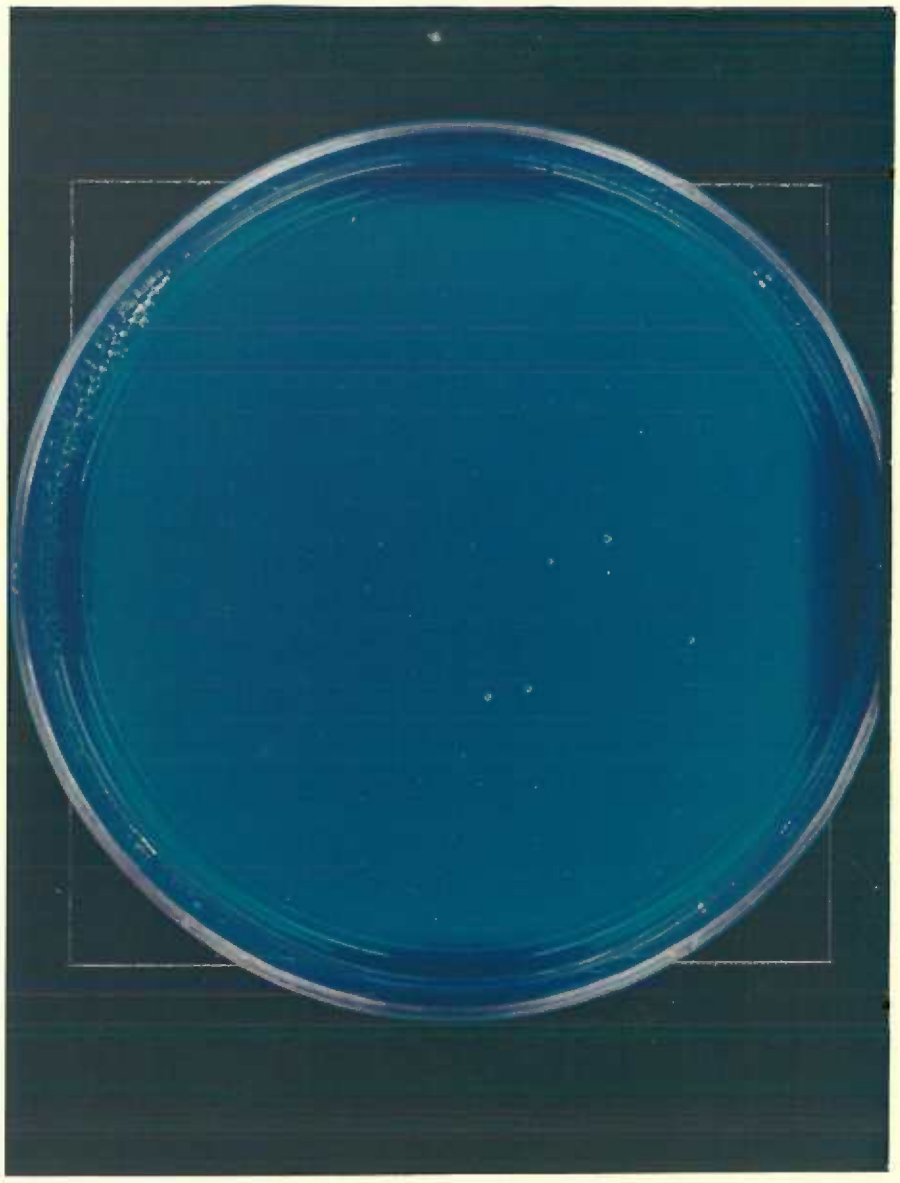
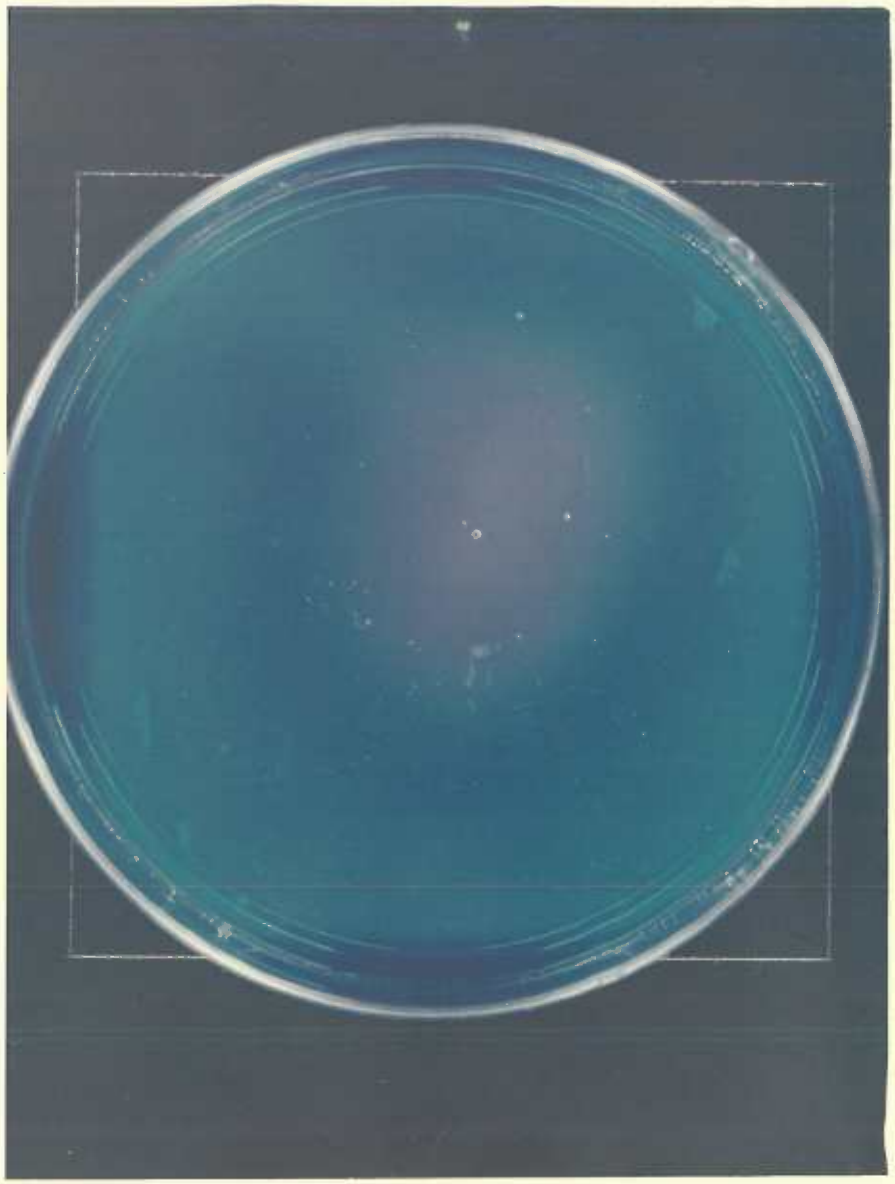


