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TITLE OF THESIS..A...STUDY...OF...THE...CONTROL, PRODUCTION AND...CHARACTERIZATION...OF...A...BACTERIAL EXTRACELLULAR...ENZYME.....

UNIVERSITY...ALBERTA.....

DEGREE.....Ph.D..... YEAR GRANTED .1969.....

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THE UNIVERSITY OF ALBERTA

A STUDY OF THE CONTROL, PRODUCTION AND CHARACTERIZATION OF A BACTERIAL EXTRACELLULAR ENZYME

by

© SHEILA A. BERRY

A THESIS

SUBMITTED TO THE FACULTY OF GRADUATE STUDIES

IN PARTIAL FULFILMENT OF THE REQUIREMENTS FOR THE DEGREE

OF DOCTOR OF PHILOSPHY

DEPARTMENT OF MICROBIOLOGY

EDMONTON, ALBERTA

Spring, 1969

UNIVERSITY OF ALBERTA

FACULTY OF GRADUATE STUDIES

The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies for Acceptance, a thesis entitled "A Study of the Control, Production and Characterization of a Bacterial Extracellular Enzyme" submitted by Sheila A. Berry in partial fulfilment of the requirements for the degree of Doctor of Philosophy.

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ABSTRACT

A nuclease produced by Micrococcus sodonensis was isolated from culture supernatants and purified by a sequence of ultrafiltration, ion exchange chromatography and gel filtration. The purified protein was homogeneous and yielded a single peak on gel filtration, electrophoresis and ultracentrifugation. The molecular weight and $S_{20,w}$ were concentration dependent and extrapolated values of 500,000 and 2.1S respectively were obtained. The concentration dependence and the sharpening of the peak in sedimentation velocity experiments were indicative of an asymmetric molecule.

Both monoesterase and diesterase activities were associated with the protein and could not be separated by any of the techniques employed. Both activities were stabilized by Ca^{++}, stimulated by various chelators and -SH containing compounds and inhibited by PCMB and various nucleotide analogues. Kinetic studies yielded a K_m of 2.9×10^{-6}M for RNA and 2.83×10^{-5}M for AMP. AMP was inhibitory to diesterase activity and an apparent K_i of 2.9×10^{-3}M was obtained.

The enzyme was released into the medium by logarithmically growing cells and production ceased when the cells entered the stationary phase. No activity could be demonstrated intracellularly and less than 1% of the total activity could be detected in isolated cell wall fractions. The enzyme was established as extracellular on the basis of the above criteria. Studies with metabolites demonstrated that enzyme production

was controlled by the intracellular pyrimidine nucleotide concentration. A cytidine nucleotide is implicated.

Amino acid analyses of the protein revealed a low number of cysteic acid residues and a relatively large amount of serine, threonine and glycine. The enzyme was shown to be a glycoprotein consisting of 21.5% carbohydrate covalently linked to the protein moiety. Glucosamine, glucose, galactose and rhamnose were identified as the component sugars and were present in a ratio of 4:2:1:1. A serine-glucosamine linkage involving at least 80% of the serine residues was demonstrated. The enzyme carbohydrate was shown to be related both chemically and immunologically to a cell wall carbohydrate. The possible role of the cell wall in enzyme assembly is discussed.

ACKNOWLEDGEMENTS

I wish to express my deepest appreciation to Dr. J. N. Campbell for his advice, encouragement and support throughout the course of this investigation. I also with to express my thanks to Dr. L. B. Smillie and Dr. C. M. Kay, Department of Biochemistry, University of Alberta, for their advice and for the use of their equipment, and to Mr. E. Paradowski and Mr. M. Aarbo for technical assistance in the amino acid and ultracentrifugal analyses. I am grateful to the members of the Department of Microbiology for their many helpful suggestions and moral support throughout my graduate programme. Thanks are also expressed to Mrs. Marilyn Vantour for the typing of this thesis.

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LIST OF ABBREVIATIONS

TCS	- Trypticase soy
OD	- Optical density
RNA	- Ribonucleic acid
DNA	- Deoxyribonucleic acid
RNase	- Ribonuclease
DNase	- Deoxyribonuclease
Poly A	- Polyadenylic acid
P_i	- Inorganic phosphate
pN,pM	- 5' nucleotides
TCA	- Trichloroacetic acid
UTCA	- 0.25% uranyl acetate in 10% trichloroacetic acid
UR,CR	- Uridine, Cytidine
AMP,UMP,CMP,GMP,XMP,IMP	- 5' monophosphates of adenosine, uridine, cytidine, guanosine, xanthosine and inosine
ADP,UDP, etc.	- 5' diphosphates of the respective nucleosides
ATP,UTP, etc.	- 5' triphosphates of the respective nucleosides
pTp	- Thymidine-3',5'-diphosphate
pTpT	- Thymidine dinucleotide
ApA	- Adenylyl-3'5'-adenosine
PCMB	- p-chloromercuribenzoate
EDTA	- Ethylenediaminetetraacetic acid
TRIS	- TRIS(hydroxymethyl)aminomethane
R-1-P	- Ribose-1-phosphate
R-5-P	- Ribose-5-phosphate

INTRODUCTION

Enzymes which degrade nucleates such as DNA and RNA are termed nucleases and are found in a wide variety of plant, animal and microbial tissue. They are classified by several criteria (Laskowski, 1959) such as type of substrate, type of attack, products formed, etc. They may be highly specific, such as pancreatic RNase and DNase, and attack only one substrate, or they may act on both RNA and DNA. The term phosphodiesterase was applied to this last group of enzymes (Heppel and Rabinowitz, 1958) although it is a broad term which is applied to any enzyme capable of breaking a phosphodiester bond.

In addition to variations in substrate specificity, there are also differences in the mode of attack. Some are endonucleolytic and will break the internal linkages in the polynucleotide chain releasing large fragments which in turn become substrates. The nuclease from Staphylococcus aureus was shown to act in this manner, releasing products with 3' phosphate end groups (Alexander, Heppel and Hurwitz, 1961; Reddi, 1959; Sulkowski and Laskowski, 1962), while pancreatic DNase releases products with a terminal 5' phosphate (Potter, Brown and Laskowski, 1952; Vanecko and Laskowski, 1961). Others are exonucleolytic and will release mononucleotides sequentially from one end of the polynucleotide chain. Phosphodiesterase I from snake venom is specific for polynucleotides with a free 3' hydroxyl group (Razzell and Khorana, 1959) and will attack from this end of the chain, releasing 5' mononucleotides. In contrast, phosphodiesterase II from spleen requires a free 5' hydroxyl group (Razzell and Khorana, 1961) and releases 3' mononucleotides.

In addition to the many plant and animal enzymes, a wide variety of microbial nucleases have been described. They have been isolated from such organisms as Pseudomonas aeruginosa (Streitfeld, Hoffman and Janklow, 1962), Serratia marcescens (Eaves and Jeffries, 1963), Bacillus subtilis (Nishimura, 1960), Group A Streptococci (Wannamaker, 1958; Winter and Bernheimer, 1964), Escherichia coli (Lehman, 1960, 1963; Lehman,Roussos and Pratt, 1962; Richardson, Sample, Schildkraut, Lehman and Kornberg, 1963) and Staphylococcus aureus (Cunningham, Catlin and Privat de Garilhe, 1956). Some have specific RNase or DNase activities, others are non specific, most are endonucleolytic and all, with the exception of the E. coli nucleases have been reported to be produced extracellularly. It is probable that E. coli also produces extracellular nucleases since at least one extracellular RNase has been demonstrated in this laboratory (unpublished data) from this species.

There are a large variety of microbial enzymes which are released into the culture medium. These enzymes are classed as "exoenzymes" and include proteinases, polysaccharidases, lipases, penicillinases, toxins and nucleases. In a review of exoenzymes Pollock (1962) has tabulated these various enzymes and divided them into two groups: those which have been definitely established as extracellular and those for which the evidence is incomplete or merely suggestive. The extracellular nature of some enzymes has been based simply on the basis of clearing around the colonies which could be obtained from surface bound enzymes which are not truly extracellular.

Others have been isolated from culture supernatants at a late stage of growth when autolysis cannot be disregarded. It is obvious then that care must be taken in designating an enzyme as extracellular. Pollock suggests the following criteria for the classification of enzymes as extracellular: a) the enzyme appears in the supernatant during the logarithmic phase of growth; b) the appearance of the enzyme does not depend upon irreversible damage to the cell; c) the proportion of total enzyme appearing in the supernatant is greater than 50%.

There are certain properties which appear to be characteristic of exoenzymes in general, the most obvious being the nature of the substrate. Nearly all the exoenzymes attack high molecular weight molecules (proteins, polysaccharides, nucleic acids) or molecules such as fats and phospholipids which aggregate and therefore would not be taken up by the cell. Other generalities have been noted although the information is far from complete. The cysteine content of most of these enzymes is very low. For example, B. subtilis RNase (Lees and Hartley, 1966) was found to lack both cysteine and methionine while Staphylococcal nuclease (Taniuchi and Anfinsen, 1966) was deficient in cysteine. Pollock (1962) suggests that this is a characteristic of bacterial exoproteins as a whole.

The molecular weights seem to be generally low, ranging from 10,000 to 90,000. The molecular weight of B. subtilis RNase has been calculated to be 10,700 (Hartley et al, 1963) and a figure of 90,000 for the molecular weight of V. cholerae neuraminidase has been reported

(Pye and Curtain, 1961). One further characteristic is the requirement for Ca^{++} for either activation or stabilization of exoenzymes and it has been suggested (Linderstrøm, Lang and Schellman, 1959) that Ca^{++} may compensate for the deficiency of disulfide bonds in providing configurational stability to the protein molecule.

Information regarding the site of formation of exoenzymes is limited but it is generally supposed that the formation or the final stages of assembly takes place on the cell surface outside the cytoplasmic membrane (Pollock, 1962). If they are formed on the surface then the only barrier to their release is the cell wall itself.

Eylar (1965) reported that the majority of extracellular proteins were glycoprotein. This survey, however, was carried out primarily on animal proteins with no information given on microbial systems. He suggested that the carbohydrate was a "label" which was involved in the transport of the protein through the membrane. Two mammalian nucleases have been reported as glycoproteins: bovine pancreatic RNase B (Plummer and Hirs, 1964) and RNase B from bovine milk (Bingham and Kalan, 1967). Pancreatic RNase B contains 2.16% glucosamine and 5.7% mannose while RNase B from milk contains 3% glucosamine, 1.2% galactosamine and 5.17% mannose.

