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# THE DEMONSTRATION AND CHARACTERIZATION OF DEOXYRIBONUCLEASES OF STREPTOCOCCI GROUP A, B, C, G AND L

By

*Roar Gudding*

GUDDING, R.: *The demonstration and characterization of deoxyribonucleases of streptococci group A, B, C, G and L.* Acta vet. scand. 1979, *20*, 102—121. — Streptococcus pyogenes, S. agalactiae, S. dysgalactiae, S. equi, S. equisimilis, S. zooepidemicus, Streptococcus group G and L were found to produce deoxyribonucleases (DNases) which were demonstrated using the Toluidine Blue DNA Agar (TDA) described for staphylococcal DNases. The activity of streptococcal DNases increased in the presence of $Mg^{++}$ and $Ca^{++}$ ions, the pH optimum was about 7.5 and native DNA was the best enzyme substrate. It is consequently recommended to modify the TDA according to these results for the demonstration of streptococcal DNases. All streptococcal DNases, except the DNase of S. zooepidemicus, were found to be heat-stable. Isoelectric focusing was a convenient technique for separation of streptococcal DNases and for estimation of the pI values of the DNases. S. agalactiae and S. dysgalactiae generally exhibited distinct species specific patterns in the isoelectric focusing experiments. The DNases produced by S. pyogenes were serologically related to the DNases of S. dysgalactiae and Streptococcus group G. A similar relationship was demonstrated between the DNases produced by S. equisimilis and Streptococcus group L.

s t r e p t o c o c c u s; d e o x y r i b o n u c l e a s e.

The production of deoxyribonucleases (DNases) by streptococci group A, B, C, F, G and L was first demonstrated by *Tillett et al.* (1948) and *Brown* (1950). Later, the DNases of group A and to some degree group C streptococci have been characterized (*Wannamaker* 1958, *Winter & Bernheimer* 1964, *Wannamaker & Yasmineh* 1967, *Wannamaker et al.* 1967 and *Smyth & Fehrenbach* 1974).

In most studies the streptococcal DNase activity has been detected by a viscosimetric method (*Mc Carty* 1949), but an agar diffusion method (*Schill & Schumacher* 1972) has also been used (*Smyth & Fehrenbach*).

DNases elaborated by staphylococci can easily be demonstrated by Toluidine Blue DNA Agar (TDA) (*Lachica et al.* 1971), and the agar described for staphylococcal DNases has also been used for the detection of Streptococcus pyogenes DNases (*Tiesler & Merklen* 1974).

The present paper deals with the demonstration of streptococcal DNases and the examination of some biochemical, biophysical and enzymoserological properties of the DNases produced by streptococci belonging to groups A, B, C, G and L.

## MATERIALS AND METHODS

### *Bacterial strains*

A total of 21 strains of streptococci were used in this study (3 strains of S. pyogenes, 6 strains of S. agalactiae, 6 strains of S. dysgalactiae, 2 strains of S. zooepidemicus and 1 strain of each of S. equi, S. equisimilis, Streptococcus group G and Streptococcus group L) (Table 2). The organisms were isolated from cases of bovine mastitis except the following ones: S. pyogenes (all strains from man), S. equi (horse), S. equisimilis (horse), S. zooepidemicus NVH* 3151 (horse) and Streptococcus group G (dog).

### *Enzymes*

The DNases were produced by cultivating the bacteria in Todd-Hewitt Broth (Difco**) for 48 hrs. at 37°C. In a preliminary study the Todd-Hewitt Broth had given the highest yield of streptococcal DNases when compared with 9 other commonly used media. The supernatants were added $(NH_4)_2SO_4$ to 80 % saturation, and the precipitates produced were dialyzed against running tap water for 18 hrs. The enzyme-containing solutions were stored at —20°C.

### *Sera*

Antibodies against DNases were produced in rabbits by 4—6 intradermal injections of equal volumes (1 ml) of adjuvant and enzyme-containing solution given at weekly intervals. Freund's Complete Adjuvant (Difco) was used in the first injection and

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* The culture collection at the Department of Microbiology and Immunology, Veterinary College of Norway, Oslo.

** Difco Laboratories Inc., Detroit, Michigan, USA.

Freunds's Incomplete Adjuvant (Difco) in the following ones. The rabbits were bled 1 week after the last injection and the sera were stored at —20°C.

*Assay of DNase activity*

Agar diffusion method. The TDA as described by *Lachica et al.* (1971) was modified for the demonstration of streptococcal DNases by the addition of $MgSO_4$ and $CaCl_2$ in concentrations of $10^{-4}$M. The pH was adjusted to 7.5 by use of Tris-HCl buffer and the agar was melted at 100°C. This test agar was used in all experiments, except those in which the influence of pH, the concentrations of $Mg^{++}$ and $Ca^{++}$ ions and heat denaturation on the enzyme activity were tested. The concentration of DNase was given in diffusion units as described by *Sandvik* (1962) for proteolytic enzymes.

Turbidimetric method. The DNase activity at different pH levels, the influence of $Ca^{++}$ and $Mg^{++}$ ions as cofactors and the effect of heat denaturation of the substrate were also tested with a modification of the turbidimetric method of *Erickson & Deibel* (1973 b). The boric acid-borax buffer was replaced with the acetate buffer, Tris-maleate buffer or Tris-HCl buffer, and the DNA* was generally resolved by heating the solution to 60°C during constant stirring.