Plate 4

Toluidine blue-DNase test agar plate inoculated with Cl. novyi Type A and incubated for 48 hr. The center pink zone shows the action of DNase. The photography does not show the pink color adequately.



D. Effect of incubation time on zone diameters of DNase activity

This experiment was designed to demonstrate the effect of incubation time on zone diameters of DNase activity, to determine a suitable time which would give maximum zone diameters and to determine the earliest time of detection of DNase production. Inoculation of the plates was performed as previously described and the plates were incubated at 37 C for a selected period, removed and examined for DNase production. The results are shown in Table 4 and Figure 1.

E. Effect of pH on zone diameters of DNase activity

The purpose of this experiment was to determine the effect of pH variation on zone diameters of DNase activity. DNase test agar plates with and without toluidine blue were prepared in the usual manner and adjusted to the desired pH as described in the Materials and Methods section. Inoculation and incubation of the plates and the determination of DNase activity were performed as previously described. The results are shown in Table 5.

F. Effect of temperature on zone diameters of DNase activity

This experiment was designed to determine whether each organism studied had an optimum temperature for zone diameter of DNase activity. DNase test agar plates with and without toluidine blue were prepared as previously described. Following the usual inoculation procedure, the test plates were incubated for 48 hr at the temperature selected. DNase activity was determined by the standard methods. The results are shown in Table 6.

TABLE 4

Effect of Incubation Time on Zone Diameters of DNase Activity of Three Clostridial Species

Incubation Time (hr)	Assay Method	Zone Diameters of DNase Activity[a]		
		Clostridium novyi A	*Clostridium capitovale*	*Clostridium tetani*
10	HCl	9.0	0.0	0.0
		9.0	0.0	0.0
10	Tol-Blue	9.0	0.0	0.0
		9.0	0.0	0.0
12	HCl	10.0	9.0	9.0
		10.0	9.0	9.0
12	Tol-Blue	10.0	9.0	9.0
		10.0	9.0	9.0
15	HCl	11.0	10.5	10.0
		11.5	10.0	10.0
15	Tol-Blue	11.0	10.0	10.5
		10.5	10.0	10.0
19	HCl	12.0	11.0	11.0
		12.0	10.0	11.0
19	Tol-Blue	12.0	11.0	11.0
		12.0	11.0	11.0
24	HCl	12.5	11.5	12.0
		13.0	11.0	11.5
24	Tol-Blue	13.0	11.0	12.0
		13.0	12.0	12.0
30	HCl	14.0	12.0	13.5
		14.5	12.0	13.5
30	Tol-Blue	14.0	12.0	14.0
		14.0	12.5	13.5

Continuation of TABLE 4

Incubation Time (hr)	Assay Method	Clostridium novyi A	Clostridium capitovale	Clostridium tetani
36	HCl	15.0	13.0	14.5
		15.5	13.5	14.5
36	Tol-Blue	15.0	13.0	15.5
		15.5	13.0	15.0
48	HCl	16.0	14.0	16.0
		16.0	14.0	16.0
48	Tol-Blue	16.0	14.0	16.0
		16.0	14.0	16.0

[a] Zone diameters are expressed in mm to the nearest 0.5 mm.

Figure 1

Effect of incubation time on zones of DNase activity as determined by the toluidine blue method.