Gottschalk (1966) defines glycoproteins as conjugated proteins containing one or more heterosaccharides with a relatively low number of sugar residues, lacking a serially repeating unit and bound covalently to the peptide chain. The carbohydrate moiety is usually highly

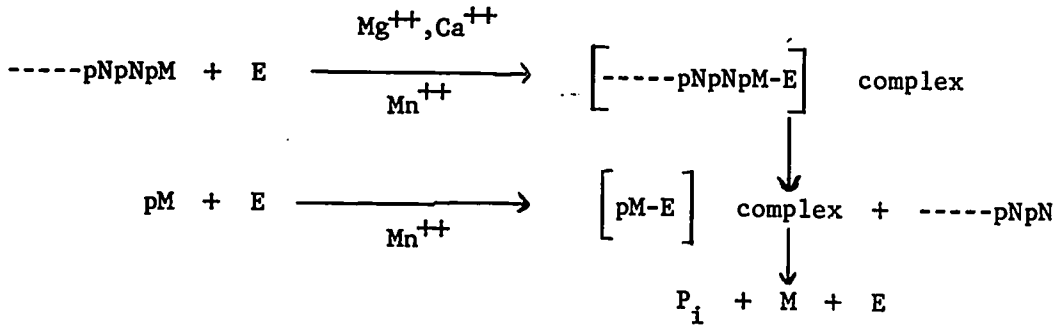
branched. In contrast, the mucopolysaccharides have a linear carbohydrate moiety with a serially repeating unit, a high number of sugar residues (150-1,000) and in some the linkage is electrostatic rather than covalent. The amount of carbohydrate in a glycoprotein can be as high as 80% and the sugars commonly found are D-mannose, D-galactose, L-fucose, D-glucosamine, D-galactosamine and D-neuraminic acid. D-glucose and D-rhamnose occur less frequently. In all glycoproteins so far investigated the linkage of carbohydrate to protein has been through an amino sugar (Neuberger et al, 1966).

Certain physical properties are common to glycoproteins (Gibbons, 1966), polydispersity, a high degree of assymetry, high intrinsic viscosity and a high molecular charge density. Gibbons suggested that physical studies, such as ultracentrifugal studies, be carried out at low protein concentrations in order to avoid interaction of the large flexible molecules.

The majority of the microbial nucleases has not been studied in great detail but a few aspects have been investigated in some systems. The mode of action of the E. coli and Staphylococcal enzymes have been well documented and requirements for activity are known. Amino acid analyses have been carried out on B. subtilis RNase (Lees and Hartley, 1966) and the complete amino acid sequence of Staphylococcal nuclease has been obtained (Tanuichi et al, 1967). Other areas, such as control of enzyme synthesis, remain relatively unexplored.

The nuclease of Micrococcus sodonensis was isolated from culture supernatants and purified 1400 fold (Berry and Campbell, 1967a). It was shown to be exonucleolytic and to act on both native and denatured DNA as well as RNA and synthetic polynucleotides such as Poly A. Monoesterase activity, specific for 5' mononucleotides, is associated with the phosphodiesterase and could not be separated from it by any of the purification techniques employed. The final purified enzyme is homogeneous on gel filtration, electrophoresis and ultracentrifugation. The two activities were shown to have identical pH optima, the same requirement for Mn^{++} and an increase in Mn^{++} concentration above the optimal level was equally inhibitory to both. The ratio of the two activities remained constant during purification from the $(NH_4)_2SO_4$ precipitation step through the final gel filtration.

On the basis of the above similarities the existence of a single protein with two activities was postulated. It was suggested that two sites were required for diesterase activity, one of which was Mg^{++} dependent and the second which was Mn^{++} dependent and which was also involved in monoesterase activity. This would obviate the necessity of the enzyme releasing and recomplexing at another separate site. The mechanism was postulated as shown below (Berry and Campbell, 1967b).



The concept of a single protein with two activities is not a unique one. Exonuclease III of E. coli (Richardson and Kornberg, 1964) has an associated phosphatase activity which serves to remove the 3' phosphate from the intact polymer thus enabling the exonuclease activity to proceed with the release of 5' mononucleotides. Loring et al (1966) isolated a fraction from Mung bean sprouts which contained both 3'-nucleotidase and RNase activity. On the basis of similarities in the effect of chelating agents and heat inactivation, these workers suggested the existence of a single protein. Cyclic phosphodiesterases possessing 3'-nucleotidase activity have been isolated from several sources such as E. coli (Anraku, 1964) and Proteus mirabilis (Center and Behal, 1968). In both cases a single protein with two sites was postulated.

The present study is an extension of the initial work and involves several aspects. Since little is known about the control of extracellular

nucleases, an attempt was made to determine what control mechanism was involved in the production of the M. sodonensis enzyme. The physical and chemical nature of the enzyme was investigated and inhibition studies were carried out in an effort to resolve the "one protein" hypothesis. And finally, information was obtained concerning the site of formation of the active nuclease.

MATERIALS AND METHODS

I. Materials

All reagents used were reagent grade and were obtained from commercial sources.

Biogel P-200 was purchased from Bio-Rad Laboratories and the Sephadex gels from Pharmacia.

Lithium salts of inosine chloromethylphosphonate and inosine fluorophosphonate were the kind gift of Dr. A. Hampton, Cancer Research Laboratories, University of Alberta. Purified cell walls were kindly provided by Mr. K. G. Johnson, Department of Microbiology, University of Alberta.

II. Organism and Growth Conditions

Micrococcus sodonensis ATCC 11880 was the organism used throughout these studies. Stock cultures were maintained on TCS Agar. All cultures were incubated at 30° C and fluid cultures were grown with vigorous aeration.

III. Culture Media

1) TCS Broth (Baltimore Biological Laboratories) pH 7.3. 1.5% agar was added when solid medium was required.

2) Synthetic Medium: The amounts indicated are per 100 ml final volume.

$CaCl_2$	1.0	mg
$(NH_4)6Mo_7O_{24}.4H_2O$	0.08	mg
$ZnSO_4$	1.0	mg
$MnSO_4.H_2O$	0.8	mg
H_3BO_3	0.4	mg
$CuSO_4.5H_2O$	0.1	mg
$CoCl_2.6H_2O$	0.1	mg
$FeSO_4$	0.4	mg
$MgSO_4.7H_2O$	20.0	mg
KCl	50.0	mg
Versene	0.05	mg
Sodium β glycerol phosphate	10.0	mg
Biotin	0.001	mg
Lactic acid	500.0	mg
TRIS	365.0	mg
Glutamic acid	500.0	mg
NH_4Cl (if present)	50.0	mg

The pH was adjusted prior to autoclaving to 8.0. Final pH = 7.3.

IV. Enzyme Production

TCS broth cultures of M. sodonensis were grown in a 10 litre capacity New Brunswick MicroFerm Laboratory Fermentor at 30° C with constant aeration and agitation. Cells were harvested in a Sharples centrifuge and the enzyme was isolated from the supernatant.

V. Purification of Nuclease

Two purification procedures were employed. The first was the $(NH_4)_2SO_4$, DEAE-cellulose, gel filtration sequence described by Berry and Campbell (1967a) except that Biogel P200 replaced Sephadex in the sequence. The second was a modification of this technique and was employed with large volumes of supernatant for ease of manipulation. The supernatant from the fermentor cultures was concentrated and dialysed by

ultrafiltration using an Amicon Model 400 cell and a Diaflo XM-50 membrane. Samples were filtered under nitrogen at 50 p.s.i. Following dialysis of the concentrate, the DEAE-cellulose, gel filtration sequence previously described was employed.

VI. Control Studies

The synthetic medium was employed in these studies as a basal medium with the various compounds under investigation being added as indicated in the text. Cultures were grown in flasks in a New Brunswick Scientific Gyrotory temperature controlled shaker.

Standard inocula were prepared from 18 hr cultures of M. sodonensis in synthetic medium $+NH_4^+$ and cells were washed 3X and resuspended in sterile distilled H_2O.

Cell growth was determined by measuring the absorption at 600 mμ and converting to dry cell weight by means of a standard curve which had been previously prepared.

Continuous culture experiments were carried out using a Dawson cyclone type continuous flow apparatus (Dawson, 1963).

VII. Preparation of Cell Free Extracts

Studies on intracellular enzymes were carried out on extracts prepared from 18 hr cultures of M. sodonensis grown in synthetic medium plus NH_4^+. Cells were washed, treated with lysozyme for 5 mins at pH 7.5 and disrupted in a Hughes press. The broken cells were resuspended in .05M TRIS, pH 8.8, and subjected to sonication at 4° C for 5 mins in a Bronwill Biosonik (Bronwill Scientific, Rochester, N.Y.).

VIII. Enzyme Assays

1) Diesterase: Standard reaction mixtures contained 2 mg RNA, 33 μmoles TRIS, 13 μmoles $MgCl_2$, 1.7 μmoles $CaCl_2$, 1.7 μmoles $MnCl_2$, 10 μmoles mercaptoethanol and 1 μmole EDTA per 0.9 ml. Final pH of the mixture was 8.8. 0.1 ml of enzyme, containing a maximum of 0.5 units of activity, was added at zero time and the reaction incubated at 37° C. Aliquots were removed at the designated times and added to equal volumes of cold UTCA. Activity was followed either by measuring the release of acid-soluble 260 mμ-absorbing material, or by the release of acid-soluble radioactivity from ^{14}C RNA. Samples were suspended in Bray's scintillation fluid and the radioactivity determined in a Nuclear Chicago Mark I Scintillation counter. 1 unit of activity is defined as that amount of enzyme which releases 1 μmole of acid-soluble nucleotide per hour. The plate assay of Berry and Campbell (1965) was employed as a qualitative test for diesterase activity.

2) Monoesterase: The reaction mixture was identical to that for diesterase activity except that AMP (2 μmoles/ml) was the substrate employed. After incubation at 37° C the reaction was stopped by the addition of an equal volume of UTCA and the amount of P_i released was determined by the Ames-Dubin colorimetric procedure. 1 unit of activity is that amount of enzyme which releases 1 μmole of P_i per hour. Qualitative assays for monoesterase in fractions from columns employed 0.5 ml of the above reaction mixture plus one drop from each fraction. After

incubation at 37° C, P_i was detected by the Ames-Dubin technique.

3) UTP Aminase: A modification of the method of Lieberman (1956) was employed. The reaction mixture contained 5 µmoles TRIS, 5 µmoles $MgCl_2$, 6 µmoles $(NH_4)_2SO_4$, 1 µmole ATP, 0.3 µmoles of UR, UMP, UDP or UTP and 0.2 ml cell extract in a final volume of 1.0 ml pH 8.5. After 30 mins incubation at 37° C the reaction was stopped by the addition of 0.5 ml of 7% perchloric acid. Activity was determined by the increase in absorption at 290 mµ.

4) Glutaminase activity: The Conway diffusion plate technique was employed to follow the release of NH_3. Reaction mixtures contained 7.5 µmoles TRIS, 7.5 µmoles $MgCl_2$, 1.5 µmoles ATP, 9 µmoles glutamine, 0.15 ml cell extract, in a final volume of 1.5 ml, pH 8.5. After incubation at 37° C, NH_3 was released by the addition of saturated K_2CO_3 and trapped in 0.1N H_2SO_4 in the center well.

5) Cytidine Deaminase: The reaction mixture contained 5 µmoles TRIS, 5 µmoles $MgCl_2$, 1 µmole ATP, 0.3 µmoles CR, CMP or CTP, and 0.2 ml cell free extract in a final volume of 1.0 ml, pH 8.5. After incubation at 37° C the reaction was stopped by the addition of 7% perchloric acid and the activity was measured by the decrease in absorption at 290 mµ.

6) Carbamyl Phosphate Synthetase: The formation of carbamyl phosphate was determined by measuring the appearance of alkali-labile phosphate in the following reaction mixture: 8 µmoles ATP, 100 µmoles $MgCl_2$, 100 µmoles TRIS, 250 µmoles NH_4 carbamate, 2 µmoles CTP, and 0.2

ml of cell free extract in a final volume of 1.5 ml, pH 8.5. After 30 mins incubation at 37° C, 0.5 ml aliquots were added to 1.0 ml of 5% trichloroacetic acid at 4° C. The precipitate was removed by centrifugation and 0.5 ml of the supernatant added to 0.2 ml of 2N KOH. After 10 mins at 25° C the hydrolyzed samples were assayed for P_i.

