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PURIFICATION AND
CHARACTERIZATION OF
STAPHYLOCOCCAL NUCLEASE

DAVID GORDON STUART

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PURIFICATION AND CHARACTERIZATION
OF STAPHYLOCOCCAL NUCLEASE

by

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B. A., B. S., Gordon College, 1961, 1963

A THESIS

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INTRODUCTION

Since its discovery by Cunningham, et al (1956), staphylococcal nuclease has become the best known of the bacterial nucleases (Hacha and Fredericq, 1966). Among the reasons for this is that, since it was the first 3'-monoester former discovered among the deoxyribonucleases (Laskowski, 1961), it has been valuable as a probe in elucidating the structure of deoxyribonucleic acid (DNA).

Staphylococcal nuclease is an exocellular, heat stable phosphodiesterase hydrolysing single stranded DNA with the production of 30% 3'-deoxyribonucleotides. Di- and higher oligonucleotides compose the remaining 70% of the digest (Cunningham, Catlin, and Privat de Garilhe, 1956). Subsequently, Reddi (1958) found that the same enzyme also has the ability to attack ribonucleic acid (RNA). Both activities proceed under identical conditions of calcium activation at a level of 0.01 M, and an optimum pH of 9.2 (Laskowski, 1961).

These peculiar properties of the enzyme, along with the success achieved through its use as a tool in probing the structure of DNA (Alexander, Heppel, and Hurwitz, 1961), have attracted research attention to its biochemical and physical chemical properties. On the other hand, any biological effects it might possess have been almost completely overlooked.

The fact that staphylococcal nuclease was discovered in cultures of pathogenic strains of Staphylococcus aureus (Cunningham, et al 1956) and that the best producers were the most pathogenic strains (Weckman and Catlin, 1957) would indicate that this enzyme might have some role in the biology of staphylococcal virulence. This indication

was further supported by the finding of Burns and Holtman (1960), that nuclease production in S. aureus was very well correlated with coagulase production and the pathogenicity of the strain producing them.

A theoretical model explaining the role of nuclease in staphylococcal pathogenicity and virulence can be constructed through consideration of mammalian host defenses. The first line of defense includes the skin, the hyaluronic acid ground substance of tissue, and fibrin clots (Elek, 1959). S. aureus is prepared to combat these with alpha toxin to necrotize the skin (Artenstein, Madoff, and Weinstein, 1963a, 1963b; Taubler, 1963), hyaluronidase to open passageways through the intercellular cement (Lack, 1956; Rogers, 1956), and staphylokinase to dissolve the fibrin-retaining walls laid down by the host (Lack, 1948). The second line of defense consists of the phagocytic action of leukocytes, (Shayegani, Kapral, and Mudd, 1964). Staphylococcal leucocidin (Woodin, 1961) and staphylococcal coagulase (Rammelkamp and Lebovitz, 1956; and Smith, 1963) produced by S. aureus diminish the effectiveness of this important defense mechanism.

The host defense not engaged by the classical staphylococcal toxins is that mediated by antibodies which neutralize toxins and agglutinate invading cells. A mechanism whereby the nuclease might play a part in effectively reducing the antibody mediated defenses of the host can be envisioned when the modern theory of antibody formation is examined. Fishman, (1961), Fishman and Adler, (1963), and Friedman, Stravitsky, and Solomon, (1965) have shown that, after antigen has been phagocytized and partially degraded, a ribonucleic acid (RNA)-antigen complex carries necessary information and stimulatory powers to the antibody forming cells. If the RNA moiety of this complex is hydrolysed,

antibody production does not take place. Although these studies were conducted in vitro, Mowbray and Scholand (1966) have obtained in vivo results which are compatible with these.

If, indeed, antibody formation is dependent upon the transfer of information via a complex containing RNA which is indispensable to the function of the complex, the exocellular staphylococcal nuclease, known to be present in the pathogenic situation, may hydrolyse enough of the RNA of the complex to effectively diminish the immune response of the host to the S. aureus involved.

It is the object of this research to purify the staphylococcal nuclease, to investigate its effect on antibody production, and thus, its role in staphylococcal virulence.

REVIEW OF THE LITERATURE

Staphylococcal nuclease was first described under the name, micrococcal nuclease (Cunningham, Catlin, and Privat de Garilhe, 1956), because the organism from which it was isolated was then known as Micrococcus pyogenes. The officially recognized name for this organism, at present, is Staphylococcus aureus (Laskowski, 1961). The most recent designation of the enzyme is ribonucleate (deoxyribonucleate) 3'-nucleotidohydrolase, EC 3.1.4.7 (Cuatrecasas, Fuchs, and Anfinsen, 1967).

Classification of the enzyme has been just as confused as its name. Nucleases are in the class of phosphodiesterases, and the meaning of this term has changed several times in the literature (Schmidt and Laskowski, 1961). Basically, the phosphodiesterases can be subdivided as follows: (Laskowski, 1961).

Phosphodiesterases

Lipophosphodiesterases

Nucleodepolymerases

- (1) Nucleases
- (2) DNases
 - (a) DNase I
 - (b) DNase II
- (3) RNases
- (4) Exonucleases

Schmidt and Laskowski (1961) list four criteria for the classification of nucleodepolymerases: (1) sugar specificity; (2) endonucleolytic activity versus exonucleolytic activity; (3) production of either, 5'-monoester fragments or, 3'-monoester fragments; (4) and the influence of certain groups, located on the polyphosphodiester chain, upon the activity of the enzyme.

Staphylococcal nuclease hydrolyses both DNA and RNA while ex-

hibiting both endo- and exonucleolytic activity. Thus, it could be classified under any of the headings above. This is a good example of why Schmidt and Laskowski (1961) state that classification of phosphodiesterases is not clearcut.

Khorana (1961) feels that the attempt to classify these enzymes is premature because most of them have not been purified sufficiently to determine substrate specificity or mode of action.

Deoxyribonucleases (DNases) are found in many biological materials. The classic DNase is pancreatic DNase which Kunitz (1950a, 1950b) crystallized and characterized. It has been well-established that many microorganisms elaborate some kind of DNase (Jeffries, et al 1957; Wagner, 1962). Widespread surveys of DNase and RNase production were made rapid and sensitive by the plate method of Jeffries, et al (1957) and a modification of it by Burns and Holtman (1962). So many positive results have been obtained that Laskowski (1961) feels inclined to doubt the negative findings, since most of the surveys have been done on the assumption that cationic requirements similar to those of pancreatic DNase would suffice for the microbial DNases.

Most of the bacterial DNases are similar to pancreatic DNase with respect to cationic requirement and heat stability, but they can be distinguished from pancreatic DNase and from each other immunologically (Weckman and Catlin, 1957; McCarty, 1949; Wannamaker, 1958).

More than one DNase may be produced by the same microorganism. Examples of this occur in Group A streptococci (Brown, 1950; Wannamaker, 1958), Alcaligenes faecalis (Catlin and Cunningham, 1958) and E. coli (Lehman, 1959). These different DNases produced by the same organism have different specificities in terms of size, shape, and degree of

nativity of the substrate molecule.

Bacterial DNases have interesting involvements in the microbial world. Wanack, Bidwell, and Oakley (1951) reported that the beta toxin of Clostridium septicum is a DNase. This is a little misleading since they observed nucleolytic effects of the enzyme on leukocytes with the toxin present in the form of a crude filtrate of Cl. septicum. It is possible that other components of the filtrate caused initial damage to the white cells, and that the DNase, in purified form, would have no effect on them.

Kozloff (1953) found that a DNase of E. coli was inhibited by an RNA-like substance which was destroyed upon infection of the bacterial cell by T₆ phage. Thus, the DNase destroyed the host DNA leaving the metabolic machinery of the cell under the direction of the phage nucleic acid. This phenomenon was confirmed by Kunkee and Pardee (1956) in their study of the role of DNase in T₂ phage development.

Pneumococcal DNase made it very difficult for McCarty and Avery (1946) to isolate the substance, (DNA), which induced transformation of the pneumococcal types, until they discovered that citrate inactivated the DNase.

While bacterial DNases are quite prevalent, there are few reports of bacterial nucleases, enzymes which hydrolyse both DNA and RNA. Exocellular RNase activity and a DNase have been reported in soil actinomycetes, but these are undoubtedly two separate enzymes, (Muggleton and Webb, 1952).

Even though production of other bonafide nucleases have been reported for Azotobacter (Stevens and Hilmo, 1960), Serratia (Leshinskaya and Bogautdinov, 1963; Eaves and Jeffries, 1963), and Bacillus subtilis

(Birnboim, 1966) nuclease from cultures of Staphylococcus aureus differs from all the other nucleodepolymerases mentioned above. Staphylococcal nuclease is rendered unique by its calcium requirement, high pH optimum, remarkable heat stability, and low molecular weight (Cunningham, Catlin, and Privat de Garilhe, 1956; Weckman and Catlin, 1957; Cuatrecasas, Fuchs, and Anfinsen, 1967; Chesbro, Stuart, and Burke, 1966).

Much research has been done on staphylococcal nuclease and each investigator has used his own methods of purifying it. For the most part, they are modifications of the original Cunningham procedure: the medium was heated and then precipitated with ammonium sulfate, 2.5% trichloroacetic acid (TCA), and 83% ethanol. Cunningham stated, in the first publication, that the nuclease activity was dialysable, and he did not use that method of purification.

Later purification schema include the ammonium sulfate step but some of the other precipitation steps have been replaced by chromatography on various types of columns.

Hacha and Fredericq (1965) found that most of the colored material in the crude preparation is not retained on calcium phosphate, while the nuclease is tightly bound. Two sequential gradient elutions of the nuclease from calcium phosphate at 0.2-0.22 M sodium phosphate yielded a purified nuclease of high specific activity and free of alkaline and acid phosphatase.

Carboxy methyl cellulose (CM-cellulose) has been used by Mukai, et al (1965); Sulkowski and Laskowski (1966); and Taniuchi and Anfinsen (1966). Taniuchi used a linear gradient from 0.05 M sodium phosphate at pH 6.1 to 0.15 M potassium phosphate at pH 7.5 to elute the nuclease. Two such fractionations resulted in a clean peak of highly active

nuclease. On the other hand, Laskowski's group, using a gradient of 0.1 to 1.0 M ammonium acetate at pH 6.0, obtained multiple peaks on CM-cellulose. Laskowski attributed this phenomenon to the formation of complexes between nuclease and a number of inactive proteins.

DEAE-cellulose has been used with, (Mukai, et al 1965) and without, (Ohsaka, Mukai, and Laskowski, 1964; Alexander, Heppel and Hurwitz, 1961) gradients in the elution process. Alexander and Ohsaka found that 30-60% of the nuclease activity runs straight through DEAE with only a little retained. Mukai observed multiple peaks of nuclease activity when he eluted DEAE-cellulose with a gradient of 0.1 to 1.0 M Tris HCl pH 9.0. The highest specific activity was in the first peak to emerge from the column.

Mukai, et al (1965) found multiple peaks in the effluent of Sephadex G75 columns also. Sulkowski and Laskowski (1966), however, found only one peak using Bio-Gel P-100.

Taniuchi and Anfinsen (1966) observed their purified nuclease to move in a single peak upon moving boundary and free electrophoresis as well as upon ultracentrifugation. The latest ultracentrifugal data indicate a molecular weight of 17,500 for staphylococcal nuclease (Heins, et al, 1967). This is in disagreement with the earlier figure of 12,000 (Alexander, Heppel, and Hurwitz, 1961; Anfinsen, Rumley, and Taniuchi, 1963).

Disc-gel electrophoresis revealed that the most highly purified, and even crystallized, nuclease separates into two components (Taniuchi and Anfinsen, 1966). Further work on these two components has shown that the two species are interconvertible. Taniuchi states that the basis of the interconversion is still unknown.

Staphylococcal nuclease has been crystallized using ammonium sulfate (Sulkowski and Laskowski, 1966) and potassium phosphate (Cotton, Hazen, and Richardson, 1966) to salt it out of solution. Tetragonal, colorless crystals with a density of 1.25-1.30, were obtained.

Anfinsen's group has worked out the amino acid content and sequence of the nuclease molecule (Taniuchi and Anfinsen, 1966; Heins, et al, 1967). No cysteine or cystine was found. The amino acid residues occur in the following ratios per mole of phenylalanine: 21.4 lysine, 3.7 histidine, 4.6 arginine, 14.2 aspartic acid, 9.4 threonine, 6.1 serine, 17.5 glutamic acid, 6.6 proline, 10.2 glycine, 13.1 alanine, 8.6 valine, 3.6 methionine, 5.2 isoleucine, 10.3 leucine, 6.9 tyrosine, 3 phenylalanine, and 1 tryptophan. The group is now working on the structure of the active site of the enzyme.

In characterizing the enzyme, Cuatrecasas, Fuchs, and Anfinsen (1967), have reconfirmed that staphylococcal nuclease has a high pH optimum (9.2), is calcium activated (0.01 M), attacks both DNA and RNA, has an ionic strength optimum of 0.025, and an isoelectric point of 9.62. They also found the enzyme to be stable at a pH as low as 0.1, and to resist heating at 100 C for 20 min when at a concentration of 0.15 mg of more per ml.

