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TRYPTOPHAN ANALOG EFFECTS AND NUCLEASE SECRETION IN *STAPHYLOCOCCUS AUREUS*

by

DAVID F. CARPENTER

B. A., University of Vermont, 1967

A THESIS

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D. F. C.

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ABSTRACT

TRYPTOPHAN ANALOG EFFECTS AND
NUCLEASE SECRETION IN STAPHYLOCOCCUS AUREUS

by

DAVID F. CARPENTER

The growth characteristics and nuclease secretion patterns of Staphylococcus aureus, strains UNH-15 and Foggi were investigated. While Foggi displayed a more rapid growth rate, it lagged behind UNH-15 in nuclease secretion.

The analysis of the crude supernatants from these strains revealed that three chromatographically and electrophoretically separable forms were present. Closer examination indicated that the forms were not produced simultaneously, and that their relative concentrations changed during the growth cycle. The forms, which eluted in three relatively distinct peaks from columns of carboxy methyl cellulose, were dubbed nucleases I, II and III, in order of their elution.

Physical-biochemical determinations indicated that the molecular weight of the I, II and III forms were approximately 7,000, 14,000, and 20,000, respectively. Electrophoretic analysis indicated that nuclease III of Foggi was synonymous with Worthington's micrococcal nuclease. Immunological data and amino acid analyses indicated that the forms were related. The II form appeared to be nuclease III minus a significant portion of its N-terminal sequence. This hypoth-

esis appeared to be confirmed by the observed specific activities of the forms. While the activity of III was routinely 200, the II form displayed an activity of only 60 to 70.

Radioactive isotope analysis showed that the forms were related via one of two possible processes. The I and II forms, which predominate during log phase growth might be precursors to the III form which appears during the early stationary phase and persists to the end of the growth cycle. Alternatively, the III form is the only nuclease molecule synthesized by the cell. Then, the I and II forms are derived from the III form by some conversion process mediated by the cell. Further experiments indicated that the conversion was not due to extracellular constituents.

The addition of tryptophan analogs to mid-log phase cultures caused the inhibition of growth by 20-30%; nuclease activity was reduced by 80-90%. The analysis of crude supernatants produced in the presence of analogs revealed that nucleases I and II were present exclusively. The accumulated data indicated that analog poisoned the mechanism(s) responsible for the appearance of the various forms. Thus, the culture was metabolically frozen in mid-log position.

INTRODUCTION

An analysis of the supernatant derived from a culture of Staphylococcus aureus reveals the presence of numerous proteins demonstrating a wide array of biological activities. The fractionation of this crude supernatant by conventional protein purification methods yields as many as twenty different proteins depending on the strain used and conditions employed during growth. It has been unequivocally established that some of these proteins are toxins. These toxins have provoked a great deal of research in an attempt to elucidate their significance in the virulence of pathogenic staphylococci.

One of the extracellular proteins (one which is not considered a toxin) is nuclease. This relatively small molecule possesses the ability to preferentially hydrolyze single-stranded nucleic acids. Practically no research has been conducted on its pathological significance. Primary interest has centered around its utility as a tool in determining base sequence in nucleic acids. Its small size and apparently simple structure have inspired studies of the molecule as a protein, the major interest being its chemical and biophysical characteristics. Relatively little has been done on the physiology of its secretion.

The preliminary analysis of a relatively pure nuclease preparation produced by a bovine strain, S. aureus UNH-15, indicated that the enzyme lacked tryptophan. It was felt that an extracellular protein of such composition would be valuable

in studying the mechanism of protein secretion. Growth of the organism in the presence of tryptophan analogs should affect all proteins containing tryptophan. If secretion is dependent on protein machinery, then one should observe an inhibition of release of nuclease activity to the medium. If proteins are not needed for the process, then nuclease release should continue, but at a reduced rate.

Thus, it was the purpose of this study to investigate the characteristics of nuclease secretion and to determine the possible effects of tryptophan analogs on the secretory process.

REVIEW OF THE LITERATURE

Since Cunningham et al. (24) described a deoxyribonuclease (DNase) in the culture medium of Staphylococcus aureus, extensive research has led to the physical and biochemical characterization of the enzyme. The enzyme was described by these workers as a specific phosphodiesterase having unusual properties: it was found to be calcium rather than magnesium activated, there was no loss of activity when the enzyme was boiled in crude supernatants, and the molecule appeared to be rather small as it passed through dialyzing membranes. Its hydrolytic action was endonucleolytic initially and enonucleolytic in the terminal stages of the reaction. Enzymic action gave rise to unusual end products, in that the mono and dinucleotides were 3' rather than 5' phosphonucleotides. Subsequently, Reddi (58) reported that the enzyme was similarly active on ribonucleic acid (RNA).

Methods Used in Purifying Nuclease

Alexander et al. (1) were the first to report on the extensive purification of staphylococcal nuclease from strain SA-B. By combining a series of ammonium sulfate and trichloroacetic acid (TCA) precipitations with column chromatography on diethyl amino ethyl (DEAE) cellulose, they achieved a 500-fold purification.

In order to eliminate contaminating phosphatases, Ohsaka et al. (49) described an extensive purification procedure for nuclease. Their experiments necessitated this pure

preparation as the nuclease was used to identify the nucleotide terminus bearing a free 5' monophosphate. Ammonium sulfate filter cakes of nuclease obtained from strain SA-B were fractionated with 50-90 percent ammonium sulfate, then precipitated with TCA. The final precipitation with 50% ethanol was followed by DEAE cellulose column chromatography. This procedure yielded nuclease which contained about 0.01% phosphatase.

Mukai et al. (47) reported that the nuclease obtained from strain SA-B could be purified to yield a product with a specific activity comparable to the staphylococcal nuclease (V-8) reported on by another laboratory (2). In addition to purification procedures which were described earlier (49), the investigators employed column chromatographic techniques to obtain a product with a much higher specific activity. Gel filtration through Sephadex G-75 yielded two poorly resolved peaks. Subsequent chromatography of the first peak on carboxy methyl cellulose (CMC) and DEAE cellulose indicated a number of nuclease peaks which had decreasing specific activities in order of their elution from the cellulose. Similar chromatography of the second Sephadex peak yielded a rather homogeneous product with a specific activity over 100. The authors concluded that the various peaks were due to formation of stable complexes between nuclease and inert protein. Thus, the elution patterns of the nuclease activity varied and the inert protein contributed to a lower specific activity.

Hacha and Fredericq (32) reported on the purification and properties of the deoxyribonuclease of S. aureus. They

indicated that their principle tool for obtaining nuclease with a high specific activity was column chromatography through calcium phosphate. Their studies revealed that the calcium activator was tightly bound to the enzyme rather than the substrate during hydrolysis.

Eventually, Anfinsen and his colleagues were conducting the most extensive research on staphylococcal nuclease. After an earlier report on the characterization of the nuclease of strain V-8 (2), they concentrated their research efforts on the nuclease of S. aureus, strain Foggi. Thus, this enzyme of Foggi has become the most extensively studied staphylococcal nuclease. Sulkowski and Laskowski (77) reported that the crude ammonium sulfate cakes derived from this strain were 10-fold richer in nuclease than those from strain SA-B. Unfortunately for their studies dealing with nucleic acid structure, the preparation was also richer in phosphatases. However, they devised a purification scheme which gave a product nearly devoid of acid and alkaline phosphatases. Purification steps involved heating which was followed by a series of precipitations with ammonium sulfate, TCA, and ethanol. After column chromatography on Bio-Gel P-100 and CM cellulose, the nuclease was crystallized from an ammonium sulfate solution. The crystals were devoid of all phosphatase activity. However, when the preparations were subjected to disc gel electrophoresis, trace impurities were noted. A prolonged enzyme assay indicated that there was some phosphatase activity, but it was less than 1×10^{-6} units/ml.

The ultimate purification scheme for nuclease, affinity chromatography, was reported by Cuatrecasas et al. (22). In this technique, a derivative of the specific substrate inhibitor (deoxythymidine-3'-5'-diphosphate) was produced and chemically linked to the porous gel, Sepharose. The addition of nuclease sample to the modified gel adsorbed the nuclease from the solution while all other components emerged in the effluent. After washing the column with appropriate buffer to rid the column of contaminants, the nuclease was eluted by lowering the pH to dissociate the enzyme from the inhibitor. Analysis of this preparation revealed apparent homogeneity.

Mikulski et al. (45) have recently reported on a purification procedure which finally rendered their nuclease completely devoid of any phosphatase activity. The procedure involved chromatography on CM cellulose, coupled with gel filtration and final purification on a phosphocellulose column.

Enzymatic Action of Nuclease

Further investigation on the mode of action of nuclease indicated that the enzyme had a preference for the 5' phosphodiester bonds adjacent to the adenylic and thymidylic residues (27). Depending on the particular substrate present, preferential action varied. However, the overall reaction was more rapid if heat denatured DNA was used. In the presence of highly polymerized DNA, enzymic action was primarily endonucleolytic, occuring at the adenine and thymine sites. However, once shorter chained oligonucleotides had been produced, the hydrolytic action changed to exonucleolytic. Here again, the site of action was preferential as deoxythymidine and de-

oxyadenine residues were the first monophosphates detected (62, 76).

Alexander et al. (1) confirmed these discoveries in reporting that the mode of action changed from endonucleolytic to exonucleolytic during the reaction. By using synthetic polymers, they found that the rate of hydrolysis for oligonucleotides ending in 3' phosphates was greater than for polymers which had no phosphate in the terminal 3 position. Polynucleotides with a 5' phosphate end group were least susceptible to hydrolysis.

The hydrolytic action of the enzyme on natural and synthetic polymers was investigated further by Sulkowski and Laskowski (78). They examined the activity on polymers of deoxyadenylic and deoxythymidylic acids, and on crab satellite DNA which has a low G C content. The investigators confirmed earlier findings in that the primary action is endonucleolytic. However, the salt concentration was critical for maximum endonucleolytic action on the synthetic polymer. When the salt concentration was raised to a critical level, endonucleolytic cleavages were reduced 4-fold, and exonucleolytic action predominated. The rate of enzymic action depended most critically on the nature of the base. At an abnormally low ionic strength, the exonucleolytic action was preferential on the polydeoxyadenylic strand of the synthetic poly $(dA)_n$ $(dT)_n$ DNA. Polydeoxythymidylic acid was degraded most rapidly; polydeoxyguanylic acid was most resistant to hydrolysis.

Mikulski et al. (45) reported that the enzyme, at near saturation levels, could digest deoxydinucleotides. These

molecules were formerly believed to be completely resistant to hydrolysis. The deoxydithymidine substrate was found to be most susceptible, while the deoxythymidinedeoxyguanidine nucleotide was most resistant.

Characteristics of the Active Site of Nuclease

A preliminary study of the active center of nuclease was conducted by Cuatrecasas et al. (18, 21). The authors reported that spectrophotometric studies on substrate enzyme interaction could be facilitated by using the specific inhibitor deoxythymidine 3' -5' diphosphate. Binding of the inhibitor, which occurred most readily at pH 7.0 did not occur in the absence of calcium or other suitable cation; however, strontium could be substituted. The nucleotide bound in a 1:1 ratio with enzyme, and the binding indicated that tyrosine residues were intimately involved. This was disclosed by the shift to higher pH values of phenolic hydroxyl ionizations upon binding of substrate. There was no change in the tryptophan absorption which indicated that this residue was probably not involved in binding. By observing the dissociation constants between the enzyme and a series of 5'-phosphoryloligothymidyl derivatives, the investigators were able to determine which oligonucleotides bound most tenaciously. They found that the active site involved a large portion of the enzyme's surface, and accomodated a trinucleotide most favorably. Binding was primarily due to ionic interaction between the phosphate groups of the substrate and the appropriate amino acids in the enzyme. The hydrolytic site was closely associated with one binding site which recognized the phosphodiester bond to

be cleaved.

Further studies revealed that, indeed, tyrosine residues at the active sites were important for hydrolytic action. By modifying the tyrosines in the nuclease with tetranitromethane, Cuatrecasas et al. (19) tested which tyrosine residues were spatially adjacent and probably contributed to binding and subsequent hydrolysis of the substrate. By nitrating the molecule in the presence and the absence of the specific inhibitor, the authors were able to deduce that 3 tyrosine residues were involved in substrate binding. One of the three residues, the tyrosine at position 85 was particularly important in catalytic activity. Its specific nitration yielded an inactive protein. The investigation also revealed that the lone tryptophan residue was within the hydrophobic region of the protein, as it could be nitrated only if the nuclease were denatured. However, the specific nitration of tyrosine 115 gave a derivative which exhibited all normal DNase activity, but only 1/2 of the normal ribonuclease (RNase) activity. Thus, it was postulated that tyrosine 115 was particularly important in binding the carbohydrate moiety of RNA.

Further chemical modification of the active site of native nuclease resulted in a more detailed characterization of the enzyme's catalytic center (20). Nuclease, with nitrated tyrosyl residues, was allowed to react with either of two cross-linking reagents, p,p'-difluoro-m,m-dinitrodiphenyl sulfone or 1,5 difluoro-2,4-dinitrobenzene. The linkage of adjacent tyrosyl residues confirmed the stereochemical proximity of the tyrosine residues at positions 85 and 115.

Anfinsen et al. (2) reported rudimentary data on the structural properties of nuclease in 1963. This communication served as an introduction for the massive investigation of the enzyme which followed. They indicated properties of strain V-8 nuclease which were very different from those which were finally advanced (80, 81). The molecule, with a sedimentation coefficient of 1.7S, had a molecular weight of 11,000 to 12,000 and contained approximately 100 amino acids.

Preliminary investigations on the chemical characteristics of strain V-8 nuclease at the molecular level were published by Taniuchi and Anfinsen in 1966. Cleavage of the protein with cyanogen bromide yielded five peptides. Alanine was reported as the amino terminal residue; glutamine was the carboxy terminal amino acid. The molecule possessed no cysteine or cystine residues and the molecular weight was calculated to be about 17,000.

Through extensive research, Taniuchi et al. (80, 81) finally resolved the complete primary structure of V-8 nuclease. The techniques involved the sequential degradation and subsequent analyses of peptides produced via proteolytic enzymes. The molecule was described as a single polypeptide chain containing 149 residues. There was no sulfhydryl or disulfide groups present. The protein contained one tryptophan residue and had an exact molecular weight of 16,807. The isoelectric point was determined to be 9.6 and the pH optimum for enzymatic activity was 9.2.

While producing active fragments of nuclease, Taniuchi and Anfinsen (82) indicated that the N-terminal end of the poly-

peptide was not necessary for enzymatic activity. Treatment of the inhibitor-enzyme complex with trypsin yielded 3 fragments composed of residues 1 through 5, 6 through 48, and 49 or 50 through 149. These fragments were dubbed respectively, P_1, P_2, and P_3. Combination of the middle and C-terminal fragments gave limited nuclease activity. No fragment alone had any catalytic activity.

Heins et al. (33) summarized the known characteristics of this nuclease and proposed that these traits made the enzyme an interesting subject for conformation and active center investigations. In contrast to their earlier report (2), ultracentrifugal measurements indicated that the molecular weight was approximately 17,650. The optical rotatory dispersion and circular dichroism of the nuclease revealed that the molecule was a compact globular protein containing approximately 45% α-helix.

When Foggi nuclease became the most prominent staphylococcal nuclease used for various studies, Cusumano et al. (25) determined its primary structure. Using the protocol employed in delineating the structure of V-8 nuclease, they found that Foggi nuclease was structurally similar to V-8 with one exception. The leucine in position 124 of the V-8 molecule was substituted by a histidine in Foggi. Thus, they concluded that the residue in this particular position was not important in biological activity. In conjunction with this discovery, Anfinsen and Corley (3) produced an active variant of nuclease which contained norleucine in place of methionine. The methionine-less enzyme exhibited normal ac-

tivity, and thus, it appeared that the four residues of methionine were simply structural components in the molecule unnecessary for enzymic activity.

The production and properties of a semi-synthetic enzyme were described by Ontjes and Anfinsen (54, 55). Via solid phase organic synthesis, they were able to produce the P_2 fragment (residues 6 through 48). When this polypeptide was mixed with an equimolar amount of P_3 (residues 49 through 149), which had been derived from native nuclease, there was limited nuclease activity. They also reported that certain regions of the synthetic polypeptide were singularly necessary for activity. If glutamine was substituted for the glutamic acid at position 43, no activity was observed when this altered fragment was mixed with P_3. However, the fragment retained the ability to bind to P_3.

Studies employing circular dichroism, optical rotatory dispersion, immunodiffusion, solvent perturbation, and fluorescence measurements indicated that the P_2 and P_3 fragments are very loose, random coiled polypeptides. However, when the fragments are combined in a equimolar ratio the complex becomes ordered and there is limited enzymic activity. These results are consistent with the theory that nearly the entire primary structure must be present before the molecule folds into its ordered native configuration (83).

The latest physical characteristic to be delineated has been the determination of a reversible structural transition which occurs between pH 3 and 4. Traversing this pH range causes a minor refolding which is very rapid. By measuring the

change in the tryptophan fluorescence, Schechter et al. (66) reported that the refolding was a two-step, sequential, first order process with 1/2 times of 55 and 350 milliseconds.

Omenn et al. (53) have concluded that the nuclease by lacking disulfide bridges and having low helix content is a highly motile or flexible polypeptide chain. They found that increasing substitution by acetylation led to parallel decreases in enzymic and antigenic activities.

Nuclease - Immunological Properties

Immunological studies revealed that antinuclease antibody could neutralize the DNase and RNase activity of the enzyme (30). However, two types of antibodies were detected.

One of these reacted with the molecule either in the presence or the absence of the specific inhibitor, whereas the other would react only when no inhibitor was present. The authors postulated that the lack of reaction of this latter antibody was due to a conformational change of the nuclease while bound to the substrate. Through this alteration, the antigenic determinant was modified so as to be unrecognizable by the antibody. However, the same investigators have since reported that the nuclease actually possesses two antigenic determinants, one of which is obscured by binding the inhibitor deoxythymidine-3',5'-diphosphate to the antigen (52).

Initial experiments using affinity chromatography have indicated that one of nucleases' two antigenic sites was located on the C-terminal end (51). After chemically binding the P_1, P_2, or P_3 fragment to Sepharose 4B, antinuclease anti-

body was passed through the column. The assay of the effluent revealed that the greatest amount of specific antibody had been bound to the P_3- Sepharose gel.

Physiology and Genetics of Nuclease Production

Since nuclease has not been considered in relation to the infectious process, there is only limited data on the enzyme as a physiological product. However, production of the enzyme is closely correlated with pathogenicity in S. aureus strains (6, 34).

Investigations by Schakmann and Blobel (64, 65) have indicated that the bulk of the nuclease activity was secreted during the late log and early stationary phases of growth. In contrast, to the apparent similarity between V-8 and Foggi nucleases, these authors noted distinct differences between staphylococcal nucleases. Upon comparing the nuclease of a human strain to that of a canine strain, the authors noted differences in heat stability, citrate activation and serological specificity. The two nucleases were quite similar in their pH optimum, cation activation and substrate specificity.

The effect of anaerobiosis on nuclease production was investigated by Fox and Holtmann (28). They found that the cell yield from an anaerobically-grown culture was one-fourth that of an aerated culture. Consequently, the total amount of enzyme in the supernatant was proportionately lowered. When grown aerobically, the culture appeared to follow deaminative pathways, as the terminal pH was 8.0. Under anaerobic conditions, metabolism was fermentative and the pH fell to 5.5.

However, if the pH were held constant at 7.5 throughout aerobic or anaerobic growth, the amount of nuclease produced per cell was constant.

Only one report has dealt with the genetic modification of a strain of S. aureus for the purpose of obtaining nuclease deficient mutants (50). All other attempts to obtain single amino acid substitutions by point mutation in the nuclease gene were unsuccessful. Rather, the nitrosoguanidine treatment yielded only nuclease deficient mutants which simultaneously lost the β-lysin and coagulase markers. Reversion with ethyl methane sulfonate restored all markers simultaneously. Thus, the authors speculated that the nuclease, as well as β-lysin and coagulase genes were linked. Their position could possibly be on an extrachromosomal element, but regardless, nitrosoguanidine affected all of the genes simultaneously.

Amino Acid Analogs

An early review of the effect of the amino acid analogs on microorganisms was conducted by Richmond (60), and dealt with the various effect of analogs on growth and protein synthesis. The earliest work was concerned with the effect of tryptophan analogs in tryptophan auxotrophs of Escherichia coli. The use of 4 or 5 methyltryptophan (MT) resulted in an immediate shift in the growth rate from exponential to linear. However, there was an eventual three to four-fold increase in optical density.

The immediate effect of p-fluorophenylalanine (FP)

on E. coli cells was a reduction in protein synthesis. However, when the decreased protein synthesis was plotted against dry weight, the proportion was the same as in normal cells. Later, it was reported that FP and other analogs were not specific inhibitors of protein synthesis but rather partical inhibitors to growth. The FP in the media of E. coli cultures is rapidly incorporated into cellular protein. If the analog was in the presence of an equimolar concentration of phenylalanine, then the natural amino acid was incorporated exclusively and the analog excluded from the cell. Only when the concentration FP was raised to four times that of the phenylalanine did any enter the cell. Once within the pool though, FP was not utilized until the phenylalanine supply had been exhausted.