7) Aspartic Transcarbamylase: Standard reaction mixtures contained 200 µmoles TRIS, 20 µmoles carbamyl phosphate, 40 µmoles aspartic acid and 0.2 ml of cell free extract in a final volume of 1.0 ml, pH 8.5. After 30 mins incubation at 37° C, 0.5 ml aliquots were assayed for the formation of carbamyl aspartic acid.

8) Ornithine Transcarbamylase: (Jones and Spector, 1960). The reaction mixture consisted of 15 µmoles carbamyl phosphate, 15 µmoles ornithine, 150 µmoles TRIS and 0.2 ml of cell free extract in a final volume of 1.5 ml, pH 8.5. After 30 mins incubation at 37° C the reaction was stopped by the addition of 5% TCA and the supernatant analysed for citrulline.

9) XMP Aminase: The conversion of XMP→ GMP was assayed by the technique described by Magasanik (1963). The reaction mixture contained 40 µmoles TRIS, 1 µmole ATP, 4 µmoles $MgCl_2$, 6 µmoles XMP, 80 µmoles $(NH_4)_2SO_4$ and .02 ml cell free extract in a final volume of 0.25 ml, pH 8.5. After 30 mins incubation at 25° C the reaction was stopped by the

addition of 2.75 ml of 3.5% perchloric acid. The increase in absorption of the supernatant at 290 mμ was measured.

IX. Analytical Methods

1) Protein: The technique of Lowry et al (1951) was employed. Standard curves were prepared using crystalline bovine serum albumin.

2) Ammonia: Quantitation of NH_3 was carried out by Nesslerization and values were obtained from a standard curve.

3) Inorganic Phosphate: The method of Ames and Dubin (1960) was employed.

4) Carbamyl Aspartic Acid: Quantitation of carbamyl aspartate was carried out according to the method of Gerhart and Pardee (1962).

5) Citrulline: The technique described by Oginsky (1957) was employed.

6) Analysis of Carbohydrates:

   a) Total neutral sugar content of the unhydrolysed protein was determined by the indole and anthrone techniques described by Ashwell (1957) and the phenol-sulfuric acid technique of Dubois (1956). Glucose was employed as a standard.

   b) Total hexose was assayed by the cysteine-sulfuric acid technique described by Ashwell (1957) using a glucose standard.

c) The cysteine-sulfuric acid technique (Ashwell, 1957) was employed for the assay of methyl pentose using a rhamnose standard.

d) Uronic acids were assayed using the carbazole technique described by Davidson (1966) and a glucuronic acid standard.

e) The thiobarbituric acid assay of Warren (1959) was employed for the detection of sialic acids. Samples were hydrolysed in 0.1N H_2SO_4 for 60 mins at 80° C. A standard curve was prepared with N-acetyl-neuraminic acid.

f) Estimation of glucose and galactose was carried out on the nuclease after hydrolysis for 3 hrs in 3N HCl at 100° C. The hydrolysed samples were evaporated to dryness and assayed by means of a coupled enzyme reaction employing the specific glucose and galactose oxidases and a peroxidase. The reaction was followed by measuring the formation of the oxidized chromogen which had an absorption maximum in the 400-425 mμ region.

g) The Morgan-Elson technique as described by Ghuysen et al (1966) was employed for the estimation of amino sugars. The N-acetyl-glucosamine standard and the protein were

hydrolysed in sealed tubes in 3N HCl at 100° C for varying times and the assays were carried out after chemical reacetylation of the samples.

h) Reducing sugars were detected by the Park-Johnson ferricyanide procedure as modified by Ghuysen et al (1966).

X. Paper Chromatography

Descending chromatography and Whatman #1 paper was used in all cases.

1. Developing Solvents:

   (a) 75% ethanol was employed for the separation of nucleosides and nucleotides (Singh and Lane, 1964).

   (b) Phenol:H_2O:NH_4OH (80:20:0:5) was the solvent system used for the separation of glutamic acid, glutamine and α-ketoglutaric acid (Smith, 1960).

   (c) Pyridine:ethyl acetate:H_2O:acetic acid (5:5:3:1) as described by Gottschalk (1966) was employed for the separation of carbohydrate components.

2. Location Reagents:

   (a) UV absorption (nucleosides and nucleotides).

   (b) Amino acids were located by means of a ninhydrin spray consisting of: 200 mg ninhydrin in 100 ml 95% ethanol + 20 ml glacial acetic acid (10 vols.); 1% $Cu(NO_3)_2$ (2 vols).

(c) α-keto acids were located by spraying with 0.05% dinitrophenylhydrazine in 2N HCl.

(d) Alkaline $AgNO_3$ reagent (Smith, 1960) was employed for detection of carbohydrates. The background was cleared with 5% sodium thiosulfate.

(e) ^{14}C isotopes were located by means of a Nuclear Chicago Actigraph III Strip Scanner.

XI. Amino Acid Composition

1) Acid hydrolysis: Samples of purified nuclease containing 0.1 mg of protein were pipetted into 150 x 18 mm pyrex tubes and evaporated to dryness in a Buchler Rotary Evapo-Mix. To each was added 1.0 ml of constant boiling 6N HCl. Tubes were evacuated and sealed under reduced pressure and hydrolysed at 100° C for 12, 24, 48 and 72 hrs. All hydrolyses were done in duplicate.

2) Performic acid oxidation: Samples for cysteic acid analysis were prepared according to the method of Moore (1963). Triplicate samples containing 0.1 mg of protein were evaporated to dryness and 2 ml of performic acid added to each tube. After 4 hrs at 0° C, 0.3 ml of 48% HBr was added and samples were dried and hydrolysed as above for 18 hrs.

3) Amino acid analysis: The hydrolysed samples were taken to dryness and resuspended in sodium citrate buffer pH 2.2 according to the method of Smillie *et al* (1966). Aliquots were analysed for their amino acid composition using the Spinco Model 120B Automatic Amino Acid Analyser.

4) Tryptophane and tryosine: The analysis was carried out spectrophotometrically using the method of Goodwin and Morton (1946). Absorption of the enzyme at pH 13 and pH 7.4 was determined at 280 mμ and 294.4 mμ using a Beckman DB-G Grating Spectrophotometer and the values for tryptophane and tyrosine calculated by means of the equation proposed by these authors.

XII. Ultracentrifugal Studies

A Spinco Model E. Analytical Centrifuge employing Schlieren optics was used for these studies. The protein was prepared at varying concentrations in phosphate buffer pH 7.3, ionic strength 0.1. Sedimentation velocity experiments were carried out at 60,000 rpm and a rotor speed of 12,000 rpm was used for the Archibald approach to sedimentation equilibrium. The $\bar{v}$ was calculated on the basis of the carbohydrate and amino acid analyses and a value of 0.713 was used in the calculations of molecular weight and $S_{20,w}$.

XIII. Serological Tests

1) Agglutination tests: Doubling dilutions of anti-sera were prepared in buffered saline (95 ml of 0.85% NaCl + 5 ml of 0.1M borate buffer pH 8.4). Equal volumes of antigen were added to each serum dilution and tubes were incubated overnight at 27° C followed by refrigeration for 24 hrs. The titre of the serum was the highest dilution which still caused visible agglutination of the antigen.

2) Tube precipitation test: Into each of a series of Durham tubes was placed 0.1 ml of antiserum. To each tube was added increasing volumes

(e.g. 10 µl, 20 µl, 30 µl) of the enzyme solution. Suitable serum and buffer controls were employed. Tubes were sealed and incubated for 30 mins at 37° C, refreigerated overnight and examined for visible precipitation. Quantitative tests were carried out using doubling serum dilutions and a constant volume of antigen.

3) Ring test: Antiserum was placed in Durham tubes (1-2 cm in depth) and carefully overlaid with a 2-4 mm layer of enzyme or buffered saline. Tubes were allowed to stand at room temperature, observing frequently for precipitation.

4) Gel diffusion: The Oudin tube method of single gel diffusion was employed. Glass tubing (internal diameter 2mm) was cut into 7 cm. lengths, coated with 0.1% agar solution and sealed at one end. A 0.6% solution of Noble Agar (Difco) was prepared in buffered saline and held at 50°C. Equal volumes of serum and agar solution were mixed and added to the tubes to form a 4 cm column. After the agar had solidified the enzyme or buffer solutions were layered on the columns and the tubes were sealed and incubated at 30° C with periodic examination for bands of precipitation.

EXPERIMENTAL RESULTS

I. Enzyme Production

The appearance of nuclease activity in culture supernatants of M. sodonensis grown in synthetic medium, synthetic medium plus NH_4^+ (10 µmoles/ml) and in TCS broth was investigated. 100 ml of each medium was incubated with a washed standard inoculum and 5 ml aliquots were removed at the designated time intervals for estimation of cell growth and diesterase activity as described in Methods. No activity could be detected in the synthetic medium without added NH_4^+. Fig. 1 shows the growth and nuclease production in TCS broth and synthetic medium plus NH_4^+. Activity appeared in both media when the cells were in the middle-to-late logarithmic phase of growth and in TCS broth the level of enzyme per unit cell remained relatively constant throughout the experiment. In the synthetic medium, however, there was an increase in activity per unit cell until 24 hrs by which time the cells were in the early stationary phase. In both media there was a slight decrease in activity after the cells had entered the stationary phase which suggested that although the cell numbers were increasing slightly, they were no longer actively producing nuclease and that the production of the enzyme was a function of rapidly growing cells.

Attempts to study nuclease production in continuous culture were unsuccessful since , although a stabilized culture was obtained, no enzyme activity could be detected in the supernatant. That the lack of activity could not be attributed to instability of the enzyme was apparent from