*Properties of the enzymes*

Cofactor requirements. By use of the agar diffusion method and the turbidimetric method the activity of the enzymes was examined in the presence of $MgSO_4$ and/or $CaCl_2$ in added concentrations of $10^{-5}$M, $10^{-4}$M and $10^{-3}$M. Controls without the addition of $MgSO_4$ and $CaCl_2$ were also included. Both DNA (Difco) and DNA (Sigma)** were used as enzyme substrates in this experiment, the former even after dialysis against distilled water for 48 hrs. at 4°C. The pH was adjusted to 7.5 with 0.05M Tris-HCl buffer.

Activity at different pH levels. The effect of pH on the DNase activity was tested by the agar diffusion method and the turbidimetric method. $MgSO_4$ and $CaCl_2$ were added in concentrations of $10^{-4}$M (agar diffusion method) and $10^{-3}$M

* Difco Laboratories Inc., Detroit, Michigan, USA.
** Sigma Chemical Company, St. Louis. Mo., USA.

(turbidimetric method) in these experiments. The pH was adjusted in the range of 5.5 to 9.5 by the use of 0.1M acetate buffer (5.5), 0.05M Tris-maleate buffer (6.0—7.2) and 0.05M Tris-HCl buffer (7.4—9.5).

Heat denaturation of DNA. The enzyme activity was also studied using native and denatured DNA as enzyme substrate in both methods. In the TDA with native DNA the enzyme substrate was added as a non-heated solution to the melted and cooled agar. The TDA with heat-denatured DNA was prepared by boiling under constant stirring for 30 min.

Thermostability of the streptococcal DNases. Aliquots of 0.2 ml of enzyme solutions were transferred into 1 ml thin-walled glass ampoules which were sealed and submerged in water (45—95°C) or glycerol (100—120°C). The temperature intervals up to 110°C were 5°C and the heating time was 2 min. The stability at temperatures of 100, 110 and 120°C was also tested by heating the samples from 1 up to 60 min. at these temperatures. The DNase of S. zooepidemicus was heated at 45, 50, 55, 60 and 65°C for 0.5 to 30 min. In a separate experiment equal parts of the enzyme solutions and 0.0005 %* trypsin in 0.05M Tris-HCl (pH 8.0) were heated in glass ampoules for 2 min. at temperatures ranging from 45 to 100°C. The ampoules were transferred to an ice bath at the end of the heat exposure period. The enzyme activity was subsequently determined by the agar diffusion method. The possible production of proteolytic enzymes by the streptococci was tested by using the Casein Precipitation Test (*Sandvik* 1962). The streptococci were cultivated in Todd-Hewitt Broth and litmus milk.

Electrophoresis. A 1 % agarose gel in 0.08 M barbital buffer, pH 8.8, was prepared on a glass plate, 5×5 cm. The same buffer was also used as running buffer. Aliquots of 5 µl of the enzyme solution were applied in 2 mm wide wells in the agar. The electrophoresis was performed using a LKB** equipment at a voltage of 10 v per cm. The running time was 90 min. For the determination of the enzymes after the electrophoretic separation the zymogram technique as described for proteolytic enzymes by *Dahle* (1970) was used by pouring melted TDA on to

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* Novo Industri A/S, Copenhagen, Denmark.

** LKB Instruments Ltd., Bromma, Sweden.

the electrophoretic gel. The plates were incubated overnight at 37°C.

Isoelectric focusing. A commercially available polyacrylamide gel (LKB pH 3.5—9.5) was used. The procedure described by the manufacturer was followed with the exception that the duration of each experiment was 18 hrs. at a voltage of 200 v. The determination of the pH gradient and consequently the isoelectric points was performed according to the following procedure: Small sections of the gel were cut from both sides at regular intervals and tested individually. Tubes with the gel sections were added a small quantity of distilled water before the pH measurements. The separation of the DNases by the isoelectric focusing technique was demonstrated by the same processing system as described for the electrophoresis.

Enzymoserological differentiation. A crosswise inhibition test as described for staphylococcal DNases by *Sandvik* (1974) was used for serological analyses of the streptococcal DNases. TDA with addition of $MgSO_4$ and $CaCl_2$ in an added concentration of $10^{-4}$ M and with a pH of 7.5 was used in the enzymoserological analyses. Antisera against the DNases of S. pyogenes, S. dysgalactiae, S. equisimilis and Streptococcus group G were also included in the TDA overlayer in the isoelectric focusing experiments.

## RESULTS

DNases produced by S. pyogenes, S. agalactiae, S. dysgalactiae, S. equi, S. equisimilis, S. zooepidemicus and Streptococcus group G and L could be demonstrated with the TDA as described by *Lachica et al.* (1971) for staphylococcal DNases. It was found that the zones in that agar as a result of DNA depolymerization did not always have the characteristic pink colour, and the size of the zones was relatively small.