● = Cl. novyi Type A

○ = Cl. capitovale

▲ = Cl. tetani

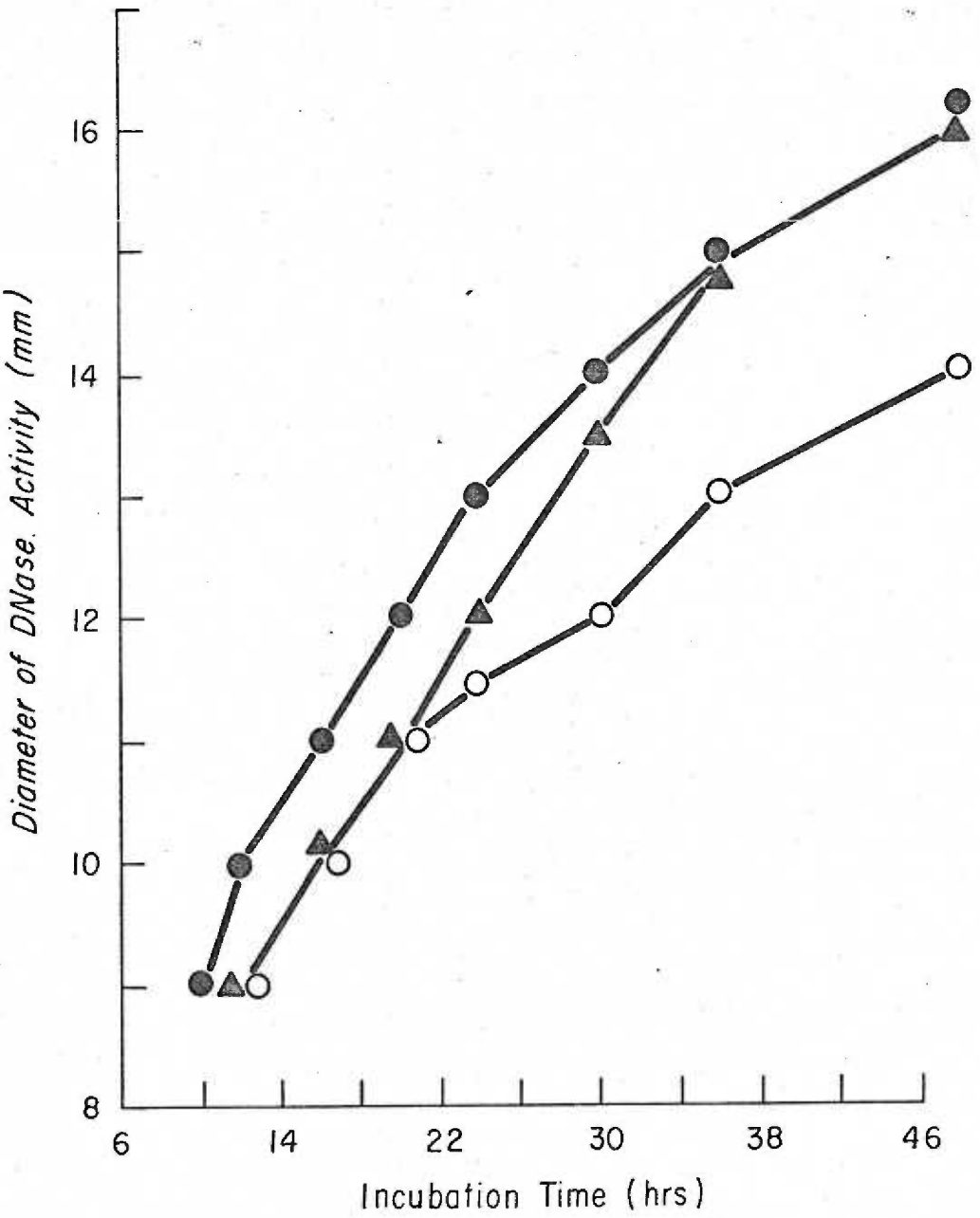

Diameter of DNase Activity (mm)
16
14
12
10
8
6
14
22
30
38
46
Incubation Time (hrs)

TABLE 5

Effect of pH on Zone Diameters
of DNase Activity of Three Clostridial Species

Assay Method	pH	Zone Diameters of DNase Activity[a]		
		Clostridium novyi A	*Clostridium capitovale*	*Clostridium tetani*
HCl	6.0	13.0 12.0	9.5 9.5	9.0 9.0
Tol-Blue	6.0	12.0 12.0	9.0 9.5	9.0 9.0
HCl	7.0	16.0 16.0	14.0 14.0	16.0 16.0
Tol-Blue	7.0	16.0 16.0	13.5 14.0	17.0 16.0
HCl	8.0	12.0 12.0	12.0 13.0	12.0 12.0
Tol-Blue	8.0	12.0 12.0	12.0 13.0	11.0 11.0

[a] Zone diameters are expressed in mm to the nearest 0.5 mm.

TABLE 6

Effect of Temperature on Zone Diameters of DNase Activity of Three Clostridial Species

Assay Methods	Temp. (C).	Zone Diameters of DNase Activity[a]		
		Clostridium novyi A	Clostridium capitovale	Clostridium tetani
HCl	30	10.0	12.0	10.0
		10.0	13.0	11.0
		11.0	12.0	11.0
		10.0	12.0	11.0
Tol-Blue	30	10.0	11.0	10.0
		10.0	12.0	10.0
		10.0	12.0	11.0
		10.0	12.0	10.0
HCl	37	16.0	14.0	16.0
		16.0	14.0	16.0
		16.0	14.0	16.0
		16.0	14.0	16.0
Tol-Blue	37	16.0	13.0	16.0
		15.0	14.0	16.0
		16.0	14.0	16.0
		16.0	14.0	16.0
HCl	42	13.0	13.0	13.0
		13.0	13.0	14.0
		13.0	13.0	14.0
		13.0	13.0	14.0
Tol-Blue	42	13.0	13.0	13.0
		13.0	13.0	13.0
		13.0	13.0	13.0
		13.0	13.0	14.0

Continuation of TABLE 6

Assay Methods	Temp. (C).	Clostridium novyi A	Clostridium capitovale	Clostridium tetani
HCl	45	11.0	10.0	9.0
		11.0	10.0	9.0
		12.0	10.0	9.0
		12.0	11.0	9.0
Tol-Blue	45	12.0	10.0	9.0
		12.0	10.0	9.0
		12.0	11.0	9.0
		12.0	10.0	9.0

[a] Zone diameters are expressed in mm to the nearest 0.5 mm.

G. Effect of $CaCl_2$ on zone diameters of DNase activity

The effect of $CaCl_2$ on zone diameters of DNase activity was determined with the HCl and the toluidine blue methods. $CaCl_2$ was incorporated into DNase test agar in various concentrations. DNase test agar plates without $CaCl_2$ were used as controls. The plates were inoculated as described, incubated for 48 hr anaerobically and assayed for DNase activity in the usual manner. The first investigation of the effect of $CaCl_2$ on DNase production using concentrations of 0.01, 0.10, 0.50, and 1.00 per cent indicated that the optimum concentration for Cl. novyi A was 0.5 per cent while the other organisms showed a progressive decrease in zone diameters of DNase activity as the concentration of $CaCl_2$ increased. Additional tests using concentrations of 0.40 and 0.60 per cent confirmed the optimum to be 0.50 per cent. The results of these experiments are summarized in Table 7 and in Figure 2.