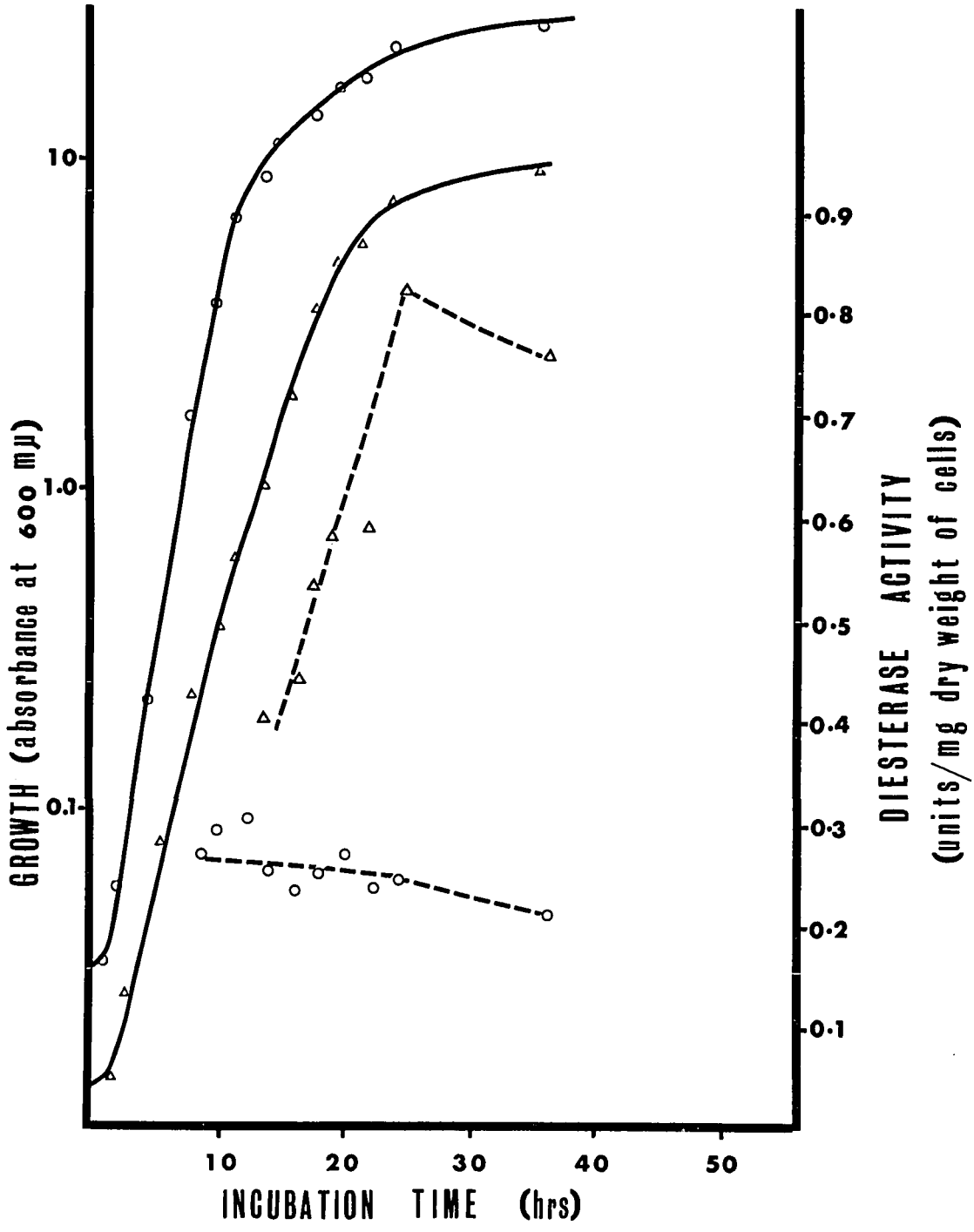

GROWTH (absorbance at 600 mμ)
10
1.0
0.1
DIESTERASE ACTIVITY
(units/mg dry weight of cells)
0·9
0·8
0·7
0·6
0·5
0·4
0·3
0·2
0·1
10
20
30
40
50
INCUBATION TIME (hrs)

FIGURE 1

GROWTH RESPONSE AND NUCLEASE PRODUCTION BY

M. SODONENSIS IN VARIOUS MEDIA

Growth in TCS broth O——————O

Nuclease production in TCS broth O- - - - - - - -O

Growth in synthetic medium Δ——————Δ

Nuclease production in synthetic medium Δ- - - - - - - -Δ

the following experiment. The supernatant from a 24 hr TCS broth culture of M. sodonensis was placed in a continuous culture apparatus under the same conditions of temperature and aeration as that employed for culture. Aliquots were removed periodically and assayed for diesterase activity and protein content. As shown in Table I, the specific activity remained constant over a 48 hr period as did that of the control sample which had been stored at 4° C. It is suspected that, under the coninuous culture conditions employed, a variant was selected since some changes were observed in the cells after stabilization. Although they retained their staining characteristics, their colonial morphology was altered with the colonies becoming sticky in consistency, suggestive of leaky cells. In broth culture a leakage of intracellular components, primarily DNA, was observed. This selective leakage of DNA has been reported previously under other culture conditions (Campbell et al 1961b). A series of experiments failed to reveal the cause of this aberrant behavior in the chemostat so this aspect was abandoned and subsequent studies on enzyme control were carried out in batch culture.

II. Physical and Chemical Characterization

1. Purity of M. sodonensis nuclease.

The enzyme was purified as previously described by Berry and Campbell (1967a). The modified purification technique described in Methods did not significantly alter the purification obtained. The

TABLE I

STABILITY OF *M. SODONENSIS* NUCLEASE UNDER CHEMOSTAT CONDITIONS

Conditions	Activity*						
	2 hr	4 hr	7 hr	22 hr	24 hr	44 hr	48 hr
Chemostat	0.4	0.4	0.4	0.44	0.44	0.4	0.4
Control	0.36	0.33	0.36	0.37	-	0.36	0.4

* Activity is expressed as units/mg dry cell wt

elution pattern obtained on Biogel P-200 is shown in Fig. 2. 4 ml fractions were collected and screened for diesterase and monoesterase activity by the qualitative procedures described in Methods and the active fractions were pooled and used for further characterization studies. As shown in Fig. 2, diesterase and monoesterase activities were not separated. Other techniques such as sucrose density centrifugation and electrophoresis also failed to separate the two activities.

2. Ultracentrifugal Analyses

Samples of the purified enzyme were prepared at different concentrations in 0.1 ionic strength phosphate buffer pH 7.2. Sedimentation velocity experiments were carried out at a rotor speed of 60,000 rpm and the $S_{20,w}$ values were calculated for each concentration according to the method of Schachman (1957). Fig. 3 shows the Schlieren patterns of a 0.25% enzyme solution at 3 different time intervals. A single homogeneous peak was obtained which was indicative of a high degree of purity.

The $S_{20,w}$ values varied with the concentration and the results obtained were plotted and extrapolated to infinite dilution. The results of one such experiment are shown in Fig. 4 in which an $S^{o}_{20,w}$ of 2.26×10^{-13} was obtained. A second experiment on a different enzyme preparation yielded an $S^{o}_{20,w}$ of 2.0×10^{-13}.

The Archibald approach to sedimentation equilibrium was employed for molecular weight determinations (Schachman, 1957). The reciprocal

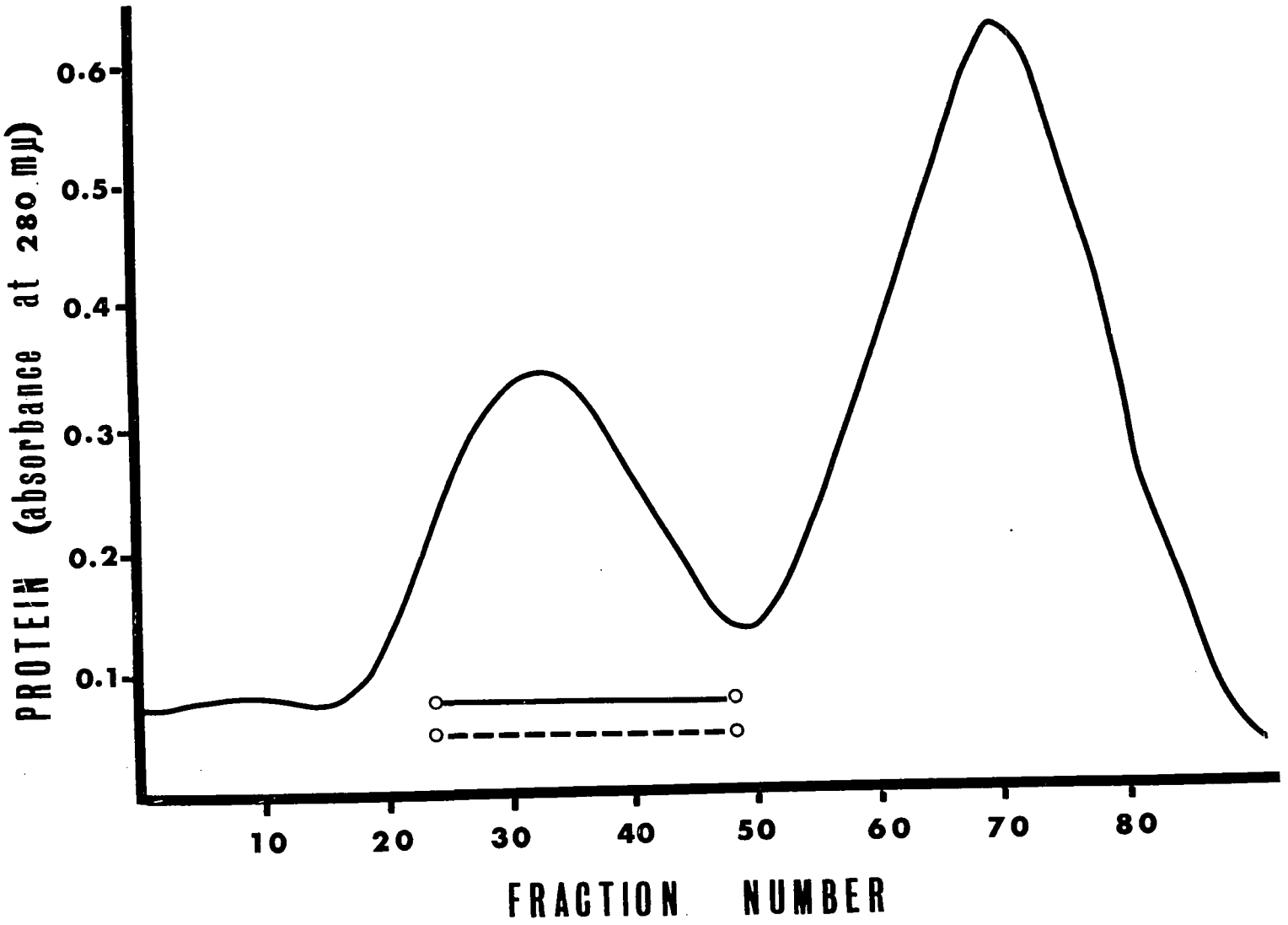

PROTEIN (absorbance at 280 mμ)
0.6
0.5
0.4
0.3
0.2
0.1
10
20
30
40
50
60
70
80
FRACTION NUMBER

FIGURE 2

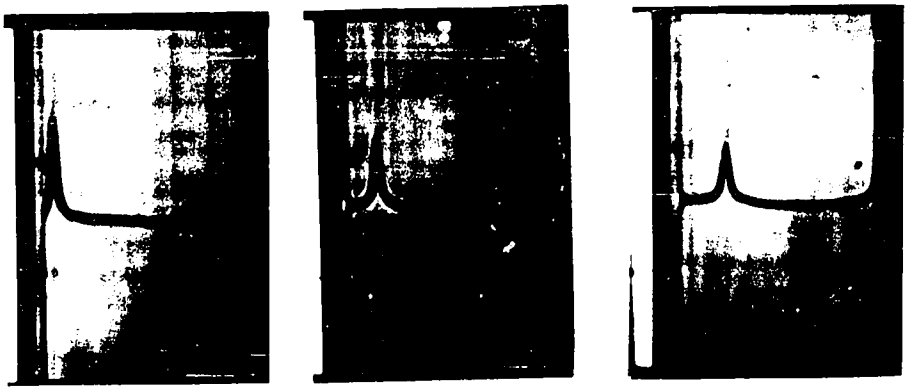
PURIFICATION OF M. SODONENSIS NUCLEASE BY GEL FILTRATION

Enzyme was eluted from a 2.5 x 80 cm column of Biogel P200 with .01M TRIS pH 8.8 and 4 ml fractions collected. Activity was determined by qualitative assay procedures.

Protein ————

Region containing diesterase activity O————O

Region containing monoesterase activity O -----O



48 112 160

FIGURE 3

SCHLIEREN PATTERN OF PURIFIED M. SODONENSIS NUCLEASE

A 0.25% solution of purified nuclease in pH 7.2 phosphate buffer (ionic strength 0.1) was sedimented at 60,000 rpm at 20° C. Bar angle = 50°. Sedimentation is from left to right. Photos were taken at 48, 112 and 160 mins after attaining full speed.