Optical rotation data measured through the far ultraviolet region indicate that the nuclease molecule is similar to compact globular proteins such as myoglobin.

The biochemical shorthand of Smith and Markham (1952) and Heppel, Whitfield, and Markham (1955) will be used in the following discussion. According to this notation, capital letters represent nucleotides, A = adenosine, G = guanosine, etc; Pu = purine nucleoside, Py = pyrimi-

dine nucleoside, X = unknown nucleoside; small p = phosphate, in the 5' position when it precedes the capital letter, in the 3' position when it follows the capital letter, and represents 3', 5'-dinucleotide linkage when it is between the capital letters.

The mode of action of staphylococcal nuclease has been approached by several means.

Some aspects of the activity of the enzyme have been determined by the use of specific substrate.

Reddi (1958) found that staphylococcal nuclease is not active on purine or pyrimidine cyclic nucleotides. On the basis of this result, he stated that there is no possibility of this enzyme cleaving nucleic acid via transphosphorylation.

Cunningham (1959) while working with poly Ap discovered that, while poly Ap was susceptible to cleavage by staphylococcal nuclease, the dinucleotide, ApAp, resisted further degradation by the enzyme. According to Sulkowski and Laskowski (1962), dinucleotides are completely resistant to this enzyme.

Further information about the mode of action of staphylococcal nuclease has been obtained by means of identifying its products and relating them to the specificity of the enzyme.

When it was found that a staphylococcal nuclease digest yielded virtually no 5'-phosphate (Cunningham, Catlin, and Privat de Garilhe, 1956), it was assumed that the 5'-phosphodiester bond was preferentially attacked by the enzyme. This assumption was confirmed by identifying the nucleoside-3'-phosphate products of digestion, using chromatography, UV spectra, and X-ray diffraction studies (Cunningham, 1958).

A more informative use of products is the isolation of oligo-

nucleotides from nuclease digests and determination of the frequency with which particular nucleosides occur at each end of the chains. This was done by treating the oligonucleotides with venom phosphodiesterase. The 5'-OH terminus appeared as a nucleoside and the 3'-phosphate terminus appeared as a nucleoside-3',5'-diphosphate. It was found that the major components of the digests had either A or T on the terminal residue that bore the 5'-OH group (Dekker, 1960; Sulkowski and Laskowski, 1962; Rushizky and Knight, 1960; Pochon and Privat de Garilhe, 1960). Therefore, it was evident that staphylococcal nuclease had a preference for 5'-phosphodiester bonds adjacent to adenylic and thymidylic residues.

RNA digests have been found to contain the corresponding products in similar quantities (Cunningham, 1959; Reddi, 1959a; Reddi, 1959b). This reaction goes under the same conditions, but at 32% of the activity found with denatured DNA as the substrate (Cuatrecasas, Fuchs, and Anfinsen, 1967).

These methods of using products of reaction to determine the specificity of an enzyme are valid only if the specificity does not change during the course of the reaction (Sulkowski and Laskowski, 1962). Examination of the products at various intervals revealed that the specificity of staphylococcal nuclease did change with prolonged incubation (Sulkowski and Laskowski, 1962; Roberts, et al, 1962; Rushizky, et al, 1962). Evidence was found for endonucleolytic attack in areas rich in A and T during the early part of the reaction. This was in harmony with the A-T specificity described above. As the reaction proceeded, however, a gradation of preference was observed. In the case of DNA as substrate, this was: dTp > dAp > dCp > dGp, i. e., as the liberation of

thymidylic acid reached a plateau, the liberation of guanylic acid accelerated.

Finally, the extent of hydrolysis, expressed in terms of total bonds broken, was determined by measuring alkali consumption at a static pH, and from relative moles of product formed (Rushizky, et al, 1962).

The extent of degradation of DNA by staphylococcal nuclease is intermediate between that by pancreatic DNase (an endonuclease) and snake venom phosphodiesterase (an exonuclease), (Cunningham, Catlin, and Privat de Garilhe, 1956; Williams, Sung, and Laskowski, 1961). Almost the maximum theoretical hypochromicity is attained, i.e., 60-70% of the bonds are broken (Dekker, 1960).

These data indicate that there is an exonucleolytic action and an endonucleolytic action involved in the mode of action of staphylococcal nuclease.

Although dinucleotides are totally resistant to further action of the nuclease, at least one-third of the total products of DNA digest appear as mononucleotides (Sulkowski and Laskowski, 1962). These mononucleotides must have come from an exonucleolytic action of the enzyme.

Trinucleotides can serve as precursors of mononucleotides, since ApApCp gave rise to ApAp Cp with no other products formed (Roberts and Dekker, 1960). This also implies an exonucleolytic activity.

The exonucleolytic action has been found to progress from the 3'-phosphate ends of the subchains picking off mononucleotides one at a time, stopping at the dinucleotide level (Dekker, 1960; Alexander, Heppel, and Hurwitz, 1961; Sulkowski and Laskowski, 1962; Reddi, 1961; Roberts and Dekker, 1960).

A mode of action which is compatible with these data is that of

a combined endo- and exonucleolytic action on both DNA and RNA. The endonucleolytic action takes place first. It loosens the secondary structure of the nucleic acid and fragments the polymer in areas of high concentrations of A and T (U). This is followed by a rapid, step-wise removal of mononucleotides from the 3'-phosphate ends by exonucleolytic action. Which of the two activities is predominant is a matter of contention (Reddi, 1959; Sulkowski and Laskowski, 1962).

This postulated mode of action explains why staphylococcal nuclease hydrolyses heat-denatured DNA up to 100 times as fast as native DNA (Robert, et al, 1962). The denaturation does not significantly change the preference of the enzyme, but it does rupture the G-C base pairing which impedes the progress of the enzyme (Dekker, 1960).

Determination of the nucleotide sequence in nucleic acid is much more difficult than the determination of the amino acid sequence in protein. Primarily this is because there are only four nucleotides versus 20 amino acids. Thus, finding enzymes capable of attacking sites specified by three or four nucleotides in sequence is of paramount importance.

Staphylococcal nuclease shows promise in this regard, for the sites of its initial attack are specified, in part at least, by several nucleotides in sequence. Amorphous and crystalline areas, i. e., easily and difficultly denaturable areas, of the polymer are differentiated by this enzyme, because of the A-T preference and G-C resistance.

Reddi (1960) used staphylococcal nuclease in conjunction with pancreatic RNase to show significant differences between RNA's of different viruses for the first time.

Staphylococcal nuclease, along with spleen DNase, was used in

Kornberg's nearest neighbor base sequence studies of his synthetic DNA (Josse, Kaiser, and Kornberg, 1961).

Trinucleotides bearing a 5'-monophosphate group can be hydrolysed at both linkages by a large dose of staphylococcal nuclease. This produces a mononucleotide 3',5'-diphosphate, a nucleotide, and a nucleoside, thus elucidating the nucleotide sequence of the trinucleotide (Sulkowski and Laskowski, 1962).

The "left" (5'-monophosphate) terminus can always be degraded to pXp, provided an excess of staphylococcal nuclease is present. Therefore, this terminus can be identified, even in heterogeneous mixtures. In highly purified homologous chains, such as specific soluble RNA, both terminals are identifiable.

Purified staphylococcal nuclease has no adverse effects on tissue culture cells, while a filtrate of S. aureus, or nuclease in conjunction with alpha toxin, will hydrolyse the nuclei of the cells. (Jeljaszewicz, et al, 1965; Korbecki and Jeljaszewicz, 1965).

Nuclease production is closely correlated with pathogenicity in the genus Staphylococcus (Weckman and Catlin, 1957; Elston, and Fitch, 1964; Jacobs, Willis, and Goodburn, 1963; Barber and Kuper, 1951; DiSalvo, 1958; Burns and Holtman, 1960). It is agreed among these authors, that coagulase is the best single test for potential pathogenicity. Several alternate tests have been used: hemolysis, fermentation of mannitol, liquefaction of gelatin, nitrate reduction, utilization of ammonium phosphate as the sole source of nitrogen, colonial pigmentation, phenolphthalein phosphatase activity, and possession of fibrinolysin. When these characteristics were tested, it was found that nearly all strains regarded as potentially pathogenic produced DNase as

well as coagulase.

To explain the high correlation between nuclease production and the other criteria of pathogenicity in S. aureus, one might look at the role of nucleic acids in antibody formation.

Nucleic acids are involved in antibody formation at three levels: (1) The specific antibody forming potential of cells is determined by DNA. (2) Transfer of information, regulating antibody specificity, to sites of protein synthesis is mediated by messenger RNA. (3) Cells capable of forming antibodies are activated by an RNA-rich product of macrophages (Braun and Cohen, 1967).

Evidence now available indicates that RNA and/or RNA complexes are important, or even essential for initiation of an immune response. (Braun and Cohen, 1967).

Garvey and Campbell (1957) reported that antigen complexed with RNA was highly effective in initiating antibody production.

Fishman (1961), and Fishman and Adler (1963) found that antibody synthesis could be initiated in lymph node cells from non-immunized rats by incubating them in vitro with a cell-free homogenate of lymph node cells from immunized rats.

Fishman's suspicion, that the active component of the homogenate was RNA, was supported by Friedman, Stavitsky, and Solomon (1965) who also found detectable antigenic fragments in the RNA preparations. No antibody synthesis occurred when the preparation was treated with ribonuclease while trypsin and alpha- or beta-amylase had no effect on the activity of the preparation (Cohen, Newcomb, and Crosby, 1965).

It has been shown that ingestion of antigen by macrophages is a crucial step in antibody formation. This ingestion of antigen is the

first step of the immune response, while nonphagocytized antigen can cause immune paralysis (Frei, Benacerraf, and Thorbecke, 1965).

Antigen is degraded within the phagocyte before antibody synthesis begins (Uhr and Weissmann, 1965). Resulting antigenic fragments are complexed with RNA. This complexing is necessary, for Askonas and Rhodes (1965) found that a simple mixture of antigen and RNA was ineffective.

The RNA-rich material, thus formed, is transferred to cells capable of producing antibody specific for the antigen in question. Upon reaching the appropriate lymphocyte stem cell, the antigenic components of the complex react with specific antibody at the surface of the cell. This antigen-antibody reaction alters the permeability of the cell allowing the RNA moiety of the complex to enter the cell. The RNA activates the cell to produce specific antibody and to multiply, forming a clone of cells which, in turn, produce this specific antibody (Braun and Cohen, 1967).

MATERIALS AND METHODS

Cultures and cultural methods

Five strains of Staphylococcus aureus from widely differing sources were studied: the Wood 46 strain; UNH-15, a bovine mastitis strain; F.D.A. 234, a food poisoning strain; and two strains isolated from human infections, one in England and the other in France.

These strains of S. aureus were maintained on trypticase soy slants. Before use, the organism was plated out on sheep blood agar, and isolated colonies showing best hemolysis were picked for experimental use and for propagation of the strain.

The special production medium (Chesbro, Heydrick, Martineau, and Perkins, 1965) was prepared by dialysing 60 g of N-Z-Amine (Sheffield Chemical, Norwich, N.Y.) and 60 g of yeast extract (Difco) in 300 ml of distilled water against three liters of distilled water containing 7.5 g NaCl and 7.5 g KCl. Five ml of chloroform were added to prevent contamination during dialysis. After one week, 0.4% K_2HPO_4 and 0.2% sodium succinate were added to the dialysate and the pH was adjusted to 7.2. The completed medium was then dispensed and autoclaved at 15 lb for 15 min.

A 20 ml starter culture was inoculated with 3-5 colonies from the sheep blood agar plates and shaken overnight at 37 C. This culture was used to inoculate 50 ml of fresh medium which, after shaking at 37 C for 2 hr, was used to inoculate six thick-walled four L flasks containing 500 ml of medium each. After the air in these flasks had been replaced with an 80% O_2 : 20% CO_2 atmosphere, the cultures were shaken at 37 C. The O_2 was replaced after 3.5 and 7 hr of growth.

The chloroform-treated cultures were held 12 hr at 5 C and the cells then removed by centrifugation.

Nuclease purification

The culture supernatant was boiled for 15 min (Alexander, Heppel, and Hurwitz, 1961) to inactivate any acid or alkaline phosphatase and deoxyribonuclease (DNase) present, to protect the nuclease against proteolytic enzymes present, and to activate the nuclease (Ohsaka, Mukai, and Laskowski, 1964). The boiled medium was recentrifuged and the supernatant retained as the boiled crude preparation.

For $(\text{NH}_4)_2\text{SO}_4$ fractionation, the crude preparation at 5 C was saturated with $(\text{NH}_4)_2\text{SO}_4$ and the precipitate collected after 24 hr by centrifugation (insoluble activity). The supernatant was made 0.3 M in trichloroacetic acid (TCA). The resulting precipitate, collected by centrifugation and neutralized with 1 N NaOH, was dissolved in distilled water (soluble activity).

The boiled crude preparation, insoluble activity and soluble activity were put on the columns without further purification in the molecular weight studies.

For some of the early experiments, the soluble activity was partially purified by electrophoresis on polyurethan foam (Davidson, 1959).