### *$Ca^{++}$ and $Mg^{++}$ requirements*

Increasing concentrations of $CaCl_2$ and $MgSO_4$ up to $10^{-4}$M and $10^{-3}$M caused a moderate increase in the enzyme activity when determined by the agar diffusion method and the turbidimetric method, respectively. Using the agar diffusion method the $Ca^{++}$ and $Mg^{++}$ addition increased the enzyme activity up to 4-fold compared with no addition of divalent ions. When tested

by the turbidimetric method the increase was up to 10-fold at a concentration of $CaCl_2$ and $MgSO_4$ of $10^{-3}$M. The greatest increase in activity was found when DNA (Sigma) and dialyzed DNA (Difco) were used as enzyme substrates. The enzyme activity of the DNases of S. pyogenes, S. agalactiae, S. dysgalactiae and Streptococcus group G increased relatively more at increasing concentrations of $Ca^{++}$ and $Mg^{++}$ than the DNase activity by the other streptococci tested. $Mg^{++}$ was generally a more active potentiator than $Ca^{++}$ and this observation was most evident for S. dysgalactiae. The original concentration of $Ca^{++}$ and $Mg^{++}$ in the DNA was found to be 7.6 mg/g and 1.4 mg/g (Difco) and 6.8 mg/g and 0.6 mg/g (Sigma), respectively.

*Enzyme activity at different pH values*

The relative activity of DNases of S. pyogenes, S. agalactiae and S. dysgalactiae at pH values from 5.5 to 9.5 as observed by the turbidimetric method is shown in Fig. 1. The DNases of all the other streptococci had a curve resembling that of the S. dysgalactiae DNase, and the pH optimum of all these DNases was about 7.5. There was close correlation between the results obtained by the turbidimetric and the agar diffusion method. However, when tested by the agar diffusion method, the pH area in which the DNase activity was maximal, was a little wider.

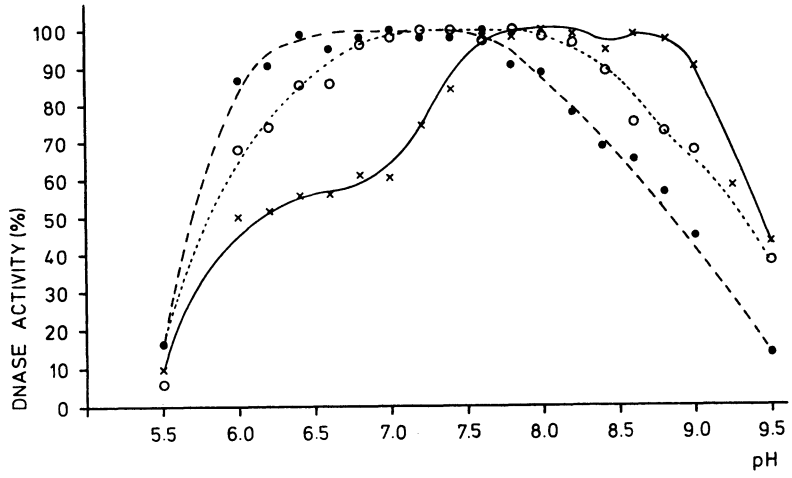



Figure 1. Relative enzyme activity of the DNases of Streptococcus pyogenes (x——x), S. agalactiae (● - - - - - ●) and S. dysgalactiae (o . . . . . o) at different pH values measured by the turbidimetric method.

*Enzyme activity on heat denatured DNA*

Native DNA was depolymerized at 4- to 8-fold higher rate than heated DNA by all the streptococcal DNases when tested by the turbidimetric method. Even in the TDA, native DNA was a more favourable substrate than heat denatured DNA as the rate was up to 4-fold higher when DNA was added as a solution after the melting of the agar than that found when the TDA was prepared with heated DNA. However, in the TDA prepared with native DNA small particles of unresolved DNA and Toluidine Blue could be seen and the colour of this TDA was generally more red compared with the blue colour of TDA prepared in the prescribed way. The colour contrast of the pink zones resulting from the cleavage of DNA was consequently less distinct when the agar was prepared with native DNA.

*Thermostability*

The heat resistance of a crude DNase solution of S. pyogenes (NVH 3144) is shown by a thermal-destruction-rate curve (Fig. 2). The thermostability of the crude streptococcal DNases is given in Table 1 expressed as D values (time at a given tempera-

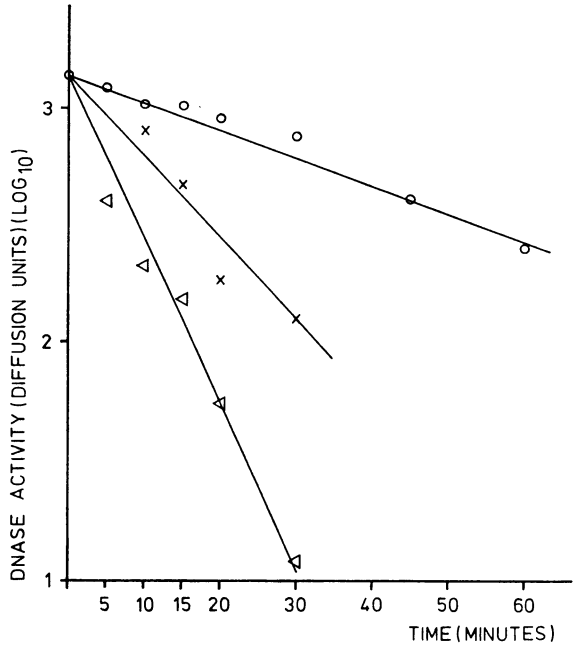



Figure 2. Thermal-destruction-rate curve for Streptococcus pyogenes DNases at 100°C (o——o), 110°C (x——x) and 120°C △——△.