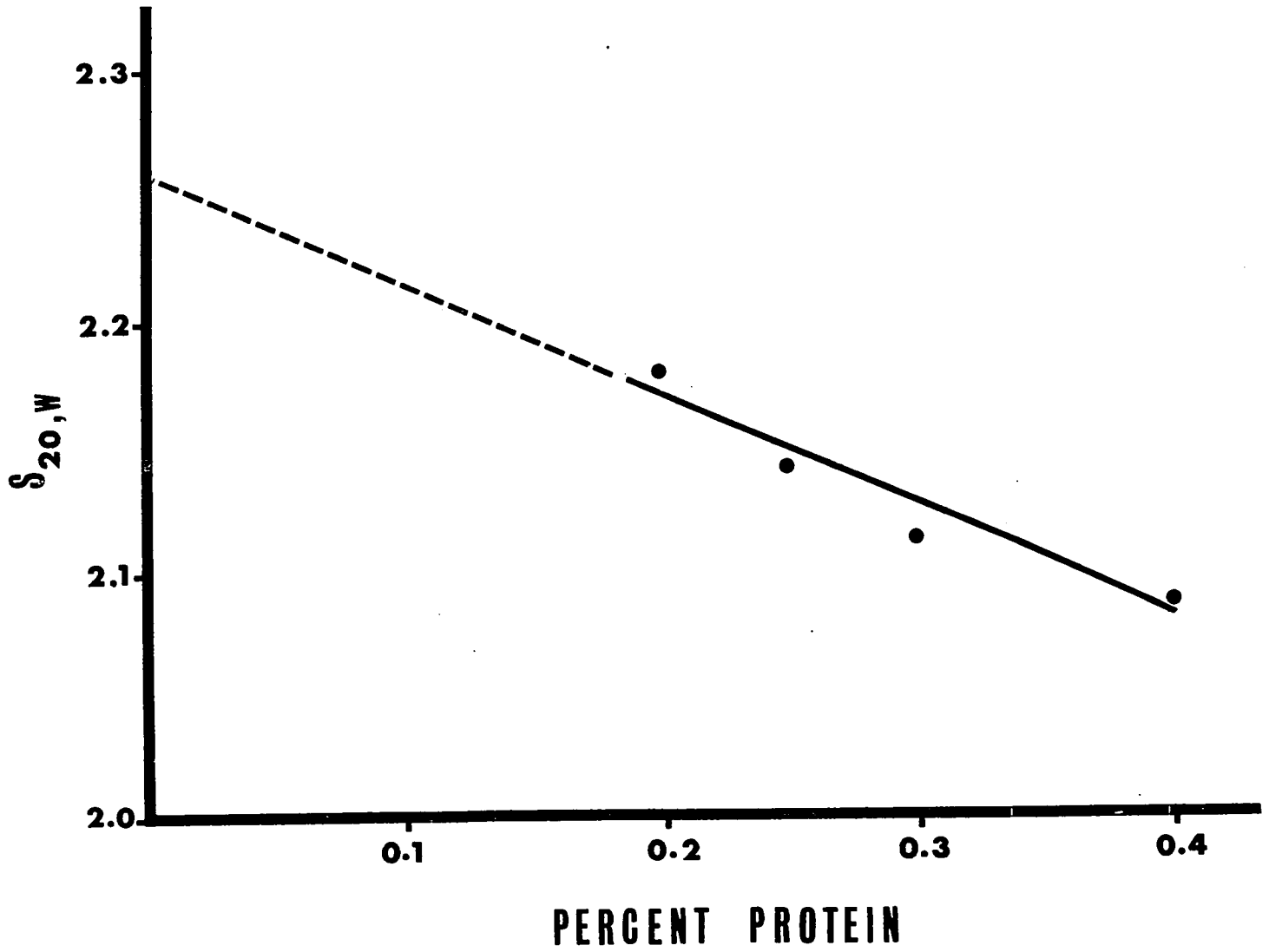

2.3
2.2
2.1
2.0
$S_{20,W}$
0.1
0.2
0.3
0.4
PERCENT PROTEIN

FIGURE 4

SEDIMENTATION COEFFICIENT OF M. SODONENSIS NUCLEASE AT VARIOUS CONCENTRATIONS

Solutions of purified M. sodonensis nuclease in pH 7.2 phosphate buffer (ionic strength 0.1) were sedimented at 60,000 rpm at 20° C. $S_{20,w}$ values were calculated at each concentration and extrapolated to infinite dilution.

of the molecular weight was plotted at each concentration and extrapolated to infinite dilution. As shown in Fig. 5, the system is extremely concentration-dependent and the value obtained for the molecular weight at infinite dilution was 500,000. This concentration-dependence plus the sharpness of the peak in the sedimentation velocity experiment (Fig. 3) are indicative of an asymmetric molecule.

3. Amino Acid Composition

Amino acid analyses of the purified enzyme were performed as described in Methods. The results are shown in Table II and are the average values calculated from the different hydrolysis times with the following exceptions. The 12 hr value was excluded from the glutamic acid results. Serine and threonine were extrapolated to zero time and the values for valine and isoleucine were obtained after hydrolysis for 72 hrs. Fig. 6 shows the results of these 4 amino acids at the different hydrolysis times. Tryptophane was determined spectrophotometrically and from the absorption data 75.5 moles of tryptophane and 130 moles of tyrosine were obtained per mole of enzyme. The value for tyrosine did not agree with that obtained in the amino acid analysis and therefore the value for tryptophane may actually be lower if one calculates it on a tyrosine:tryptophane ration of 1.7 using the analyser value.

4. Carbohydrate Analyses

Initial observations had shown a discrepancy between the protein values obtained by the Lowry technique and total nitrogen and this

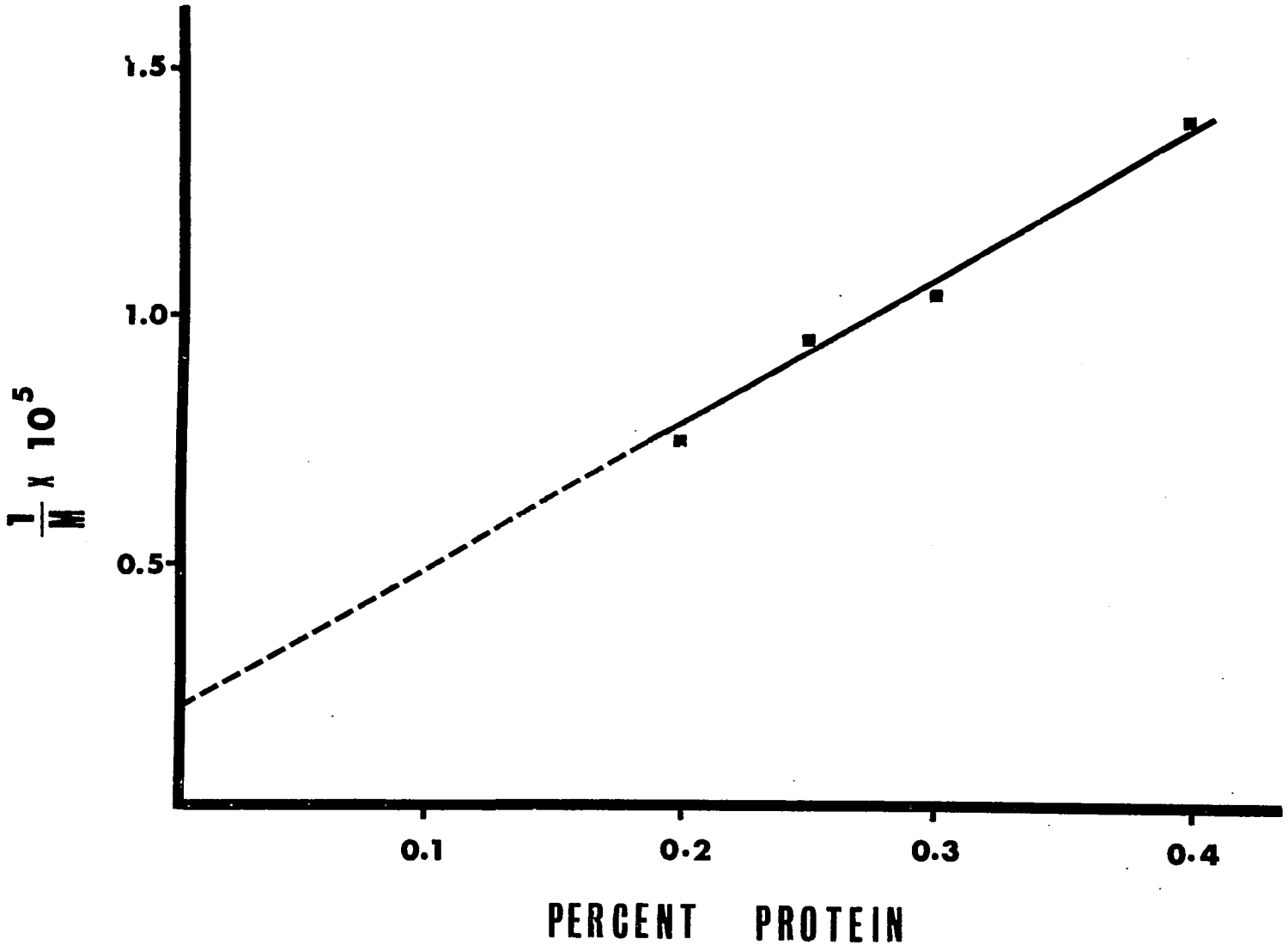

1.5
1.0
0.5
$\frac{1}{M} \times 10^5$
0.1
0.2
0.3
0.4
PERCENT PROTEIN

FIGURE 5

CONCENTRATION DEPENDENCE OF THE APPARENT MOLECULAR WEIGHT OF M. SODONENSIS NUCLEASE

Solutions of purified M. sodonensis nuclease in pH 7.2 phosphate buffer (ionic strength 0.1) were centrifuged at 12,000 rpm at 20° C. $\frac{1}{M}$ values were calculated at each concentration and extrapolated to infinite dilution.

TABLE II

AMINO ACID COMPOSITION OF M. SODONENSIS NUCLEASE

Amino Acid	Moles/100 gms	Moles/mole	Grams/100 gms
Lysine	.0226	112.9	3.31
Histidine	.0083	41.4	1.29
Arginine	.0164	82.1	2.86
Tryptophane*	.0151 (.0065)	75.5 (30.25)	3.08 (1.24)
Aspartic acid + asparagine	.0658	328.8	8.77
Threonine	.0575	287.5	6.85
Serine	.055	275.0	5.78
Glutamic acid + glutamine	.0574	287.0	8.45
Proline	.0441	220.6	5.08
Glycine	.0704	352.0	5.28
Alanine	.0834	417.1	7.43
Valine	.064	320.0	7.50
Methionine	.0075	37.5	1.12
Isoleucine	.025	125.0	3.28
Leucine	.0405	202.5	5.31
Tyrosine	.0103	64.3	1.87
Phenylalanine	.0156	78.2	2.58
Cysteic acid	.0075	37.5	.91
		Total	80.75 (78.91)

*Values in brackets are calculated on the basis of the amino acid analyser value for tyrosine — assuming a tyr: tryp ratio of 1.7.