Polyurethan foam blocks (5 x 10 x 20 cm) were filled with boiling agar, cooled, and sliced to a thickness of one cm on a bacon slicing machine. After slicing, the blocks were boiled, washed in Haemo-Sol, rinsed seven to eight times in tap water, and five to six times in distilled water. After storage overnight at 4 C in distilled water, the blocks were rinsed seven to eight times in distilled water,

and wrung out ready for use.

Electrophoresis was carried out in a Shannon chamber modified by placing a sheet of polypropylene over the partitions of the tray. Wicks cut from Whatman 3 mm filter paper were laid under the ends of the foam strips and against sponges standing in buffer.

The buffer used was 0.5% glycine adjusted to a conductivity of 5.6 millimho with NaCl. The pH ranged from 6 to 11 depending upon the experiment.

The origin blocks were loaded by placing them in plastic bags containing the sample, and squeezing while holding them submerged in the sample. A block of Agarose was poured at each end of the origin block to prevent the crude preparation from diffusing out into the purified material.

A Beckman Duostat was used to supply 30-40 mA of constant current at 300-400 volts for 12 hr per run. The chamber was cooled with cold running water.

Preliminary to the amino acid composition and in vivo studies, the insoluble and soluble activities were chromatographed on carboxy methyl cellulose (CMC) and DEAE-cellulose (Schleicher and Schuell, Keene, N.H.).

The CMC was prepared by washing it in 0.1% phosphoric acid and then adjusting the pH to 6.5 by rinsing 0.01 M Na_2HPO_4 buffer, pH 6.5, and a conductivity, (C) = 1.5 millimho, through the column until the pH stabilized at 6.5. The conductivity of the nuclease sample was lowered from 50-70 millimho to 0.2-4.0 millimho by filtration through Bio-Gel P-4. The sample was then rinsed onto the CMC column with the same phosphate buffer used to equilibrate the pH of the column. The nuclease

was removed from the column by gradient elution with NaCl using a 9-chamber gradient maker (Varigrad) in the following manner:

Tank #	Distilled water	Buffer	C	pH
1	100 ml	-	-	-
3	100 ml	-	-	-
5	100 ml	-	-	-
2	-	100 ml 0.01 M Na ₂ HPO ₄	60	6.5
4	-	"	100	7.0
6	-	"	100	7.5

The column (Pharmacia, Upsala, Sweden) was serviced by L K B pump and fraction collector. Ten ml fractions were collected by a siphon arrangement.

The DEAE was washed in 0.1% NH₄OH, distilled water, 0.01 N HCl, and then adjusted to pH 6.5 with a pH meter before packing the column. The nuclease collected from the CMC column was rinsed through the DEAE with distilled water. The nuclease remaining on the column was removed by gradient elution with NH₄OH using the Varigrad. Tanks 1 and 3 contained 50 ml of distilled water and tanks 2 and 4 contained 50 ml of 15% NH₄OH.

After column chromatography, the nuclease was lyophilized, dissolved in 0.4% TCA, then filtered through Bio-Gel P-10 in 0.4% TCA, pH 1.5. The proper molecular weight peaks were cut from the effluent of this column and re-filtered through Bio-Gel P-2 in 0.8% NaCl and 0.01 N HCl.

Measurement of the nuclease

Nuclease activity was measured by the method of Alexander, et al (1961) using heat-denatured calf thymus deoxyribonucleic acid (DNA, A grade, Calbiochem, Los Angeles, Calif.) as substrate. The assay was

carried out with the following reagents: 250 ul of 0.18 M glycine buffer pH 8.6; 25 ul of the enzyme, or dilution thereof; 25 ul of 2.24% CaCl_2 ; 200 ul of DNA which had been boiled 15 min in glycine buffer at pH 7.0, cooled rapidly in running cold water, and readjusted to pH 8.6. The reaction mixture and the substrate were warmed at 37 C separately. The reaction was started by adding the substrate at zero time. The reaction was stopped after 30 min by the addition of 0.5 ml cold 7% perchloric acid and 3 ml of cold distilled water. The unhydrolysed DNA, thus precipitated, was centrifuged at 3,000 g for 10 min. The oligonucleotides, released by the nuclease, were measured by reading the optical density of the supernatant at 260 millimicrons in a Beckman DU spectrophotometer. Nuclease activity was reported in OD units of 260 absorbance produced by the enzyme in 30 min under these conditions.

Column fractions were screened for the presence of nuclease by placing 5 ul of the fraction on DNase test agar (Difco), incubating for 2 hr at 37 C, and flooding the plate with cold 7% perchloric acid. The presence of nuclease was revealed by a clearing in the DNA precipitated by the cold perchloric acid.

Molecular weight studies on the nuclease

Ultracentrifugal analysis was performed by Mr. James J. Burke, II on a 2-3% sucrose gradient by the sedimentation velocity method of Kuff, Hogeboom, and Striebich, (1955). The sample was centrifuged at 39,500 x g for 10-12 hr in the SW39 rotor of the Spinco Model L preparative ultracentrifuge. After bottom-puncturing the centrifuge tubes, three drop fractions were collected and assayed for nuclease activity.

Gel filtration was performed with Sephadex G75 and three spherical polyacrylamide gels, Bio-Gel P-10, P-6, and P-4 (Bio-Rad laboratories, Richmond, Calif.), with approximate molecular weight fractionating ranges

of 3,000-70,000; 5,000-17,000; 1,000-5,000; and, 500-4,000, respectively. The gels were equilibrated and eluted with the 0.18 M, pH 8.6, glycine buffer used in the enzyme assay system. Sephadex G75 and Bio-Gel P-10 were standardized with muramidase and ribonuclease, proteins whose basicity approximate that of the nuclease and which would reveal any tendency of the columns to retard polycations. Bio-Gel P-6 and P-4 were standardized with bacitracin and raffinose. The void volume (V_0) and permeable volume ($V_0 + V_1$) of the columns were determined with blue dextran (Pharmacia Chemicals, Piscataway, N.J.) and methyl red (Merck, Rahway, N.J.), respectively.

Study of the amino acid composition of the 818 molecular weight form of nuclease

Hydrolysis was accomplished by acidifying the sample to 6 N with concentrated HCl, sealing it in a ignition tube, and heating it at 121 C for 12 hr.

Electrophoresis was performed on S. and S. 589 Green Ribbon C paper using an ammonium acetate buffer adjusted to pH 5.0 with acetic acid and diluted with distilled water to a conductivity of 1.0 millimho. A gradient of 16.5 V per cm was used.

One dimensional descending chromatography was performed with S. and S. 507 C paper qualitatively, and S. and S. 589 Green Ribbon C preparatively, using butanol:acetic acid:water (55:15:20). The same solvent was used in the first direction for two-dimensional chromatography and 80% phenol:concentrated NH_4OH (98:1) was used in the second direction. The two-dimensional chromatograms were washed with 300 ml of ether and acetone (1:1) and hung in flowing air to dry, on two successive days, before they were sprayed. Amino acids were identified

by the use of standard amino acids in conjunction with a polychromatic spray (Moffat and Lyttle, 1959).

Analysis of the amino acid composition of the 13,000 molecular weight nuclease

After the ammonium sulfate insoluble fraction had been chromatographed on Bio-Gel P-4, CM Sephadex C50, and DEAE-cellulose, it was lyophilized and filtered through Sephadex G75. The 13,000 molecular weight peak was cut out and precipitated with TCA after it had been rotary evaporated and reconstituted in 0.5% Na_2HPO_4 . One mg of the TCA precipitated 13,000 molecular weight nuclease was dissolved in 6 N HCl and hydrolysed for 20 hr at 110 C. This hydrolysate was then lyophilized. The lyophilized powder was dissolved in 300 ul of 0.1 N HCl plus 100 ul of norleucine as a control. Of this 400 ul sample, 200 ul were applied to the column of the Technicon Autoanalyzer.

In order to determine acid labile amino acids, a sample of the 13,000 molecular weight nuclease was hydrolysed in barium hydroxide according to the method of Ray and Koshland (1962): 500 mg of freshly ground $\text{Ba}(\text{OH})_2 \cdot 8\text{H}_2\text{O}$, with 2 ml of starch solution (1:10 dilution of 360 mg of washed (0.1 N HCl) potato starch boiled for 2 min in 1 ml of 0.5 N HCl) was added to 10 mg dry protein in 12 ml heat-resistant polyethylene tubes. The solution was stirred and the stirring rod washed with 0.1 ml of starch solution. The tubes were placed in a vacuum desiccator, flushed with N_2 , and then a vacuum of 40 mm of Hg was drawn. After 20 min, the vessel was refilled to atmosphere pressure with N_2 , and stoppered with a gum rubber stopper. The desiccator was reopened and flushed one min with N_2 before final stoppering. It was then clamped and heated at 115 C for 10 min, swirled, and returned to room

temperature for 16 hr.

One ml of 20% glacial acetic acid was added to each tube. The contents were transferred to a small beaker with 6.0 ml of water. One N NH_4OH was added to a faint phenolphthalein color. One ml of freshly prepared $(\text{NH}_4)_2\text{CO}_3$ (15.5 g of solid $(\text{NH}_4)_2\text{CO}_3$ was added to 15 ml of NH_4OH in 60 ml of water, then diluted to 100 ml) was added dropwise. The resulting pink solution was then filtered through a medium porosity funnel into a 100 ml round bottomed flask containing one ml of ammonium acetate. The precipitate in the funnel was washed several times with 4 ml of a 1:100 dilution of $(\text{NH}_4)_2\text{CO}_3$. When rotary flask evaporation had yielded a syrup, a few crystals were added to promote crystallization, and evaporation was continued to solidification. A cold finger condenser with a vacuum bypass was inserted into the flask and the ammonium acetate sublimed at 40 C. The residue was dissolved in 4.5 ml of pH 2.2 buffer and 0.025 ml of Na_2SO_4 (200 mM) added to insure complete barium precipitation. After one hr at 0 C, the pH was adjusted to 2.2 ± 0.1 with 0.05 ml concentrated HCl. If turbid, the sample was centrifuged before 20 ml went to the analyzer.

The actual analysis was kindly done by Dr. Norbert P. Newman, protein chemist at Rutgers University, New Brunswick, N.J.

The peaks on the recording were analysed by drawing the base line, finding the half height of the peak, and counting the dots in the upper half of the peak. The number of dots were multiplied by the net height and divided by a correction factor to yield the number of micro-moles present in the sample.

Correlation of DNase production with other characteristics of staphylococci

The organisms studied were received from Dr. Bartley on blood plates that had been streaked with milk from cattle which were being tested for incidence of mastitis.

The DNase test was performed by streaking the freshly isolated staphylococci on Difco DNase agar as suggested by Burns and Holtman (1962). After being incubated for 18-20 hr at 37 C, the plates were flooded with cold 7% perchloric acid to precipitate the unhydrolysed DNA. DNase production was evident by clearing around the streak of organisms.

Hemolysin production was examined by streaking the organisms on sheep blood agar plates and observing for alpha and beta hemolysin after 18-20 hr at 37 C.

Pigment production was observed on the various plates.

Coagulase production was determined by inoculating 0.1 ml of whole rabbit blood, mixed with 0.1 ml trypticase soy broth, and incubating at 37 C for 2 hr. A firm clot at this time was read as a positive result.

A leukocyte count on one million or more per ml of milk was considered to be indicative of a clinical involvement of the staphylococci in the udder of the cow.

Preparation of leukocytes*

A method similar to that of Skoog and Beck (1956) was used with some success. Fresh blood drawn from a cow aseptically was mixed in a

*All vessels and solutions were sterile, and aseptic techniques were used throughout.

serum bottle 1:3 with 2.25% dextran (4-5 million molecular weight). After mixing with care not to cause foaming, this solution was drawn into a syringe which was left standing on the plunger overnight. The white cells suspended in serum above the erythrocyte pack were injected into test tubes fitted with rubber diaphragms. These tubes were centrifuged at 300 x g for 0.5 min, pooled (using serum to suspend the pellet), and centrifuged again at 300 x g for 0.5 min. Leukocytes in this pellet were suspended in serum and used in the tests.

Another method of combining dextran precipitation of red blood cells with hemolysis by osmotic shock was also used with reasonable success. One part of fresh blood was mixed with two parts of 0.75% dextran (4-5 million molecular weight). To this was added 3 parts of cold distilled water. Thirty seconds later, 3 parts of cold 1.8% NaCl were added and the resulting suspension of leukocytes was centrifuged 20 min at 400 x g. These leukocytes were washed in wash solution, (0.9% NaCl, 0.5% dextran, and 0.1% glucose), and suspended in tissue culture medium 199 for examination.

As reported by Derbyshire (1964), leukocytes can be induced into milk by injecting sterile distilled water into the cows' udder, via the teat canal, 12 hr before milking. Samples of this "traumatic milk" were centrifuged 30 min at 20,200 x g in plastic tubes. The cell pack was washed at 400 g once for 20 min, twice for 10 min and twice for 5 min, all in the same wash solution described above.

Estimation of the quality of the prepared leukocytes

Determination of the types of leukocytes present and their ratio to erythrocytes was made by the use of Wright's Stain.

The viability of the leukocytes was determined by supravital

staining with neutral red and methylene blue.