Table 1. D values at 100°C, 110°C and 120°C and Z values for streptococcal DNases.

| Bacteria | | D values (minutes) at 100°C | 110°C | 120°C | Z values (°C) |
|---|---|---|---|---|---|
| Streptococcus pyogenes | (NVH 3144) | 84 | 29 | 14 | 27 |
| Streptococcus agalactiae | (NVH 3148) | 50 | 18 | 8 | 25 |
| Streptococcus dysgalactiae | (NVH 3153) | 58 | 21 | 11 | 26 |
| Streptococcus equi | (NVH 3150) | 32 | 14 | 5 | 26 |
| Streptococcus equisimilis | (NVH 3159) | 42 | 18 | 6 | 26 |
| Streptococcus group G | (NVH 3155) | 61 | 20 | 11 | 25 |
| Streptococcus group L | (NVH 3157) | 15 | 6 | | 24.5 |

ture to effect 1 log decrease in enzyme activity) and Z values (increase in degrees centigrade required to cause a 1 log decrease of the D values) (*Erickson & Deibel* 1973 a). The D values of the DNase of S. zooepidemicus (NVH 3161) at 55 and 60°C were 8 and 2 min., respectively.

With the exception of the DNase of S. zooepidemicus, all crude enzymes were thermostable when heated for 2 min. in the temperature range of 45—100°C. When the S. zooepidemicus DNase was heated under these conditions a partial reappearance of DNase activity as indicated in Fig. 3 could be observed.

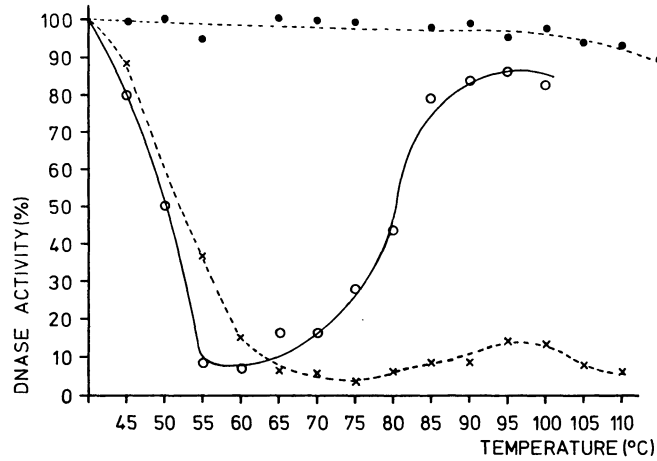



Figure 3. The DNase activity after heating for 2 min. at 5°C intervals at temperatures from 45 to 110°C. Streptococcus dysgalactiae DNase without addition of trypsin ●----●, Streptococcus dysgalactiae DNase added trypsin o——o, Streptococcus zooepidemicus DNase without addition of trypsin x----x.

When aliquots of crude DNase and 0.0005 % trypsin were heated for 2 min. at temperatures ranging from 45 to 100°C, the DNases of all streptococci were heat-sensitive in the area 55—75°C, but when heated at higher temperatures the enzyme activity reappeared more or less (Fig. 3). The curve based on the results of the heating of the other streptococcal DNases had a shape resembling that of the S. dysgalactiae DNase, but the inactivation and/or the reappearance seemed more moderate.

None of the streptococcal strains was found to produce proteolytic enzymes when grown in Todd-Hewitt Broth or in litmus milk.

*Electrophoresis*

The DNases of all streptococci tested migrated anodically in the electrophoretic system used in the present experiment (Fig. 4). The DNases of S. agalactiae, S. zooepidemicus and 1 of the

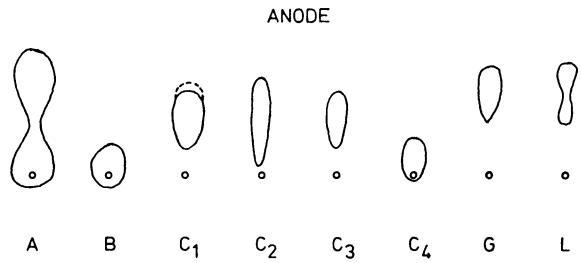
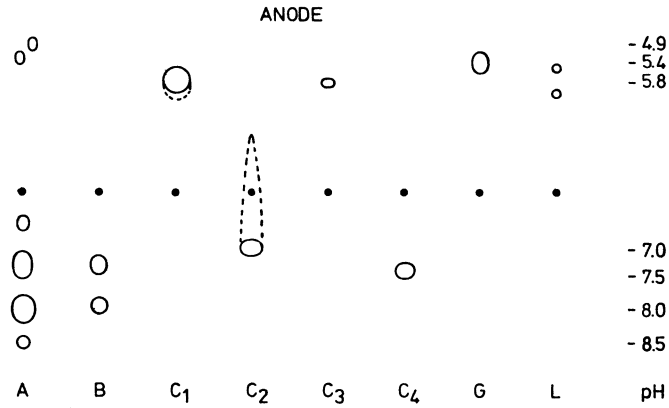



Figure 4. Gel electrophoresis zymogram developed in TDA of DNases produced by strains of streptococci. Samples: A: Streptococcus pyogenes; B: Streptococcus agalactiae; $C_1$: Streptococcus dysgalactiae; $C_2$: Streptococcus equi; $C_3$: Streptococcus equisimilis; $C_4$: Streptococcus zooepidemicus; G: Streptococcus group G; L: Streptococcus group L.