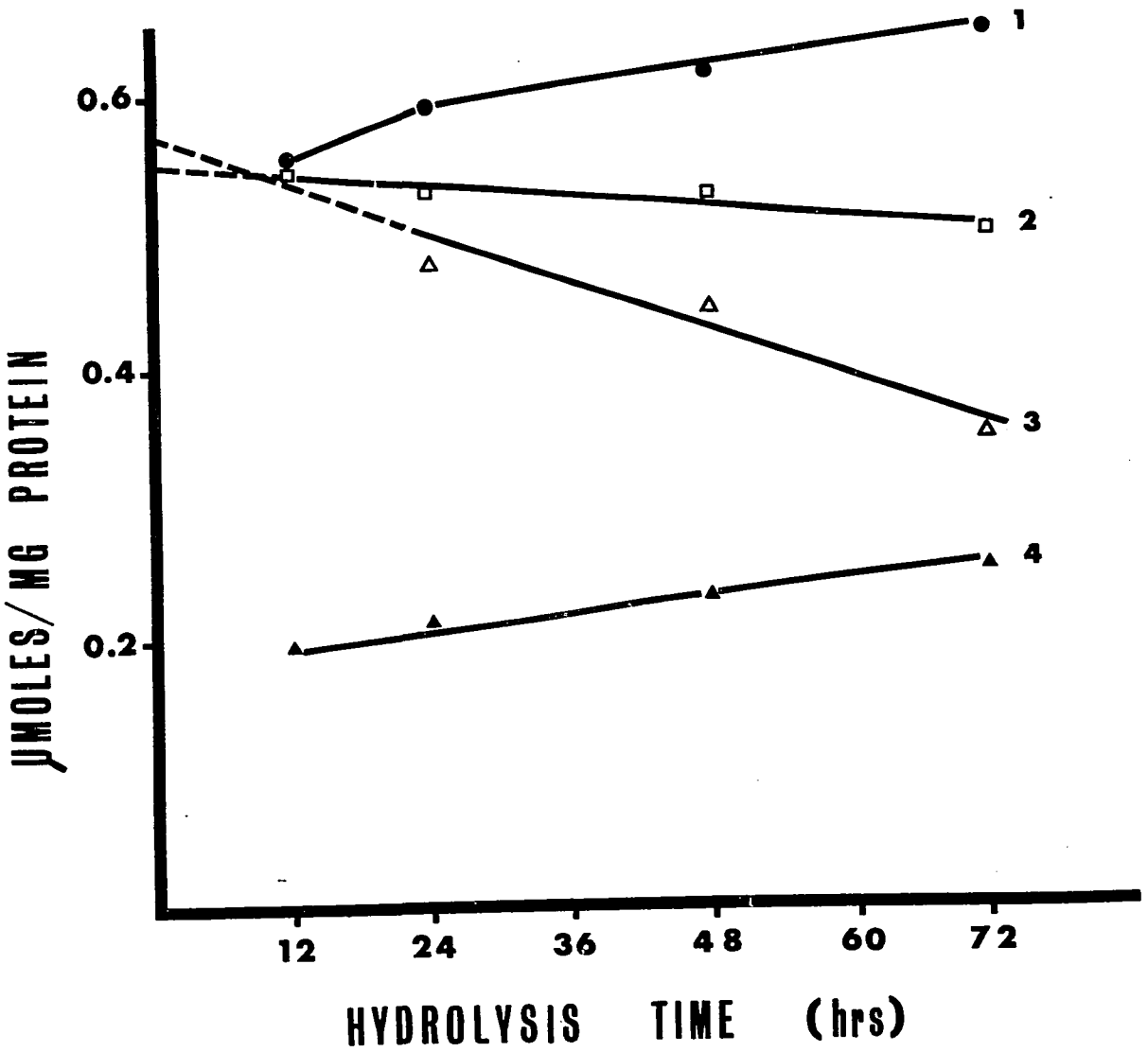

0.6
0.4
0.2
μMOLES/MG PROTEIN
1
2
3
4
12
24
36
48
60
72
HYDROLYSIS TIME (hrs)

FIGURE 6

EFFECT OF TIME OF ACID HYDROLYSIS OF M. SODONENSIS NUCLEASE ON CONCENTRATION OF SEVERAL AMINO ACIDS

The enzyme was hydrolysed in 6N HCl at 110° C for 12, 24, 48 and 72 hrs.

Curve 1 - valine

Curve 2 - threonine

Curve 3 - serine

Curve 4 - isoleucine

suggested either that the protein was low in aromatic residues or that something else was present which was contributing to the nitrogen value. The amino acid analysis indicated that aromatic amino acids were present at relatively normal levels, however other aspects of the analysis led to the consideration of a possible carbohydrate moiety. As well as a darkening and charring of the sample with acid hydrolysis, an abnormally high value was obtained for tryptophane which did not agree with the spectrophotometric data. The peak decreased with increasing hydrolysis time and at 72 hrs had disappeared (Fig. 7). Since tryptophane is highly unstable to acid, and normally is completely destroyed by 12 hrs hydrolysis, the "tryptophane" peak must be due to some other compound which behaves as tryptophane on the column. The third observation was that in the 12 hr hydrolysate the serine and glutamic acid peaks could not be resolved. The high initial value attributed to glutamic acid fell to a stable level at 24 hrs as shown in Fig. 7. Since glutamic acid is very stable to acid hydrolysis, such a decrease was unexpected. These anomalies could be explained, however, if an amino sugar were present. Glucosamine will peak in the same region as tryptophane in this system and therefore any free glucosamine released by acid hydrolysis will contribute to the tryptophane value. Two amino sugar-peptide linkages which have been demonstrated in glycoproteins involve the hydroxyl group of serine or the γ-carboxyl of glutamic acid. Any amino sugar which was still linked to either amino acid could be responsible for the high value and the "smearing" of the serine-glutamic acid peak.

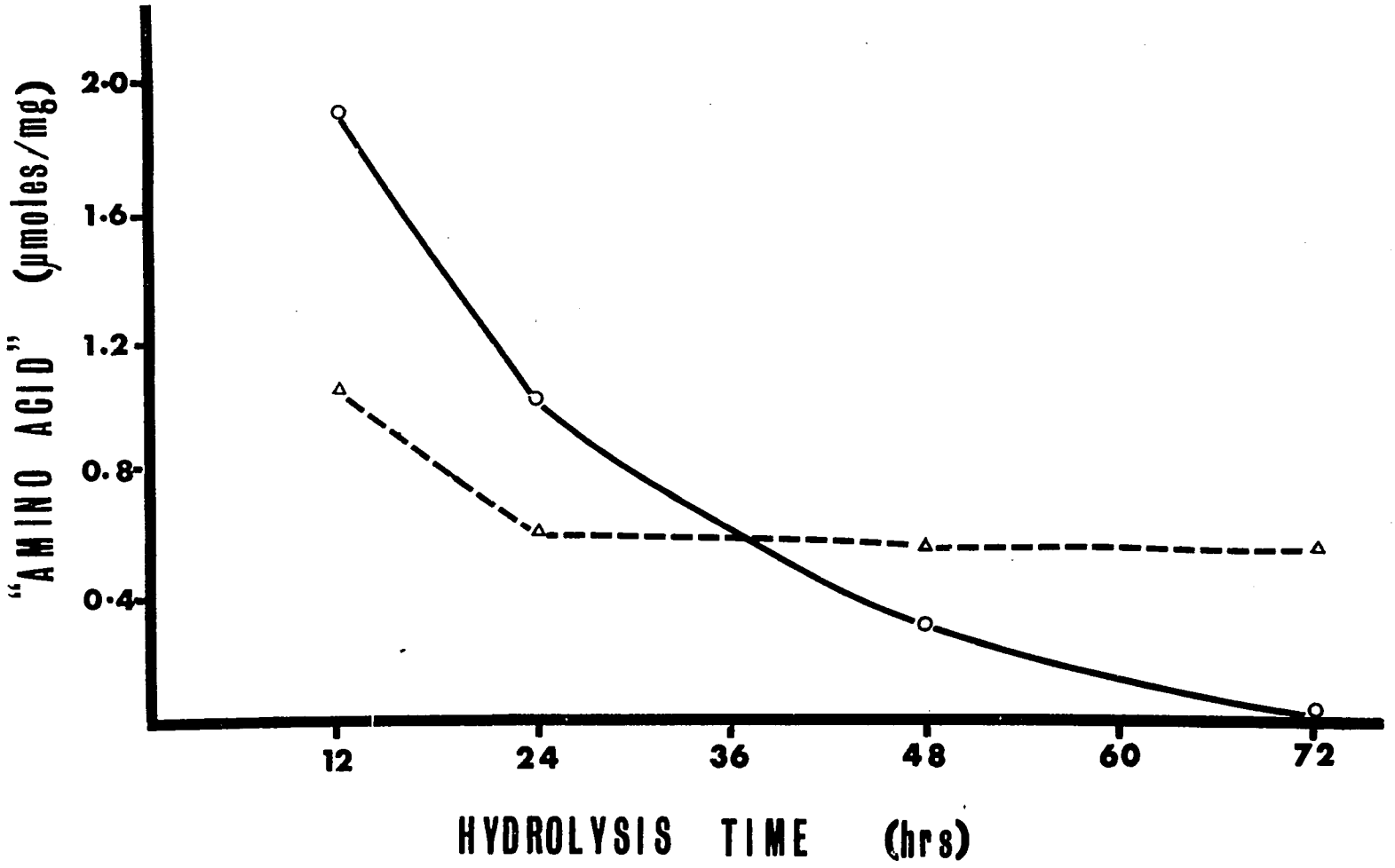

"AMINO ACID" (μmoles/mg)
2·0
1·6
1·2
0·8
0·4
12
24
36
48
60
72
HYDROLYSIS TIME (hrs)

FIGURE 7

EFFECT OF TIME OF HYDROLYSIS ON APPARENT CONCENTRATIONS OF TRYPTOPHANE AND GLUTAMIC ACID IN AMINO ACID ANALYSES OF *M. SODONENSIS* NUCLEASE

Tryptophane O———O

Glutamic acid Δ--------Δ

The enzyme was analysed for the presence of carbohydrate by 3 different assay techniques. As indicated in Table III virtually identical results were obtained.

The individual carbohydrate components were identified by controlled acid hydrolysis and paper chromatography. Two ml aliquots of enzyme (0.5 mg/ml) were hydrolyzed in 3N HCl in vacuo at 100° C for 3 and 12 hrs, evaporated to dryness and resuspended in 1 ml of H_2O. 100 µl of each hydrolysate were spotted on Whatman #1 paper and developed for 12 or 24 hrs in the solvent system described in Methods. Standard sugar solutions were run with each chromatogram. Fig. 8 shows a chromatogram which had been developed for 12 hrs. Three spots were detectable in both the 3 and 12 hr hydrolysates with the 2 lower ones corresponding to the glucose and rhamnose standards. The third spot was subsequently identified as glucosamine. As shown in Fig. 9, after 24 hrs development rhamnose had run off the paper but the apparent single glucose spot was resolved into 2, one of which corresponded to galactose. The third spot corresponded to glucosamine. The presence of glucose and galactose was confirmed enzymatically by means of the Worthington Glucostat and Galactostat reagents. Further confirmation of glucose and glucosamine was obtained by co-chromatography with ^{14}C labelled standards. 100 µl of a 3 hr hydrolysate, containing an estimated 34 and 52 mµmoles of glucose and glucosamine respectively, were applied to 4 cm Whatman #1 filter paper strips together with 1 µl of the ^{14}C standards (representing about .01 mµmoles of each) and the strips

TABLE III

TOTAL NEUTRAL SUGAR CONTENT OF *M. SODONENSIS* NUCLEASE

Method	µmoles*/mg protein
Phenol-H_2SO_4	0.746
Indole	0.756
Anthrone	0.750

* Expressed as glucose equivalents

GLUCOSE

FUCOSE

RHAMNOSE

3 HR HYDROLYSATE

12 HR HYDROLYSATE

GALACTURONIC ACID

GLUCURONIC ACID

FIGURE 8

CARBOHYDRATE COMPOSITION OF M. SODONENSIS NUCLEASE

100 µl of the 3 hr and the 12 hr acid hydrolysates were spotted and the chromatogram developed for 12 hrs in the pyridine: ethyl acetate:H_2O:acetic acid solvent. Spots were located with $AgNO_3$ reagent.