Viability and general health of the white cells were ascertained by microscopic observations of granular motion, locomotion, and phagocytosis.

Estimation of damage done to leukocytes by the exocellular products of staphylococci

The effects of the various reagents used were determined by the use of vital stains and by microscopic observation of cellular activity, as well as the loss of structural integrity and lysis of the cells.

These observations were conducted on hemocytometers and formvar-coated slides, mounted in a warm stage at 37 C.

The materials being tested on the leukocytes were either mixed with the leukocyte suspension just prior to being placed on the slides, or they were applied at one edge of the cover slip and results were observed as the test material diffused across the field.

The cover slips were ringed with either tisuemat or candle wax to prevent desiccation of the preparation.

Propagation of tumor in mice.

After consulting with Dr. Leroy Stevens and three of his associates at the Jackson Laboratory in Bar Harbor, Maine, it was decided that the best system to use was sarcoma I tumor with strain A/J as the susceptible host and B10D2 as the resistant strain.

The sarcoma I tumor was chosen because it is impervious to circulating antibody, and because of the possibility of carrying it either intraperitoneally or subcutaneously. The A/J and B10D2 strains were selected on the basis of their relative sensitivity to the tumor. The A/J strain is completely susceptible while B10D2 is relatively resistant.

A completely resistant strain was not considered because only a drastic effect of the nuclease could be measured in such a system. The experimental system selected makes possible the measurement of moderate effects of nuclease on tumor proliferation. Mice bearing this tumor and four week old female mice of these two strains were purchased from the Jackson Laboratory.

The sarcoma I tumor can be carried either subcutaneously, as a tough white growth, or intraperitoneally as an ascites growth. The tumor obtained from the Jackson Laboratory was the subcutaneous growth and was transplanted by the following procedure: The host was sacrificed by holding a spatula behind its head and giving a sharp jerk on the tail, breaking its neck. The growth area was swabbed with alcohol and all instruments were alcohol flamed prior to use. The mouse was pinned to a dissecting board and the skin was laid back. The growth was excised and minced in a sterile petri dish by rapid opposing slices of two scalpels. This minced tumor was loaded into a sterile 14 gauge needle fitted with a stylet (trocar).

For subcutaneous implants, this trocar was inserted in the hip region, (previously swabbed with alcohol) and moved forward under the skin to the axillary region. The implant was pushed out with the stylet and the needle was withdrawn through a constriction formed by the thumb and forefinger placed at the tip of the needle. The point of insertion was pinched for a short time and then swabbed with alcohol to prevent abscess formation.

For intraperitoneal implants, the loaded trocar was inserted into the peritoneum, the implant pushed out, and the point of insertion treated as above after withdrawal of the needle.

Because the implant could be more accurately measured in the liquid form, the tumor was carried as the ascites growth. The ascites fluid was withdrawn from the peritoneum of the host, after seven days of incubation, through an 18 gauge needle and placed in sterile screw cap tubes for treatment. A yield of 6-8 ml was obtained per mouse.

Subcutaneous implantation of the ascites fluid was carried out by depositing 0.1 ml of the fluid under the skin, by the method described above, using a one ml tuberculin syringe fitted with a 1.5 inch 20 gauge needle.

Measurement of the proliferation of tumor in mice

The parameter used to measure the proliferation of tumor in mice was the size of the tumor with respect to the time elapsed after the administration of a standardized implant. The sarcome I was palpable after two or three days in the A/J strain. Subsequent to the three days, measurements were made along two axes of the tumor. At 14 days, the animals were sacrificed and the tumor excised and weighed on an analytical balance.

Estimation of the effect of the nuclease on antibody production in mice

Five hundred units of nuclease were given intravenously to A/J mice 16 hr before they were immunized with an intravenous injection of 0.1 ml of 10 per cent sheep red blood cells, (Mowbray and Scholand, 1966). After 6 days the mice were anesthetized with ether and exsanguinated by cutting the axillary artery. The blood was collected with a sterile Pasteur pipette, the serum separated, and complement inactivated by heating at 56 C for 30 min. Serial dilutions of the serum were made in hemagglutination buffer (Difco).

To obtain hemagglutination titres, 0.1 ml of each dilution

and 0.02 ml of 2% washed sheep red blood cells were mixed in 1 x 7.5 cm test tubes. Results were read after incubation at 37 C for one hr, and again after 12 hr at 4 C.

Hemolysin titres were obtained by use of the protocol shown in Table 1 (Metcalf, 1961).

TABLE 1
 PROTOCOL FOR HEMOLYSIN TITRATION

Tube #	Saline (0.85%)	Hemolysin Dilutions (0.5 ml)	Complement (1-20)	Sheep red blood cells (2%)
1	1.5 ml	1-500	0.5 ml	0.5 ml
2	1.5 ml	1-1000	0.5 ml	0.5 ml
3	1.5 ml	1-2000	0.5 ml	0.5 ml
4	1.5 ml	1-3000	0.5 ml	0.5 ml
5	1.5 ml	1-4000	0.5 ml	0.5 ml
6	1.5 ml	1-5000	0.5 ml	0.5 ml
7	1.5 ml	1-6000	0.5 ml	0.5 ml
8	1.5 ml	1-8000	0.5 ml	0.5 ml
9	1.5 ml	1-10,000	0.5 ml	0.5 ml
10	2.0 ml	1-500	--	0.5 ml
11	2.0 ml	--	0.5 ml	0.5 ml
12	2.5 ml	--	--	0.5 ml

RESULTS

Purification of the nuclease

Staphylococcal nuclease was originally obtained from heated culture supernatants (Cunningham, Catlin, and Privat de Garilhe, 1956), and it has been reported that heating actually activates the nuclease, (Ohsaka, Mukai, and Laskowski, 1964). Since research was being done on nuclease in connection with a food project, sliced ham was used as the growth medium for the Staphylococcus aureus. After inoculation and incubation, the ham was made 60% in $(\text{NH}_4)_2\text{SO}_4$ and homogenized in an Omnimixer. The homogenate was boiled for 5, 10, 20 and 40 min, cooled quickly in cold running water, and centrifuged. The precipitate was discarded, the supernatant made 0.3 M in trichloroacetic acid (TCA), and recentrifuged. This precipitate was dissolved in distilled water, neutralized with NaOH, and examined for nuclease activity with results as shown in Figure 1. Upon obtaining this information, the crudes were boiled for 10 min as the first step in the purification.

For preliminary purification of the nuclease, Davidson's (1959) technique of electrophoresis on polyurethan foam was to be used. In order to use this technique, the isoelectric point of the nuclease had to be known. This information was obtained by loading nuclease in the middle of separate polyurethan blocks which had been filled with buffers of differing pH. After 12 hr at 200 volts (constant voltage), the blocks were laid on DNase test agar and incubated 2 hr at 37 C. The agar was then flooded with 7% perchloric acid and the migration of the nuclease was determined by measuring from the center of the origin block to the center of the band of clearing in the agar. Figure 2 shows that the isoelectric point of nuclease, prepared under these conditions,

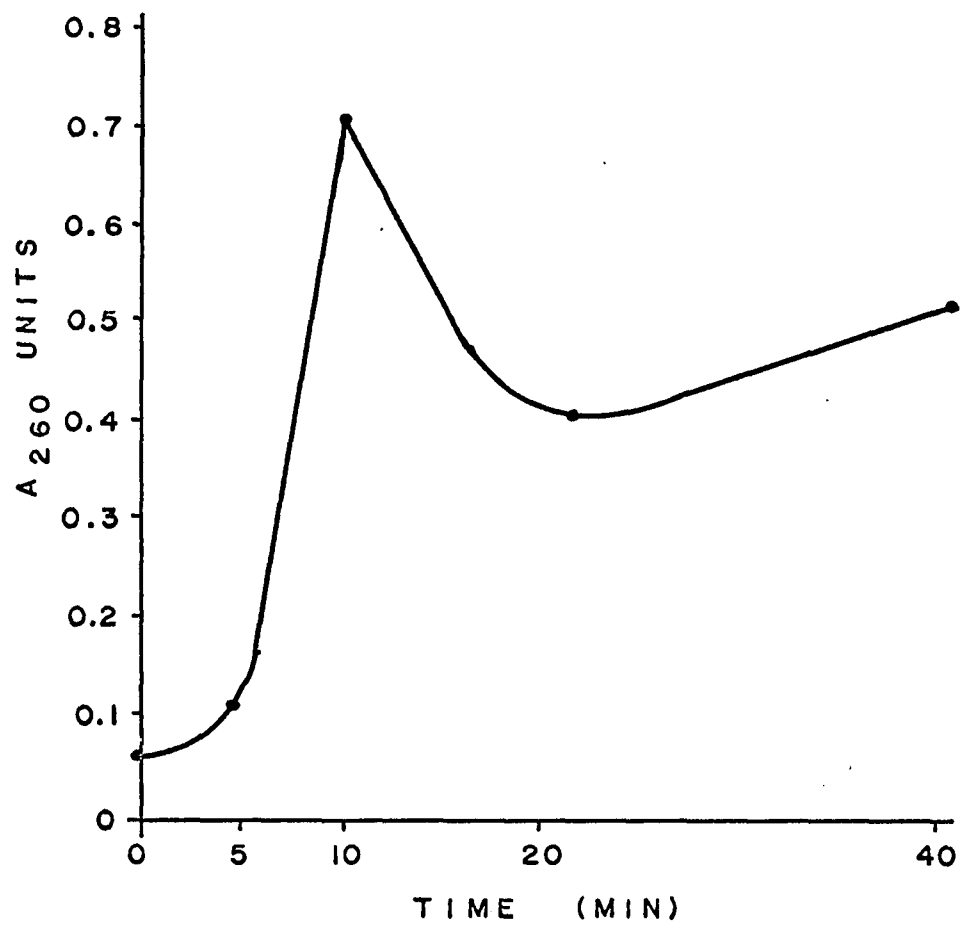


FIGURE 1. Heat activation of nuclease at 100 C in ham homogenate made 60% saturated with $(\text{NH}_4)_2\text{SO}_4$.

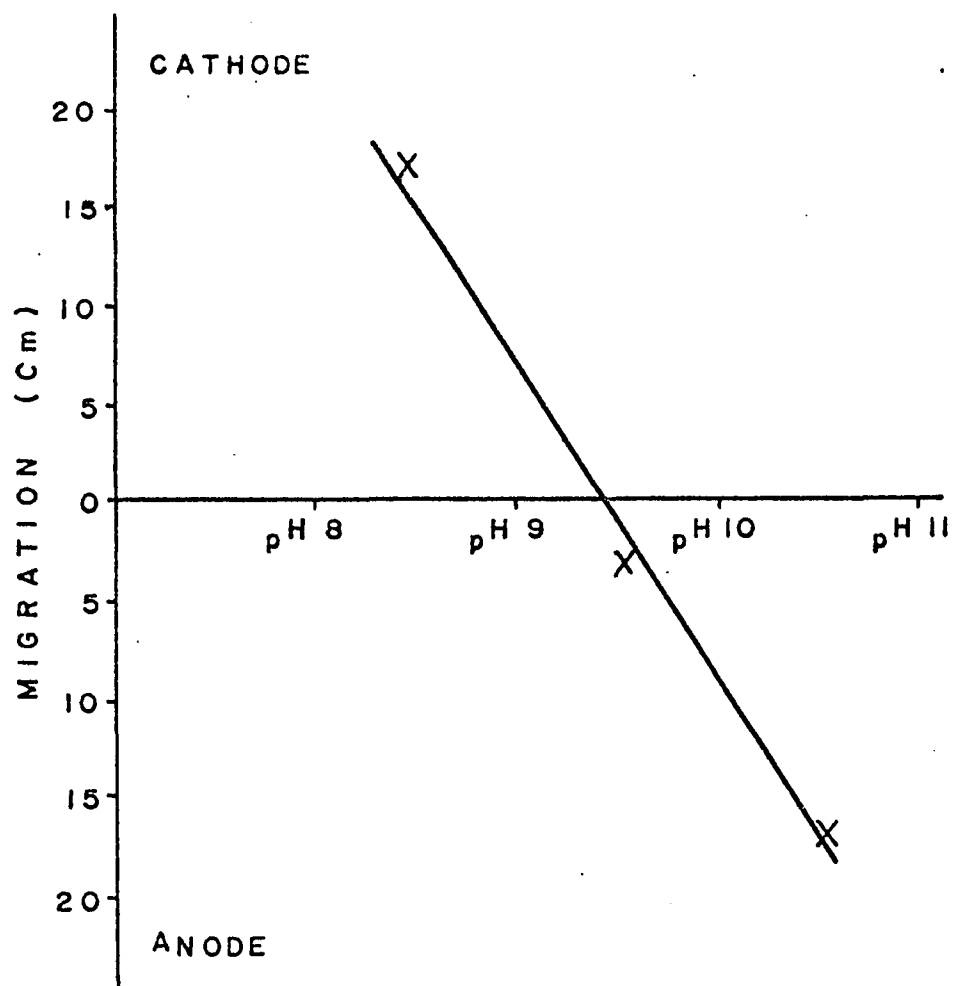


FIGURE 2. The isoelectric point of nuclease as determined from electrophoretic migration on polyurethan foam with varying pH. Electrophoresis was carried out in 0.5% glycine with a conductivity of 5.6 millimho for 12 hr at 200 V constant voltage. Nuclease activity was assayed on DNase plates.

was approximately 9.5. On the basis of this information, the nuclease was placed at the anode end of the block and allowed to migrate the maximum distance toward the cathode at pH 6.8 in glycine buffer. It was found that pouring Agarose at both ends of the origin block retarded diffusion of the colored material from the origin into the wings and made possible a reasonably pure preparation of nuclease. This partially purified nuclease was free of staphylococcal antigens, as measured by the Ouchterlony technique (1962). The material caused no overt ill effects when injected into mice.