TABLE 7

Effect of $CaCl_2$ on Zone Diameters of DNase of Three Clostridial Species

Conc. of $CaCl_2$ (%)	Assay Method	Zone Diameters of DNase Activity[a]		
		Clostridium novyi A	Clostridium capitovale	Clostridium tetani
0.00	HCl	16.0[b]	14.0	16.0
0.00	Tol-Blue	16.0	14.0	16.0
0.01	HCl	18.0	14.0	16.0
0.01	Tol-Blue	18.0	14.0	16.0
0.10	HCl	19.0	14.0	15.0
0.10	Tol-Blue	19.0	14.0	15.0
0.40	HCl	20.0	-[c]	-
0.40	Tol-Blue	20.0	-	-
0.50	HCl	22.0	14.0	12.5
0.50	Tol-Blue	22.0	14.0	12.5
0.60	HCl	21.0	-	-
0.60	Tol-Blue	21.0	-	-
1.00	HCl	18.0	11.5	11.0
1.00	Tol-Blue	18.0	12.0	11.0

a Zone diameters are expressed in mm to the nearest 0.5 mm.

b Values are the average of four determinations. In no case was the variation greater than 1.0 mm from the average.

c Not done.

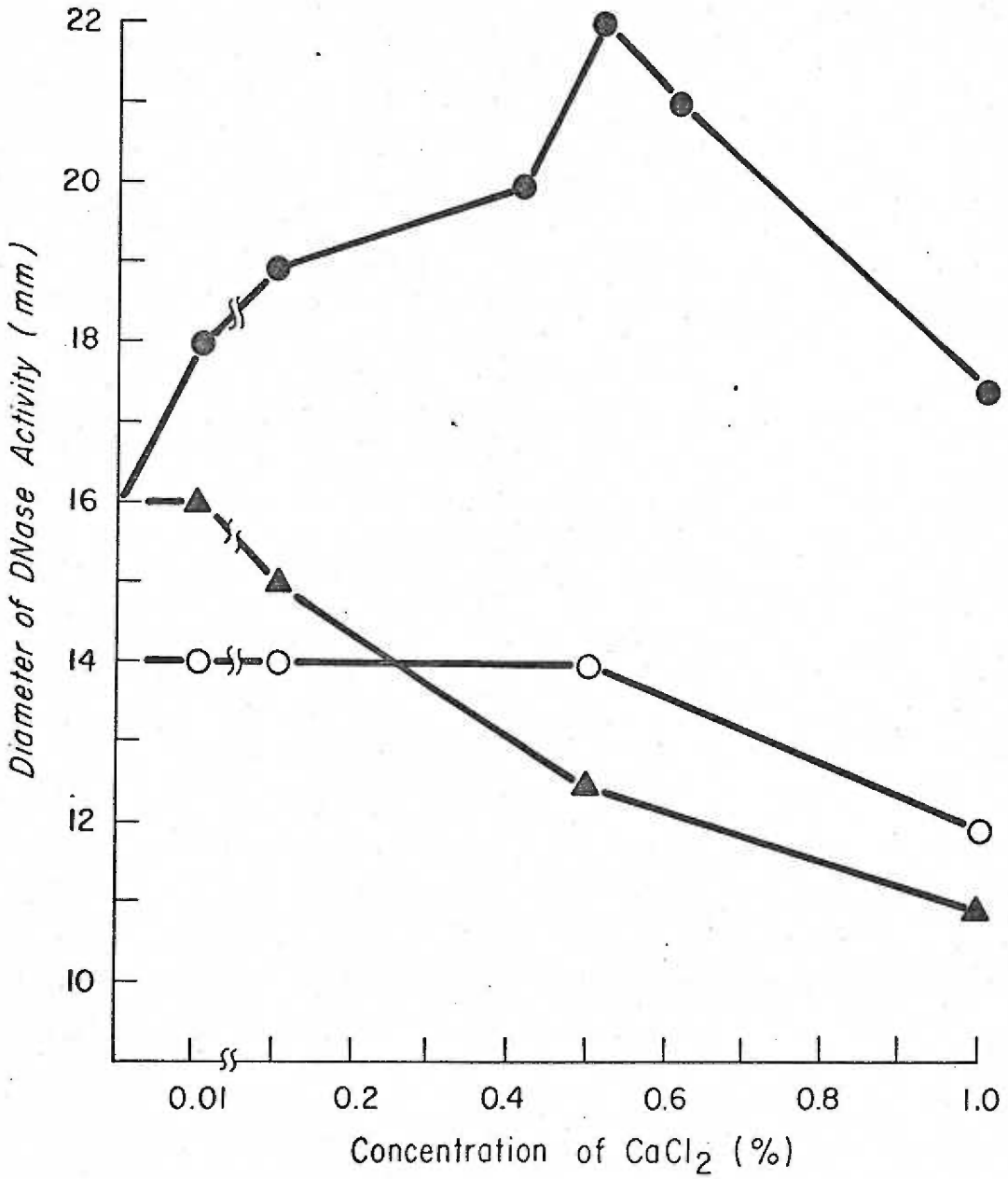
Figure 2

Effect of $CaCl_2$ on zones of DNase activity as determined by the toluidine blue method.

● = Cl. novyi Type A

○ = Cl. capitovale

▲ = Cl. tetani


22
20
18
16
14
12
10
Diameter of DNase Activity (mm)
0.01
0.2
0.4
0.6
0.8
1.0
Concentration of CaCl2 (%)

H. Effect of $MgCl_2$ on zone diameters of DNase activity

Mg^{++} and other divalent cations have been shown to exert a definite effect on DNase activity (18, 30, 42). For this reason, it was decided to investigate the effect of $MgCl_2$ on zone diameters of DNase activity of the clostridial species studied. $MgCl_2$ was incorporated into DNase test agar in varying concentrations. The initial investigation using concentrations of 0.01. 0.10, 0.50, and 1.00 per cent indicated that the optimum concentration for Cl. novyi A was 0.01 per cent. The other organisms showed a decrease in zone diameters of DNase activity as the concentration of $MgCl_2$ increased. Further tests using concentrations of 0.005 and 0.05 per cent confirmed the optimum to be 0.01 per cent. The results are shown in Table 8 and in Figure 3.