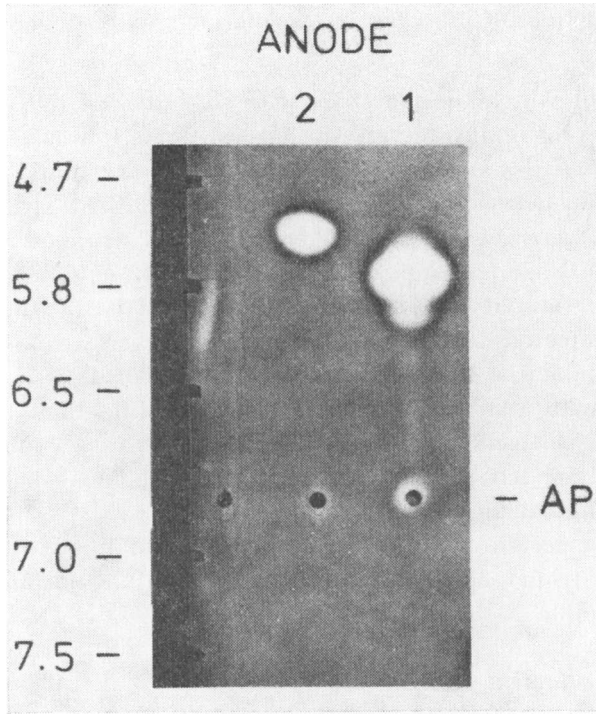
DNase fractions of S. pyogenes moved for a shorter distance than did the other streptococcal DNases tested. The DNases of S. pyogenes, S. dysgalactiae (NVH 3216) and Streptococcus group L were separated into 2 fractions by the electrophoresis.

*Isoelectric focusing*

By an isoelectric focusing technique a separation of the streptococcal DNases was performed (Fig. 6). As seen from Table 2 and Fig. 5 the DNases of S. pyogenes were separated into 5 (6)




**F i g u r e 5.** Isoelectric focusing zymogram developed in TDA of DNases produced by strains of streptococci. Samples: Same as Fig. 4.




**F i g u r e 6.** Isoelectric focusing zymogram developed in TDA of DNases produced by Streptococcus dysgalactiae NVH 3160 (1) and Streptococcus group G NVH 3155 (2). AP indicates the points of applications.

Table 2. Estimated isoelectric points (pI values) for streptococcal DNases.

| Bacteria | | pI values | | | | | |
|---|---|---|---|---|---|---|---|
| Streptococcus pyogenes | (NVH 3144) | 8.5* | 8.0 | 7.5 | 6.9* | 5.1* | 4.7* |
| Streptococcus pyogenes | (NVH 3145 and 3154) | | 7.8 | 7.2 | 6.7* | 5.1* | 4.7* |
| Streptococcus agalactiae | (NVH 3146, 3147, 3148, 3156 and 3215) | | | 8.0** | 7.5** | | |
| Streptococcus agalactiae | (NVH 3149) | | | 7.4** | 6.7** | | |
| Streptococcus dysgalactiae | (NVH 3152, 3153, 3160, 3162 and 3163) | | | 5.7 | | | |
| Streptococcus dysgalactiae | (NVH 3216) | | | 5.7** | 5.1** | | |
| Streptococcus equi | (NVH 3150) | | | 7.0 | | | |
| Streptococcus equisimilis | (NVH 3159) | | | 5.7 | | | |
| Streptococcus zooepidemicus | (NVH 3151 and 3161) | | | 7.5 | | | |
| Streptococcus group G | (NVH 3155) | | | 5.4 | | | |
| Streptococcus group L | (NVH 3157) | | | 5.9** | 5.6** | | |

* Minor fractions.
** Two fractions of the same strain with apparently similar strength.

fractions of which the fractions with pI values of 8.0 (7.8) and 7.5 (7.2) were quantitatively the largest ones. It was possible to separate 1 of the strains (NVH 3144) into 6 fractions, the sixth one with an isoelectric point at about pH 8.5. The strains of S. agalactiae were generally homogeneous as all strains were found to have 2 DNase fractions with approximately equal strength of the enzyme activity. With 1 exception (NVH 3216) the DNases of S. dysgalactiae had 1 main fraction. However, in most strains of S. dysgalactiae tested a smaller fraction of DNase could be observed with a slightly higher isoelectric point (Figs. 5 and 6). A distinct isoelectric point for the S. equi DNase could not be demonstrated, but the main fraction of the enzyme had an isoelectric point at approx. pH 7.0.