A large amount of nuclease was partially purified, by this technique. Upon re-electrophoresis of rinsings from the blocks used in the preparative run, nuclease activity was found in two well-separated peaks, (Figure 3).

Taniuchi, et al (1965) reported that the nuclease occurs in two forms with differing molecular weights. Thus, upon observing that the nuclease fractionated electrophoretically on polyurethan foam, as well as displayed bimodal solubility in $(\text{NH}_4)_2\text{SO}_4$ (80-90% of the activity precipitated between 60 and 70% saturation, while the remainder of the activity was soluble in saturated $(\text{NH}_4)_2\text{SO}_4$), an attempt was made to establish if different molecular weight forms were being separated by $(\text{NH}_4)_2\text{SO}_4$ treatment.

Molecular weight studies

Ultracentrifugal analysis of the insoluble activity of the UNH-15 strain indicated a major component having molecular weight of 15,000, while analysis of the soluble activity indicated a major component of molecular weight 7,000. However, both activities were poly-disperse.

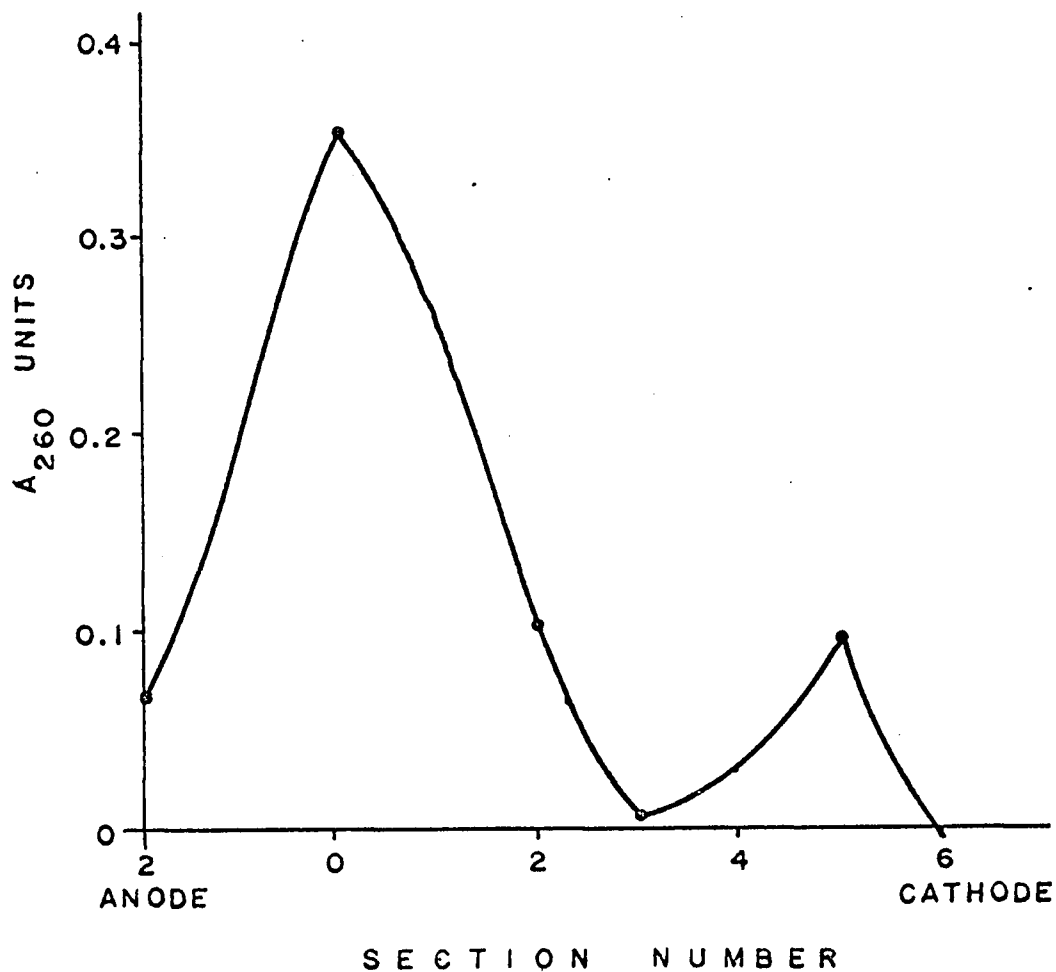


FIGURE 3. Electrophoretic fractionation of nuclease on polyurethan foam. Electrophoresis was carried out in 0.5% glycine, pH 9.0, with a conductivity of 5.6 millimho for 12 hr at 200 V constant voltage. Twenty μ l from each section were assayed for nuclease activity.

The two types of activities were subjected to filtration through Sephadex G75 (Figure 4). The insoluble activity eluted in two major peaks, corresponding to molecular weights of 15,500 and 13,500. The soluble activity resolved into two peaks, one corresponding to a molecular weight of 6,500, while the other was a broad peak leaving the column at the termination of the permeable volume, indicating that it contained unresolved components with molecular weights of 3,000 or less.

To establish the size of the smaller members of this apparent series of molecular weight forms, and also to establish if these lower molecular weight forms could be demonstrated in the culture supernatants of other strains, the soluble activity from five strains was filtered through Bio-Gel P-6. Any activity eluting at the lower operational limit of this gel (approximate molecular weight 1,000) was re-filtered through Bio-Gel P-4.

Except for the F.D.A. 234 strain, all strains produced nuclease activity having indicated molecular weights of 5,000 or less, with the minimal form having a molecular weight of 2,800. The F.D.A. 234 strain produced a form that, on refiltration through the P-4 gel, had an indicated molecular weight of 900.

To determine if these multiple forms were present in the crude preparations, or were a result of the $(\text{NH}_4)_2\text{SO}_4$ and TCA treatments, crude preparations from the F.D.A. 234 and UNH-15 strains were filtered through Sephadex G75. The results, shown in Figure 5, demonstrated that the multiple forms were present in the culture supernatants before either precipitating treatment was employed. There were, in fact, more peaks in the crude UNH-15 than had been found in both precipitated

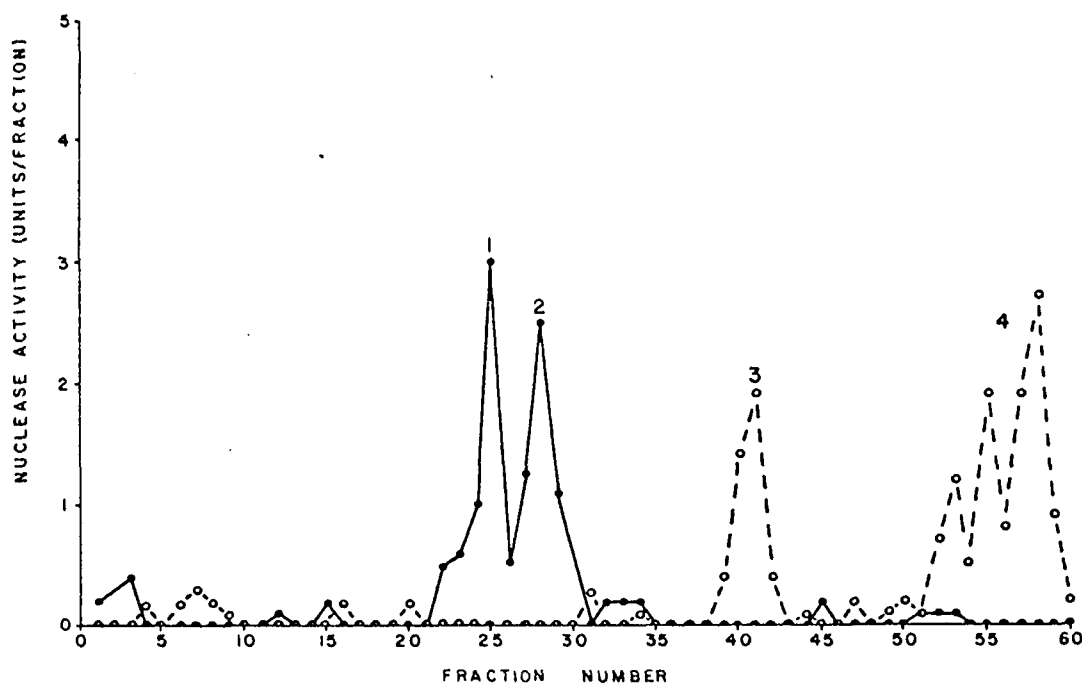


FIGURE 4. Sephadex G75 filtration of $(\text{NH}_4)_2\text{SO}_4$ - fractionated nuclease activity from *Staphylococcus aureus* UNH-15. The $(\text{NH}_4)_2\text{SO}_4$ insoluble activity (O—O) from two ml of culture supernatant, and the $(\text{NH}_4)_2\text{SO}_4$ soluble activity (O---O) from four ml of culture supernatant were applied at 4 C to a three cm ID column with a void volume (V_0) of 40.5 ml, a total permeable volume ($V_0 + V_1$) of 121.5 ml, and a flow rate of 8.4 ml per hr. The eluting buffer is described in the text. Fractions of 1.4 ml were collected. The peaks correspond to forms with approximate molecular weights of 15,500 (1), 13,000 (2), 6,500 (3), and 3,000 or less (4).

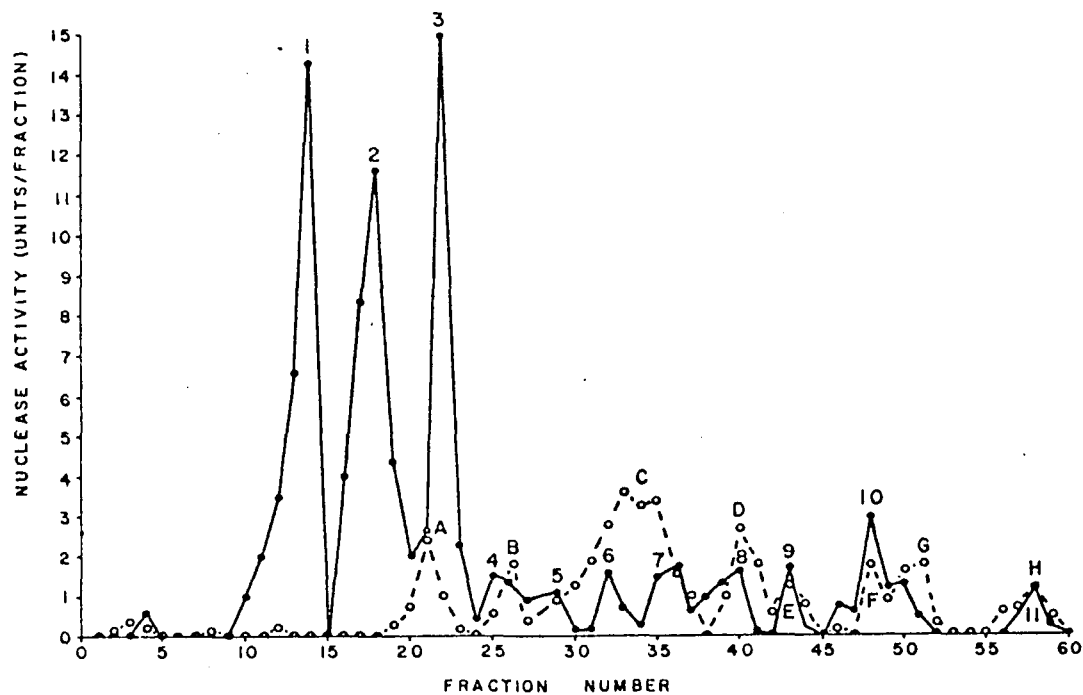


FIGURE 5. Sephadex G75 filtration of the nuclease activity in crude culture supernatants of *Staphylococcus aureus* strains F.D.A. 234 (●—●) and UNH-15 (○—○). Two ml of culture supernatant were filtered as described in Figure 4. The peaks correspond to forms with approximate molecular weights of 29,000(1), 23,500(2), 18,600(3), 15,500(4), 12,500(5), 10,500(6), 8,500(7), 6,600(8), 5,600(9), 4,200(10), 3,000 or less (11); and 19,700(A), 14,800(B), 10,000-8,500(C), 6,600(D), 5,600(E), 4,200(F), 3,500(G), 3,000 or less (H).

fractions of this strain.

Observation that fewer forms were detectable in the precipitated fractions than in the crude supernatant suggested that the forms were interconvertible, and indicated the possibility of providing evidence that the multiple forms observed were aspects of one enzyme, rather than several, by regenerating the forms of either fraction from the other.