The treatment of Bacillus cereus with FP caused no observable change in the culture for 20 minutes. Growth then slowed, and eventually ceased in 4 to 5 hours. In competition experiments with FP and phenylalanine, it was noted that the concentration of FP had to be 10 times that of phenylalanine in order for the analog to enter the cell. From this data, it appeared that the analog and natural product utilized the same transport system, but that the system discriminated against the analog.

The endogenous synthesis of amino acids within the cells is controlled by feedback inhibition or repression. Through the use of radioactive amino acids, it was shown that the amino acid pools in E. coli are in rapid equilibrium with the protein synthesizing sites of the cell. Thus, once analogs

get into the cell the endogenous supply is affected immediately. Through repression or inhibition, the analog works to effectively lower the concentration of natural product within the pool. Thus, its incorporation is facilitated.

Investigations have shown that the amount of analog incorporation depends on the organism and the particular antimetabolite used. Thienylalanine or FP can replace 75% of the phenylalanine in E. coli and B. cereus proteins (16, 60). Of all systems studied, this replacement is perhaps the most efficient. Norleucine or ethionine can replace 50% of the methionine in E. coli protein, whereas 7-azatryptophan acts to replace only 30% of the tryptophan.

In general, the mode of action of analogs is inhibitory if one sees an immediate effect after adding the analog; repression on the other hand, causes effects slowly as the natural product continue to be produced by the existing enzymes.

Early investigations of the effects of methyltryptophan analogs on E. coli tryptophan synthetase revealed that all of the isomers were not equally effective. The 2-methyl derivative had no effect; while a definite effect was seen with the other derivatives with the effects of 4-MT > 5-MT > 6-MT > 7-MT. Reports have indicated that 6-MT is a repressor of many enzymes in the tryptophan pathway. The 4-methyl derivative is a strong inhibitor of pure tryptophan synthetase, while 5-MT inhibits the enzymes responsible for the conversion of shikimic-5-phosphate to anthranilic acid (46). Other reports confirmed that the methyl substituted tryptophans pass into the cell, but act only a repressors and inactivators. In conjunction

with this observation, some investigators (48, 73) noted that the analogs are not activated or incorporated into protein. However, azatryptophan and trypazan entered the cell by a normal transport mechanism and then were incorporated into protein.

More recent investigations have largely confirmed the earlier discoveries on analog effects. Attention has turned from studying the gross overall effects of analogs on growth to the specific alterations caused by their incorporation into proteins. Of particular significance in this respect, are the findings which have been reported for E. coli alkaline phosphatase. By using a series of E. coli amino acid auxotrophs, Schlesinger has produced alkaline phosphatase which has nearly all of a particular amino acid replaced by an appropriate analog. The active alkaline phosphatase of E. coli is a dimer. When a histidine auxotroph is grown in media containing 1,2,4, triazole-3-alanine, protein synthesis and the production of the alkaline phosphatase subunits occurs. However, the incorporation of the analog prevents the association of the subunits into a dimer, and the active enzyme is never formed (70).

In studies with an arginine auxotroph of E. coli, the same investigators found that replacement of the basic amino acid with canavanine led to the production of a limited amount of active alkaline phosphatase. Thus, they concluded that the actual presence of arginine in alkaline phosphatase was not essential for enzymatic activity (72).

The use of a tryptophan auxotroph of E. coli confirmed the earlier work reported by Richmond (60). Schlesinger (71)

found that a tryptophan auxotroph of E. coli can produce active alkaline phosphatase when the mutant is grown in the presence of 7-azatryptophan or trypazan.

The presence of natural tryptophan is important for activity in a variety of proteins. Tryptophan is an intregal part of the active site of the egg white lysozyme (36). The chemical modification of one-half or more of the tryptophan residues yielded an inactive protein. In investigating the active site of yeast alcohol dehydrogenase, Schellenberg (67) reported strong evidence for the participation of a tryptophan residue during enzymic activity. Data indicated that the indole side chain underwent a reversible dehydrogenation to an indolenine during the transfer of the hydrogen atom between NAD and the substrate.

Recent endeavors by some investigators have challenged earlier reports (48, 73) which stated that the methyl tryptophan derivatives were neither activated nor incorporated into protein. In using a tryptophan auxotroph of E. coli 15T$^-$, Lark (39) has indicated that 5-MT will satisfy the tryptophan requirement of the mutant in order to initiate another round of DNA replication. The use of tritiated 5-MT in the growth medium and the subsequent fractionation of the cells led to the isolation of a radioactive hot TCA insoluble fraction. This incorporation could be eliminated when tryptophan was added to the growth medium which contained ^{3}H 5-MT. Pronase digestion of the radioactive hot TCA insoluble fraction resulted in the isolation of approximately 70% of the radioactivity in a product which was indistinguishable from 5-MT,

and which co-chromatographed with added 5-MT.

Evidence for the incorporation of 5-MT into the proteins of B. subtilis was presented by Barlati and Ciferri (5). After growing a tryptophan auxotroph in the presence of the analog, they isolated the cellular protein and recovered the 5-MT from the protein after alkaline hydrolysis. Chromatography of the hydrolysate indicated that no tryptophan was present.

Jensen (35) has indicated that 5-MT acts on other enzymes of the tryptophan pathway in addition to the shikimate to anthranilate enzymes. His data, accrued on B. subtilis, showed that the addition of indole almost completely counteracted the effect of 5-MT, whereas anthranilate was about 1/4 as effective. The author noted that the basis for this ineffectiveness was not known. The work of Leboeuf-Trudeau et al. (40) found that S. aureus reacted differently to tryptophan analogs than did B. subtilis. The addition of L-tryptophan, indole or anthranilic acid completely reversed the inhibitions caused by tryptophan analogs. This same report also revealed that the effects produced by 5-MT, 4-MT, or 7-AT were quite comparable. These analogs suppressed growth by 50% and caused the production of culture supernatants which were nonlethal for mice. The authors speculated that the possible incorporation of the analogs caused the synthesis of inactive toxic material, but offered no evidence indicating that the analogs were actually incorporated into proteins.

An investigation of the effect of amino acid analogs

upon exocellular protein secretion in Bacillus has been reported by Welker and Campbell (12, 87). In studying the tryptophanless alpha amylase of B. stearothermophilus, they found that the enzyme was preferentially synthesized in the presence of 5-MT. However, growth in the presence of FP inhibited active alpha amylase formation by greater than 90%. This, apparently, is the only analog-secretion study which has been conducted.

Secretion of Exocellular Enzymes

Studies on the secretion of extracellular enzymes has largely been confined to microbial systems. Within this group of organisms, investigations on the enzymes of the genus of Bacillus have surpassed all others. Within the Bacillus species, an overwhelming amount of information has been accrued on the secretion of penicillinase.

Pollock (57) proposed the first model for penicillinase secretion. His studies on B. subtilis revealed that a large portion of the penicillinase was actually cell-bound. Growth inhibiting concentrations of chloramphenicol only partially inhibited the release of the enzyme. Initial experiments indicated that glucose was far more effective as an inhibitor of enzyme secretion. However, subsequent experiments with glucose revealed that the noted effect was due to the lowered pH of the medium. If a culture was grown in the presence of glucose while the pH was held constant at 7.8, then the culture elicited as much penicillinase as the control. An experiment was conducted to determine if a proteinase was involved. The results indicated that near saturation concen-

trations of the protease inhibitor, diisopropylfluorophosphate had no effect on penicillinase release. Pollock concluded that the penicillinase secretion was dependent upon a non-proteolytic, pH dependent, enzymic cleavage which occurred at the surface of the cell envelope.

The studies on B. subtilis were extended to its alpha amylase by Coleman and Elliott (15). In this instance, they reported that alpha amylase secretion depended upon a carbohydrate source, but not glucose, and ferric ion at a concentration of 0.01 mM. Respiratory enzymes were also important, as no secretion occurred when a culture was placed under N_2 gas or in the presence of 2,4 DNP. However, the pathway of secretion of this enzyme was apparently very different from that of penicillinase. The addition of chloramphenicol completely inhibited the production and secretion of alpha amylase.

Lampen (38) published a concise review on the known characteristics of enzyme secretion. In this symposium, he discussed three rather well-characterized systems: bacillary alpha amylase, yeast invertase and the penicillinase of B. licheniformis. In general, he noted that most, if not all, of the bacterial exoenzymes are relatively small molecules with few or no disulfide cross-links. He thus speculated that this rather loose structure could be important in permitting the proteins to pass through cell walls and other barriers. In support of this, he noted that the Gram negative organisms secrete far less or fewer exoenzymes. This, Lampen felt, was due to the higher lipid content of the wall in these organisms, which made it more difficult for hydrophilic molecules, such

as enzymes, to pass through. Also, the higher concentration of mesosomal membrane in Gram positives could be of significant importance since some workers had reported that the cytoplasmic membrane was an important site for general protein synthesis in E. coli and B. megaterium.

In summarizing the previously mentioned work of Coleman and Elliott and other workers, Lampen stated that secretion of bacillary α-amylase is consistent with de novo synthesis on the membrane. Release may require the partial breakdown of the cell wall by autolysins or the enzyme may simply diffuse through the wall.

Yeast invertase is cell-bound and external to the membrane. Protoplasting releases it immediately. The production of invertase by protoplasts yields no cell-bound form. Since this enzyme is a glycoprotein, Lampen stated that its mode of secretion approached that of the transport of cell wall fragments to the external environment.

Lampen stated that the secretion of penicillinase from B. licheniformis represented an entirely different process. Over 1/2 of the penicillinase is attached to the cell. The enzyme is preferentially produced in log growth, and this production is sensitive to chloramphenicol. The enzyme is external, as 98% of the activity is released when osmotically protected protoplasts are treated with trypsin. This released protein is indistinguishable from the native exopenicillinase. As was the case with B. subtilis α-amylase, the liberation of the enzyme was dependent on temperature and pH, and the inhibition of the proteases had no effect on release. In pro-

posing a mechanism, Lampen felt that secretion of penicillinase was a facet of membrane synthesis. The penicillinase is bound to a carrier, which is placed within the cell envelope during membrane synthesis. This incorporation most probably occurs within the mesosomes, however, mesosomes are not necessary for the incorporation of the carrier-penicillinase complex within the membrane. Since the penicillinase is now external to the membrane, the appropriate hydrolytic action results in release of the enzyme. Data indicated that the carrier and penicillinase were held together via a peptide link.

Since the publication of the review, various authors have reported results as determined on other systems. The transfer of incomplete polypeptide chains across the microsomal membrane was witnessed in rat liver cells (59). The production of nascent proteins was induced by puromycin, which caused release of the polypeptide from the ribosome. The transfer was not accompanied by a change in the phospholipid metabolism and occurred in the absence of ATP and at 0 C. Similar experiments with gougerotin, which stops the elongation of the polypeptide but does not cause release from the ribosome, resulted in no transfer of polypeptide to the microsome. Thus, Redman concluded that release from the ribosome was all that was needed to effect the transfer of protein.

Protease secretion in streptococci, however, appears to be dependent on arginine and other co-factors (13). The investigators found that the effect of EDTA in halting secretion

could be reversed by the addition of zinc ions. If incubated in the presence of EDTA for more than 60 min, zinc and arginine had to be added in order to get protease release. This secretion represented de novo synthesis as chloramphenicol, puromycin, and actinomycin D halted secretion.

The secretion of penicillinase in S. aureus has received a great deal of attention by Richmond and his colleagues. By using a strain containing 2 structural genes for penicillinase, he proposed a new restriction on enzyme secretion. Experiments revealed that whether a strain was haploid or diploid for penicillinase genes, the yield of exoenzymes was constant. Thus, it was concluded that synthesis was restricted to certain regions within the cell, and synthesis occurred only when the genome and synthesizing site were in correct juxtaposition. The two genomes competed for the particular site when both were present, and thus, each genome contributed to approximately 1/2 of the total activity (61).

Another secreted staphylococcal protein which has been extensively studied is enterotoxin. Markus and Silverman (42, 43) have reported their findings on the secretion of enterotoxins A and B. By employing non-replicating cells, they defined a number of conditions which were necessary for enterotoxin production. The optimum pH for enterotoxin B production was 0.5 to 1.0 units above the optimum for enterotoxin A. Enterotoxin B, which is produced during late log and early stationary growth, continues to be secreted into nitrogen-free media. This secretion is not affected by chloramphenicol, which argues strongly for a precursor of enterotoxin B.

Enterotoxin A, however, is produced during log growth, apparently has no precursor, and is sensitive to chloramphenicol. The authors concluded that the toxins were quite similar in chemical structure and differed only in being primary or secondary metabolites.

In studying the excretion of the extracellular proteinase from a Sarcina species, Bissell et al. (8) noted that the final active enzyme was apparently transformed from an inactive precursor produced in early log. Calcium ion was required for the stability of the enzyme rather than its activity. In the absence of the divalent cation, the investigators could not detect any proteinase in the culture. Through other experiments the authors concluded that the calcium substituted for -S-S- bridges in stabilizing the tertiary structure. Thus, the active proteinase acted upon the precursor to form more active proteinase. In the absence of the calcium, the enzyme underwent self-destruction.

Evidence has now been presented which suggests that secretion of the bacillary penicillinase represents a unique secretory system. In studying the exocellular penicillinase, nuclease, and alkaline phosphatase of Bacillus species, Chesbro and Lampen (14) indicated that nuclease and alkaline phosphatase do not exist in a long-lived, cell-bound form. In B. licheniformis, over 95% of the activities are in the medium during growth. However, 50-80% of the penicillinase is cell-bound. In penicillinase secretion, the rate is dependent on synthesis and level of cell-bound enzyme; and these parameters vary during cellular growth.

The extensive data on the B. licheniformis penicillinase

system has resulted in a proposed secretion model for the enzyme. The enzyme produced on the polysomes within the cell, is immediately inserted into a growing point in the membrane. The molecule, in this state, is hydrophobic, which facilitates its transport through the membrane. From this conformation, the enzyme converts to a hydrophilic form and is stable in an aqueous environment. Since this conversion is favored by high pH, exoenzyme secretion is very pH dependent. This route which occurs rapidly, is chloramphenicol sensitive since it is directly dependent on protein synthesis. The hydrophobic penicillinase which is not secreted must polymerize into the plasma membrane at the growing point. This polymer which, it is believed, retains the ability to undergo the conformational change, can be secreted when growth conditions become adverse or otherwise vary. This route is chloramphenicol insensitive. In summary, the authors contend that the hydrophobic to hydrophilic change is the crucial step for enzyme secretion in microorganisms (63).

MATERIALS AND METHODS

Cultures

The UNH-15 and Foggi strains of S. aureus were used throughout this investigation. The UNH-15 strain was originally isolated from a case of bovine mastitis; the Foggi strain was obtained from Dr. Hiroshi Taniuchi of the National Institutes of Health.

The cultures were stored, lyophilized, on porcelin chips contained within cotton-plugged tubes. These storage tubes were placed within larger screw cap tubes containing a small amount of drierite (Hammond Co., Xenia, Ohio), in order to maintain low moisture content. The entire storage vehicle was maintained at 4 C.

A working culture was obtained by inoculating a chip into trypticase soy broth (BBL) and incubating overnight at 37 C. The culture was then streaked onto sheep blood agar and incubated for 18-24 hrs. Two or three isolated colonies, showing good zones of hemolysis, were used as the inoculum.

Cultural Conditions

Whenever a culture was grown for production of the maximum amount of nuclease in the milieu, incubation was conducted in a dialyzable complex medium, the pH of which was held constant throughout growth. The medium consisted of 30 g of N-Z amine, type A (Sheffield Chemical Co, Norwich, N.Y.) and 30 g of yeast extract (Fisher Scientific or Difco Labs) suspended in 150 to 175 ml of distilled water. This mixture was dissolved by boiling for 3-5 min. The solution was then placed in 3/4 in diameter dialysis tubing (Fischer Scientific).

Tubing and contents were placed within a Fernbach flask so that the tubing formed a circle around the periphery of the flask's bottom. One liter of salt solution containing 4 g of KH_2PO_4, 2.5 g of NaCl, and 2.5 g of KCl (Fisher Scientific) was added to the flask, along with a magnetic stirring bar. The flask was capped and autoclaved at 121 C for 12 min. The sterilized medium was cooled to cultivation temperature by immersing the flask in a 37 C water bath. The cooled flask was then placed in a water-filled Nalgene tub set on top of a magnetic stirrer. The water was held at 37 C by circulating it through an adjacent water bath (P.M. Tamson, Holland).

The Fernback flask was fitted with a No. 13 four-hole stopper. These holes accomodated one 20 cm capillary pipette, and two 0.5 x 10 cm sections of glass tubing. The electrode and the 25 cm pipette extended below the surface of the medium. The pipette was used for introducing oxygen into the culture. One of the tubing sections served as the port for addition of the 5N KOH titrant; the other tubing section was cotton-plugged and capped with a serum bottle cap. With the cap removed, this port served as the exit vent during oxygen gassing.

The medium dialyzing '<u>in situ</u>' was inoculated with 50 ml of an 18-20 hr culture in 5% yeast extract. The stopper and accessories were replaced, and the magnetic stirrer was started. The pH was immediately adjusted to 7.25 by activating the pH meter-titrator system (pH meter 28c and titrator TTT 11b, Radiometer, Copenhagen). This system served to hold

the pH at 7.2 to 7.3 throughout growth. Concurrently, approximately 3 liters of pure oxygen (Matheson Gas Products, Rutherford, N.J.) was flushed into the flask at 0.5 to 1.0 psi. At one hr intervals, through 8 hr and at 9 1/2 and 11 hr, approximately one liter of oxygen was flushed into the medium under the conditions of the initial gassing.

At 12 hr, the system was turned off, the stopper removed and the flask, capped with aluminum foil, was removed to a 37 C incubator. At 22-24 hr, the culture was killed by adding 10 ml of chloroform, and the supernatant was harvested by centrifugation at 7,000-8,000 x g for 30 min.

A modification of the tryptophanless, semi-synthetic medium of de Repentigny et al. (26) was used in the majority of experiments on the secretion process. Hy-Case, S.F. (Sheffield, Chemical Co., Norwich, N.Y.) was substituted for the casamino acids used by de Repentigny. In addition to thiamine and niacin, Ca panthothenate and biotin were added at final concentrations of 12.5 ng/ml and 50.0 pg/ml, respectively. The pH was adjusted to 7.2 with concentrated HCl. Concentrated vitamin and $MgSO_4$ solutions, separately autoclaved, were added to the medium after autoclaving. Under no circumstances was glucose or any other carbohydrate added to the media.

The inoculum for experiments employing this medium was 3 or 10% of a 16-18 hr culture grown in the same media. Except in cultures grown as an inoculum, the volume of media in a flask always represented 10% of the total flask volume.

Determination of Growth

Growth of the organisms was determined turbidimetrically on a Klett-Summerson colorimeter fitted with a #54 filter. Growth curves were obtained by monitoring the change in turbidity of the culture while incubating in 500 ml Klett sidearm flasks. (Bel-Art Products, Vineland, N.J.). Flasks were aerated by shaking at 200-225 RPM on a rotatory shaker (New Brunswick Scientific, New Brunswick, N.J.) held at 37 C.

Amino Acid Analogs

Five-methyltryptophan, 4-methyltryptophan, and 7-azatryptophan were obtained from Sigma Chemicals, St. Louis, Mo. Five-hydroxytryptophan (5-HT) and ^{14}C 5-HT were obtained from Calbiochem. One gram of 5-methyltryptophan was tritiated by New England Nuclear, Boston, Mass. In all experiments employing the analogs, the compounds were added to a final concentration of 10^{-3} M when a culture of 200 Klett units was achieved.

Protease Inhibitors

The proteolytic enzyme inhibitors, N-tosyl-L-phenylalanine chloromethane (TPCK) and N-tosyl-L-lysyl chloromethane (TLCK) were purchased from Calbiochem. When added to cultures, the final concentration in the medium was 10^{-6} M.

Assay For Nuclease

Qualitative Plate Test: DNase test agar (BBL) was prepared as instructed on the container. Wells were cut in the agar using a cork borer (I.D. 2mm) and the agar plugs removed by suction. The wells were filled with 15-20 microliters

of the samples to be tested. The petri plate was then incubated for 3 hr at 37 C. At the end of incubation, the plate was flooded with ice-cold 7% perchloric acid (Fisher Scientific). The plate was then placed at 4 C and the acid was allowed to diffuse into the agar for 15-20 min. At the end of this precipitation period, zones of hydrolysis around the wells containing nuclease activity were clearly evident.