The isoelectric points for the DNases of the streptococci, estimated from repeated isoelectric focusing experiments, are given in Table 2.

*Enzymoserological analysis*

The activity of DNases produced by the 21 streptococcal strains was tested in the presence of antibodies against the DNases of 6 different streptococcal species. The results are presented in Table 3.

Table 3. Enzymoserological analyses of DNases of strains of streptococci.

| DNases produced by | Antibodies against DNases from: S. pyogenes (NVH 3144) | S. agalactiae (NVH 3148) | S. dysgalactiae (NVH 3152) | S. dysgalactiae (NVH 3153) | S. dysgalactiae (NVH 3216) | S. equi (NVH 3150) | S. equisimilis (NVH 3159) | Streptococcus group G (NVH 3155) |
|---|---|---|---|---|---|---|---|---|
| Streptococcus pyogenes (NVH 3144) | +++* | — | — | — | ++ | — | — | (+) |
| Streptococcus pyogenes (NVH 3145 and 3154) | +++ | — | — | (+) | ++ | — | — | (+) |
| Streptococcus agalactiae (NVH 3146, 3148, 3156 and 3215) | — | ++ | — | — | — | — | — | — |
| Streptococcus agalactiae (NVH 3147 and 3149) | — | — | — | — | — | — | — | — |
| Streptococcus dysgalactiae (NVH 3152, 3153, 3160, 3162 and 3163) | ++ | — | +++ | +++ | +++ | — | — | — |
| Streptococcus dysgalactiae (NVH 3216) | +++ | — | — | — | +++ | — | — | + |
| Streptococcus equi (NVH 3150) | — | — | — | — | — | + | — | — |
| Streptococcus equisimilis (NVH 3159) | — | — | — | — | — | — | ++ | — |
| Streptococcus zooepidemicus (NVH 3151 and 3161) | — | — | — | — | — | — | — | — |
| Streptococcus group G (NVH 3155) | ++ | — | — | — | ++ | — | — | +++ |
| Streptococcus group L (NVH 3157) | — | — | — | — | — | — | + | — |

* (+), +, ++, +++ different degrees of inhibition by the antibodies against the DNases.

— enzymes not inhibited by the antibodies against the DNases.

When antibodies against the DNases of S. pyogenes (NVH 3144) were included in the TDA overlayer after separation of the DNases by isoelectric focusing, a complete inhibition of the DNases of S. pyogenes and S. dysgalactiae (NVH 3216) and a partial inhibition of the DNases of S. dysgalactiae (except. S.

dysgalactiae (NVH 3216)) and Streptococcus group G was observed. The antibodies against the DNase of S. dysgalactiae (NVH 3153) neutralized the activity of the homologous enzyme, and also the fraction of the DNase of S. dysgalactiae (NVH 3216) with the same isoelectric point as the DNase of S. dysgalactiae (NVH 3153). A slight inhibition of S. pyogenes (NVH 3144) DNase fractions with low pI values could be seen. When antibodies produced against S. dysgalactiae (NVH 3216) were added to the TDA overlayer, the DNases of S. dysgalactiae (NVH 3153 and 3216), Streptococcus group G and the fractions of the DNases of S. pyogenes (NVH 3144) with the lowest pI were neutralized and the other fractions of the S. pyogenes DNase were only inhibited. The antienzymes against Streptococcus group G neutralized, in addition to the homologous enzyme, also the activity of the fraction of S. dysgalactiae (NVH 3216) and the fractions of S. pyogenes (NVH 3144) with the lowest pI's. The activity of the fraction of the DNase of Streptococcus group L with the lowest pI was neutralized by the S. equisimilis antienzymes.

## DISCUSSION

Enzyme analyses may successfully be performed by agar diffusion methods which due to their sensitivity, simplicity and reproducibility have advantages compared with tube methods. In the TDA, described for examinations of staphylococcal DNases, the pink zones which develop in the agar are a result of a specific activity of enzymes which depolymerize DNA. However, both the size of the zones in the agar and even the colour of the zones reflect the composition of the agar in relation to the properties of the enzyme. In order to improve the TDA for the demonstration of streptococcal DNases the following properties were given special attention: the influence of $Ca^{++}$ and $Mg^{++}$ cofactors, the pH of the reaction and the state of the DNA.

The requirement for $Mg^{++}$ in addition to $Ca^{++}$ for maximal activity is a common characteristic for all streptococcal DNases included in this experiment. A concentration of $10^{-3}$M was found to be optimal when the turbidimetric method was used. This is in accordance with the data presented by *Wannamaker & Yasmineh* (1967) who studied the DNases of S. pyogenes using a viscosimetric method. In the TDA the addition of $Ca^{++}$ and $Mg^{++}$ to a concentration of $10^{-4}$M was found to be sufficient for maxi-

mal activity. The enzyme acts on the substrate for a longer time in the agar diffusion method than in the tube method and this may explain that a lower concentration of cofactors is sufficient.

The content of $Mg^{++}$ and especially of $Ca^{++}$ in the DNA generally used in this work (DNA, Difco) was so high that the TDA could be used with acceptable results even without the addition of cofactors. As there are differences between DNA of various origins and possibly also between different batches of production, the addition of both $Ca^{++}$ and $Mg^{++}$ is generally recommended for streptococcal DNase analyses.