I. Effect of glucose on zone diameters of DNase activity

Working with cultures of Cl. perfringens and Cl. septicum, Mukhin et al. (42) were able to demonstrate that glucose caused an increase in DNase activity. A concentration of 0.50 per cent was optimal. It seemed worthwhile to determine if a similar effect occurred using the clostridial species studied in this experiment. When glucose was added to the DNase test agar and the usual inoculation and incubation procedures were followed, the data presented in Table 9 were observed.

J. Effect of dextran on zone diameters of DNase activity

The effect of dextran on zone diameters of DNase activity of Cl. perfringens and Cl. septicum has been investigated by

TABLE 8

Effect of $MgCl_2$ on Zone Diameters of DNase Activity of Three Clostridial Species

Conc. of $MgCl_2$ (%)	Assay Method	Zone Diameters of DNase Activity[a]		
		Clostridium novyi A	Clostridium capitovale	Clostridium tetani
0.00	HCl	16.0[b]	14.0	16.0
0.00	Tol-Blue	16.0	14.0	16.0
0.005	HCl	17.0	-[c]	-
0.005	Tol-Blue	17.0	-	-
0.010	HCl	18.0	14.0	16.0
0.010	Tol-Blue	18.0	14.0	16.0
0.050	HCl	17.0	-	-
0.050	Tol-Blue	17.0	-	-
0.10	HCl	16.0	14.0	15.0
0.10	Tol-Blue	16.0	14.0	15.0
0.50	HCl	16.0	13.0	14.0
0.50	Tol-Blue	16.0	13.0	14.0
1.00	HCl	16.0	12.0	13.0
1.00	Tol-Blue	16.0	12.0	13.0

[a] Zone diameters are expressed in mm to the nearest 0.5 mm.

[b] Values are the average of four determinations. In no case was the variation greater than 1.0 mm from the average.

[c] Not done.

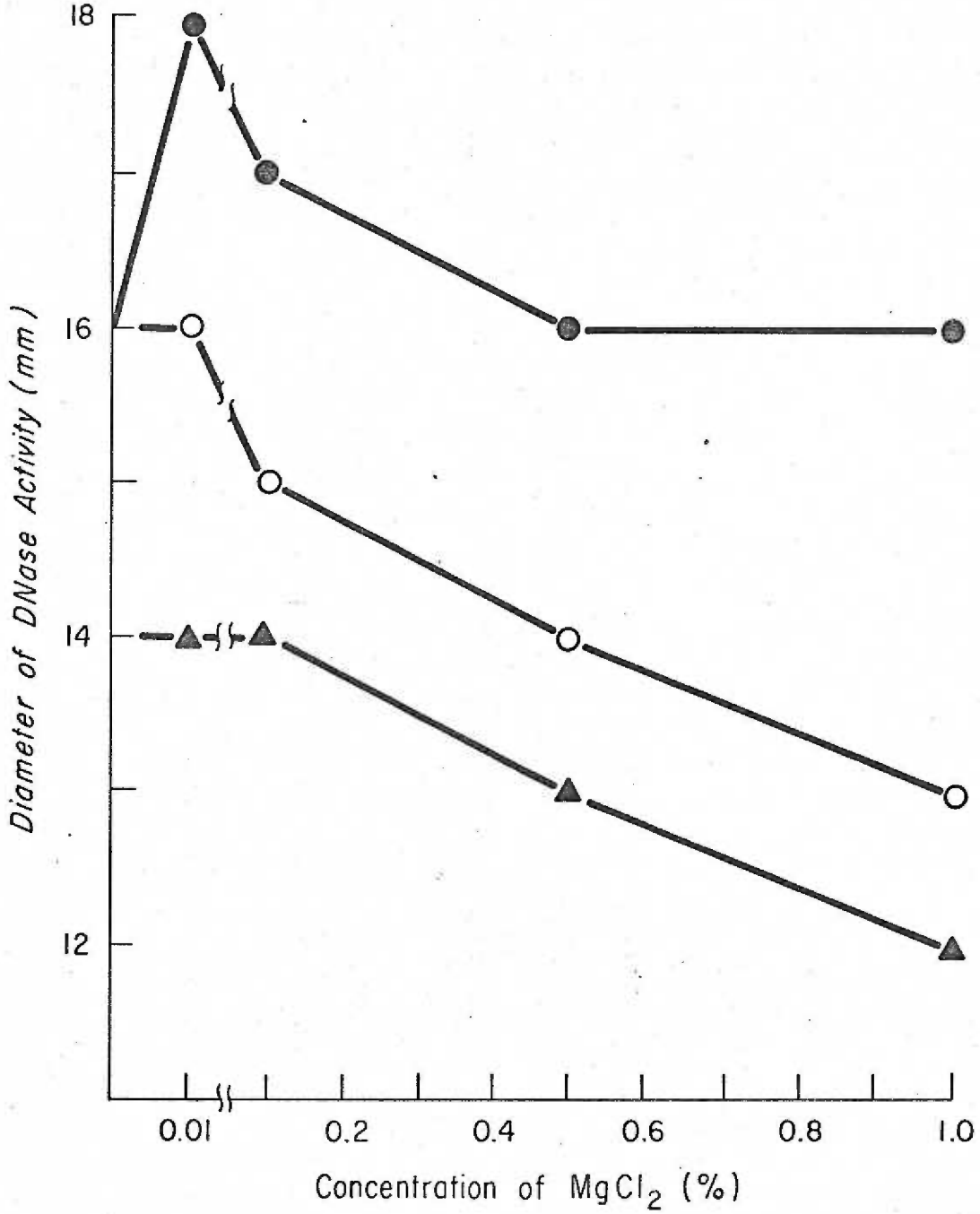

18
16
14
12
Diameter of DNase Activity (mm)
0.01
0.2
0.4
0.6
0.8
1.0
Concentration of MgCl2 (%)