Quantitative Tube Assay: Nuclease activity was measured by using a modification of the method of Alexander et al. (1). Highly polymerized salmon sperm DNA (Catalog #2620, Calbiochem) was dissolved in distilled water at a concentration of 2.0 mg/ml. The DNA was heat denatured by holding the solution in a boiling water bath for 10 min. The tube was then cooled rapidly by swirling in an ice bath. This stock substrate solution was stored at -20C. The stock buffer solution was 0.05 M tris-(hydroxymethyl) aminomethane (Tris)adjusted to pH 8.8 with concentrated HCl. The stock calcium chloride solution was 0.2 M in distilled water. The assay mixture contained 0.200 ml of substrate, 0.250 ml of stock Tris buffer, and 0.025 ml of stock $CaCl_2$. This mixture was allowed to equilibrate at 37 C for at least 10 min. At 0 time, 25 microliters of enzyme, or a dilution thereof, was added to the reaction mixture. If dilutions of enzyme were required, 0.05 M Tris-Cl, pH 7.5 was used as diluent. At the end of 30 min the reaction was stopped by adding 0.1 volume of 10% citric acid to the 0.5 ml reaction mixture, and then removing the assay tube to an ice bath. In order to subtract the acid soluble nucleotides which were present in the reaction mixture before any

nuclease was added, a blank was used. Its composition was identical to that of a reaction mixture, except that no enzyme was added. The unhydrolyzed DNA was precipitated with the addition of 0.5 ml of ice cold 7% perchloric acid. After adding three ml of cold distilled water to the assay tubes, they were centrifuged at 3,000-4,000 x g at 4 C for 10 min. The amount of acid soluble nucleotides in the supernant was measured by reading its absorbance at 260 mu in a B & L 600 spectrophotometer (Bausch & Lamb, Rochester, N.Y.). An absorbance of 1.0 was considered to represent 1.0 unit of nuclease activity in the 25 microliters added to the reaction mixture.

The heat stable nuclease present in crude supernatants was assayed in the same way. The samples to be assayed for heat stable activity were placed in a boiling water bath for 10 min. Any precipitate which formed was removed by centrifugation at 10,000 x g for 10 min.

Preparation of Cell-Free Extracts

Extracts were prepared by enzymatically digesting the cell walls with lysostaphin using the method of Schindler and Schuhardt. (68, 69). Cells, derived from 1.0 ml of culture, were washed twice with 0.85% NaCl. The washed cells were resuspended in 0.9 ml of 0.05 M Tris-Cl, pH 7.5, and equilibrated at 37 C. At 0 time, 0.1 ml of a lysostaphin solution containing 10 units of enzyme activity/ml in 0.05 M. Tris-Cl, pH 7.5, was added to the cells. After 30 min of incubation, the tubes were removed to an ice bath, and then centrifuged at 10,000

x g for 15 min. The supernatant was used as the source of enzyme in the glucose-6-phosphate dehydrogenase assay.

Assay For Glucose-6-Phosphate Dehydrogenase

Dehydrogenase activity was measured by a modification of the procedure published by the Worthington Biochemical Corporation, Freehold, N.J. (89). The stock buffer solution was 0.05 M Tris-Cl, pH 7.8 which was also 0.005 M with respect to $MgCl_2$. The stock substrate was 0.1 M glucose-6-phosphate (Calbiochem) dissolved in distilled water. The buffer and substrate solutions were stored at 4 C; the nucleotide cofactor was prepared immediately before use.

The reaction mixture consisted of 2.5 ml of Tris-Cl, $MgCl_2$ buffer, 0.1 ml of substrate, 0.3 ml of enzyme and 0.1 ml of NADP solution. The reaction mixture, excluding the NADP, was allowed to equilibrate at 30 C within the water-jacketed sample chamber of a Beckman DU spectrophotometer. At 0 time, the NADP was added to the sample; 0.1 ml of distilled water was added to the blank. Enzyme activity was measured by recording the increase in O.D. at 340 mu in the 1 to 5 min interval. The reduction of one micromole of NADP per min was considered to represent one unit of enzyme activity.

Assay For Protease

Five ml of crude supernatant, osmodialyzed to one-tenth its original volume, was used in the protease assay. The substrate, azocoll, was obtained from Calbiochem. The stock buffer solution was 0.2 M KH_2PO_4-$KHPO_4$, pH 6.8. The

stock $CaCl_2$ solution was 0.01 M. The reaction mixture, contained in a 1.0 ml volume, consisted of 20 mg of azocoll, 0.25 ml of stock phosphate buffer, 0.25 ml of the $CaCl_2$ stock, and 0.5 ml of enzyme. The substrate, buffer, and calcium were equilibrated at 37 C for at least 10 min. At 0 time, the 0.5 ml of enzyme was added. During the reaction, the azocoll was kept in suspension by stirring the mixture with a 0.3 x 1.0 cm magnetic stirring bar powered by a submersible magnetic stirrer. (Tri-R Instruments, Rockville Center, N.Y.) At the end of 1.0 hr the reaction was stopped by adding 1.0 ml of 10% citric acid. The undegraded azocoll was removed by centrifugation at 3,000 x g for 10 min. The supernatant was pipetted away from the loose pellet and its absorbance was measured at 540 mu.

Protein Determination

Protein was determined according to the method of Lowry et al. (41). Twice crystallized egg white lysozyme (Pentex Corp.) was used as the standard for all samples except the cell lysates. Bovine serum albumin (Nutritional Biochemicals) was the standard in determining the intracellular protein.

Specific activities of the intra- or extracellular enzymes were expressed in terms of units of activity per microgram of protein. Determinations which were conducted in Tris buffer were properly blanked, as this salt contributes a significant amount of color in this measurement.

Dialysis and Osmodialysis

All dialysis and osmodialysis procedures were conducted at 4 C. The pores in the dialyzer tubing (Fisher Scientific) used for the dialysis of all samples containing nuclease, were made smaller by boiling the tubing in 1.0% $NaHCO_3$ (Fisher Scientific) for 10 min (80). Crude supernatants to be loaded onto CM cellulose columns, were dialyzed against four volumes of 0.01 M β-glycerophosphate (B-GP) (Calbiochem), pH 6.8-7.0. The samples were dialyzed for 12 hr, and the dialysate was replaced at 4 and 8 hr.

Column effluents containing nuclease were dialyzed against 0.05 M NH_4HCO_3 (Fisher Scientific). The use of this volatile salt assisted subsequent operations in that nuclease samples dissolved in this buffer could be rendered salt-free through lyophilization.

Osmodialysis was performed by coating the dialysis tubing with soluble CMC (Aquacide I, Calbiochem). Dehydration was allowed to occur until the desired amount of solution, usually 2-10 ml, remained within the tubing.

Ion-Exchange Chromatography

Carboxy-methyl cellulose (CM 32, Whatman) was obtained from H. Reeve Angel (Clifton, N.J.). Approximately 5 g of dry powder was pre-cycled by first washing in 0.5N NaOH for 30 min, rinsing with distilled water on a Buchner funnel, and then washing in 0.5 N HCl for 30 min. After rinsing with distilled water, the exchanger was suspended in 200 ml of 0.01 M B-GP and the pH was adjusted to 6.8 with 0.05 N

NaOH. The cellulose was again rinsed with distilled water. The fines were removed by suspending the media in 100 ml of the B-GP buffer in a 100 ml graduated cylinder. After inverting the capped cylinder several times in order to produce a homogeneous suspension, the powder was allowed to settle for 15 min. At the end of this time, the supernatant and fines were removed by suction. A sufficient quantity of B-GP buffer was added to the cylinder in order to bring the volume to 100-110 ml and the process was repeated. After the second fines removal, the volume of the cylinder was brought to approximately 80 ml, and the settled cellulose was resuspended by inverting the capped cylinder. This suspension was poured into a 1.9 x 45 cm glass chromatography column containing 15-20 ml of B-GP buffer. The residual particles in the cylinder were rinsed into the column with buffer. Once settling had produced approximately 1.0 cm of bed, the stopcock was fully opened and the cellulose was packed at maximum flow rate. When a uniform bed was formed, the column was placed in a 4 C cold room and equilibrated with the B-GP buffer until the conductivity and pH of the effluent was equal to that of the buffer. (Approximately 200 ml were usually required).

The conductivity of the crude dialyzed supernatants was determined with a Serfass Conductance Bridge (Model RCM15B1, Arthur H. Thomas, Philadelphia, Pa.). The supernatants, having a conductivity of 2-4 millimhos/cm, were loaded onto the equilibrated columns. The loaded column was then washed with at least 100 ml of B-GP buffer. The protein was eluted with a

non-linear NaCl gradient in 0.01M B-GP, pH 6.8. The gradient was produced by using a 9-chamber gradient maker (Autograd, Technicon Corporation). Eight chambers were loaded, sequentially, with buffer having a conductivity of 10, 10, 15, 15, 20, 35, 50 and 80 millimhos/cm. The ninth chamber was not used. The column was serviced by an LKB pump and Redi-Rac fraction collector (LKB, Rockville, Md.). A flow rate of 40 ml per hour was maintained by attaching a Thermo-watch (I^2R, Cheltenham, Pa.) to the column. Fractions were collected at 12 min intervals.

Gel Filtration Chromatography

Sephadex G-75 Superfine was purchased from Pharmacia Fine Chemicals, Piscataway, N.J. The dextran was swollen in 0.05 M NH_4HCO_3 at 4 C for 24 hr. The chromatography column (K25/40, Pharmacia) was fitted with two flow adaptors (Pharmacia). These enabled reverse-flow chromatography to be used. The column was packed in the direction of flow to be used during the studies.

The column was serviced by a peristaltic pump (LKB, ReCyChrom Pump), and an Ultro Rac fraction collector (LKB). The column effluent was monitored at 280 mu by a Uvicord II (LKB), connected to a Heath recorder. The drop counting mode of collection was used. Fractions containing fifty drops were collected; the flow rate was adjusted to four drops per minute.

The void volume (Vo) was determined with blue dextran (Pharmacia). The column was standardized with ovalbumin (M.W. 45,000, Mann), myoglobin (M.W. 17,800, Calbiochem), RNase

(M.W. 13,600 Worthington Biochemical) and cytochrome c (M.W. 12,400, Calbiochem). The molecular weights of unknowns were determined according to Whitaker (88).

Electrophoresis

Analytical polyacrylamide disc gel electrophoresis was conducted in the 15% pH 4.3 gel described by Canalco (Rockville, Md.). The Canalco model 1200 apparatus was serviced by a Beckman Duostat power supply. Electrophoresis in a 0.35 M B-alanine-acetic acid buffer, was conducted at 5 ma per tube for 3.5 to 4.0 hr. The gels were stained with 0.5% buffalo black in 7% acetic acid for 1.0 to 1.5 hr. They were then electrophoretically destained at 10 ma/tube.

Gels which were run for the subsequent assay of nuclease were not stained. At the end of the electrophoresis, the gels were removed from their tubes and sliced laterally into approximately 1.0 mm thick discs. Each of the discs was then placed in a tube and eluted for 12 hr with 0.5 to 1.0 ml of 0.05 M Tris-Cl, pH 7.5. Twenty-five microliters of this solution, or a dilution thereof, was used for the quantitative tube assay described earlier.

Preparative polyacrylamide electrophoresis was conducted with a Fractophorator (Buchler Instruments, Fort Lee, N.J.) The instrument, fitted with a 13 cm diameter water-cooled column, was serviced by the LKB Redi-Rac fraction collector. The 15% pH 4.3 gel was 3.5 cm long. The stacking gel was 1.0 cm long. A constant current of 10 ma was supplied by the Duostat. The buffer was 0.1 M B-alanine-acetic acid,

pH 4.4 (11). Fractions were collected at 15-min intervals.

The molecular weights of the nucleases, as determined by SDS-polyacrylamide gel electrophoresis, were calculated by the method of Weber and Osborn (86).

Radioactive Measurements

The radioactivity of CM cellulose column effluents was measured by placing 1.0 ml of a fraction into 10.0 ml of Aquasol (New England Nuclear, Boston, Mass.) contained within low background glass vials (NEN). The vials were counted in a Packard Tri-Carb Liquid Scintillation Counter (Oak Grove, Illinois) equipped with automatic external standardization.

The radioactivity of polyacrylamide discs, derived after analytical gel electrophoresis, was determined by placing the disc in 10.0 ml of Aquasol. The cocktail completely dissolved the disc. The samples were then counted as described above.

Immunodiffusion

Immunodiffusion was carried out using a modification of the method described by Ouchterlony (56). Petri dishes (50 mm) were prepared by cleaning with acetone (Fisher, A.C.S. certified) and then allowed to air dry. Four ml of 1.3% Noble agar (Difco) in half strength veronal buffer (LKB), pH 8.6, preserved with 0.01% sodium azide, was added to each dish. The plates were allowed to dry at room temperature for 24 hr; then they were stored at 4 C. Wells (I.D. 8 mm) were cut with an Auto-gel well cutter (Grafar, Inc. Detroit, Mich-

igan), so that the distance between adjacent wells, center-to-center, was 1.0 cm. The plugs were removed by suction. The capacity of these wells was 50 microliters.

Nuclease preparations were reacted with antiserum produced in dairy cattle against a toxoid made from a 50-80% $(NH_4)_2SO_4$ precipitate of the supernatant of 4 strains: UNH-15, -10, -7 and Geraldine. Diffusion was allowed to take place at 30 C. The plates were examined daily for precipitation lines.

The diffusion was halted by rinsing the petri dish with 0.85% NaCl for 5 days with frequent changes. The salt was removed by rinsing the plate with distilled water. The wells were then filled with distilled water, and the agar was covered with a wet 50 mm diameter filter paper disc (#3, Whatman). The agar was allowed to dry for 1-2 days at room temperature. The paper disc were then removed and the lines were stained by flooding the plate with 0.5% buffalo black (Allied Chemical) in 7% acetic acid. After staining for 5 min, the dye was poured off and the plate was rinsed with a methanol-acetic acid-water (45:10:45) solution, with several changes. The lines stained blue on a clear background.

Amino Acid Analysis

Purified preparations of nuclease were hydrolyzed in 6N HCl containing 0.1% β-mercaptoethanol (Eastman Chemicals) (37, 44). Fifty microliters of 0.5 M hydrazine (Eastman) was also added to prevent chlorination of the ty-

rosine residues. The hydrolysis tubes, made from 10 mm I.D. glass tubing, were sealed in vacuo, after N_2 gas (Matheson) had been bubbled through the sample for 5 min. Hydrolysis was conducted at 110 C for 24 hr. The HCl was removed by evaporation in vacuo, over NaOH pellets. The hydrolysate, dissolved in 0.2 M citrate buffer (Pierce Chemicals) pH 2.2, was anlayzed in a Beckman 120 C amino acid analyzer, equipped with a Beckman #125 integrator.

R E S U L T S

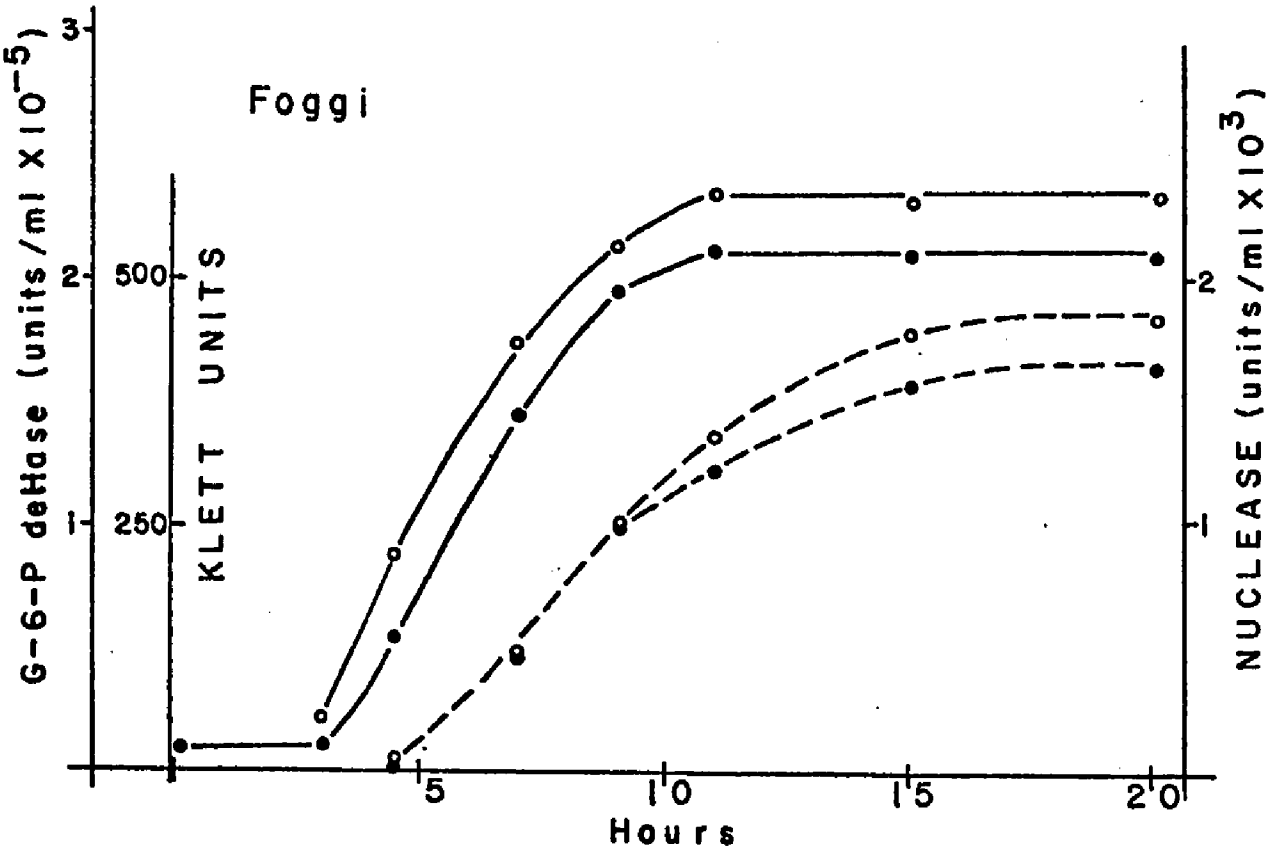
Growth and Nuclease Secretion

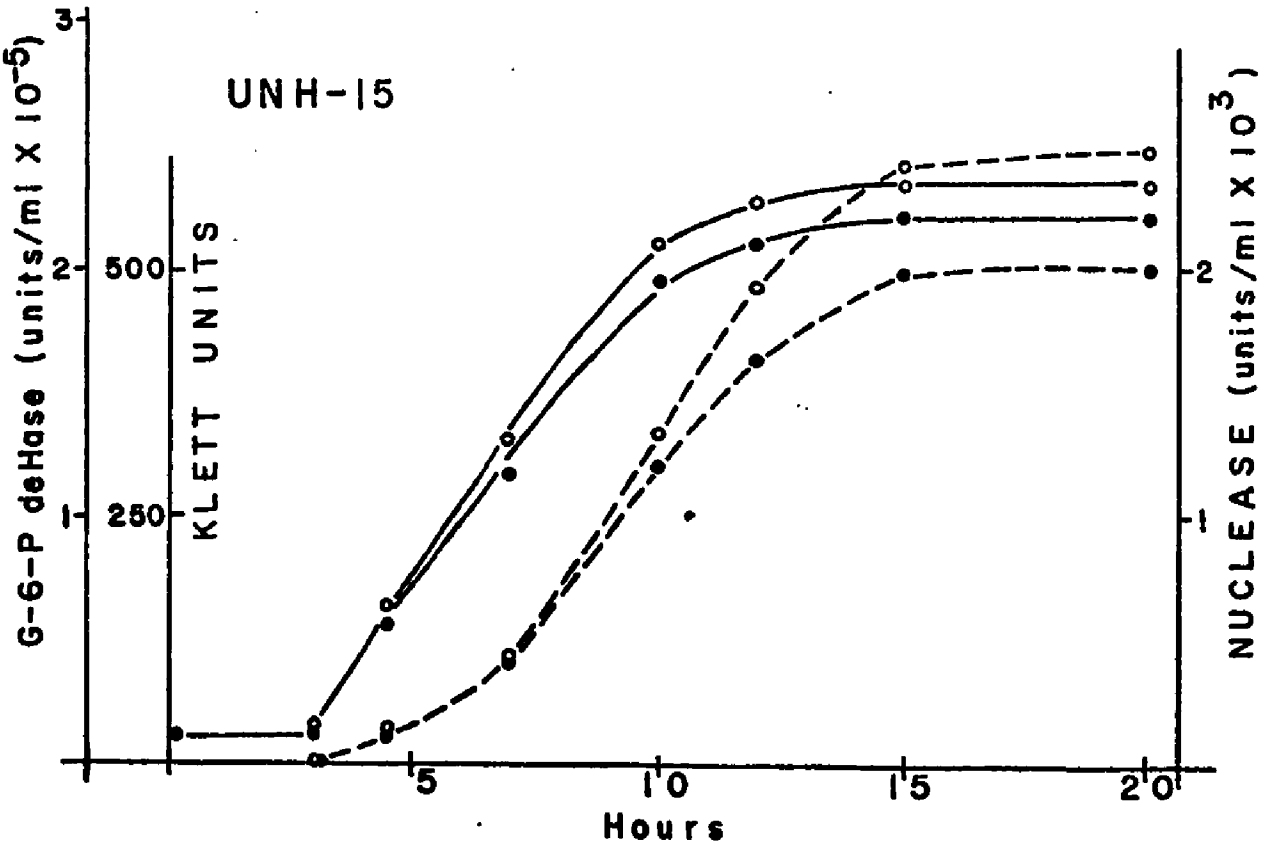
Sixteen to eighteen hour cultures of Foggi and UNH-15 were inoculated into 500 ml Klett sidearm flasks containing semi-synthetic media. The use of a 3% Foggi inoculum and a 10% UNH-15 inoculum permitted similar experiments on the two organisms to be conducted concurrently. A separate experiment was performed to determine what differences could be noted when a 3% UNH-15 inoculum was used. Results indicated that after a 6-7 hr lag phase, the growth curves were superimposable.