When DNases produced by different streptococci are to be examined a pH of 7.5 is recommended. In the TDA with pH 9.0 all streptococcal DNases except the S. agalactiae DNase may be demonstrated successfully. The latter enzyme may, however, give zones which are distinctly smaller and with less evident pink colour. The shape of the pH curve of S. pyogenes DNases (Fig. 1) probably reflects the pH optima and the relative enzyme activity of the different fractions of the enzyme. According to *Wannamaker* (1958) and *Wannamaker et al.* (1967) the DNases designated A, B and D all have pH optima in the range pH 7—9 and these 3 fractions, or 2 of them, seemed to predominate in the S. pyogenes strains examined in the present work.

Native DNA is a more favourable substrate than heat-denatured DNA for all streptococcal DNases tested. This property is of special importance for DNase analyses with the turbidimetric method.

The TDA is originally prepared for staphylococcal DNases which prefer heated DNA as enzyme substrate. A successful preparation of TDA with native DNA is, however, very difficult as the blue colour of TDA is a result of interactions between Toluidine Blue, DNA and agar (*Lachica et al.* 1971). When the unheated DNA is mixed with the agar after it has been melted, small particles often appear, and the agar medium does not get the characteristic blue colour because the heat treatment also seems to be essential for establishing the interactions, first of all between the agar and DNA. The practical consequence of this is that TDA prepared for the detection of streptococcal DNases should not be heated to more than 100°C and the heating time should be as short as possible.

The D values for streptococcal DNases as presented in Table 1, are indicative of a high heat stability. The thermostability of

the streptococcal DNases is not unique, as the D values at 100°C of Staphylococcus aureus DNases was 180 min. (*Erickson & Deibel* 1973 a). The thermostability of the S. aureus DNases has been suggested to be used for diagnostic purposes. The present results of heat stable DNases produced by streptococci show that a diagnosis should not be based on DNase thermostability as the only criterion.

According to *Wannamaker & Yasmineh* who examined the thermostability of the DNases of 3 strains of S. pyogenes, the enzymes were generally sensitive to heat, as 65°C for 10 min. inactivated 2 of the fractions of the enzyme completely. In the present experiment the heat sensitivity of S. zooepidemicus DNase was found to be comparable with the results of *Wannamaker & Yasmineh.*

The instability of the DNases at temperatures between 55°C and 80°C when the enzyme solutions were heated in mixture with small amounts of trypsin may be related to similar observations obtained in experiments with staphylococcal α-toxin (*Fulton* 1943), proteolytic enzymes (*Sandvik* 1962) and Bacillus cereus haemolysin (*Fossum* 1964). An explanation for the saddle-shaped heat inactivation curve may be that the trypsin at temperatures from 55 to 80°C acts on the DNase molecules at a higher rate than at lower temperatures and gives a rapid inactivation or digestion of the DNases. At higher temperatures the trypsin itself is irreversibly inactivated before it can hydrolyze and inactivate the DNases. In their experiments *Sandvik* (1962) and *Fossum* found that other proteolytic enzymes than trypsin, including proteinases of bacterial origin, could have the same effect. The saddle-shaped heat inactivation curve may contribute to an explanation of the great discrepancy between the thermostability of S. pyogenes DNases found by *Wannamaker & Yasmineh* and the results in the present work. It is reasonable to believe that strains of S. pyogenes producing proteolytic enzymes or other biocatalysts appear to be heat sensitive if they are tested at temperatures up to 65°C as done by *Wannamaker & Yasmineh.*

From Fig. 3 it may also be seen that a partial reappearance of the activity of S. zooepidemicus DNase occurred at temperatures above 80°C. Neither S. zooepidemicus nor the other streptococcal strains tested were, however, found to produce proteolytic enzymes. If the theory of interference from biocatalysts is a correct explanation for the disappearance and partial reappear-

ance of S. zooepidemicus DNase at increasing temperatures, this seems consequently to be due to other enzymes than caseinolytic ones.

The results of this experiment show that the thermostability of streptococcal DNases is dependent on the experimental conditions used, and interpretations of results concerning thermostability should consequently be done with this in mind.

By use of gel electrophoresis combined with the zymogram technique the DNases of S. pyogenes, S. dysgalactiae (NVH 3216) and Streptococcus group L were separated into 2 distinct fractions. However, zone electrophoresis and starch gel electrophoresis have previously been used for differentiation of 4 fractions of the S. pyogenes DNases (*Wannamaker* 1958, 1962). The method used in this experiment is simple and inexpensive. The results of the separation of streptococcal DNases should, however, be interpreted critically, as 1 spot may represent more than 1 DNase fraction. The electrophoresis may be used for identification and diagnostic purposes, as S. agalactiae may be separated from CAMP positive strains of group G streptococci, and S. pyogenes may be separated from other β-haemolytic streptococci by use of the electrophoretic separation pattern. However, other methods, for instance coagglutination, are more rapid for identification of streptococci.

Gel isoelectric focusing combined with the zymogram technique is a convenient tool for separation of streptococcal DNases. Isoelectric focusing seems to provide higher resolution and thus more accurate information on the charge properties of proteins than gel electrophoresis.