Consequently, to establish that the higher molecular weight, $(\text{NH}_4)_2\text{SO}_4$ insoluble forms could be derived from the lower molecular weight, soluble forms, the soluble activity from one L of UNH-15 crude preparation was neutralized following TCA precipitation and brought to a volume of 10 ml with distilled water. At this point, gel filtration indicated the absence of any form with a molecular weight greater than 10,000. The syrupy solution remained fluid at -5°C and it was held at this temperature for two months. Part of the sample was then saturated with $(\text{NH}_4)_2\text{SO}_4$ and the amounts of insoluble and soluble activities present determined. A second portion of the sample was filtered through Sephadex G75.

Sixty per cent of the activity had become insoluble in saturated $(\text{NH}_4)_2\text{SO}_4$, and the major portion of the activity in the gel filtered sample was associated with a peak having an indicated molecular weight of 17,000.

To establish that the lower molecular weight, $(\text{NH}_4)_2\text{SO}_4$ soluble forms could be derived from the higher molecular weight, insoluble forms, and to obtain enough of the putative monomer of molecular weight 900 for a determination of its amino acid residues, advantage was taken of the inability of DEAE-cellulose to retain the lower molecular

weight forms. Insoluble activity from the F.D.A. 234 strain (185,000 units) was dissolved in distilled water and kept at 5 C for two weeks. The solution was then absorbed on a 2 x 10 cm column of DEAE-cellulose in the chloride form. The column was eluted with distilled water and the nuclease activity in the effluent (17,000 units) precipitated with TCA. The precipitate was neutralized, redissolved in distilled water, and filtered through Bio-Gel P-4. The central fractions of the peak migrating through the column at a rate corresponding to a molecular weight of 900 were pooled, yielding 8,000 units of nuclease. It was thus possible to obtain the monomer from a mixture of the heavier forms.

Amino acid composition of the ultra-light nuclease from the F.D.A. strain 234

The pooled activity was taken to dryness, redissolved in the electrophoretic buffer, and subjected to electrophoresis. Guide strips were used to locate the nuclease activity, which was then eluted from the paper and taken to dryness.

A portion of this material was chromatographed in one dimension. Five ninhydrin reacting components were found in a guide strip and the nuclease activity in a second guide strip had an Rf corresponding to that of one of the ninhydrin positive components (0.10). The balance of the electrophoretically purified activity was preparatively chromatographed in one dimension. The band containing the nuclease activity was located in guide strips and then eluted from the main body of the chromatogram. The eluted material was reduced to a volume of one ml, made 6 N in HCl, and held for 16 hr at 120 C. After being taken to dryness and redissolved in 10% isopropanol, the hydrolysate, containing approximately 12 ug of amino nitrogen, was chromatographed in two dimensions.

Hydrolysis released eight amino acids from the active peptide, alanine, aspartic and glutamic acids, glycine, leucine, lysine, serine, and valine. The calculated molecular weight of the peptide, assuming the residues were present in equimolar amounts, was 818.

Determination of the amino acid composition of the 13,000 molecular weight nuclease

After 98,000 units of ammonium sulfate insoluble nuclease had been chromatographed on CM-Sephadex C50, DEAE-cellulose, and lyophilized, 80,000 units of nuclease activity were recovered. Sixteen thousand units of this activity were filtered through a Sephadex G75 column, and rotary evaporated. This was reconstituted in 0.5% Na_2HPO_4 and precipitated with TCA. One ml of this TCA precipitate was dissolved in 6 N HCl and hydrolysed for 20 hr at 110 C. The hydrolysate was lyophilized, reconstituted, and analyzed in the Technicon Autoanalyzer. Dr. Norbert P. Newman, at Rutgers University, New Brunswick, N. J., kindly performed the actual analysis. Fifteen amino acids were found in the amounts shown in Table 2. The absence of tryptophan was determined by analysis of the barium hydroxide hydrolysate. The molecular weight of this form of nuclease, as calculated by summation of molecular weights of the estimated number of moles of each constituent amino acid per mole of nuclease (minus the water eliminated in the formation of peptide bonds), was 11,363. This figure is low because glycine is undoubtedly present, although its determination was precluded by technical difficulties.

TABLE 2
AMINO ACID COMPOSITION OF THE 13,000 MOLECULAR WEIGHT NUCLEASE

Amino acid	uMole	*Calculated number of residues per mole of enzyme	Calculated weight of residues per mole of enzyme
Lysine	0.1136	18.0	2,630
Histidine	0.0213	3.5	465
Arginine	0.0329	5.0	870
Aspartic acid	0.0824	13.0	1,730
Threonine	0.0311	5.0	595
Serine	0.0301	5.0	525
Glutamic acid	0.0684	11.0	1,165
Proline	0.0256	4.0	460
Alanine	0.0562	9.0	800
Valine	0.0619	10.0	1,170
Half-cystine	0.0	0.0	0
Methionine	0.0064	1.0	149
Isoleucine	0.0398	6.0	786
Leucine	0.0383	6.0	786
Tyrosine	0.0158	2.5	408
Phenylalanine	0.0253	4.0	660
Tryptophan**	0.0	0.0	0

Sum of residues = 13,199
Water of hydration = 1,836
Calculated molecular weight = 11,363

*Calculated on the basis of one mole of methionine per mole of enzyme.

**Determined on the basis of barium hydroxide hydrolysis.

Preparation of low molecular weight nuclease for animal injection

Since DEAE-cellulose chromatography did not convert nuclease to the lighter forms with very high yields, other methods of dissociating the nuclease and keeping it dissociated were tried.

Ammonium sulfate insoluble nuclease which had been chromatographed on CM-Sephadex C50 and DEAE-cellulose was filtered over Sephadex G75 at different pH values to determine if changes in pH would dissociate the nuclease molecules. The pH values used were 6.0, 7.0, 9.5, and 11.0 in 30 millimho ammonium acetate buffer. The sample volume placed on the column was 0.1 ml and 0.5 ml fractions were collected. Five microliters of the even-numbered fractions were tested on DNase plates. Nuclease activity started eluting at the void volume, peaked approximately half way through the working volume, and fell off at the total volume of the column. Although the intensity of the reaction on the DNase plates varied, the elution patterns were very uniform at the various pH values. The distribution of nuclease activity in the effluent of the Sephadex G75 is shown in Figure 6.

The peak activity from each run (fractions 14-36) were pooled and quantitated. As can be seen in Table 3, nuclease activity in this system is much lower at higher pH values.

Upon observing that high pH depresses nuclease activity, a comparison with low pH and several solvents was made. Ten mg amounts of the lyophilized nuclease (ammonium sulfate insoluble activity which had been chromatographed on CM-Sephadex C50 and DEAE-cellulose) were weighed out and dissolved in one ml of distilled water, 0.4% TCA at pH 1.5, 10% and 50% dimethylformamide (DMF), 10% and 50% dimethyl sulfoxide (DMSO), 0.1 M citric acid, and 1.3% glycine at pH 8.6. The relative activity of

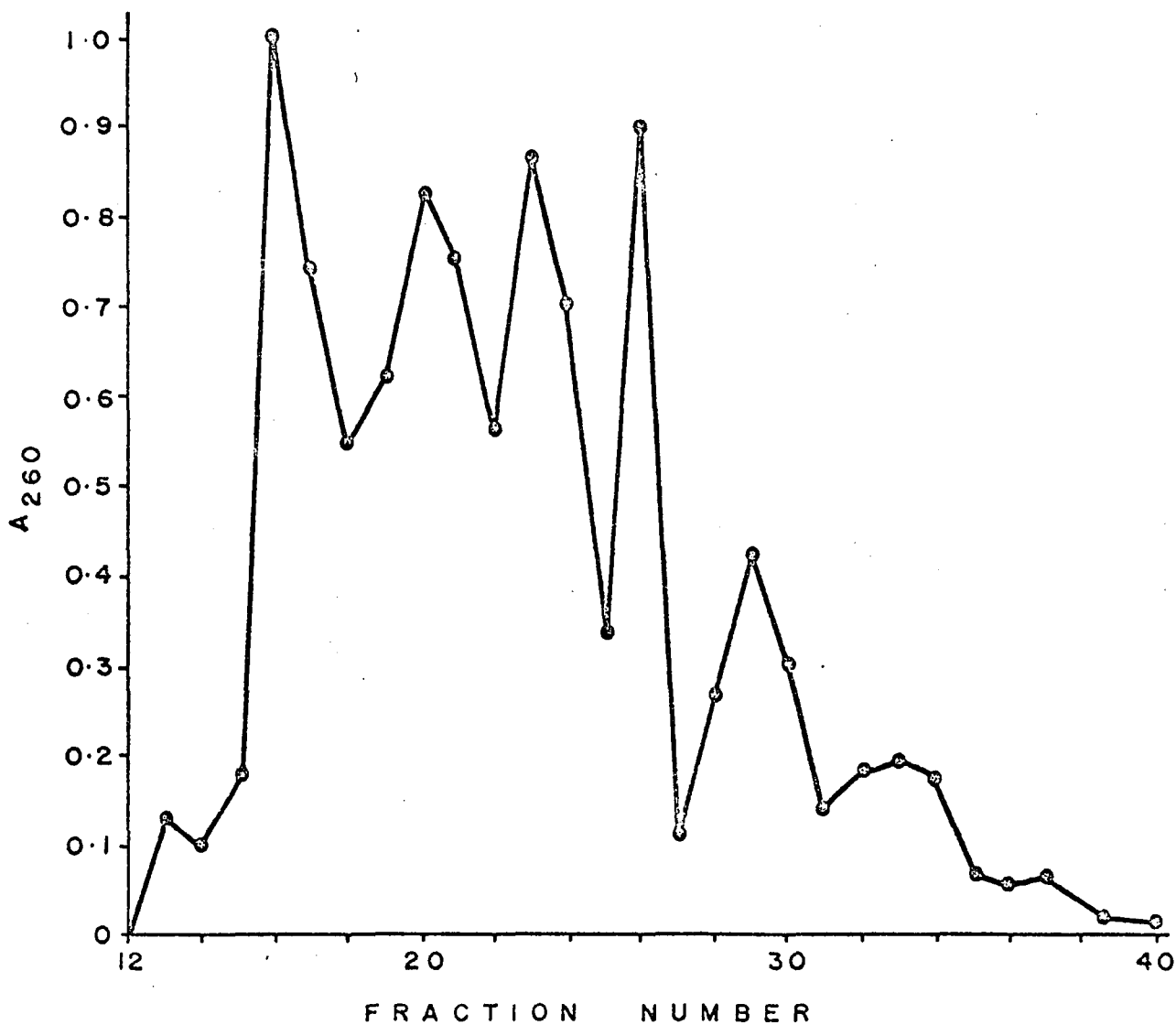


FIGURE 6. Typical distribution of nuclease activity in the effluent of Sephadex G75 when eluted with a buffer of pH greater than 6. A 0.2 ml sample of 0.1 g lyophilized nuclease dissolved in one ml ammonium acetate pH 7.0, conductivity = 30 millimho was applied to a 1.5 x 25 cm column using a flow rate of 10 ml per hr collecting 0.5 ml fractions. The eluting buffer was ammonium acetate, pH 9.5, conductivity = 30 millimho. Twenty ul from each fraction were assayed.

TABLE 3
ACTIVITY OF NUCLEASE POOLED AFTER FILTRATION ON SEPHADEX G75 AT
DIFFERENT pH VALUES

pH	A ₂₆₀ Units per ul
6.0	0.950
7.0	1.030
9.5	0.140
11.0	0.057

Twenty-one fractions were pooled in each case.

nuclease dissolved in these various solvents is seen in Table 4.

To determine if the relatively high activity obtained by dissolving the nuclease in TCA was due to disaggregation, 0.1 g of the same lyophilized nuclease was dissolved in one ml of 0.4% TCA pH 1.5 and filtered through Bio-Gel P-10 in 0.4% TCA. For a comparison of the effects of TCA against those of glycine, the experiment was re-run with 1.3% glycine (pH 8.6) replacing the TCA. The comparative distributions of nuclease activity in the effluent of these columns are shown in Figure 7.

Since 0.4% TCA seemed to activate the nuclease by dissociation, a preparative column was set up using 9 cm of Bio-Gel P-2 above 29 cm of P-10 in a 2.5 cm ID column. One g of lyophilized nuclease was dissolved in 5 ml of 0.4% TCA, and applied to this column using a flow rate of 10 ml per hr. Two ul samples from even-numbered tubes were analyzed for nuclease activity. Odd-numbered tubes were analyzed for protein by A_{280} in a Beckman DU spectrophotometer and by the Lowry technique. The results of these analyses are shown in Figure 8.

The activity located in the major peak was pooled (21,000 units) and lyophilized. The lyophilized powder was dissolved in four ml of 0.85% NaCl plus 0.01 M sodium succinate $\cdot 6H_2O$, then filtered through Bio-Gel P-2 to remove the TCA and obtain the nuclease in a solution suitable for animal injection.