TABLE 9

Effect of Glucose on Zone Diameters of DNase Activity of Three Clostridial Species

Conc. of Glucose (%)	Assay Method	Zone Diameters of DNase Activity[a]		
		Clostridium novyi A	*Clostridium capitovale*	*Clostridium tetani*
0.00	HCl	16.0[b]	14.0	16.0
0.00	Tol-Blue	16.0	14.0	16.0
0.01	HCl	16.0	14.0	16.0
0.01	Tol-Blue	16.0	14.0	16.0
0.10	HCl	16.0	14.0	16.0
0.10	Tol-Blue	16.0	14.0	16.0
0.50	HCl	16.0	14.0	16.0
0.50	Tol-Blue	16.0	14.0	16.0
1.00	HCl	16.0	14.0	16.0
1.00	Tol-Blue	16.0	14.0	16.0

[a] Zone diameters are expressed in mm to the nearest 0.5 mm.

[b] Values are the average of four determinations. In no case was the variation greater than 1.0 mm from the average.

Mukhin et al. (42). When dextran was added to the basic substrate in 0.5 per cent concentration, they were able to demonstrate a marked increase in zone diameters of DNase activity, even greater than that produced by the addition of glucose. In view of this, it was decided to investigate the effect of dextran on zone diameters of DNase activity of the organisms studied. The results of this investigation are shown in Table 10.

TABLE 10

Effect of Dextran on Zone Diameters of DNase Activity of Three Clostridial Species

Conc. of Dextran (%)	Assay Method	Zone Diameters of DNase Activity[a]		
		Clostridium novyi A	Clostridium capitovale	Clostridium tetani
0.00	HCl	16.0[b]	14.0	16.0
0.00	Tol-Blue	16.0	14.0	16.0
0.01	HCl	16.0	14.0	16.0
0.01	Tol-Blue	16.0	14.0	16.0
0.10	HCl	16.0	14.0	16.0
0.10	Tol-Blue	16.0	14.0	16.0
0.50	HCl	16.0	14.0	16.0
0.50	Tol-Blue	16.0	14.0	16.0
1.00	HCl	16.0	14.0	16.0
1.00	Tol-Blue	16.0	14.0	16.0

[a] Zone diameters are expressed in mm to the nearest 0.5 mm.

[b] Values are the average of four determinations. In no case was the variation greater than 1.0 mm from the average.

K. DNase production on a defined medium

The data in Tables 9 and 10 demonstrated that neither glucose nor dextran exerted any effect on zone diameters of DNase activity of the organisms studied. Because of the work of Mukhin et al. (42), and the possibility that the commercial medium was contaminated with glucose or dextran, it was decided that a defined medium should be prepared that did not contain glucose or dextran. This defined medium was prepared as described in Materials and Methods. The usual inoculation and incubation procedures were followed. The results are shown in Table 11.

TABLE 11

DNase Production on Defined Medium

Expt. No.	Assay Method	Zone Diameters of DNase Activity[a]		
		Clostridium novyi A	Clostridium capitovale	Clostridium tetani
1	HCl	16.0[b]	14.0	15.5
	Tol-Blue	16.0	14.0	16.0
2	HCl	16.0	14.0	16.0
	Tol-Blue	16.0	14.0	16.0
3	HCl	16.0	14.0	16.0
	Tol-Blue	16.0	14.0	15.5
4	HCl	16.0	14.0	16.0
	Tol-Blue	16.0	14.0	16.0

[a] Zone diameters are expressed in mm to the nearest 0.5 mm.

[b] Values are the average of four determinations. In no case was the variation greater than 1.0 mm from the average.

L. Effect of glucose on zone diameters of DNase activity as detected using defined medium

This experiment was designed to study the effect of glucose on zone diameters of DNase activity using a defined medium. Glucose was added in varying concentrations to the defined medium and the usual inoculation and incubation procedures were followed. The results are shown in Table 12.

TABLE 12

Effect of Glucose on Zone Diameters of DNase Activity as Detected Using a Defined Medium

Conc. of Glucose (%)	Assay Method	Zone Diameters of DNase Activity[a]		
		Clostridium novyi A	Clostridium capitovale	Clostridium tetani
0.00	HCl	16.0[b]	14.0	16.0
0.00	Tol-Blue	16.0	14.0	16.0
0.01	HCl	16.0	14.0	16.0
0.01	Tol-Blue	16.0	14.0	16.0
0.10	HCl	16.0	14.0	16.0
0.10	Tol-Blue	16.0	14.0	16.0
0.50	HCl	16.0	14.0	16.0
0.50	Tol-Blue	16.0	14.0	16.0
1.00	HCl	16.0	14.0	16.0
1.00	Tol-Blue	16.0	14.0	16.0

[a] Zone diameters are expressed in mm to the nearest 0.5 mm.

[b] Values are the average of four determinations. In no case was the variation greater than 1.0 mm from the average.

M. Effect of dextran on zone diameters of DNase activity as detected using defined medium

The effect of dextran on zone diameters of DNase activity was studied using the defined medium previously described. Dextran was added in varying concentrations and inoculation and incubation was performed in the usual manner. The results are shown in Table 13.

TABLE 13

Effect of Dextran on Zone Diameters of DNase Activity as Detected Using Defined Medium

Conc. of Dextran (%)	Assay Method	Zone Diameters of DNase Activity[a]		
		Clostridium novyi A	Clostridium capitovale	Clostridium tetani
0.00	HCl	16.0[b]	14.0	16.0
0.00	Tol-Blue	16.0	14.0	16.0
0.01	HCl	16.0	14.0	16.0
0.01	Tol-Blue	16.0	14.0	16.0
0.10	HCl	16.0	14.0	16.0
0.10	Tol-Blue	16.0	14.0	16.0
0.50	HCl	16.0	14.0	16.0
0.50	Tol-Blue	16.0	14.0	16.0
1.00	HCl	16.0	14.0	16.0
1.00	Tol-Blue	16.0	14.0	16.0

[a] Zone diameters are expressed in mm to the nearest 0.5 mm.