GALACTOSAMINE
GLUCOSAMINE
3 HR HYDROLYSATE
GALACTOSE
GLUCOSE

FIGURE 9

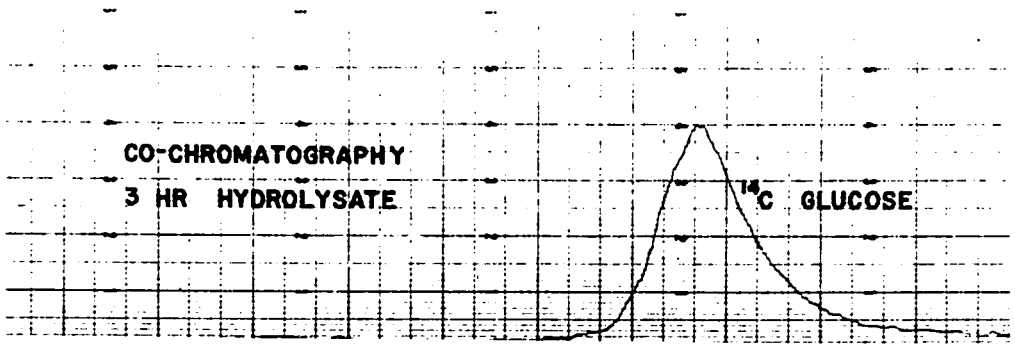
CARBOHYDRATE COMPOSITION OF M. SODONENSIS NUCLEASE

100 µl of the 3 hr acid hydrolysate was spotted and the chromatogram developed for 24 hrs in the pyridine:ethyl acetate: H_2O:acetic acid solvent. Spots were located with $AgNO_3$ reagent.

developed for 24 hrs. As shown in Fig. 10, the spots previously identified as glucose and glucosamine co-chromatographed exactly with the respective radioactive standards.

The individual carbohydrate components were quantitated as described in Methods and the results are shown in Table IV. Glucose, galactose and rhamnose accounted for 95% of the neutral sugar, therefore if any other sugar is present it must be a very minor component. No sialic acid nor uronic acid could be detected either chemically or chromatographically. The value for hexosamine was obtained after 12 hrs hydrolysis which gives maximum release of hexosamine and the relationship of hexosamine release to hydrolysis time is shown in Fig. 11. It must be emphasized that this 12 hr value can only be a minimum one since it was apparent from the amino acid analysis that some amino sugar was still bound to the amino acid (ie. serine or glutamic acid). However with longer hydrolysis times degradation of free hexosamine occurred more rapidly and the net result was a decrease in the hexosamine value.

The reducing power of the hydrolysed and unhydrolysed protein was determined. A glucose standard was employed and the standards and test samples were hydrolysed in 3N HCl in sealed tubes for the indicated times. As can be seen from the results in Table V, hydrolysis for 1 hr under these conditions is sufficient to release all of the reducing power.


CO-CHROMATOGRAPHY
3 HR HYDROLYSATE
^{14}C GLUCOSE

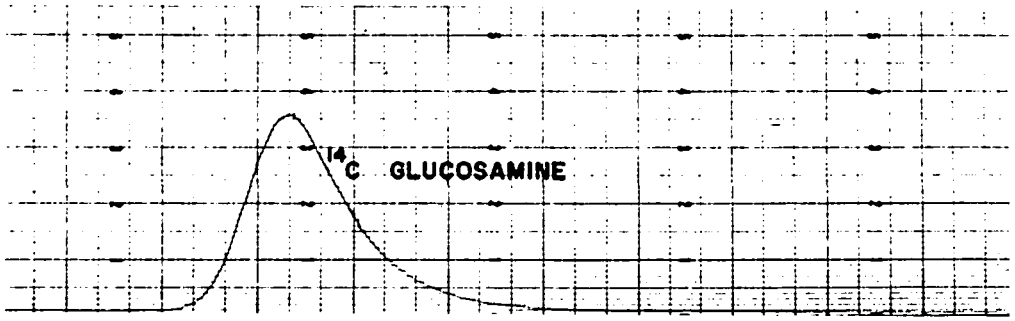

^{14}C GLUCOSAMINE

FIGURE 10

CO-CHROMATOGRAPHY OF A 3 HOUR ACID HYDROLYSATE OF M. SODONENSIS NUCLEASE WITH ^{14}C LABELLED GLUCOSE AND GLUCOSAMINE

100 µl of the 3 hr acid hydrolysate was spotted with 2 µl of each ^{14}C standard. The chromatogram was developed for 24 hrs in the pyridine: ethyl acetate:H_2O:acetic acid solvent and spots located with $AgNO_3$ reagent. Radioactivity was detected by means of a Nuclear Chicago Actigraph III Strip Scanner.

TABLE IV

CARBOHYDRATE COMPOSITION OF *M. SODONENSIS* NUCLEASE

Component	µmoles/mg protein	%
Glucose	0.355	5.0
Galactose	0.17	2.4
Methyl Pentose	0.19	2.4
Hexosamine	0.678	11.7
Total	1.393	21.5

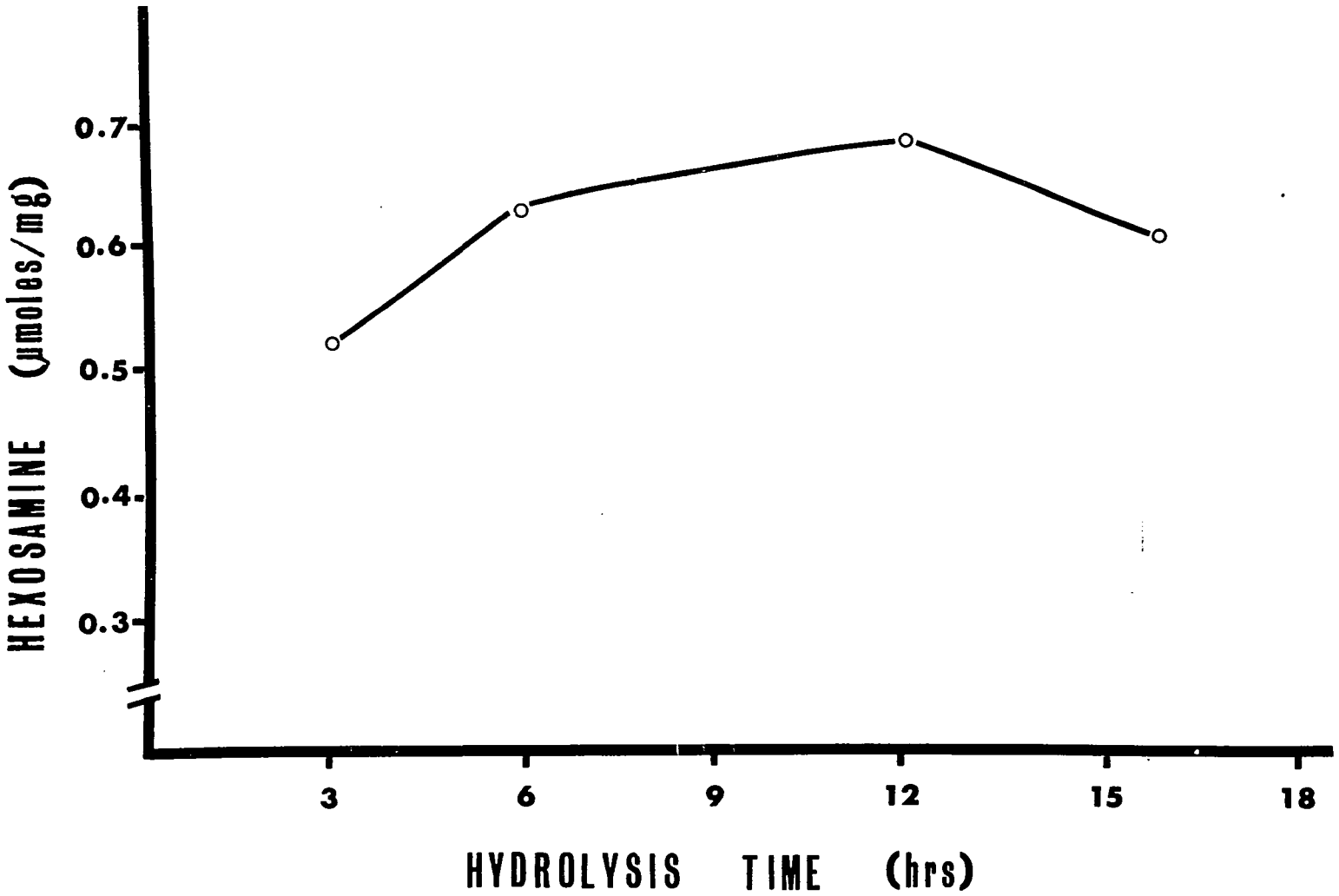

HEXOSAMINE (µmoles/mg)
0.7
0.6
0.5
0.4
0.3
3
6
9
12
15
18
HYDROLYSIS TIME (hrs)