TABLE 4
RELATIVE ACTIVITY OF NUCLEASE DISSOLVED IN DIFFERENT SOLVENTS

Solvent	A ₂₆₀ Units per ul
10% DMF	0.400
50% DMF	0.646
10% DMSO	0.515
50% DMSO	0.515
0.1 M Citric acid	0.095
0.4% TCA	0.720
1.3% Glycine pH 8.6	0.302
Distilled water	0.312

Ten mg of lyophilized nuclease were dissolved in one ml of each solvent.

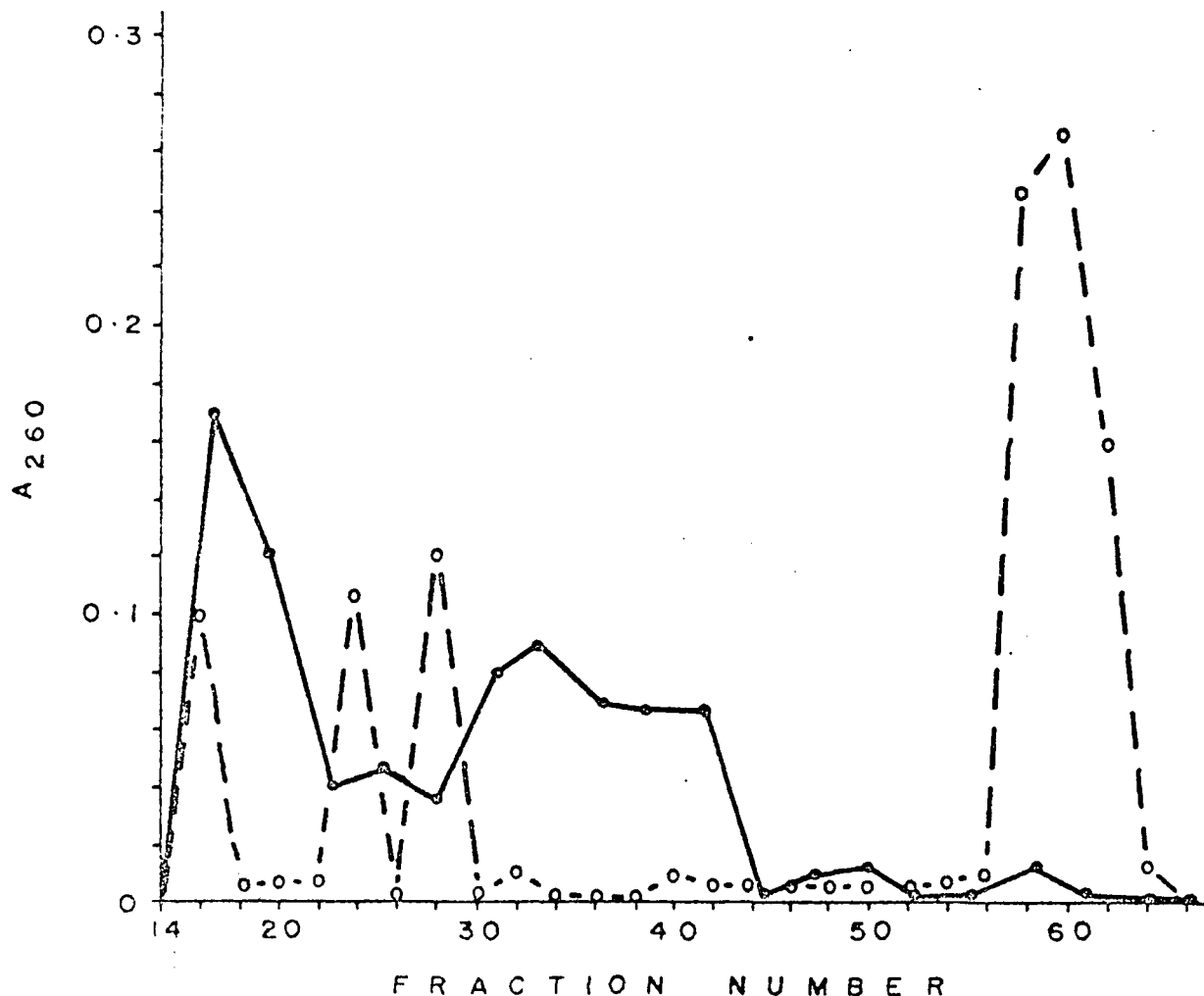


FIGURE 7. Distribution of nuclease activity on a Bio-Gel P-10 column having a void volume of 7 ml. A 0.2 ml sample of 0.1 g of lyophilized nuclease dissolved in one ml of each solvent was applied to a 1.5 x 25 cm column using a flow rate of 10 ml per hr collecting 0.5 ml fractions. Activity run in 1.3% glycine, pH 8.5, (●—●). Activity run in 0.4% TCA, pH 1.5, (○---○). Ten ul from the even-numbered fractions were assayed.

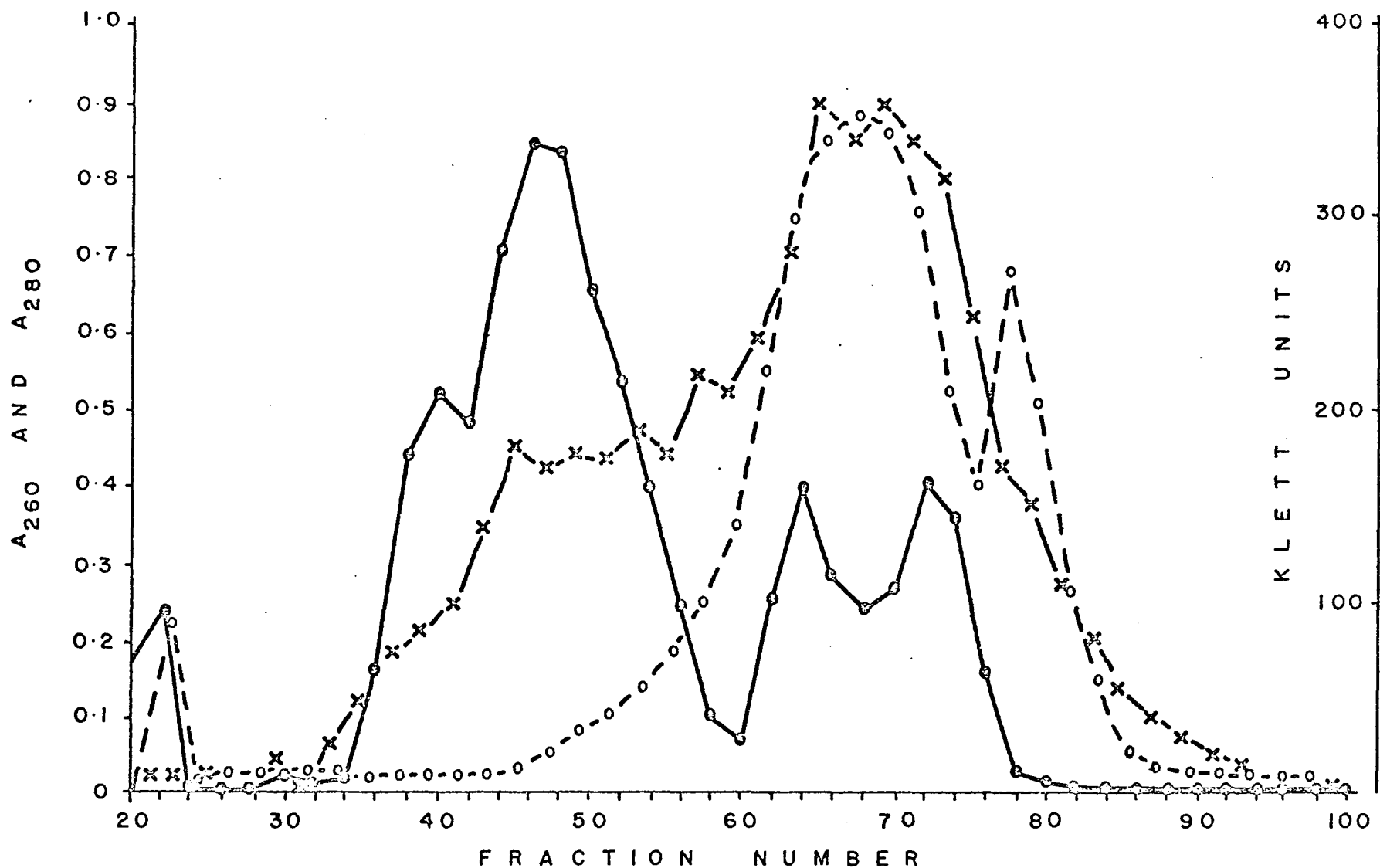


FIGURE 8. Purification of nuclease on a double column. Nine cm of Bio-Gel P-2 above 29 cm of Bio-Gel P-10 in a 2.5 cm ID column, having a void volume of 51 ml, was eluted with 0.4% TCA. One g of lyophilized nuclease dissolved in 5 ml of 0.4% TCA was applied to the column using a flow rate of 10 ml per hr collecting 0.5 ml fractions. Two ul from even-numbered fractions were assayed for nuclease (●—●). A₂₈₀ of the odd-numbered fractions was read on a Beckman DU (○---○). One-half ml samples of the odd-numbered fractions were tested for protein by the Lowry method (x—x).

Correlation of DNase production with other characteristics of staphylococci

Staphylococci isolated from the milk of cows being tested for the incidence of mastitis were used in a study of the correlation of nuclease, hemolysin, and coagulase production with pathogenicity. A positive leukocyte response by the cow, as measured by a count of greater than one million leukocytes per ml of milk, was used as an indicator of pathogenicity. In Table 5, the production of these extracellular products of staphylococci is correlated with respect to the leukocyte response of the host.

Effect of staphylococcal nuclease on bovine leukocytes

The addition of 0.1 ml of staphylococcal filtrate to 0.4 ml of leukocyte suspension caused immediate cessation of granular motion, rounding up of the cells, and heavy staining with methylene blue. Lysis of the cells soon followed.

Leukocytes treated with purified nuclease survived for four hours or more, as did the control cells. These cells migrated about exhibiting granular motion and active extension and retraction of pseudopodia all this time.

Effect of staphylococcal nuclease on antibody formation in mice

Preliminary studies indicate that the nuclease does diminish the immune response of the mouse. Agglutination titres of sera from mice treated with and without nuclease are shown in Table 6.

Investigation of the effect of nuclease on tumor proliferation in mice has been complicated by the incompatibility of tumor cells with the solutions in which the nuclease is usually carried. Methods are being worked out to remedy this situation.

TABLE 5

CORRELATION OF DNASE PRODUCTION WITH HEMOLYSIN AND COAGULASE PRODUCTION
WITH RESPECT TO THE LEUKOCYTE RESPONSE OF THE HOST

Characteristics of Strains		Leukocyte Response*	
DNase	Coagulase	# of + Strains	# of - Strains
+	+	18	25
	-	8	14
-	+	0	0
	-	15	62

DNase	Hemolysin	# of + Strains	# of - Strains
+	+	15	7
	-	11	34
-	+	1	5
	-	15	55

DNase	Coagulase and Hemolysin	# of + Strains	# of - Strains
+	+	11	3
	-	4	6
-	+	0	0
	-	9	61

* A leukocyte count greater than one million per ml of milk was taken as a positive leukocyte response.

TABLE 6

THE EFFECT OF NUCLEASE ON THE PRODUCTION OF SHEEP RED BLOOD CELL
AGGLUTININS IN THE MOUSE

Dilution of sera	Treatment*														
	Sheep red blood cells					Sheep red blood cells incubated 30 min in nuclease‡						Nil			
	1	2	3	4	5	Mouse #						12	13	14	15
1:10	+	+	+	+	+	+	+	+	±	+	+	-	-	-	-
1:100	+	+	+	+	+	+	+	+	-	+	+	-	-	-	-
1:200	+	+	+	±	±	+	-	+	-	±	+	-	-	-	-
1:400	+	+	+	-	-	+	-	-	-	-	+	-	-	-	-
1:800	±	±	-	-	-	-	-	-	-	-	-	-	-	-	-
1:1200	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-

*Sheep red blood cells with and without nuclease were injected I.V. six days before the sera were collected and titred. (+) indicates agglutination of sheep red blood cells by that serum dilution.

‡The nuclease used was TCA precipitated $(\text{NH}_4)_2\text{SO}_4$ soluble activity purified on cellulose phosphate and Bio-Gel P-10 in 0.01 M succinate at pH 6.0.

DISCUSSION

Although nucleases have been found in Azotobacter (Stevens and Hilmo, 1960), Serratia (Leshinskaya and Bogautdinov, 1963; Eaves and Jeffries, 1963), and Bacillus subtilis (Birnboim, 1966) staphylococcal nuclease is the only one that is heat stable. Heat activation of the nuclease is shown in Figure 1. Maximal activity is achieved after 10 min boiling in the crude mixture, which is similar to the findings of Ohsaka, Mukai, and Laskowski (1964).

Taniuchi, et al (1965) have reported that staphylococcal nuclease occurs in two forms with molecular weights of approximately 10,000 and 20,000, and contains nearly the usual array of amino acids. Since then, Heins, et al (1967) have further purified the enzyme and have assigned it a molecular weight of 17,000. Even this highly purified nuclease displays two interconvertible forms. Mukai, et al (1965) also found evidence of multiple forms of the nuclease.