The observation of at least 4 fractions of S. pyogenes DNase is in accordance with the previous presentations in which the 4 DNases are designated DNase A, B, C and D (*Wannamaker* 1958, 1962, *Winter & Bernheimer* 1964 and *Smyth & Fehrenbach* 1974). It is reasonable to interpret the observation of the 2 fractions with pI 5.1 and 4.7 as a result of a division of the fraction named DNase C in the presentation by *Smyth & Fehrenbach*. The possible sixth fraction in 1 of the tested strains of S. pyogenes in the present work is quantitatively a small fraction and it seems to be a new and previously unreported fraction even if its pI is similar to that of S. pyogenes DNase B reported by *Smyth & Fehrenbach*.

The isoelectric points of the 2 other alkaline fractions of S. pyogenes DNase was 0.5—1.0 pH units lower in this work than

in the experiments of *Smyth & Fehrenbach* who also examined a group C streptococcus. A further comparison with the results of their work on group C streptococci is not possible since the species name of the group C streptococci was not mentioned in their presentation.

Based solely on the results of the isoelectric focusing experiments there might be a relationship first of all between S. dysgalactiae, S. equisimilis and Streptococcus group G and L. Two of the fractions of the S. pyogenes DNases might even be similar with DNases of S. agalactiae and S. dysgalactiae (NVH 3216), respectively.

Although 1 strain each of S. agalactiae and S. dysgalactiae, respectively, differed from the other S. agalactiae and S. dysgalactiae strains examined, these 2 species were generally found to exhibit distinct species specific patterns. However, the strains of these organisms were all isolated from cases of bovine mastitis, and isolates from other species or even other tissues might have deviating properties.

Isoelectric focusing is generally a too expensive method to be used for routine identification of streptococci. The differentiation between the β-haemolytic group C streptococci could possibly be performed successfully by this method, but as the DNase production of these streptococci is scarce, a concentration procedure may be necessary in order to obtain visible spots in the TDA. In addition, there might be strain variations in the properties of the DNases like those seen for the DNases of S. pyogenes, S. agalactiae and S. dysgalactiae, and consequently isoelectric focusing cannot be recommended for differentiation of β-haemolytic streptococci.

Non-reproducible spots, multiple bands and artefacts occurred in a few experiments, but these spots or bands were generally easily distinguished from those produced by the DNase fractions. However, whenever artefacts are suspected, repeated analyses should always be performed in order to avoid erroneous interpretations of the observations. The DNase of S. equi did not form a single spot in any of the isoelectric focusing experiments, but a reasonable explanation to this was not found.

The results of the enzymoserological analyses by the crosswise inhibition test indicate a one-way cross-reaction between the DNases of S. pyogenes on the one hand and the DNases of S. dysgalactiae (except S. dysgalactiae (NVH 3216)) and Strepto-

coccus group G on the other as the DNases of S. pyogenes were not or very slightly inhibited by the antibodies against the DNases of group C and G streptococci. The experiments in which antibodies against different streptococci were included in the TDA overlayer after isoelectric focusing separation, supplied more precise information on the relationship between streptococcal DNases. The DNases of S. pyogenes represent a complex system with several fractions, and the cross-reactions with the DNases of S. dysgalactiae and Streptococcus group G seem primarily to be related to the fractions with low pI values. The results of the experiments with the DNases of S. dysgalactiae (NVH 3216) and antibodies against this enzyme indicate that some S. dysgalactiae strains may be more closely related to S. pyogenes than others. By combining isoelectric focusing and enzymoserological examinations it could also be demonstrated that the relationship between certain strains of S. dysgalactiae (NVH 3216) and Streptococcus group G and between Streptococcus group L and S. equisimilis was related to the DNase fractions of S. dysgalactiae and Streptococcus group L with the lowest pI's. The results of the present paper consequently confirm the conclusion of *Dahle & Sandvik* (1971) that a separation procedure of the enzyme complex and a subsequent serological neutralization seem to be a useful tool in the elucidation of taxonomical questions.

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SAMMENDRAG

*Påvisning og karakterisering av deoxyribonuklease produsert av streptokokker gruppe A, B, C, G og L.*

Streptococcus pyogenes, S. agalactiae, S. dysgalactiae, S. equi, S. equisimilis, S. zooepidemicus, Streptococcus gruppe G og L produserte deoxyribonukleaser (DNaser) som ble påvist ved bruk av toluidinblått -DNA-agar. Aktiviteten av disse DNasene økte i nærvær av $M^{++}$- og $Ca^{++}$-ioner, pH-optimum var ca. 7,5, og nativ DNA var det beste enzym-substratet. Ved undersøkelse av DNaser produsert av streptokokker bør sammensetningen av TDA endres i samsvar med disse resultatene. DNaser fra alle streptokokkene, unntatt S. zooepidemicus, var varme-stabile. Isoelektrisk fokusering av DNasene var en hensiktsmessig metode for separering av disse enzymene og for å anslå pI-verdiene. DNasene fra S. pyogenes var serologisk beslektet med DNasene fra S. dysgalactiae og Streptococcus gruppe G. Et lignende slektskap ble påvist mellom DNasene fra Streptococcus equisimilis og Streptococcus gruppe L.

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