One ml samples of the culture were taken at the same time the turbidity was measured. The supernatants from these samples were used to determine the total and heat stable nuclease activity. The cells were lysed in order to determine the glucose-6-phosphate dehydrogenase activity. The growth curves and enzyme patterns of both organisms are illustrated in Fig 1. The faster growing Foggi lagged behind UNH-15 in nuclease secretion. Whereas terminal turbidities were essentially identical, the final nuclease titer of UNH-15 was invariable 20% higher than that of Foggi. The increase in glucose-6-phosphate dehydrogenase activity closely paralled growth.

It is important to point out here that all cultures in the semi-synthetic medium were not pH-stated. However, since the medium was heavily buffered with Tris-Cl (0.2 M) the change in pH during growth was slight. With the pH at

Fig. 1. Enzyme activity and growth patterns of Foggi and UNH-15 under normal conditions. Fifty ml cultures were grown in Klett sidearm flasks. Turbidity (●——●) was determined with a 54 filter. One ml samples were withdrawn from the flask when the turbidity was determined. The supernatant was assayed for total (o---o) and heat stable (●---●) nuclease activity. The cells from the 1.0 ml samples were washed and lysed with lysostaphin. The intracellular protein was examined for glucose-6-phosphate dehydrogenase activity (o——o).


Foggi
G-6-P deHase (units/ml X 10⁻⁵)
KLETT UNITS
NUCLEASE (units/ml X 10³)
Hours
3
2
1
500
250
2
1
5
10
15
20


UNH-15
G-6-P deHase (units/ml X 10⁻⁵)
KLETT UNITS
NUCLEASE (units/ml X 10³)
Hours
3
2
1
500
250
2
1
5
10
15
20

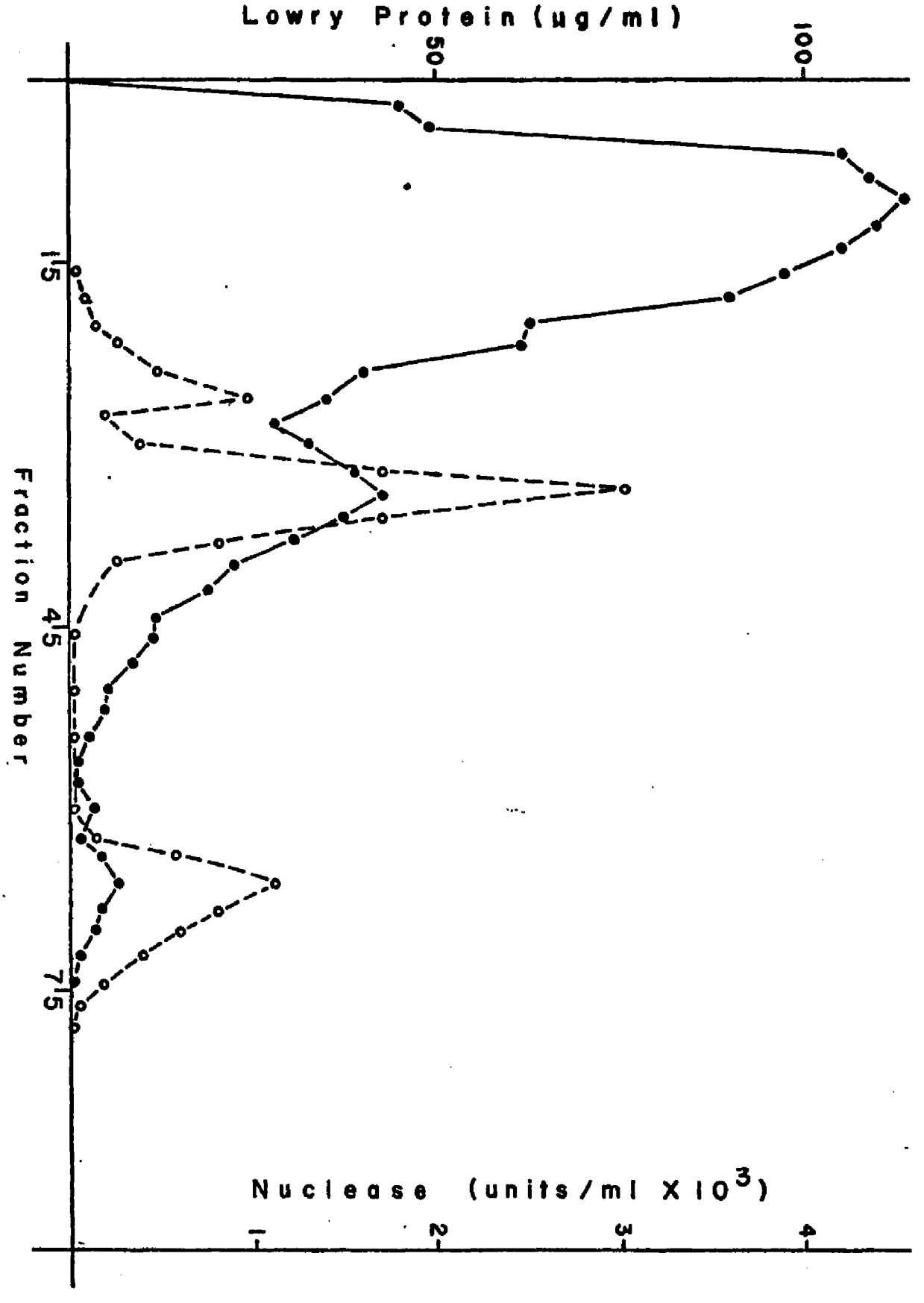
7.2 to 7.3 at the onset of growth, both Foggi and UNH-15 caused the pH to be lowered to 6.9 to 7.0 during exponential growth. In early stationary, the pH ceased to drop and eventually returned to the original value, and often rose slightly. However, a terminal pH greater than 7.4 was never observed.

Production of Various Nuclease Forms With Time

The nuclease activity which was released into the milieu during growth was due to the production of three chromatographically separable forms. In separating the supernatants derived from other cultures, I found that when I employed the same chromatographic condition, the elution pattern of the nucleases was very reproducible. On this basis, successive peaks were identified as I, II, and III. These designations will be used subsequently in this discussion. The production of these forms did not occur simultaneously during growth. The major nuclease component in fully-grown stationary phase cultures was III. This was preceded by the appearance of I and II in mid-log growth. The manifestation of the terminal nuclease form occurred with the simultaneous disappearance of the earlier forms.

The dialyzed supernatant from 400 ml of culture grown in semi-synthetic media, was fractionated on CMC as described in Materials and Methods. Fig 2 illustrates the elution pattern of protein and the nucleases present in a UNH-15 culture harvested at 375 KU. At this particular point in growth, the dominant form is nuclease II. The

Fig. 2. CMC elution pattern of nuclease activity derived from the supernatant of a UNH-15 culture at 375 KU. The nuclease activity from 400 ml of culture was fractionated on this column. The column had a flow rate of 40.0 ml per hr. Eight ml fractions were collected. The eluting buffer is described in the text. Lowry protein (●——●) and nuclease activity (o----o) were determined on the even-numbered fractions.


Lowry Protein (ug/ml)
50
100
15
45
75
Fraction Number
Nuclease (units/ml X 10^3)
1
2
3
4

fractionation of a supernatant derived from Foggi revealed that a similar elution pattern could be obtained from a culture at approximately 510 KU.

Analysis of a supernatant harvested later in growth indicated that the relative abundance of the particular forms had shifted. The nuclease and protein pattern of a UNH-15 culture at 450 KU is depicted in Fig 3. Here one can see that the major form is nuclease III, whereas I and II have diminished considerably. In the case of Foggi, a culture which has just attained maximum turbidity at 550 KU, contains nucleases which form an analogous pattern.

Greater than 99% of the nuclease in an early stationary phase culture of UNH-15 could be attributed to nuclease III. The distribution of the activity derived from such a culture is shown in Fig 4. At this point, nucleases I and II contribute less than 100 units/ml of activity in the culture. In Foggi, this condition does not exist until mid to late stationary.

To explore the implications of these secretion characteristics upon the apparent heat stability of nuclease, the effect of heating the crude supernatants was determined. As shown in Fig 1, heat labile nuclease begins to be produced in the mid log phase of either organism. Four hundred ml cultures were grown to appropriate turbidities, then divided into 2 aliquots. One aliquot was heated, and then fractionated; the other was fractionated without heating. The CM cellulose chromatograms indicated that the heat stability of the various forms changed during growth. In a 375 KU

Fig. 3. CMC elution pattern of nuclease activity derived from the supernatant of a UNH-15 culture at 450 KU. Two hundred ml of supernatant was used to derive this chromatogram. The column had a flow rate of 40.0 ml per hr. Eight ml fractions were collected. The eluting buffer is described in the text. Lowry protein (●——●) and nuclease activity (o----o) were determined on the even-numbered fractions.

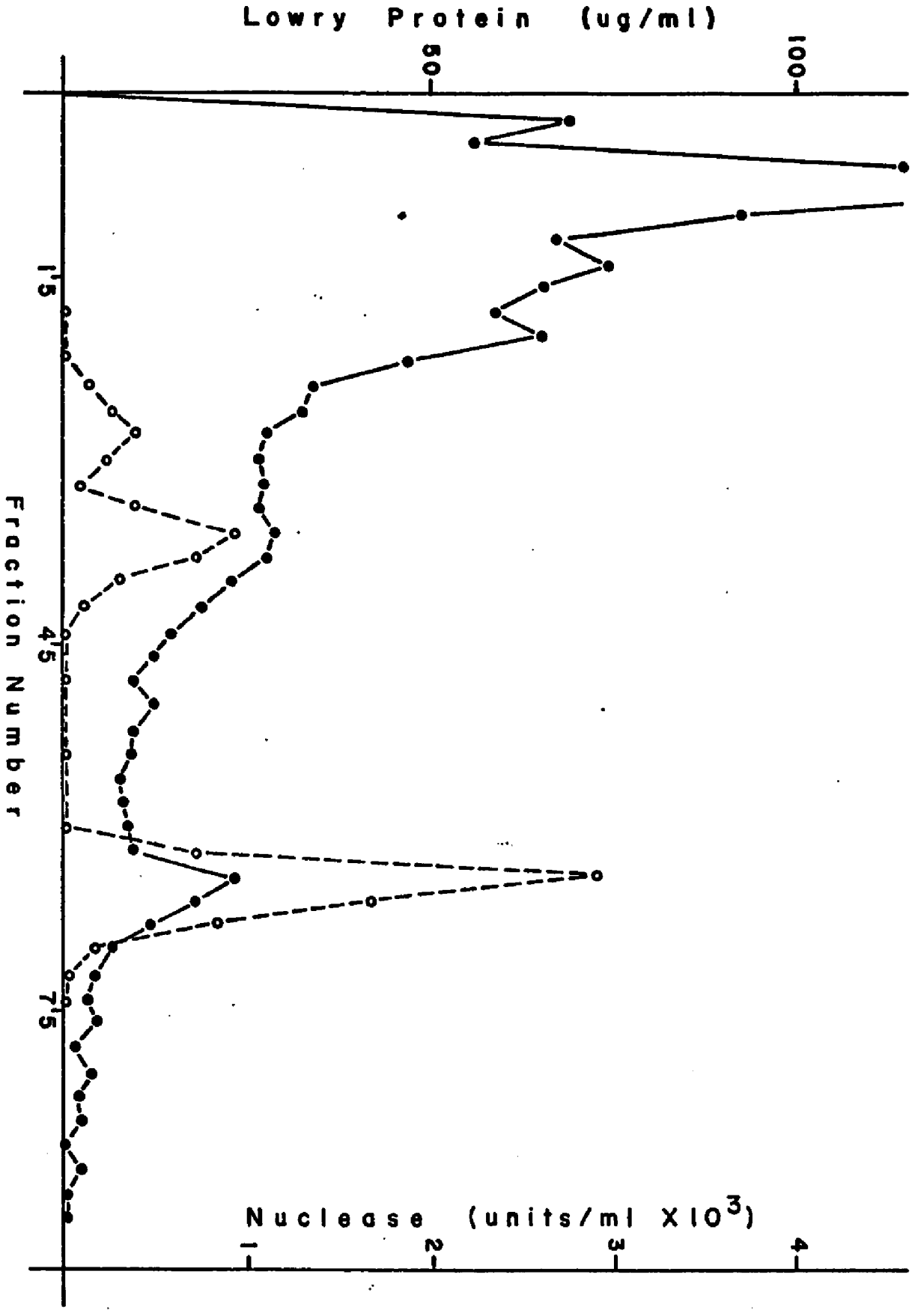
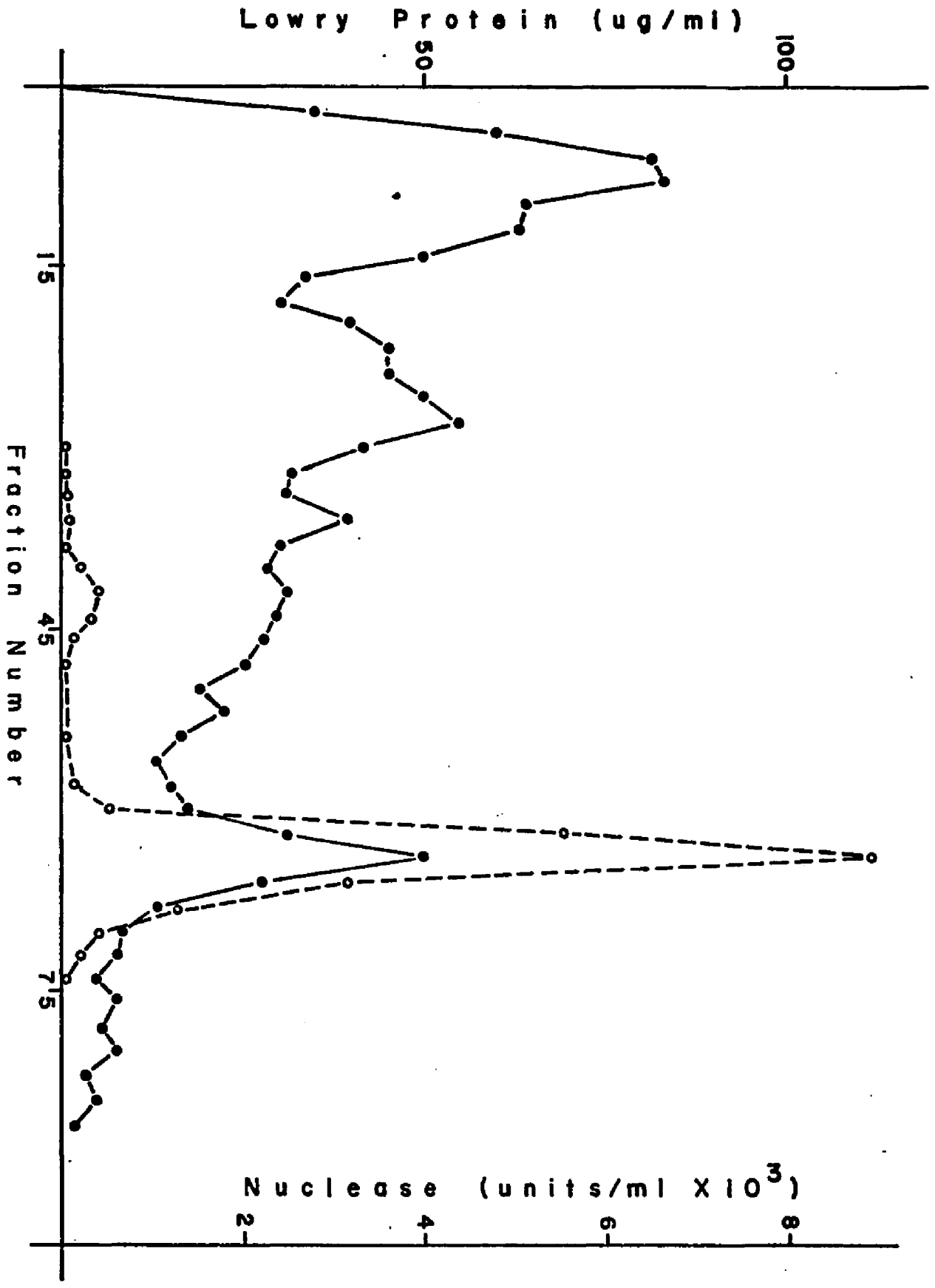

Lowry Protein (ug/ml)
50
100
1'5
4'5
7'5
Fraction Number
Nuclease (units/ml X 10^3)
2
3
4

Fig. 4. CMC elution pattern of nuclease activity derived from the supernatant of a UNH-15 culture at 515 KU. The nuclease activity here was derived from 200 ml of supernatant. The column had a flow rate of 40.0 ml per hr. Eight ml fractions were collected. The eluting buffer is described in the text. Lowry protein (●——●) and nuclease activity (o----o) were determined on the even-numbered fractions.


Lowry Protein (ug/ml)
50
100
15
45
75
Fraction Number
Nuclease (units/ml X 10^3)
2
4
6
8

culture, the activity of nuclease II remained about the same after heating, while the activity of nuclease I apparently increased and that of nuclease III decreased. However, heating a crude supernatant, 95 to 99% of whose activity could be attributed to nuclease III, indicated that all forms were heat labile. In this case the activity of the remaining nucleases I and II dropped to nearly 0 after heating while the activity of nuclease III was reduced by 15-20%.

The various forms of nuclease were also electrophoretically separable. When a 7% pH 2.3 gel was employed, all of the forms migrated in a single band immediately following the tracking dye. The use of a 15% pH 4.3 gel resulted in separation of the nucleases in a pattern predictable from the elution behavior of the isozymes on CM cellulose.

The fractions of a chromatogram which contained abundant amounts of nucleases I and II were pooled and lyophilized for electrophoretic analysis. Since these forms are poorly resolved on CM cellulose, the leading slope and peak of nuclease I were collected; while the peak and tailing edge of nuclease II were combined. In a CM cellulose separation containing abundant nuclease III, all fractions with nuclease activity over 500/ml were pooled.

Loading and subsequent separation of the concentrated material on polyacrylamide gave the pattern illustrated by the stained gels in Fig 5. Nucleases I, II, and III were run on the gels from left to right, respectively. Since nuclease III elutes well after nuclease I and II on CM cellulose,

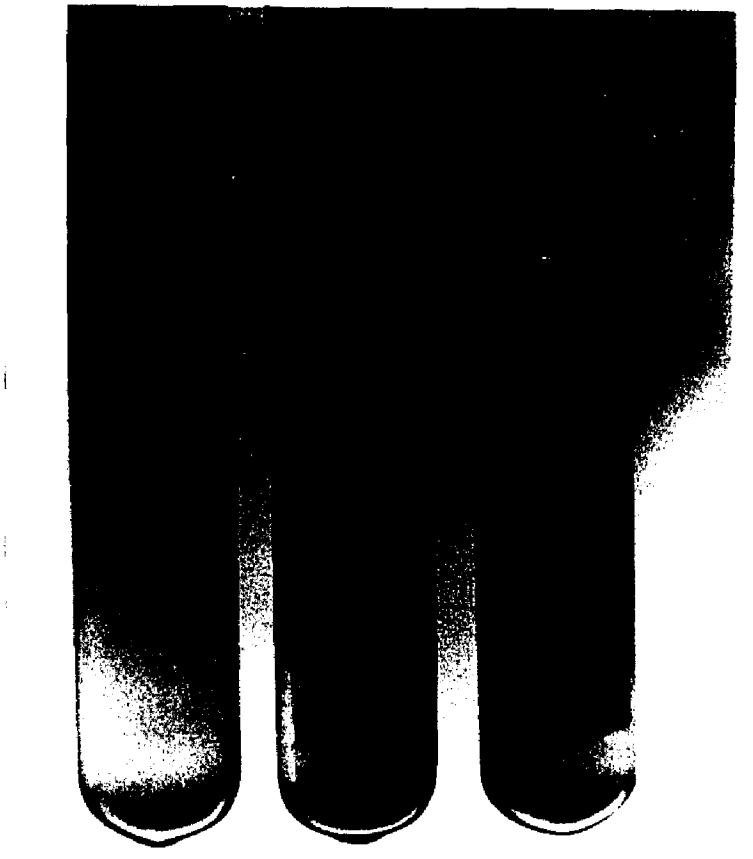


Fig. 5. Electrophoretic mobility of UNH-15 nuclease forms I, II and III. The methods used in the purification of the forms for this analysis are described in the text. Nucleases I, II and III were loaded, respectively, onto 15% 4.3 gels from left to right. Samples of 0.05 to 0.20 mg of Lowry protein were applied and run at a constant current of 4 ma per tube for 3.5 to 4.0 hr.

one would suspect that the isoelectric point of III is much higher than that of I and II. This is indeed borne out in electrophoresis. Nuclease III migrated the greatest distance in this system; whereas the mobility of I and II were very similar, with II leading I. This analysis also indicated that there were some proteins which co-chromatographed with the nucleases.