Since these workers purified the enzyme from an $(\text{NH}_4)_2\text{SO}_4$ precipitate, they were presumably analyzing what corresponds to the insoluble fraction used in the present study, which contains only higher molecular weight forms.

The data obtained in this study on the insoluble fraction are in good agreement with those found by other workers. In strain UNH-15, the nuclease activity of the insoluble fraction, after chromatography on CMC and DEAE-cellulose, occurs primarily in forms of 13-14,000 and 22-24,000 molecular weights. The amino acid composition of the lighter of these two forms, as shown in Table 2, agrees well with that obtained by Taniuchi and Anfinsen (1966), and Heins, et al (1967). The iso-

electric point of 9.5 (Figure 2) as determined by electrophoresis on polyurethan foam, is close to that of 9.6 reported by Heins, et al (1967). The nuclease from this strain also hydrolyses RNA as well as DNA. (J. J. Burke II, personal communication).

Fractionation on polyurethan foam electrophoresis (Figure 3) and bimodal solubility in $(\text{NH}_4)_2\text{SO}_4$ indicated that the nuclease under investigation in this study also possessed a multiplicity of forms. Closer examination of the $(\text{NH}_4)_2\text{SO}_4$ soluble and insoluble fractions by gel filtration through Sephadex G75 revealed the several peaks of activity shown in Figure 4. This figure clearly indicates that nuclease with molecular weights less than 6,000 are not precipitated by $(\text{NH}_4)_2\text{SO}_4$. Figure 5 shows that the relative amounts of high and low molecular weight forms of the nuclease produced vary between strains of staphylococci. Even strains which produce predominantly heavy forms, also exhibit a certain amount of the lighter forms.

Other workers have not reported these low molecular weight forms because their purification procedures preclude their finding them. Cunningham, in his first paper on the nuclease (1956) stated that the activity was dialyzable and he did not use dialysis in his purification scheme. Hence, when workers in the field discard the $(\text{NH}_4)_2\text{SO}_4$ supernatants and dialyze their preparation several times, it can not be expected that they will find the lighter forms.

The 818 molecular weight nuclease, since it occurs in only one of the five strains examined, would appear to be a special, if not a trivial case. Because of its small size, however, it provides an interesting model for investigating the fundamental nature of enzymatic action. It should be possible, for instance, to utilize this form of the nu-

lease to estimate the minimum structure required to constitute an enzymatically active site. A knowledge of its amino acid sequence should simplify the synthesis of an active enzyme because of the few residues involved.

Certainly, polymers derived solely from the 818 molecular weight form could not display an array of amino acids such as that found in the heavier forms. This indicates that there may be several active peptides, some of which are larger and possess a greater variety of amino acids. This is also suggested by the finding that four of the five strains studied produced a minimal subunit of molecular weight 2,800. Alternatively, the higher molecular weight forms may be heterogeneous aggregates (Mukai, et al, 1965), since the nuclease can be readily demonstrated to combine with other proteins: mixed with bovine serum, both the insoluble and soluble activity migrate through Sephadex G75 with the serum proteins. Also, when the heavier forms were dissolved in solvents, such as dimethylformamide, dimethyl sulfoxide, or 0.4% TCA, (Table 4), the activity was higher than when they were dissolved in glycine buffer at pH 8.6, or in distilled water. Figure 7 indicates that this increased activity in the 0.4% TCA is due to dissociation of the enzyme aggregates into smaller molecular weight forms, since a larger portion of the activity has been retarded on the Bio-Gel P-10. As shown in Figure 8, the bulk of the 280 absorbing and Lowry reacting material migrated with the lighter components of the nuclease activity on Bio-Gel P-10 in 0.4% TCA. This resulted in a high protein peak corresponding to a relatively low nuclease activity peak. Since it has been suggested that the activity of nuclease, as well as beta hemolysin (Dr. W. R. Chesbro, personal communication) and alpha hemolysin (Dr. A. W. Bernheimer,

personal communication), rises with lowering molecular weight (depolymerization), the results shown in Figure 8 indicate that the TCA might have dissociated heterogeneous aggregates of nuclease and other inert compounds.

Isolation of the minimal subunits from several strains in amounts adequate for analysis should distinguish between these alternatives.

The 3,000 molecular weight subunit is also relatively small in terms of enzyme molecules. It is possible that this lack of size and corresponding low order of sophistication of structure might be responsible for the remarkable heat stability of staphylococcal nuclease. If this is the case, one would expect the nucleases of thermophilic bacteria to be low molecular weight molecules, or composed of interconvertible multiple molecular weight forms with heat resistance conferred by the low degree of complexity of the subunits. This hypothesis could well be tested by examining the bacteria which inhabit the hot springs in Montana and Wyoming.

Staphylococcal nuclease was discovered in a pathogenic strain of S. aureus, and has been found to be correlated with pathogenicity of whatever strain is producing it, (Weckman and Catlin, 1957; DiSalvo, 1958; Burns and Holtman, 1960; Jacobs, Willis, and Goodburn, 1963; and Elston and Fitch, 1964).

Table 5 shows the results of an attempt to correlate DNase production with pathogenicity of staphylococci implicated in bovine mastitis. Although DNase production, alone, does not correlate well with pathogenicity, as measured by the leukocyte response in the milk of the infected cow, DNase production with simultaneous coagulase and hemolysin production is a very good indicator of pathogenicity. More signifi-

cantly, however, it may be seen from Table 5 that if coagulase production, or simultaneous coagulase and hemolysin production are taken as indicators of pathogenicity (as is commonly done), DNase is always present in the pathogenic situation. This is shown by there being no occurrence of a DNase (-) coagulase (+) or a DNase (-), coagulase and hemolysin (+) Staphylococcus. These results would seem to indicate that DNase (or staphylococcal nuclease) plays some important role in the pathogenicity of the staphylococci.

This role can be theoretically deduced when consideration is given to the sequence of events which are postulated by Braun and Cohen (1967) for the formation of antibody. If, indeed, there is an indispensable RNA-antigen complex transferred from the phagocytizing cell to the antibody producing cell, then staphylococcal nuclease in the immediate area should diminish the immune response of the host by hydrolysing some of the RNA of this RNA-antigen complex.

Since the nuclease caused no apparent adverse effects when injected into mice, or on bovine leukocytes in vitro, it was possible to test the nuclease for its effects on antibody formation in vivo.

An indication that the nuclease does diminish the immune response was obtained with staphylococcal vaccine injected into cows (Dr. W. R. Chesbro, personal communication). When the nuclease was mixed with the vaccine prior to injection, the antibody pattern usually produced by the cows was obliterated.

Preliminary investigation on the effect of nuclease on the production of antibodies in mice indicates that it is effective in this case also. As shown in Table 6, agglutinin titres of mice treated with sheep red blood cells which had been incubated in nuclease are generally

lower than those of mice which had received untreated sheep red blood cells.

This is in agreement with Dr. Werner Braun's finding (personal communication) that a similar preparation of UNH-15 nuclease diminished antibody formation in mice, as measured by the Jerne technique. In this case, the nuclease was $(\text{NH}_4)_2\text{SO}_4$ insoluble activity purified on CM-Sephadex C50 in 0.01 M phosphate and DEAE-cellulose in distilled water.

Based on these various results, it seems that the nuclease does play a role in the pathogenicity and virulence of staphylococci.

SUMMARY

Upon examination of five strains of staphylococci from widely differing sources (Wood 46 strain; F.D.A 234, a food poisoning strain; UNH-15, a bovine mastitis strain; and two strains isolated from human infections, one in England and the other in France), it was found that staphylococcal nuclease occurs in interconvertible forms varying in molecular weight from 3,000 to 23,000. One of the five strains (F.D.A. 234) possessed a minimal subunit of nuclease with a molecular weight of 818.

While the forms of molecular weight greater than 7,000 were insoluble in saturated $(\text{NH}_4)_2\text{SO}_4$, the lighter forms were precipitated with 0.3 M TCA. Since other workers in the field purify the enzyme from an $(\text{NH}_4)_2\text{SO}_4$ precipitate and dialyze their preparations, the lighter forms have not been found previous to this study.

Staphylococcal nuclease was found to be remarkably heat stable with maximal activity of crude mixtures being obtained only after 10 min of boiling. It hydrolyzed RNA as well as DNA, has a high pH optimum, and exhibited an isoelectric point of 9.5, as determined by electrophoresis on polyurethan foam. Based on gel filtration using Sephadex G75 and Bio-Gel P-10, the $(\text{NH}_4)_2\text{SO}_4$ insoluble activity, after chromatography on CMC Sephadex C50 and DEAE-cellulose occurred primarily in forms of 13-14,000 and 22-24,000 molecular weight. Using a Technicon Autoanalyzer for amino acid analysis, the lighter of these two forms was found to be composed of the usual array of amino acids except for cysteine and tryptophan. All these data agree with what has been published on the characteristics of the staphylococcal nuclease.

The low molecular weight subunits of the nuclease are believed to be responsible for the heat stability of the enzyme because of their lack of structural sophistication.

In a study of the correlation of DNase production with pathogenicity, DNase was found to be produced by all staphylococci which produced coagulase or coagulase and hemolysin, simultaneously. Since production of these exoenzymes is often used as the criterion of pathogenic staphylococci, these data suggest that DNase (or nuclease) might play an important role in the pathogenicity of staphylococci.

Since RNA and/or RNA-antigen complexes are considered to be important, if not essential to the transfer of information between cells during antibody formation, it would be expected that nuclease in the vicinity would diminish the immune response by damaging the RNA involved in this intermediate step.

The nuclease has been found to cause no observable adverse effects upon injection into mice or on bovine leukocytes in vitro. Therefore, it was possible to test the nuclease in vivo for its effects on antibody formation.

Preliminary investigation indicates that the nuclease does retard antibody formation in mice. Thus, it seems that the nuclease does play a role in staphylococcal pathogenicity and virulence.

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ABSTRACT

PURIFICATION AND CHARACTERIZATION OF STAPHYLOCOCCAL NUCLEASE

by

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Upon examination of five strains of staphylococci from widely differing sources (Wood 46 strain; F.D.A. 234, a food poisoning strain; UNH-15, a bovine mastitis strain; and two strains isolated from human infections, one in England and the other in France), it was found that staphylococcal nuclease occurs in interconvertible forms varying in molecular weight from 3,000 to 23,000. One of the five strains (F.D.A. 234) possessed a minimal subunit of nuclease with a molecular weight of 818. This molecule contained the amino acids: alanine, aspartic and glutamic acids, glycine, leucine, lysine, serine, and valine, which were assumed to be present in equimolar amounts.

While the forms of molecular weight greater than 7,000 were insoluble in saturated $(\text{NH}_4)_2\text{SO}_4$, the lighter forms remained in the $(\text{NH}_4)_2\text{SO}_4$ supernatant and were precipitated with 0.3 M TCA. Since other workers in the field purify the enzyme from an $(\text{NH}_4)_2\text{SO}_4$ precipitate and dialyze their preparations, the lighter forms have not been found previous to

this study.

Staphylococcal nuclease was found to be remarkably heat stable with maximal activity of crude mixtures being obtained only after 10 min of boiling. It hydrolyzed RNA as well as DNA, had a high pH optimum, and exhibited an isoelectric point of 9.5, as determined by electrophoresis on polyurethan foam. Based on gel filtration using Sephadex G75 and Bio-Gel P-10, the $(\text{NH}_4)_2\text{SO}_4$ insoluble activity, after chromatography on CMC Sephadex C50 and DEAE-cellulose, occurred primarily in forms of 12-13,000 and 22-24,000 molecular weight. Using a Technicon Autoanalyzer for amino acid analysis, the lighter of these two forms was found to be composed of the following number of amino acid residues per mole of nuclease: 18.0 lysine, 3.5 histidine, 5.0 arginine, 13.0 aspartic acid, 5.0 threonine, 5.0 serine, 11.0 glutamic acid, 4.0 proline, 9.0 alanine, 10.0 valine, 0.0 half-cystine, 1.0 methionine, 6.0 isoleucine, 6.0 leucine, 2.5 tyrosine, 4.0 phenylalanine, 0.0 tryptophan. The relative numbers of amino acid residues were calculated on the basis of one methionine residue per mole of enzyme. These data agree with what has been published on the characteristics of the staphylococcal nuclease.

The low molecular weight subunits of the nuclease are believed to be responsible for the heat stability of the enzyme because of their lack of structural sophistication.

In a study of the correlation of DNase production with pathogenicity, DNase was found to be produced by all staphylococci which produced coagulase or coagulase and hemolysin, simultaneously. Since production of these exoenzymes is often used as the criterion of pathogenic staphylococci, these data suggest that DNase (or nuclease) might play an important role in the pathogenicity of staphylococci.

Since RNA and/or RNA-antigen complexes are considered to be important, if not essential to the transfer of information between cells during antibody formation, it would be expected that nuclease in the vicinity would diminish the immune response by damaging the RNA involved in this intermediate step.

The nuclease has been found to cause no observable adverse effects upon injection into mice or on bovine leukocytes in vitro.

Preliminary investigation of the effect of nuclease on antibody production in mice indicates that the nuclease does lower the immune response of the mouse.