[b] Values are the average of four determinations. In no case was the variation greater than 1.0 mm from the average.

N. Modification of the DNase test procedure

A modified procedure for detecting extracellular DNase was developed. DNase test agar was prepared according to the commercial formulation except that 100 mg of toluidine blue were added per liter of test solution (34). After gentle heating to dissolve the agar, 10-ml aliquots were dispensed into 12 X 115 mm screw-cap tubes. The tubes were autoclaved at 115 C for 15 min, removed and cooled in slanting racks until solidified. They were stored at 4-6 C until used. To detect DNase activity, an inoculating needle was dipped into a broth culture or touched to a colony, and was streaked lightly up the slant. The tubes were incubated at 37 C for 12 hr, and examined for a bright pink coloration which appeared along the line of inoculation. If negative at 12 hr, the tubes were reincubated for 24 hr and examined again.

DISCUSSION

The DNase test has been shown to be very useful for identifying bacterial species (32, 33, 34). DNase activity has been reported for Cl. perfringens and Cl. septicum (30, 42). This study is the first demonstration of DNase activity of Cl. novyi Type A, Cl. capitovale and Cl. tetani.

A standard inoculum size was developed using a 24-hr growth of the test organism in liquid medium. After 24 hr of growth, the number of viable cells present was shown to be nearly identical whether the inoculum had been 1, 2, 3, or 5 drops of liquid culture from a 1-ml tuberculin syringe. Because of the nearly identical results obtained, it was felt that differences in DNase activity in the presence of any test variable, i.e., pH, were not due to inoculum size.

DNase activity of the clostridial species was first determined by the HCl precipitation method (45). Repeated determinations of DNase activity by this method demonstrated that Cl. novyi, Type A and Cl. tetani produced identical zones while Cl. capitovale produced a smaller zone.

It was decided to investigate a second method of detecting DNase activity, the toluidine blue method, simply because the method was reported to be easier to use (34). Because toluidine blue was present during the time of DNase production, an investigation was made to determine whether toluidine blue had any inhibitory effect on the zone diameters of DNase activity. DNase activity was measured by the toluidine blue method (34)

and a comparison was made with the results obtained by HCl precipitation. The results were essentially identical, indicating no inhibitory effect by toluidine blue.

Before inoculation, the agar plates with their covers ajar, were incubated at 37 C for 20-30 min. This step is essential if measurement of zone diameters is to be performed. If the plates are not dried, organisms spread on the agar surface and zones are produced which cannot be accurately measured.

A minimum incubation time of 10 hr was required to detect DNase activity of Cl. novyi, Type A. Both Cl. capitovale and Cl. tetani required a minimum of 12 hr incubation to detect DNase production. The zone diameters were larger and easier to measure at 48 hr. In addition, increases in the size of the zones of DNase activity due to variation of environmental factors would be more obvious at 48 hr than at 12 hr. Therefore, in the remaining experiments, 48 hr was used as the incubation time.

The effects of variation of environmental factors on the DNase of these clostridial species have been shown in the Results section. Each of these factors, i.e., pH, temperature, etc., could have affected the amount of bacterial growth, the amount of enzyme produced by each cell, the activity of the enzyme or the rate of enzyme diffusion. No attempt was made to determine the specific mode of action of each factor; however, the effect of each factor on the zone diameter of DNase activity was measured so that a more favorable medium could be made.

pH appears to have a definite effect on the zone diameter

of DNase activity. The optimum pH for each species studied was estimated to be 7.0. A change in pH to 6.0 or 8.0 was reflected by a substantial decrease in the zone diameters of DNase activity. The manner of inhibition caused by an increase or decrease beyond the optimum is not known. It is likely however, that the zone diameter of DNase activity decreased due to a decrease in cell population, since pH changes are known to cause inhibition of growth (59). When detecting DNase activity of the species studied, a pH of approximately 7.0 is recommended.

The greatest zone diameters of DNase activity occurred at a temperature of 37 C for the clostridial species studied. An increase in temperature resulted in smaller zone diameters of DNase activity. The reason for this has not been established, however, since the optimum temperature for growth is 37 C for each organism, it is likely that the amount of DNase produced decreased as the growth rate decreased. Jeffries et al. (45) and Valu (32) have reported that the optimum temperature for DNase activity for S. marcescens is 30 C. The effect of temperature on DNase activity does not appear to have been investigated by the other workers mentioned in this thesis.

Mukhin et al. (42) were able to show that glucose caused an increase in DNase activity of Cl. perfringens and Cl. septicum and that dextran caused an even greater increase in DNase activity. Based on their report of the effect of glucose on Cl. perfringens and Cl. septicum, it seemed logical to investigate the effect of glucose and dextran on Cl. novyi Type A,

Cl. capitovale and Cl. tetani. When these species were tested, no change in measurable DNase was observed. It is possible that, unlike the basal medium of Mukhin et al. (42), the commercial DNase test agar medium used in the present research was contaminated with glucose or dextran. For this reason, a defined medium was prepared which contained no glucose or dextran. Measurements of zone diameters of DNase activity on this defined medium were shown to be identical to the zone diameters produced on the commercial medium. When glucose or dextran was added to the defined medium, there were no changes in the zone diameters of DNase activity of Cl. novyi Type A, Cl. capitovale and Cl. tetani. It is not known why increases in DNase activity were observed with Cl. perfringens and Cl. septicum when glucose or dextran was added. However, it is possible that the basal medium of Mukhin et al. (42) was nutritionally poorer than my own defined medium. When they added glucose, the growth rate increased with a concomitant increase in DNase production. It is difficult to be certain of this since the composition of the basal medium used by Mukhin et al. was not reported.