Electrophoretic analyses were conducted to determine the possible relationship among the nuclease forms and the commercially available Worthington nuclease. Fig 6 illustrates the cumulative results of those tests. The gels, from left to right, represent UNH-15 III, derived from a 50-80% $(NH_4)_2SO_4$ precipitate and CM cellulose fractionation, Worthington nuclease, and a mixture of the two preparations. The first two gels were run in parallel; while the last gel is the product of another electrophoretic separation which operated under the same conditions. As depicted, the nucleases from the two sources appear to be electrophoretically identical.

Effect of Analogs on Growth and Secretion

The use of the various analogs indicated that they were not equally effective on the organisms. The effect of 5-MT was most pronounced; 5-HT was least effective in inhibiting growth and secretion. The relative effects of the antimetabolites are illustrated in Fig 7. In all cases, the analogs were more effective in inhibiting the appearance of nuclease activity rather than growth. Seven azatryptophan

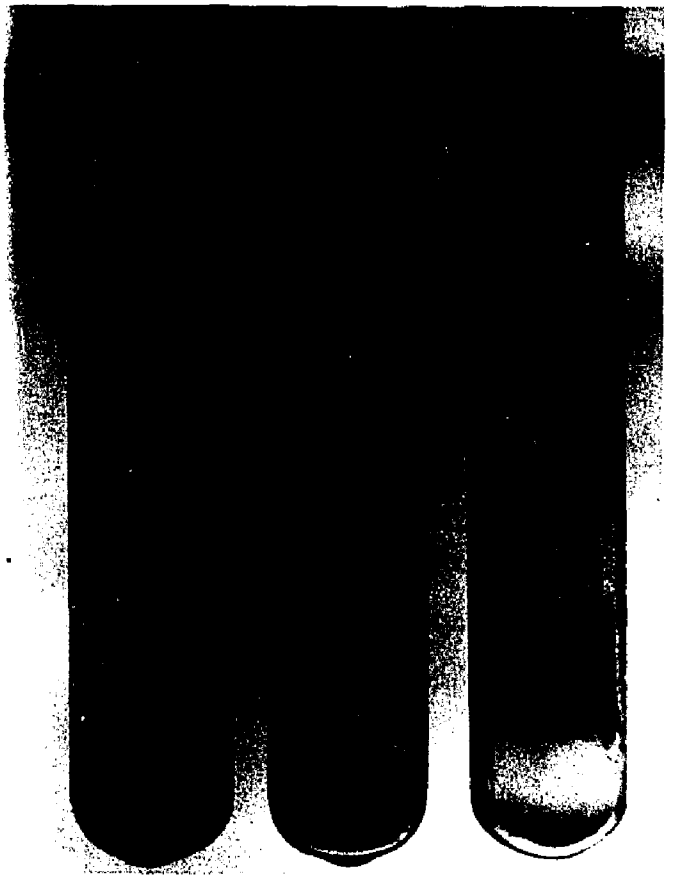
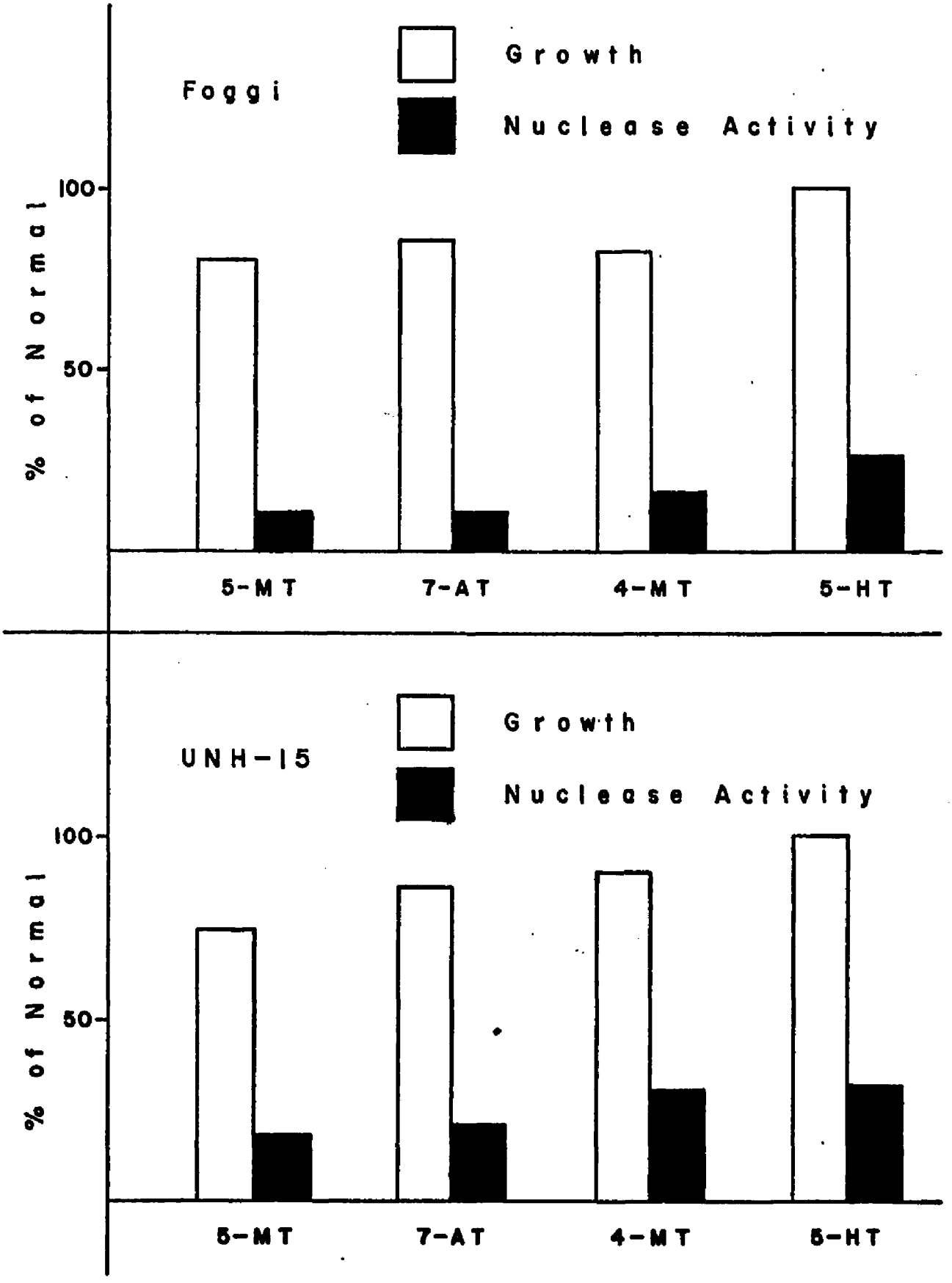


Fig. 6. Comparative electrophoretic mobility of UNH-15 III and Worthington nuclease. The 15% 4.3 gels represent, from left to right, UNH-15 III, Worthington nuclease and a combination of the two samples. Samples of 0.01 mg were applied to the left and middle gels. These gels were run in parallel. A composite sample of both III forms, representing approximately 0.017 mg of protein, was run on the right gel in a later experiment.

Fig. 7. Comparative effects of tryptophan analogs on growth and nuclease production. The appropriate analog, at a concentration 10^{-3} M, was added to the culture at approximately 200 KU. The data represented in this figure was determined on cultures which had attained maximum turbidity.

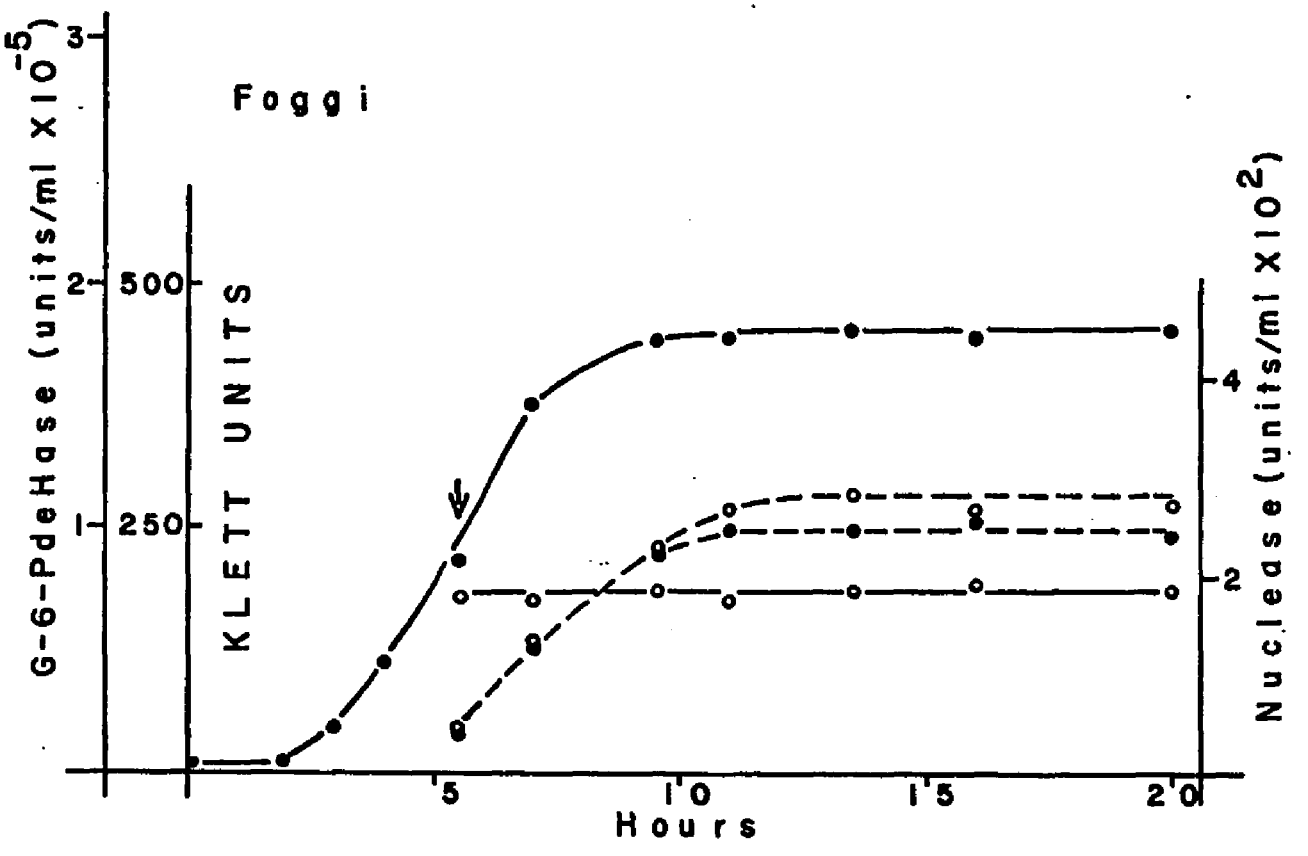

Foggi
Growth
Nuclease Activity
% of Normal
100
50
5-MT
7-AT
4-MT
5-HT
UNH-15
Growth
Nuclease Activity
% of Normal
100
50
5-MT
7-AT
4-MT
5-HT

and 4-methyltryptophan showed approximately equal effects on growth. However, 7-AT was more effective in reducing nuclease activity.

Since 5-MT had the greatest overall effect upon the organisms, it was used in the more extensive investigations which were conducted. The effect of the analog on growth and enzyme activity is represented in Fig 8. The analog affected the growth of the slower-growing UNH-15 more adversely. The final turbidity of the culture was 70-75% of normal; the Foggi culture, however, was always 80-85% of normal. On the other hand, the final UNH-15 nuclease titre was 15-20% of normal, while that of Foggi was approximately 10%. The activity of the intracellular enzyme in Foggi failed to increase soon after analog addition, whereas, the cessation of the increase of the activity of the UNH-15 enzyme was preceded by a short lag. Most significantly, the heat stable and total nuclease activities were nearly equal.

In order to determine what effect(s) the 5-MT had on the various forms, the nucleases in an analog-poisoned culture were fractionated on CM cellulose. The growth and subsequent analysis of 400 ml batch culture of either UNH-15 or Foggi in 10^{-3} M 5-MT gave the nuclease elution pattern depicted in Fig 9. The supernatant for this chromatogram was derived from a stationary phase culture of UNH-15. As opposed to the condition in a normal stationary phase culture, nuclease III is absent here. Rather,

Fig. 8. Enzyme activity and growth patterns of Foggi and UNH-15 in the presence of 5-methyltryptophan. Fifty ml cultures were grown in Klett sidearm flasks. Five-methyltryptophan (10^{-3}M) was added at the point indicated by the arrow. Turbidity (●——●) was determined with a 54 filter. One ml samples were withdrawn from the flask when the turbidity was determined. The supernatant was assayed for total (o---o) and heat stable (●---●) nuclease activity. The cells from the 1.0 ml samples were washed and lysed with lysostaphin. The intracellular protein was examined for glucose-6-phosphate dehydrogenase activity (o——o).


Foggi
G-6-PdeHase (units/ml X10^{-5})
KLETT UNITS
Nuclease (units/ml X10^{2})
Hours
3
2
1
500
250
4
2
5
1'0
1'5
2'0

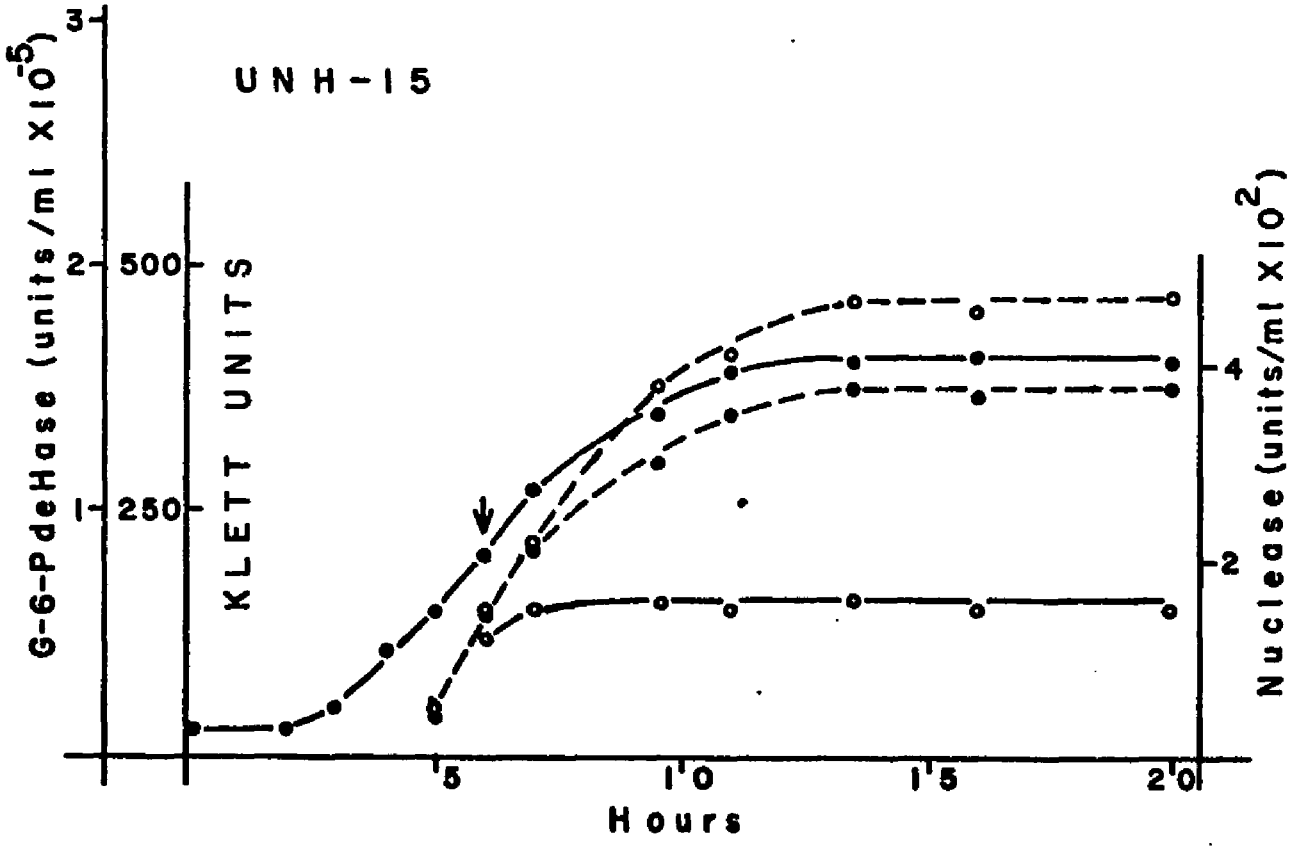
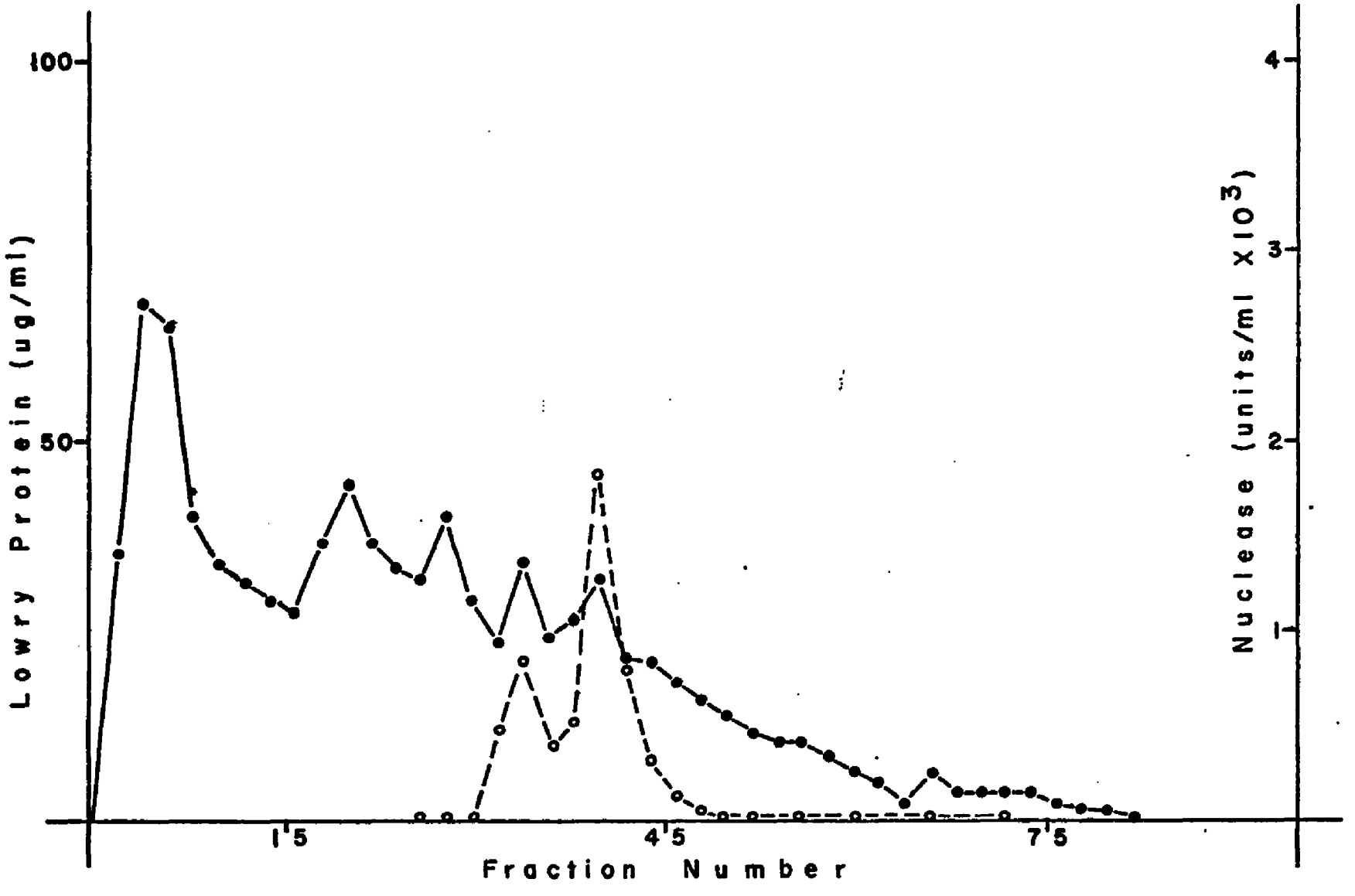

UNH-15
G-6-PdeHase (units/ml X10^{-5})
KLETT UNITS
Nuclease (units/ml X10^{2})
Hours
3
2
1
500
250
4
2
5
1'0
1'5
2'0

Fig. 9. CMC elution pattern of nuclease activity derived from the supernatant of a UNH-15 culture grown in the presence of 5-MT. The nuclease activity in this chromatogram was obtained from 400 ml of supernatant. The column had a flow rate of 40.0 ml per hr. Eight ml fractions were collected. The eluting buffer is described in the text. Lowry protein (●——●) and nuclease activity (o---o) were determined on the even-numbered fractions.


100
50
Lowry Protein (ug/ml)
4
3
2
1
Nuclease (units/ml X10^3)
1'5
4'5
7'5
Fraction Number

there is the predominance of nucleases I and II.

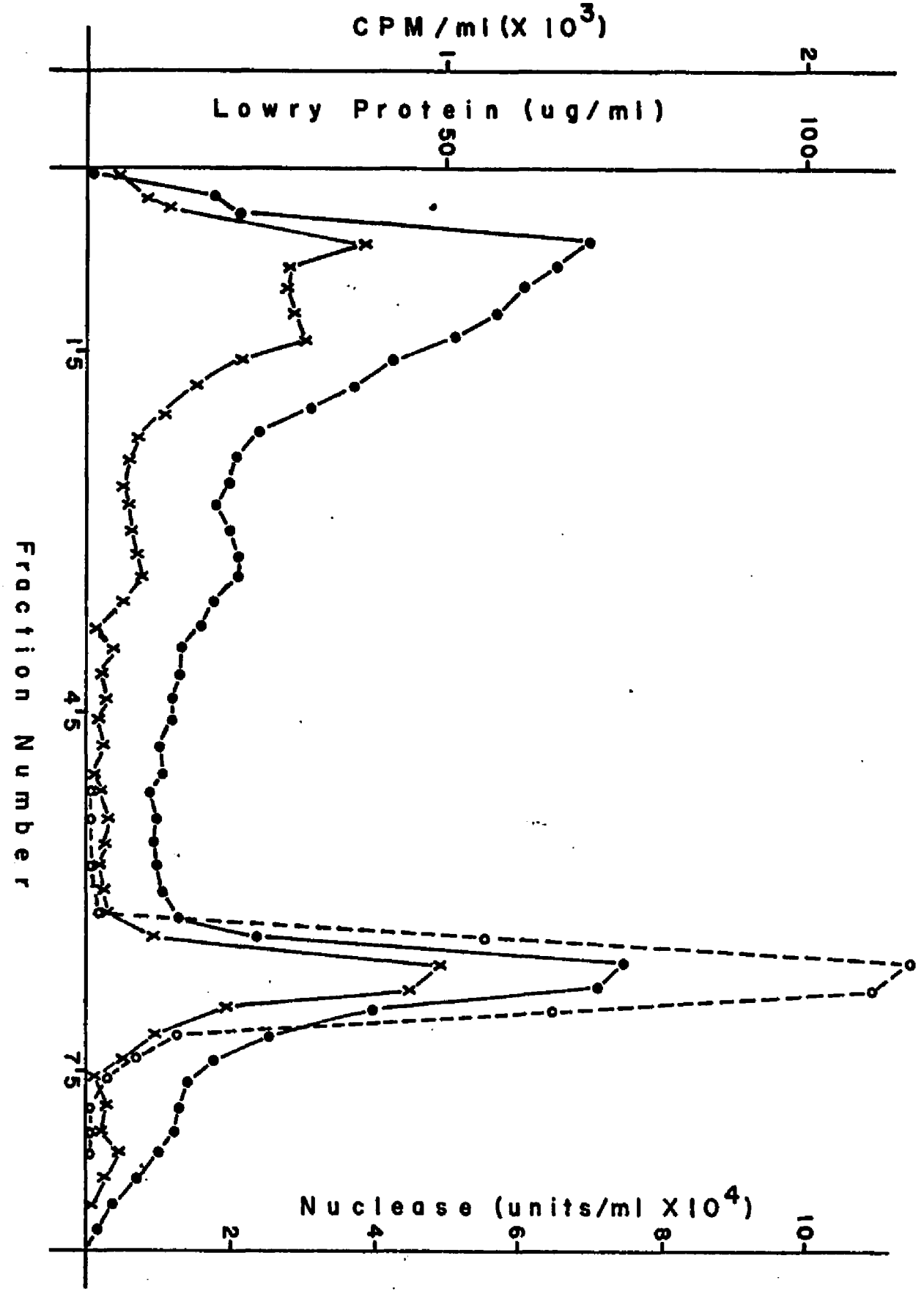
The effect of heating nucleases produced in the presence of analog was investigated by growing additional batch cultures to stationary. One-half of the supernatant was heated and fractionated; the other half was fractionated without heating. In the case of both organisms, the activitiesof nucleases I and II decreased slightly. This observation was in sharp contrast to the results obtained with normal mid-log cultures described in the previous section. The significance of these apparent anomalous results are described in the discussion.

Relationship of the Nuclease Forms

The secretion characteristics of the forms suggested a relationship through some sort of precursor function. To test this hypothesis, a batch of UNH-15 was grown to stationary in complex media, as described in Materials and Methods. The medium within the tubing was supplemented with 35 uC of $_{14}C$ mixed amino acids. The supernatant was harvested, dialyzed, and fractionated on CM cellulose. The elution pattern of the radioactive protein derived from this culture is shown in Fig 10. This chromatogram is typical of pH-stated culture grown in complex media. The nuclease activity and protein derived from this culture is approximately twice that of a semi-synthetic culture on a volume:volume basis.

Radioactive nuclease III was obtained by pooling fractions 63 through 77. After dialysis, osmodialysis and lyophilization, the radioactive enzyme was redissolved and assayed.

Fig. 10. CMC elution pattern of nuclease activity derived from the supernatant of a UNH-15 culture grown in complex media at constant pH. This chromatogram was obtained from 1 liter of supernatant. Thirty-five microCuries of ^{14}C amino acids were added to the culture medium used to produce the protein in this fractionation. The column had a flow rate of 40.0 ml per hr. Eight ml fractions were collected. The eluting buffer is described in the text. Lowry protein (●——●) and nuclease activity (o----o) were determined on the even-numbered fractions. The distribution of radioactivity (X——X) was also determined on the even-numbered fractions.

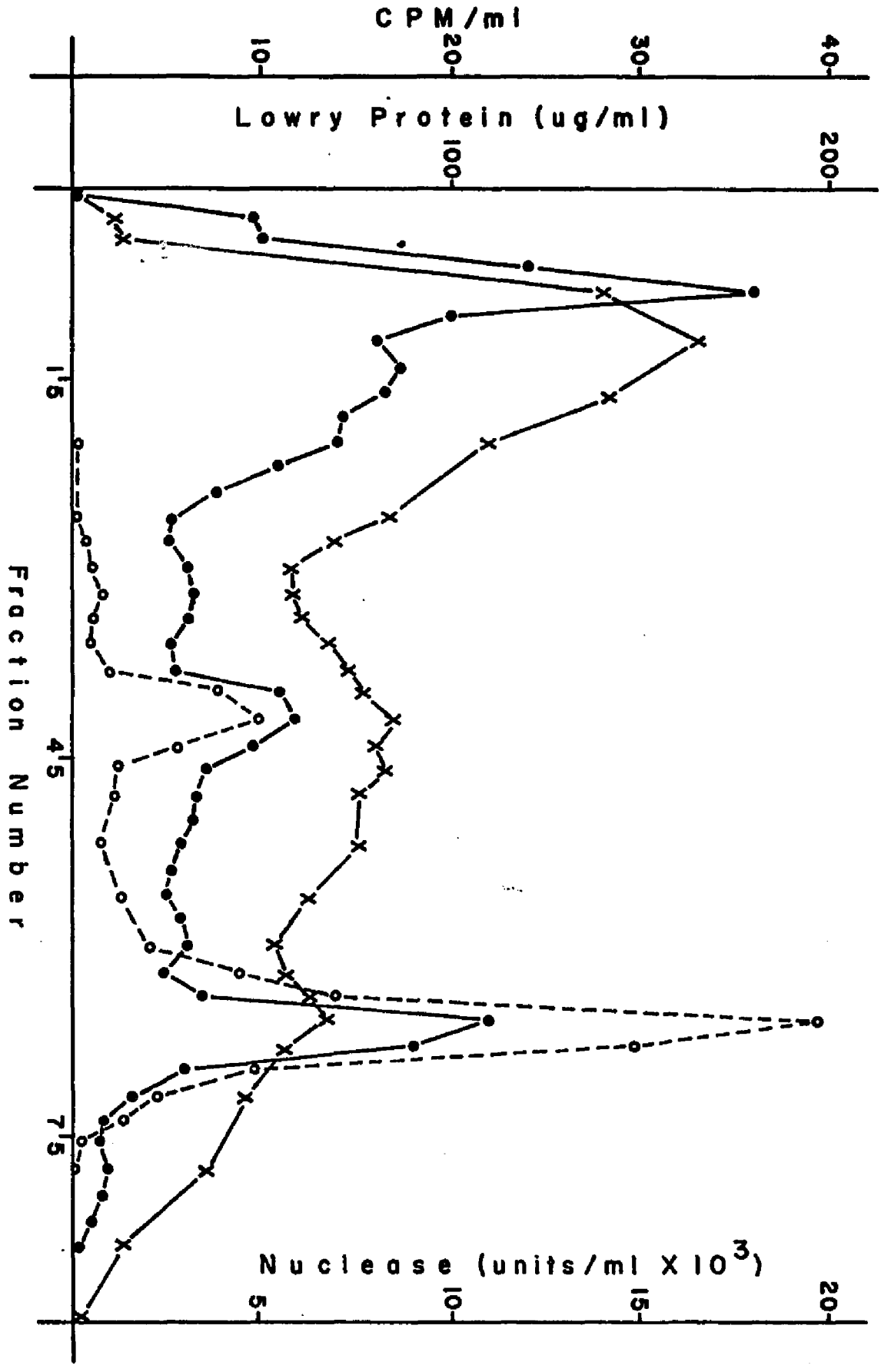

CPM / ml (X 10^3)
1
2
Lowry Protein (ug/ml)
50
100
Fraction Number
1'5
4'5
7'5
Nuclease (units/ml X10^4)
2
4
6
8
10

In order to determine if nuclease III was a precursor for the other forms, approximately 20.0 mg of protein (Lowry), obtained from the nuclease III peak, was added to a semi-synthetic UNH-15 culture at 100 KU. At 375 KU, the culture was harvested and the supernatant was fractionated and analyzed. The distribution of the added radioactivity is described in Fig 11. The nuclease III, normally absent from such a culture, represents about 25% of the III activity added. Calculation of specific radioactivities based on protein reveal that the nuclease III of Fig 10 has an activity of about 1.3 counts per microgram of protein. The activity of III in Fig 11 has dropped to about 0.12, while that of the other protein on the chromatogram is approximately 0.40. The notable exception is nuclease II, with an activity of about 0.30.

Examination of this data indicates that there apparently was no preferential labelling of nucleases I and II, which indicates that the radioactive nuclease III was not converted into I and II specifically. The significant drop in the specific radioactivity of III in Fig 11 would indicate that a considerable amount of cold III was produced in the interval following the addition of the ^{14}C III. The significance of this observation in light of other experiments will be expanded upon in the Discussion.

Since it was quite apparent that III was not a precursor to I and/or II, the converse experiment was performed. A 400 ml batch culture of UNH-15, in semi-synthetic media, was grown in the presence of ^{14}C mixed amino acids. At 350 KU, the supernatant was harvested and fractionated. Radioactive

Fig. 11. Distribution of radioactivity derived from ^{14}C nuclease III added to an early log phase culture. Four-hundred ml of supernatant were loaded onto this chromatogram. The column had a flow rate of 40.0 ml per hr. Eight ml fractions were collected. The eluting buffer is described in the text. Lowry protein (●——●) and nuclease activity (o----o) were determined on the even-numbered fractions. The distribution of radioactivity (X——X) was also determined on the even-numbered fractions.


CPM/ml
10
20
30
40
Lowry Protein (ug/ml)
100
200
Fraction Number
15
45
75
Nuclease (units/ml X 10^3)
5
10
15
20

I and II were obtained by pooling fractions 28 through 46 of the chromatogram represented in Fig 12. This material was dialyzed, osmodialyzed, and lyophilized. All of this material representing approximately 6.2 mg of protein and approximately 52,000 CPM, was introduced into a semi-synthetic culture of UNH-15 at 350 KU. The culture was incubated to the end of its growth cycle. The elution pattern derived from this supernatant is illustrated in Fig 13. This chromatogram indicates that nucleases I and/or II may be precursors to nuclease III. The specific radioactivity in this peak is approximately 2.0 times that of the rest of the protein.

The labelling of the other protein may have occurred through the degradation and re-utilization of the protein which elutes with nucleases I and II from CMC.

Other evidence has suggested that nuclease I and/or II may be precursors for III. Cultures of UNH-15 or Foggi were harvested shortly after the onset of the conversion process. The cells from these cultures were washed in 0.2 M Tris-Cl buffer (pH 7.2) and resuspended in an equal volume of new media. The cultures were then incubated for an additional 6 hr. At the end of this incubation, the supernatant was harvested and assayed for nuclease activity. While there was no increase in turbidity during this interval, Foggi had produced an additional 500 units of nuclease/ml. The fractionation and analysis of the supernatants disclosed that all of the activity could be attributed to nucleases I and II.

An experiment was conducted to determine if constitu-

Fig. 12. CMC fractionation of ^{14}C nucleases I and II. Thirty-five microCuries of carbon-14 amino acids were added to the medium used to produce the protein in this pattern. This chromatogram was produced from 400 ml of UNH-15 culture supernatant at 350 KU. The column had a flow rate of 40.0 ml per hr. Eight ml fractions were collected. The eluting buffer is described in the text. Lowry protein (●——●) and nuclease activity (o----o) were determined on the even-numbered fractions. The distribution of radioactivity (X——X) was also determined on the even-numbered fractions.

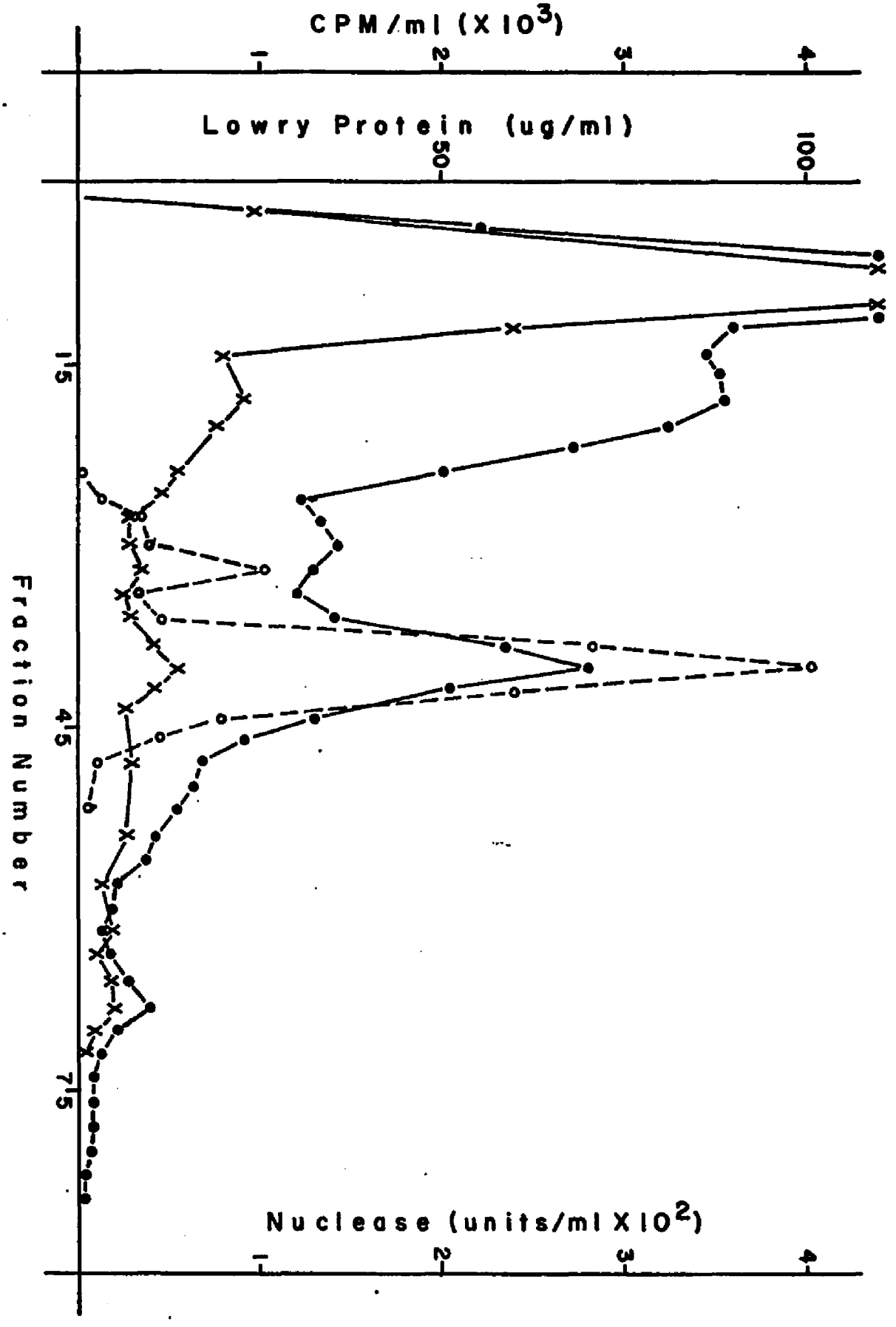
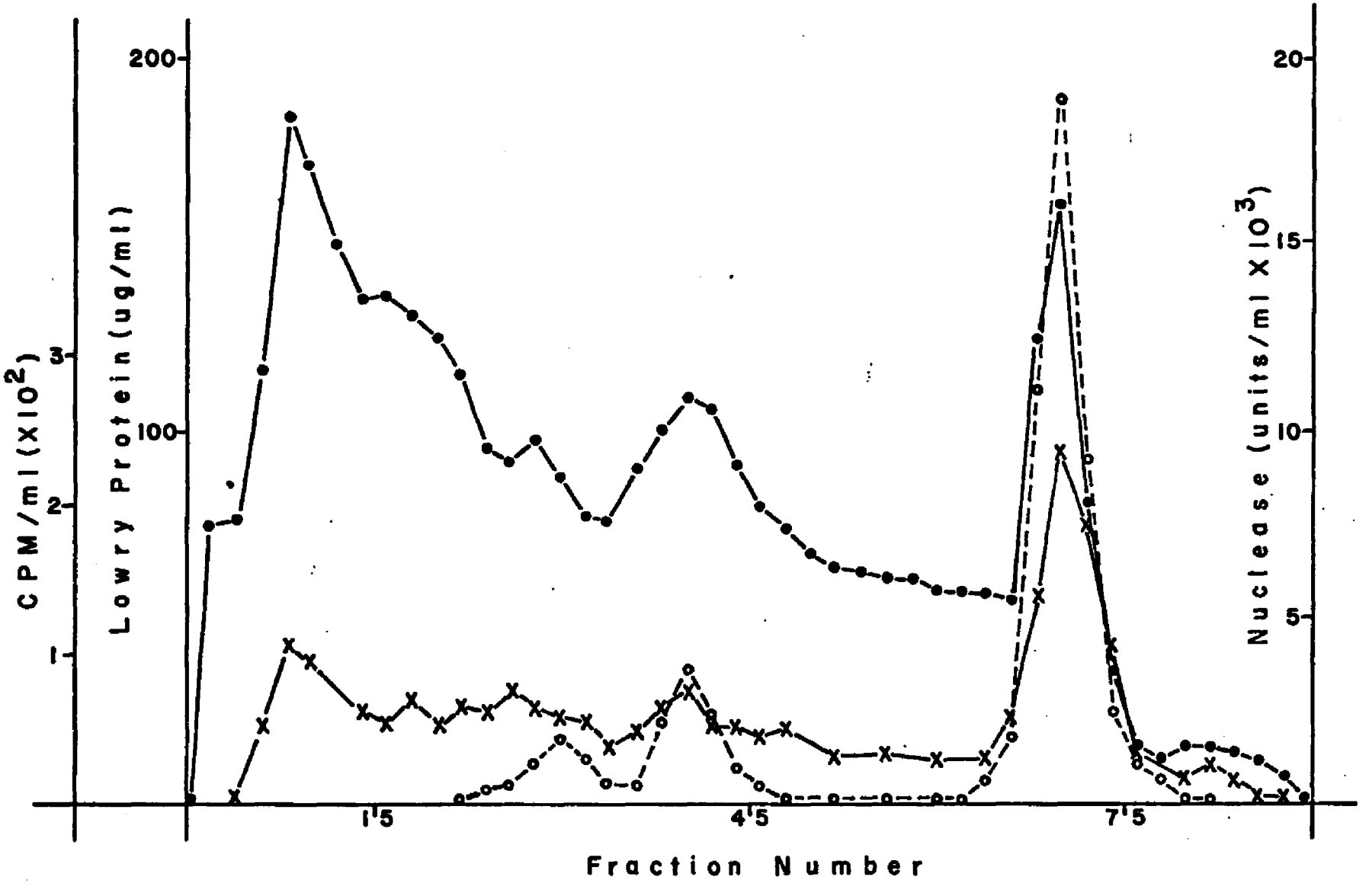

CPM/ml (X 10³)
1
2
3
4
Lowry Protein (ug/ml)
50
100
Fraction Number
15
45
75
Nuclease (units/ml X 10²)
1
2
3
4

Fig. 13. Distribution of radioactivity derived from ^{14}C nucleases I and II added to mid-log phase culture. Four-hundred ml of late stationary phase supernatant were loaded onto this column. The column had a flow rate of 40.0 ml per hr. Eight ml fractions were collected. The eluting buffer is described in the text. Lowry protein (●——●) and nuclease activity (o---o) were determined on the even-numbered fractions. Radioactivity (X——X) was also determined on the even-numbered fractions.