Price, Stein and Moore have described the effect of divalent cations on DNase activity (58). They concluded that these are necessary for maintaining the tertiary structure of the enzyme by protecting disulfide bonds. Divalent cations promote the reactivation of reduced DNase and protect active DNase from reduction and subsequent inactivation. Because of their findings and the earlier reports of other workers (9, 14, 42, 44),

an investigation of the effect of divalent cations was performed. Ca^{++} greatly increased the zone diameters of the DNase activity of Cl. novyi Type A. The optimum concentration was 0.50 per cent. On the other hand, Ca^{++} concentrations as small as 0.10 per cent decreased the zone diameters of DNase activity of Cl. tetani. In the case of Cl. capitovale, no change in DNase was observed until slight inhibition occurred at concentrations greater than 0.50 per cent. I do not know why Ca^{++} increased the zone diameters of DNase activity of Cl. novyi Type A while it inhibited the DNase of Cl. capitovale and Cl. tetani. One possible explanation is that the DNase of Cl. capitovale and Cl. tetani require a different divalent cation to maintain tertiary structure. A second explanation is that the medium already contained an adequate concentration of Ca^{++} for Cl. capitovale and Cl. tetani and that an increase inhibited bacterial growth. In addition, the enzyme diffusion rate may have decreased because of binding Ca^{++} to the DNase or to the agar medium.

The effect of Mg^{++} on DNase activity has been investigated by several workers (9, 14, 18, 42, 44). In this study, Mg^{++} slightly increased the zone diameters of DNase of Cl. novyi Type A. On the other hand, Mg^{++} inhibited the zone diameters of DNase activity of Cl. capitovale and Cl. tetani. The optimum concentration of Mg^{++} for Cl. novyi Type A was 0.01 per cent. The data presented is not sufficient to explain why Mg^{++} inhibited the DNase of Cl. capitovale and Cl. tetani while slightly increasing the zone diameters of DNase activity for

Cl. novyi Type A. The possible explanations given for the effect of Ca^{++} may also apply to Mg^{++}.

Because of the clinical laboratory's need for a rapid DNase test and a need for a test medium with a longer storage time, an investigation of such a procedure was undertaken. The modified DNase test using toluidine blue agar in screw-cap tubes has an important advantage over the agar plate-DNase test methods: The medium can be stored at 4-6 C for at least 60 days. In addition, the time required to detect DNase activity of the clostridial species studied is 10-12 hr, the same as is required for the agar plate methods. Based upon these facts, the method is highly suitable for routine use in the clinical laboratory.

Our results indicate that the commercial test medium is suitable for detecting DNase activity of Cl. novyi Type A, Cl. capitovale and Cl. tetani. No change is proposed in the test medium; however, a change in procedure is recommended. This method is as follows:

1. DNase test agar is prepared according to the manufacturer's directions, except that 100 mg of toluidine blue are added before autoclaving (34).
2. The dissolved DNase test agar solution is dispensed into screw-cap tubes, and the tubes are autoclaved and cooled in slanting racks until solidified. The tubes may be stored at 4-6 C for as long as 60 days.
3. An inoculating needle is dipped into a broth culture or touched to a colony and is streaked lightly up the slant.

4. The tubes are incubated anaerobically at 37 C for 12 hr, removed and examined for a bright pink coloration along the line of inoculation.[1]
5. If DNase has been produced, a change from royal blue to bright pink will occur. A negative result is indicated by an absence of pink color along the line of inoculation.
6. If negative at 12 hr, the tubes are reincubated for 24 hr and examined again for the color change.

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[1] Upon removal from the anaerobe jar, the toluidine blue tubes may be colorless due to the reduction of toluidine blue by the anaerobic environment. Exposure of the agar to air for a few minutes restores the blue color.

SUMMARY AND CONCLUSIONS

This is the first demonstration that Cl. novyi Type A, Cl. capitovale and Cl. tetani produce extracellular DNase. When production of extracellular DNase was measured on DNase test agar plates, Cl. novyi Type A, and Cl. tetani produced larger zones of activity than did Cl. capitovale.

Two methods were used to detect DNase activity, namely, the HCl precipitation method of Jeffries et al. (45) and Schreier's toluidine blue method (34). Toluidine blue did not inhibit the production or the activity of the DNase's of the species studied. Zone diameters of DNase activity were shown to be nearly identical when either method was used.

In the case of Cl. novyi Type A, 10 hr were required to detect DNase activity, whereas Cl. capitovale and Cl. tetani required 12 hr of incubation before their DNase's were detectable.

The optimum pH for production of DNase by each species was 7.0, and the zones of DNase activity decreased as the pH was changed to 6.0 or to 8.0. The manner of inhibition is not known but a possible explanation was discussed.

A temperature of 37 C was shown to be optimal for DNase activity for every species. When the temperature was varied above or below this optimum, enzymatic activity decreased markedly. An explanation of this effect was presented.

Other workers (42) have found that DNase activity of Cl. perfringens and Cl. septicum increased when glucose or dextran

were added to the culture media. On the other hand, experiments described herein indicate that these substances do not affect the activity of the enzyme produced by Cl. novyi Type A, Cl. capitovale and Cl. tetani. Reasons for these differences were discussed.

Ca^{++} or Mg^{++} were shown to be necessary for maximal zones of DNase activity of Cl. novyi Type A. The optimal concentrations of these ions were determined. It was further demonstrated that Ca^{++} and Mg^{++} inhibited the DNase's of Cl. capitovale and Cl. tetani.

An evaluation was made of the commercial DNase test medium, and it was concluded that the medium is suitable for routine use in the clinical laboratory.

A modification of the DNase test was devised in which screw-cap tubes containing Schreier's toluidine blue-DNase test agar were used rather than petri dishes. The advantages of this method were discussed.

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