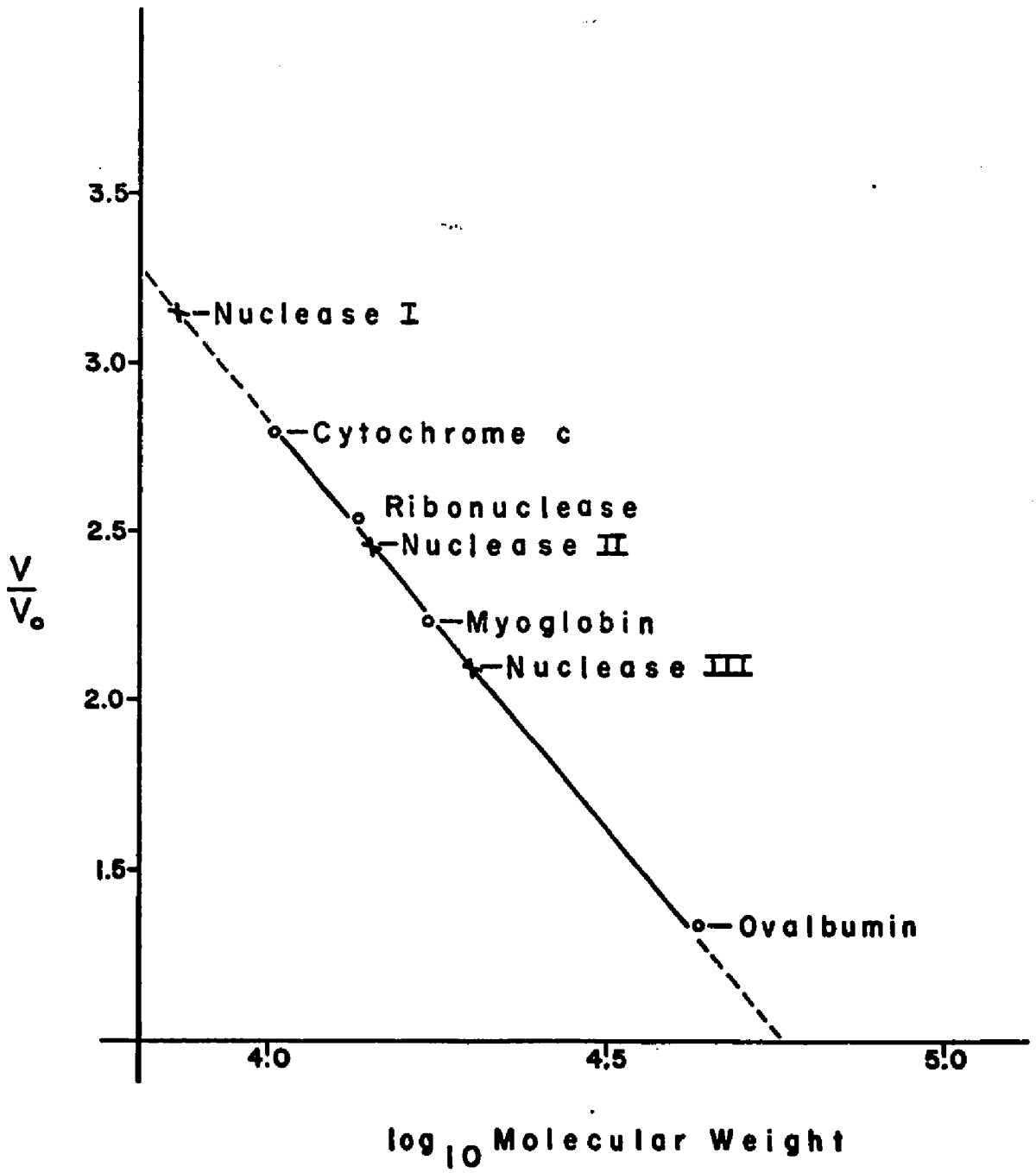

200
100
3
2
1
CPM/ml (X10²)
Lowry Protein (ug/ml)
20
15
10
5
Nuclease (units/ml X10³)
1'5
4'5
7'5
Fraction Number

ents in the supernatant of a mid-log culture were responsible for the conversion. A 400 ml batch of UNH-15 was grown to 300 KU. The supernatant was then harvested and divided into two aliquots. Approximately 1200 micrograms of ^{14}C III was added to one aliquot; the other served as control. Both supernatants were then incubated at 37 C for 3 hr. The samples were then dialyzed and analyzed via CM cellulose chromatography. The elution patterns of the two chromatograms indicating the expected amounts of I and II, were nearly identical. The exception was the nuclease III peak which had been added to the respective supernatant. Also, all of the radioactivity was associated with this peak. This indicated, rather conclusively, that the cells and not the supernatant were responsible for the conversion.

Gel filtration analysis of the forms was then conducted. The use of Blue Dextran and the protein standards indicated that the G-75 column and the system were functioning properly. A plot of the elution volume of the proteins against their respective molecular weights is depicted in Fig 14. The elution positions of the three nuclease forms of UNH-15 is also included in this illustration.

The molecular weights of the forms, as calculated from their elution positions, varied considerably. Nuclease III of both organisms was apparently the largest form with an approximate weight of 20,000. The smallest form appeared to be nuclease I with a calculated molecular weight of 7,200. The size of nuclease II was intermediate at 14,500. Subsequent experiments indicated that Foggi I was 7,050; while the

Fig. 14. Molecular weight determinations of nuclease forms on G-75 Sephadex. A 2.5 x 35.0 cm column of Sephadex was used in reverse flow. The elution position of the proteins was monitored at 280 nm. (See text for details.)


3.5
3.0
2.5
2.0
1.5
V/Vo
Nuclease I
Cytochrome c
Ribonuclease
Nuclease II
Myoglobin
Nuclease III
Ovalbumin
4.0
4.5
5.0
log10 Molecular Weight

II form was 13,800.

From these results, it seems that the putative precursor forms of nuclease III are smaller than the final product. The only anomaly in these results is the size of nuclease I. A protein with such a low molecular weight should not be retained by dialysis tubing. Thus, the apparent weight is probably artifactual, created by some sort of retardation of the enzyme on the Sephadex.

The CM cellulose fractions, containing nucleases I and II which were derived from each organism, were pooled into two respective pools. An attempt to separate the forms from each other and the contaminating protein by preparative electrophoresis in the Fractophorator was not very successful. This procedure served mainly to rid the nucleases of the contaminating protein. A distinct separation between I and II was achieved by running the enzymes in numerous analytical polyacrylamide electrophoresis columns. This cleaner separation was apparently achieved via the action of the discontinuous buffer system, which was not used in the preparative apparatus. The nucleases, eluted from discs formed by slicing the gels laterally, were subsequently lyophilized. In order to quantitate the amount of active protein recovered, a nuclease assay and Lowry determination were performed. Based on this determination, the appropriate amount of nuclease was used for the immunodiffusion assay.

The antiserum was obtained by immunizing a cow with the toxoided 50-80% $(NH_4)_2SO_4$ fraction obtained from the crude supernatants of 4 strains as indicated in Materials and Methods.

Antiserum, placed in the center well, was surrounded with the various forms. The UNH-15 nuclease III was not extensively purified. The preparation used was obtained by pooling, dialyzing and freeze drying the fractions of a CMC chromatogram. Worthington's micrococcal nuclease which is prepared from Foggi, was used in lieu of purified Foggi nuclease III. Electrophoretic data has indicated that the Worthington nuclease and Foggi nuclease III are identical.

The reaction of the various forms with the antiserum is represented in Fig 15. The lines between the antiserum and UNH-15 II and III, and between Worthington and Foggi II, were evident after 9.0 hr. The most significant trait of this pattern is the line of identity extending from UNH-15 nuclease III through Worthington. This apparently confirms the relationship of the forms, as a common antigenic determinant is shared by nucleases II and III of both strains.

The reaction of Foggi I with the antiserum indicates that it is a serologically distinct molecule. There is no indication of any identity with Worthington nuclease.

Effect of Protease Inhibitors

In an attempt to determine if the conversion of the nucleases was mediated by the staphylococcal protease, batch cultures of UNH-15 and Foggi were grown to the point where nucleases I and II predominate. The cultures were then divided into two 200 ml aliquots. Each culture received the appropriate protease inhibitor, and incubation was continued in 2 liter flasks. The supernatants derived from the cul-

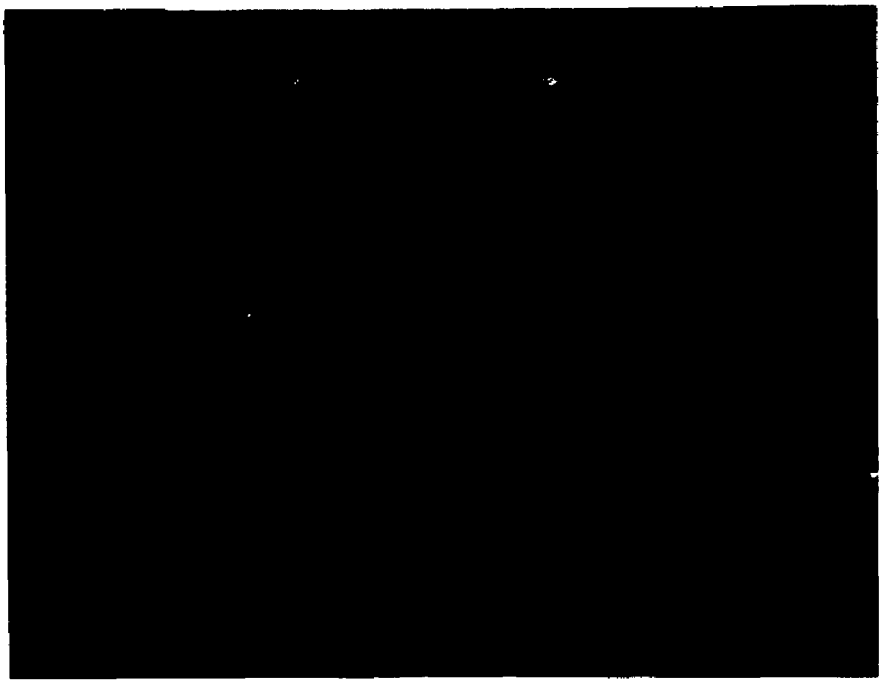


Fig. 15. Immunodiffusion analysis to determine relatedness of nuclease forms. Wells were filled with 50 ul of each nuclease form. Each well contained 5-10 ug of protein per 50 ul. The symbols in the wells represent the respective forms. 'W' represents the Worthington nuclease. The center well contained undilute cow antiserum produced against the toxins in the crude supernatant of S. aureus cultures. (See text for details.)

tures in late stationary were fractionated and analyzed on CMC. Results indicated that, with one possible exception, the inhibitors did not hinder the conversion. The one exception was the effect of N-tosyl-L-phenylalanine chloromethane on UNH-15. There was a significant amount of nucleases I and II left in this culture. However, the overall effect of the inhibitors was to reduce growth in both strains.

In order to determine if the UNH-15 protease was being affected by the inhibitors, 15 ml of crude supernatant was reduced to one-tenth the volume. The material was divided into three equal samples. Two were made 10^{-6} with each of the inhibitors; the third sample served as the blank. All of the samples were incubated for 30 min at 37 C. Their protease activity was then determined. Table I summarized the effect of the inhibitors on the UNH-15 protease, and on the growth of the two organisms. Since neither of the inhibitors was effective against the enzyme, the significance of the protease in the conversion of the nucleases could not be determined.

Amino Acid Analyses

In an attempt to delineate the chemical relationship among the forms, an appropriate amount (200-400 micrograms of protein) (Lowry) of Worthington nuclease, Foggi II, UNH-15 III and UNH-15 II was analyzed to obtain the amino acid composition. Due to the erratic results obtained in the apparent glycine and lysine content of the samples, no conclusive data could be accrued. However, the analyses of both Foggi

TABLE 1

EFFECT OF PROTEASE INHIBITORS[a]

Inhibitor	Protease[b] Activity	UNH-15[c] growth	Foggi[c] growth	Nuclease[d] UNH-15 I	UNH-15 II	UNH-15 III	Foggi I	Foggi II	Foggi III
TLCK[e] (10^{-6}M)	0.75	445 (81)	400 (73)	17	22	58	17	22	96
TPCK[e] (10^{-6}M)	0.67	440 (80)	400 (73)	0	0	95	0	0	95
Control	0.71	550 (100)	550 (100)	0	0	96	0	0	97

[a]All values determined on cultures in stationary phase.

[b]O.D. units at 640 nm.

[c]Klett units with a 54 filter. Percent of control in ( ).

[d]Value is % of total activity in supernatant which eluted in the respective peak. (Numbers do not add to 100, as 3-5% of the activity did not adsorb to CM cellulose).

[e]TLCK = N-tosyl-L-lysine chloromethane; TPCK= N-Tosyl-L-phenylalanine chloromethane.

II and UNH-15 II indicated that these molecules possessed a lower molecular weight than the respective III form. The most striking difference between the chromatograms of II and III was the threonine to serine ratio. The III ananlysis indicated that there was a greater percentage of threonine than serine, which indeed is the case in Foggi III (25). However, in II, the percentage of threonine residues had declined below that of the serine.

SDS-Polyacrylamide Electrophoresis

The actual molecular weights of the nuclease forms were determined in SDS-polyacrylamide gels. Cytochrome c (M.W. 12,400), lysozyme (M.W. 14,500) and myoglobin (M.W. 17,800) were used as standards.

The mobility of Worthington nuclease and UNH-15 indicated that both molecules were of comparable size. The indicated molecular weights were 20,000-22,000. UNH-15 II appeared to be slightly smaller than III, with an indicated molecular weight of 16,000-18,000.

However, the reliability of the method in determining molecular sizes appeared somewhat dubious, as lysozyme migrated a distance greater than that traversed by cytochrome c. Assuming a true value for cytochrome c and myoglobin, the molecular weight of lysozyme was approximately 10,500.

DISCUSSION

The extensive research conducted on staphylococcal nuclease has led to the description of many of its physicochemical properties. Early reports from Anfinsen's laboratory indicated that there were two forms of nuclease which had approximate molecular weights of 10,000 and 20,000 (2). A later publication (33) disposed of this apparent discrepancy by stating that there was only one form which had an approximate weight of 17,000 by ultracentrifugal measurements. The eventual determination of the complete amino acid sequence and composition of the 17,000 form indicated that the actual size was 16,807 (81). Further purification and subsequent analyses, however, have shown that even highly purified preparations exhibit two bands on polyacrylamide disc gel electrophoresis (45). The authors suggest that this is apparently due to an internal conversion which is characteristic of nuclease's motility.

In analyzing the crude supernatant of strain UNH-15 on G-75 Sephadex, Stuart (75) found numerous nuclease peaks with apparent molecular weights ranging from 20,000 to less than 3,000. Analysis of an ammonium sulfate insoluble fraction, however, indicated that many of the low molecular weight forms were absent.

Upon examining ammonium sulfate precipitates, Mukai et al. (47) found a wide variety of chromatographically separable nuclease forms. They felt that this simply represented the formation of numerous complexes between nuclease and inert

protein. In conjunction with these findings, the investigators noted that the formation of these complexes contributed to the lowered specific activity of some forms. Although nuclease forms with an approximate specific activity of 35 were present, the use of proper chromatographic techniques could yield an enzyme with an activity over 100.

Wadström (85) reported that isoelectric focusing techniques revealed the presence of numerous nuclease forms. The specific activities of these forms, however, were not indicated.

The results obtained in the present study confirm many of the above reports, and clarify some of the anomalies. The analyses of dialyzed crude staphylococcal culture supernatants, which had not been precipitated with $(NH_4)_2SO_4$, revealed that three chromatographically separable forms were present. The various forms, which were not present simultaneously, changed relative concentrations during the growth cycle. Whereas nucleases I and II predominated in the culture during log phase growth, III was present exclusively in late stationary phase cultures.

The subsequent characterization of these forms indicated that these nucleolytic proteins possessed different physical and biochemical properties. The sizing of III on G-75 Sephadex indicated that the molecular weight was approximately 20,000. The nuclease II form of UNH-15 sized at 14,500, while the same form of Foggi had an elution volume corresponding to a molecular weight of 13,800. The nuclease I form may be complexed with some inert material, because of its centra dictory behavior in gel filtration and dialysis systems. Though it is retained by

dialysis tubing, the UNH-15 enzyme has an indicated molecular weight of 7,200. The Foggi I enzyme appears to be slightly smaller at 7,050.

These findings were basically confirmed in the SDS-polyacrylamide electrophoresis experiments. The migration characteristics of the nuclease II form indicated its having a molecular weight of 16,000 to 18,000. However, the size of III in this system appeared to slightly more than 20,000.

Examination of the chromatograms depicted in Fig 2, 3 and 4 shows that the specific activities of the nuclease forms varies considerably. The activity of the nuclease I forms, from both strains, is routinely 25 to 30. The activity of the II forms is approximately 60 to 70. Nuclease III exhibits the highest specific activity which is invariably 170 to 210. Worthington's nuclease preparation is routinely 200 in our assay system.

These nuclease forms are roughly equivalent to three of the G-75 peaks which Stuart noted as obtainable from crude culture supernatants of UNH-15 (75). The specific activity of I is similar to one of the enzyme forms which Mukai described (47). The major constituent of old cultures, nuclease III, is probably synonymous with the form which had a specific activity over 100. Electrophoretic data has indicated that Foggi's nuclease III and Worthington nuclease are the same by this criterion. The calculated molecular weight of UNH-15 III is close to that reported for the Worthington nuclease (25).

The further examination of the various forms by other criteria seemed to indicate that the forms were related. The

amino acid analyses of the forms indicated that II represented III minus a considerable portion of its N-terminal sequence. Commercially available Foggi nuclease has ten threonine residues and five serine residues (25). Consideration of the N-terminal and C-terminal ends of the molecule disclosed that 70% of the threonine residues were within the first 50 residues, whereas only 20% of the serine was in this portion. Conversely, the 50 residues of the C-terminal end, while containing only 10% of the threonine in the molecule, contained 60% of the serine residues. Thus, one can easily see that the absence of even a small portion of the N-terminal end would shift the threonine-serine ratio to favor serine.

The lower specific activity of II is consistent with the interpretation that the absence of some enzymatically unimportant portion of the polypeptide could yield a smaller molecule displaying limited enzymic activity. In fact, the combination of the P_2 and P_3 segments of Foggi nuclease, which were originally derived from the native molecule, gives an enzymatically active enzyme (54). These segments represent residues 6 through 149 of the native protein.

The reaction of the nucleases with antiserum revealed that there was at least one antigenic determinant which was common to the II and III forms of both strains. The immunoprecipitin line indicated that UNH-15 II and III had all determinants in common. Although Foggi II was related to UNH-15 II via the single determinant, there was also an indication of one difference between the II forms. Foggi II apparently had an antigenic site which was at least only partially identical to the similar

site in UNH-15 II. The Worthington nuclease (Foggi III) while also possessing the common determinant appeared to have other sites unique to itself.

The gel diffusion indicated no relationship between the I and II forms. Close examination of the precipitin lines between the antiserum and the I and III Foggi forms indicated some possible relatedeness here. The single precipitin line between Foggi I and the antiserum appeared to form a continuous line with one of the lines between the antiserum and Worthington nuclease. This line, however, indicated no relatedness to any of the antigenic determinants in Foggi II. This reaction may correspond to the reactivity of nuclease's second antigenic site which is reportedly located on its N-terminal end (52).

In light of the sizing data discussed above, these immunological studies appear to reinforce the concept that II is III lacking a portion of its primary structure. Furthermore, these studies, in conjunction with the amino acid analyses seem to indicate that the N-terminal portion is that sequence which is absent. The evidence for this hypothesis rests on the studies which have been conducted on the Foggi nuclease. These investigations have indicated that the major antigenic determinant on the molecule is located at the C-terminal end (52,53).

Reports dealing with precursor extracellular enzymes and/or their interaction are few. Lampen (38) has commented on the work which Japanese investigators conducted on the bacillary alpha amylase. They contended that the production of the enzyme was preceded by the synthesis of a low molecular weight precursor. However, subsequent work on alpha amylase, reported by other laboratories, offered evidence that the precursor func-

tion was artifactual. However, Markus and Silverman (42) have indicated that there is strong evidence pointing to the synthesis of a precursor for staphylococcal enterotoxin B. Non-replicating cells suspended in a nitrogen -free medium continue to release the toxin. This release is insensitive to chloramphenicol but is very pH dependent.

Most recently, Bissell et al. (8) have indicated that the proteinase of Sarcina is secreted as an inactive extended form. The presence of calcium in the medium confers a stable tertiary configuration upon the molecule. Having been stabilized, the proteinase undergoes self-activation via the cleavage of some of its amino acid residues. In the absence of calcium, the molecule remains in a configuration which is susceptible to proteolytic attack, and undergoes self-destruction.

Studies dealing with the release of bacillary penicillinase disclosed that a significant amount is cell-bound prior to release, and has a long residence time in the membrane (14, 38, 57, 63). The eventual release of the bound form is pH dependent, and occurs at specific points in the growth cycle.

The radioactive studies performed with the nuclease forms of UNH-15 gave evidence for the possible existence of nuclease precursors. The data depicted in Fig 12 and 13 can be interpreted to mean that the molecules constituting nucleases I and II might by converted into the final form, nuclease III. The specific radioactivity of the nuclease III peak, which was derived from the added ^{14}C I and II, is 2.3 times higher than any of the other protein present on the chromatogram.

But if I and II are precursors to III, then the final

form, III, should be quite stable when added to a culture at any point in the growth cycle. However, this did not occur. The addition of ^{14}C III to an early log phase culture resulted in what appeared to be a scrambling of the radioactivity throughout the exoproteins retained by the CM cellulose column. Since the specific radioactivities of the protein in Fig 11 indicate widespread distribution of radioactivity, the added III was apparently degraded, and the low molecular weight products were then re-incorporated into new protein.

The low specific radioactivity of III seemed to indicate the rapid dilution of this pool with cold, newly synthesized enzyme. However, this presented an anomalous result as the data shown in Fig 2 and 3 indicated that this particular form was not synthesized at this point in growth. Thus, this data led to the assumption that perhaps the III form was the only form produced by the cell. However, its presence was not detected during log phase growth because of its rapid alteration to II and/or I.

In order to determine which processes were actually occurring, subsequent experiments were conducted. The results of these experiments could be interpreted to strengthen the possibility of either process occurring.

The experiments which showed the production of more I and II in lieu of III when these forms were removed from the milieu, could indicate that I and II are necessary for the production of III. However, the data may also be interpreted to indicate that the log phase cells, when resuspended in new medium, actively synthesized and then rapidly altered III. Thus, the

putative products, II and I, represented all of the nuclease activity in the supernatant.

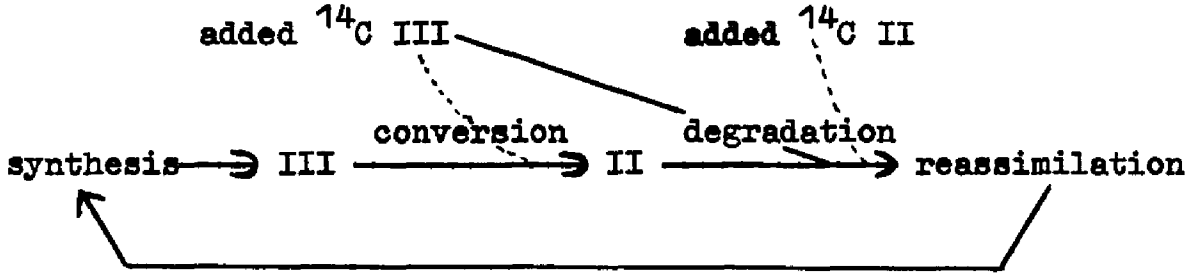
The experiments conducted to determine if the noted effects were due to constituents in the supernatant indicated that this was not the case. ^{14}C III was added to the supernatant derived from 200 ml of a UNH-15 culture at 300 KU. The subsequent incubation, dialysis and fractionation of this supernatant fluid, revealed that the added III had undergone no alteration during the subsequent incubation. The III form, which was totally absent from the control supernatant, was recovered in its normal elution position from the test supernatant. Also, all of the radioactivity was associated with this peak only.

The attempts to determine if the staphylococcal protease caused the alteration yielded inconclusive data. The protease inhibitors, TPCK and TLCK, though quite effective against chymotrypsin and trypsin, respectively, are also quite specific. They are effective inhibitors against the serine hydrolases of the chymotrypsin-trypsin family only. It has been pointed out recently that serine hydrolases outside of the trypsin-chymotrypsin family, such as the bacillary subtilisns, are unaffected by TPCK and TLCK (90). Thus the inhibitors did not affect the usual change in the nuclease forms which occurs during the growth cycle.

However, the inhibitors were not totally ineffective as some inhibition of growth occurred. This would indicate that the cell may possess proteolytic type enzymes which resemble trypsin and chymotrypsin, and these enzymes are necessary for

growth. The implications of these observed effects warrant further investigation.

In light of the above discussion, the rather complicated schema depicted below may be used to interpret the effects of a log phase culture on added III.



All of the nuclease could be produced on one gene coding for III. Thus, the cell synthesizes only one type of nuclease molecule. This is a reasonable interpretation of genetic data, since the attempts to isolate a point mutant of Foggi which produced an altered nuclease were unsuccessful (50). Every attempted mutation yielded an organism which had eliminated its nuclease gene.

During log growth, III could be synthesized and rapidly altered via a conversion to yield II and possibly I, either in a sequential or simultaneous reaction. The subsequent degradation of II and I yields low molecular weight compounds which are readily reassimilated. The evidence for this is presented in Fig 10 and 11.

In the three hour interval following the addition of the radioactive III, its specific activity had dropped by an order of magnitude. Also, this specific radioactivity was the lowest of any protein fractionated on the chromatogram. The

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specific activity of the other protein was approximately three times higher than the III.

This data seems to indicate that the addition of the radioactive III (a protein derived from a stationary phase culture) temporarily disrupted the conversion-degradation system. The added III (not normally present in log cultures) might have saturated the system, so that newly synthesized III was released to the milieu. In essence, the added ^{14}C III protected the cold III from the conversion process.

Thus, one might explain the apparent preferential labelling of nuclease III from radioactive nuclease II. The addition of radioactive I and II to the mid-log culture resulted in the production of labelled protein throughout the chromatogram. The specific radioactivity of III is higher than any other protein because it is undergoing active synthesis at that time as evidenced by the increase in extracellular nuclease activity (Fig 1). Since I and II are undergoing degradation, the ^{14}C low molecular weight products are being incorporated into the III being synthesized, most of which will not be converted, since the culture is approaching stationary phase. Thus, its specific radioactivity is higher than the other protein.

The conversion and degradation, which might operate at the surface of the membrane, are therefore responsible for the reshuffle of the added III. The particular nuclease forms predominate at various times because the rate of the conversion is variable. It appears to be rapid during log phase, and thus apparently associated with cell division. In static cultures the activity is absent.

The degradation process, which appears to operate at a lower, constant rate, is responsible for the destruction of I and/or II. Soon after I and II reach maximum concentration in late log, the rate of conversion of III decreases as the cells approach stationary, then the continued degradative activity eventually renders the culture free of I and II. Eventually, the degradative activity also ceases.

The foregoing arguments have conclusively established a relationship between the II and III forms. However, the evaluation of all data required that one propose two possible mechanisms, either of which can explain the relationship.

The data accrued in this investigation is closely analogous to the situation which Boyer and Carlton observed in B. subtilis (9). In their system they noted the production of two proteolytic enzymes by the organism. One of the proteases, a basic protein, was produced during log phase. A second protease, distinctly acidic, was produced by stationary phase cultures.

The further characterization of the enzymes indicated that they were not related. However, there are points of similarity between this system and the staphylococcal nuclease system. The log phase form was of a lower molecular weight and lower specific activity than the stationary phase form. As described above, this is the case with staphylococcal nucleases II and III. As for the nuclease I, there may be an analogous form in the protease system. However, it may by of such a size and configuration as to be inert and thus undetectable.

Even though the results obtained in the bacillary system do not indicate a relationship between the proteases, the authors presented no evidence to dismiss the concept. The smaller

log phase form could serve as precursor to the larger form or could represent a conversion product of the stationary phase form. This assumption might, in fact, be substantiated if the determination of the primary structure of the larger form indicated that an unusually large percentage of acidic amino acids was concentrated in the N-terminal end, or for that matter, in the C-terminal end. Thus, the loss (or addition) of this particular sequence could account for the change in the isoelectric point, specific activity, and molecular weight of the molecule.

The significance of nuclease I in relation to the II and III forms was not established. Based on the apparent molecular weight and specific activity, it would appear that the form might be (a) nuclease III with a larger portion of its amino acids lost than is represented in II, or (b) a degradation product of II. The serological studies indicate that if I represents partially altered II or III, the alteration occurred in the C-terminal region, as there was no identity with nuclease III. Alternatively, nuclease I, or more precisely, that form which elutes first from CMC columns, is simply a small amount of II and/or III bound to some non-nucleolytic protein or polypeptide whose properties dictate that it elute at that particular point on the chromatogram. Another interpretation would be that I is a distinct molecule, the product of another gene.

The use of many amino acid auxotrophs of E. coli enabled Schlesinger (70, 71, 72) to determine the relative importance of various amino acids in the alkaline phosphatase.

Whereas, arginine was needed for active enzyme, tryptophan analogs incorporated into the protein had little effect on its activity.

Early investigators argued that the methyl tryptophans were not incorporated into protein (60). Since then, there have been reports that these analogs are incorporated into protein (5, 39, 71). Five-methyltryptophan satisfies the requirement for tryptophan in the initiator protein of the auxotroph E. coli 15T⁻. Thus, another round of DNA replication is able to proceed (39).

Leboeuf-Trudeau et al. (40) noted the adverse effects of tryptophan analogs on the production of staphylococcal α-toxin. In the presence of the analogs, crude supernatants were no longer lethal for mice. The fluid was also non-hemolytic on rabbit blood agar, and the immunoprecipitin reaction was absent. The authors, however, did not indicate whether the analogs were incorporated to form inactive protein or not.

Five-methyl tryptophan affected the growth and enzyme activities of Foggi and UNH-15 to varying degrees. The analog had a slightly greater effect on the increase of cellular mass in UNH-15 than in Foggi. On the other hand, however, the intracellular dehydrogenase of Foggi was affected sooner than that of UNH-15. This would indicate that actively growing strains accumulate certain growth precursors, with faster growing strains accumulating a greater amount of the precursors. However, the more rapidly a given strain grows, the more rapid is the utilization and turnover of these macromolecules. Re-

plenishment of the utilized molecules occurs through incorporation of low molecular weight nutrients. Hence, the addition of analog to the medium of Foggi results in its rapid uptake and utilization into proteinaceous material. Thus, the intracellular enzyme is affected more rapidly in Foggi than is UNH-15. The turbidity of Foggi, however, terminated higher because of the large amount of macromolecular growth precursors. The final nuclease yield is higher in UNH-15 because more nuclease is present here than in Foggi at the time of analog addtion. Although the titer is only 20% of normal, it still represents twice the nuclease activity in an analog-grown culture of Foggi.

The nuclease activity produced in the presence of analog is shown in Fig 9. A full-grown culture contains only nucleases I and II. The culture appears to be metabolically frozen shortly after the antimetabolite is added. Intracellularly, the effect of the analog is almost immediate as evidenced by the data accrued on the glucose-6-phosphate dehydrogenase. However, there is less effect on the extracellular nuclease activity. This activity, which is increasing rapidly at the time of analog addition, continues to increase after analog has affected the intracellular processes. In light of the possible mechanisms discussed earlier, the observed effects require that either the I and II forms or the nuclease III continue to be synthesized or be present in a cell bound pool. Thus, after analog addition, the bound I and II forms, if indeed they exist in such a state, are slowly released to the milieu.

Alternatively, the cell bound III undergoes rapid conversion, which presumably occurs in non-analog log phase cultures, and the products, I and II, are released to the medium. Eventually, the degradative mechanism is analog-poisoned, and the I and II forms persist.

In either case, the production of the initial products eventually ceases as synthesis is analog-poisoned, or the cell bound pool is exhausted. This, in addition to the analog poisoning of the secondary processes, causes the nuclease pattern to be "frozen" in mid-log position.

This analog data compares favorably with the work of Leboeuf-Trudeau et al. (40) on the α-toxin of S. aureus Wood 46 strain. In their experiments, 5-methyltryptophan, 7-azatryptophan and 4-methyltryptophan were quite comparable to each other in restricting growth and active α-toxin formation. As shown in Fig 7, Foggi appears to parallel Wood 46 more closely than UNH-15 does in the reaction to the analogs. Whereas 5-MT, 7-AT, and 4-MT had approximately equal effects on Foggi, only 5-MT had the comparable effect of UNH-15. Results accumulated in this lab have indicated that, indeed, the growth characteristics of Wood-46 are distinctly similar to those of Foggi.

The apparent anomalous result, however, in that active nuclease was produced by Foggi, whereas no active α-toxin was produced by Wood 46 is due to differing experimental procedure. Tryptophan analog was introduced into the UNH-15 and Foggi cultures at 200 KU, and growth and enzyme activity was monitored from that point. In the Wood 46 experiments, the

organism was never propagated in the absence of tryptophan analog.

The heating of crude supernatants indicatied that nucleases I and II were rather heat stable, whereas nuclease III was more heat labile. In fact, in normal mid-log cultures I and II appeared to be heat activated. However, close examination of the data presented in Fig 3, 8 and 9 resolves the discrepancy.

In a normal mid-log culture, which contains predominantly nucleases I and II, there is also a detectable amount of nuclease III. The heating of this culture results in an 80% decrease in III and an apparent increase in I and II.. The decrease associated with III represents the loss of far more activity than is apparently gained in I and II. Thus, there is a significant difference between the total and heat stable activity in crude supernatants which contain III. This is primarily due to the heat lability of III.

The apparent heat activation of I and II in cultures which contain III may be due to some contribution which the III form makes under the influence of heating. However, it should be pointed out that even though the I and II forms gave generally higher levels of activity, the increases were not to the extent which would suggest a distinct activation effect.

The anomalous effect noted in the heating experiments was the changing stability of the III form. Whereas, as mentioned above, it was quite labile in mid-log cultures (to the extent of 75-80% inactivation), the molecule became quite stable in stationary phase cultures (only 15-20% heat inactivated). This may represent a situation which is quite analogous to Sargent and Lampen's model for exopenicillinase release in B. li

cheniformis (63). The III form, whether it is derived by direct synthesis or by way of the precursor forms, may be a partially extended, loosely coiled molecule as it begins to appear during late log growth. This would correspond to the hydrophobic form of the bacillary penicillinase. However, by virtue of the presence of its complete primary structure, the form possesses its characteristic charge, and thus elutes at the normal position on CM cellulose. However, due to its incomplete secondary and/or tertiary structure during this transition period, it is more heat labile than when it obtains its complete configuration. The constant specific activity of the form, which is observed at any point in growth, may simply be due to the influence of the calcium and substrate in the assay system. These components may confer the complete, correct, and stable configuration on the molecule in order for it to exhibit total enzymic activity. In this case, the system is analogous to the proteinase of Sarcina (8) where calcium confers a stable configuration upon the molecule in order that it may demonstrate its enzymatic activity.

In the nuclease form, the configuration which the substrate and calcium confer onto the molecule, is not achieved in the culture until stationary phase. This is noted by the increased heat stability of the form. The observed results do not indicate exactly what, in the milieu, confers the stabilization. However, the stabilization appears to be due to the changes associated with the culture as it enters stationary phase.

The heating of a culture produced in the presence of analog had little or no effect, primarily because no III, which

is quite heat labile at this point, was present. In fact, in some experiments the total and heat stable activities were virtually the same.

Some ancillary experiments indicated the apparent profound effect of pH on the production of staphylococcal nuclease. The early studies which Pollock (57) conducted on Bacillus subtilis indicated that secretion was dependent on pH. The addition of glucose to the medium sufficed to lower the pH enough to inhibit penicillinase release. Controlling the pH, however, led to the normal production of the enzyme.

When the protein elution pattern of Fig 10 is compared to that of Fig 4, a significant difference can be noted. The control of the pH in the culture used for the experiment in Fig 10 resulted in fewer protein peaks than in Fig 4. Nuclease III presents the most significant peak, and the amount of 'general' protein, which elutes early in the chromatogram, represents a significantly lower proportion in Fig 10. This effect of constant pH on nuclease production was noted by Fox and Holtman (28), but it is a general characteristic connected with the secretion of other staphylococcal proteins. (Dr. I. Wamola, personal communication).

However, the overall effects of constant pH on growth and nuclease secretion were not investigated. The effects noted above project interesting implications on the effects of constant pH on the production of the various nuclease forms, and perhaps, on similar forms of other enzymes produced in other microbial systems.

SUMMARY AND CONCLUSIONS

The growth characteristics and nuclease secretion patterns of Staphylococcus aureus strains UNH-15 and Foggi were investigated. While Foggi displayed a more rapid growth rate, it lagged behind UNH-15 in nuclease secretion.

The analysis of the crude supernatants from these strains revealed that three chromatographically and electrophoretically separable forms were present. Closer examination indicated that the forms were not produced simultaneously, and that their relative concentrations changed during the growth cycle. The forms, which eluted in three relatively distinct peaks from columns of carboxy methyl cellulose (CMC), were dubbed nucleases I, II and III, in order of their elution.

The molecular weights of the forms, determined by gel filtration on G-75 Sephadex and by SDS-polyacrylamide gel electrophoresis, indicated that the I, II and III forms were approximately 7,000, 14,000 and 20,000, respectively. Using these systems, Foggi III appeared to be synonymous with Worthington's micrococcal nuclease.

Immunological data indicated that the II forms were at least partially identical with the III forms. There was also an indication of some relationship between I and III.

Amino acid analyses of the forms indicated that the threonine: serine ratio in the II form was the reverse of the III form. Examination of the published data on the primary structure of the Foggi III form, led to the hypothesis that

the absence of some portion of the N-terminal end of III yielded II. This finding appeared to reinforce the immunological data. Published reports have indicated nuclease's major antigenic determinant is on the C-terminal end of the molecule.

Also, the specific activities of the purified forms (60 to 70 for II; approximately 200 for III) may indicate that the absence of the N-terminal end of III yields II. Thus, nuclease II displayed only limited enzymic activity.

Radioactive isotope analysis showed that the forms were related via one of two possible processes. The I and II forms, which predominate during log phase growth might be precursors to the III form which appears during the early stationary phase and persists to the end of the growth cycle. Alternatively, the III form is the only nuclease molecule synthesized by the cell. Then, the I and II forms are derived from the III form by some conversion process.

Further experiments revealed that the mechanism responsible for the appearance of the various forms was cell associated. Nuclease III (the stationary phase form) was completely degraged when it was added to a log phase culture. However, the addtion of this form to the supernatant of a log phase culture indicated that no alteration had occurred.

Attempts to determine if the staphylococcal protease was responsible for the conversion were not successful. The general protease inhibitors, N-tosyl-L-phenylalanine chloromethane and N-tosyl-L-lysyl chloromethane, did not inhibit the conversion. A subsequent investigation revealed that the in-

hibitors were ineffective against the protease.

The addition of tryptophan analogs to mid-log phase cultures caused the inhibition of growth by 20-30%; nuclease activity was reduced by 80-90%. The analysis of crude supernatants produced in the presence of analogs revealed that nucleases I and II were present exclusively. The accumulated data indicated that this was due to the inactivation of the conversion process by the analog. Thus, the overall effect of the analog was to metabolically freeze the culture in mid-log growth.

Thus, the findings indicate that the I and II forms are present only in log phase because (a) they represent precursors of the final form, nuclease III or (b) they are the degradation products of III which might be the only form synthesized. The mechanism which is responsible for the appearance of the forms appears to be cell-bound and is integrally associated with cell growth.

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