FIGURE 11

EFFECT OF HYDROLYSIS TIME ON HEXOSAMINE RELEASE FROM

M. SODONENSIS NUCLEASE

TABLE V

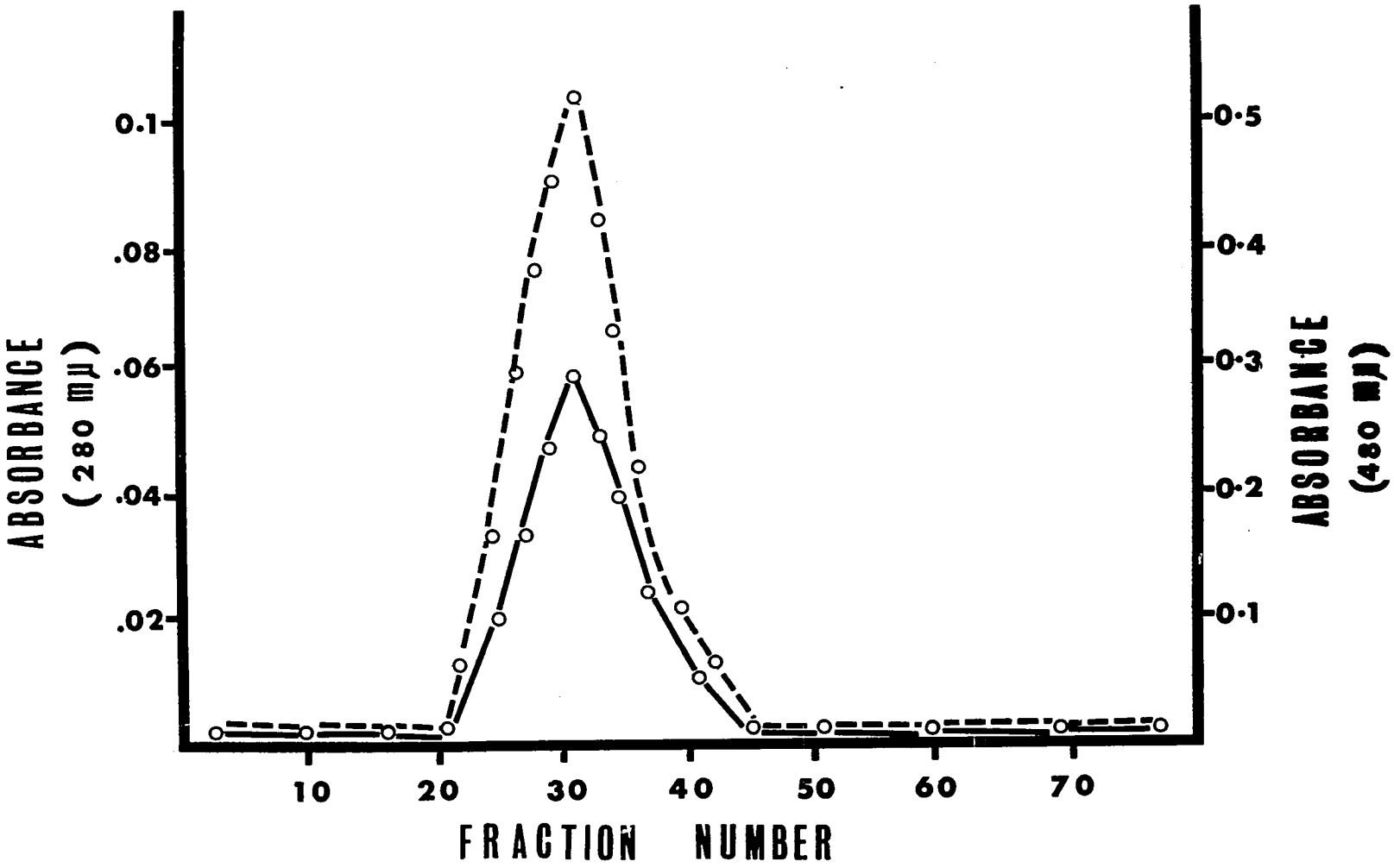
REDUCING POWER OF M. SODONENSIS NUCLEASE

Hydrolysis time (hrs)	Reducing Power* (mμmoles/mg protein)
0	25
1	1500
3	1415

* Relative to glucose

Attempts to separate the carbohydrate moiety from the protein by various methods such as dialysis, ion exchange, gel filtration, precipitation, high salt concentrations and treatment with urea were unsuccessful. Fig. 12 shows the results of one such experiment in which the enzyme was dialysed against 8M urea and applied to a 2.5 x 32 cm column of Sephadex G-100. Fractions were eluted with .01M TRIS buffer, pH 8.8, and the absorption at 280 mμ determined. Each fraction was assayed for carbohydrate by the indole technique and the color development read at 480 mμ. As shown, the carbohydrate remained associated with the protein peak. The inability to separate the two fractions by any of the above means coupled with the fact that prolonged acid hydrolysis was required to release all the amino sugar was indicative of a stable covalent linkage rather than ionic or H-bonding.

The results of the amino acid analysis suggested the possibility of an amino sugar linkage to serine or glutamic acid. Since both of these linkages are reported to be alkali-labile, an attempt was made to separate the two moieties by means of alkaline hydrolysis and to come to some conclusion regarding the linkage. Carubelli et al (1965) followed the release of N-acetyl-galactosamine from serine in ovine submaxillary gland protein by measuring the increase in absorption at 241 mμ. The sugar was released by β elimination and the α-aminoacrylic acid derivative of serine which was formed concurrently, absorbs strongly at this wavelength. M. sodonensis nuclease was suspended


ABSORBANCE
(280 mμ)
0.1
.08
.06
.04
.02
0·5
0·4
0·3
0·2
0·1
ABSORBANCE
(480 mμ)
10
20
30
40
50
60
70
FRACTION NUMBER

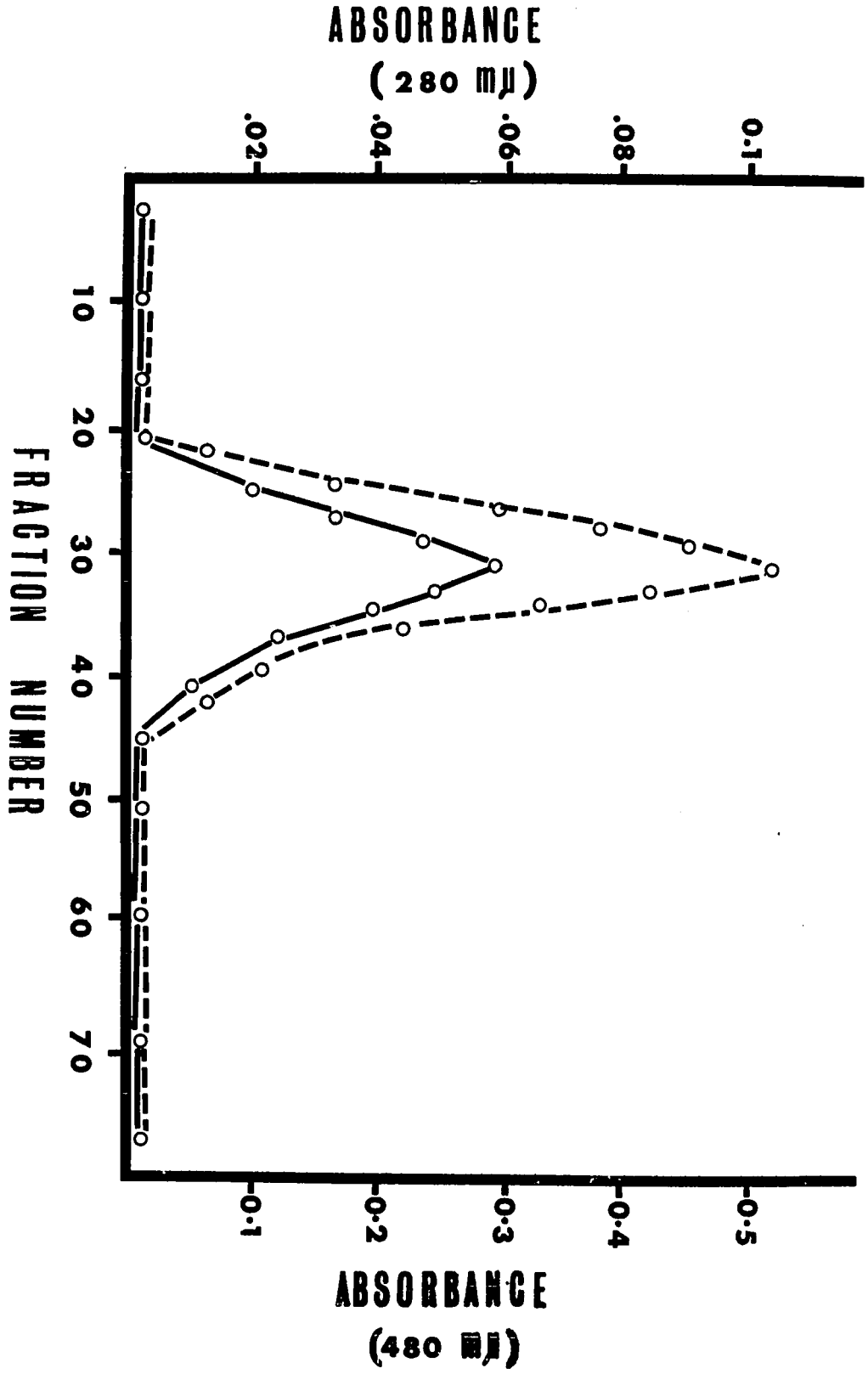

ABSORBANCE
(280 mμ)
.02
.04
.06
.08
0.1
10
20
30
40
50
60
70
FRACTION NUMBER
0.1
0.2
0.3
0.4
0.5
ABSORBANCE
(480 mμ)

FIGURE 12

GEL FILTRATION OF M. SODONENSIS NUCLEASE

AFTER TREATMENT WITH 8M UREA

The enzyme was dialysed for 2 hrs against 8M urea and applied to a 2.5 x 32 cm column of Sephadex G-100. Protein was detected by measuring OD at 280 mμ. Color development at 480 mμ with the indole technique was a measure of the carbohydrate.

Protein O————O

Carbohydrate O--------O

in 0.5 N NaOH for 1 hr and the increase in absorption at 241 mμ was measured. Assuming a molar extinction coefficient of 5300 for the aminoacrylic acid derivative, 3 mμmoles of the derivative was formed in one hour. From the amino acid analyser data the total amount of serine present in the digest sample was 3.65 mμmoles. These data suggest that at least 80% of the serine residues of the enzyme are involved in this protein-carbohydrate linkage.

III. Mode of Action

1. Substrate Specificity

Earlier experiments (Berry and Campbell, 1967b) had shown that the purified enzyme fraction was active on Poly A, RNA and both native and denatured DNA. All 5'-ribo and deoxy-ribonucleotides were dephosphorylated with higher activity on the purine nucleotides. No activity could be demonstrated on ADP, ATP, pTp or the 3' nucleotides.

Further studies have shown that ApA and pTpT will also serve as substrates. Table VI compares the reaction rates on ApA, pTpT and AMP. Several other compounds were tested, none of which would serve as substrates. These included bis(p-nitrophenyl)phosphate, p-nitrophenyl-thymidine-5'-phosphate, adenosine-2',3'-cyclic phosphate, adenosine-3',5'-cyclic phosphate, ribose-1-phosphate and ribose-5-phosphate.

TABLE VI

ACTIVITY OF M. SODONENSIS NUCLEASE ON VARIOUS SUBSTRATES

Substrate	Initial Reaction Rate (μmoles/mg/hr)
AMP	15
ApA	15
pTpT	12.3
RNA	18

2. Effect of Enzyme Concentration

Fig. 13 illustrates the results obtained when varying concentrations of enzyme protein were added to reaction mixtures with AMP and RNA as substrates. The expected linear relationship was obtained for both mono and diesterase activity indicating that the reaction velocity was proportional to enzyme concentration.

3. Effect of Substrate Concentration

The Michaelis constants of the enzyme for AMP and RNA were determined. The reaction velocities at several substrate concentrations were calculated and the Km obtained from Lineweaver-Burk plots of the data. Fig. 14 shows the results obtained with varying concentrations of AMP. The Km for AMP was calculated to be 2.83×10^{-5}M. Fig. 15 is a reciprocal plot with RNA as substrate. Assuming the molecular weight of 5×10^5 for the RNA, the Km was calculated to be 2.9×10^{-6}M or, on the basis of weight, 1.45 mg/ml.

4. Effect of AMP on Diesterase Activity

The inhibitory effect of AMP was determined at several RNA concentrations. ^{14}C RNA was added to give a specific activity of 0.125 μc/mg and the release of UTCA soluble radioactivity was measured in the presence of varying amounts of cold AMP. The data were plotted using the graphical method described by Dixon and Webb (1965) as shown in Fig. 16,

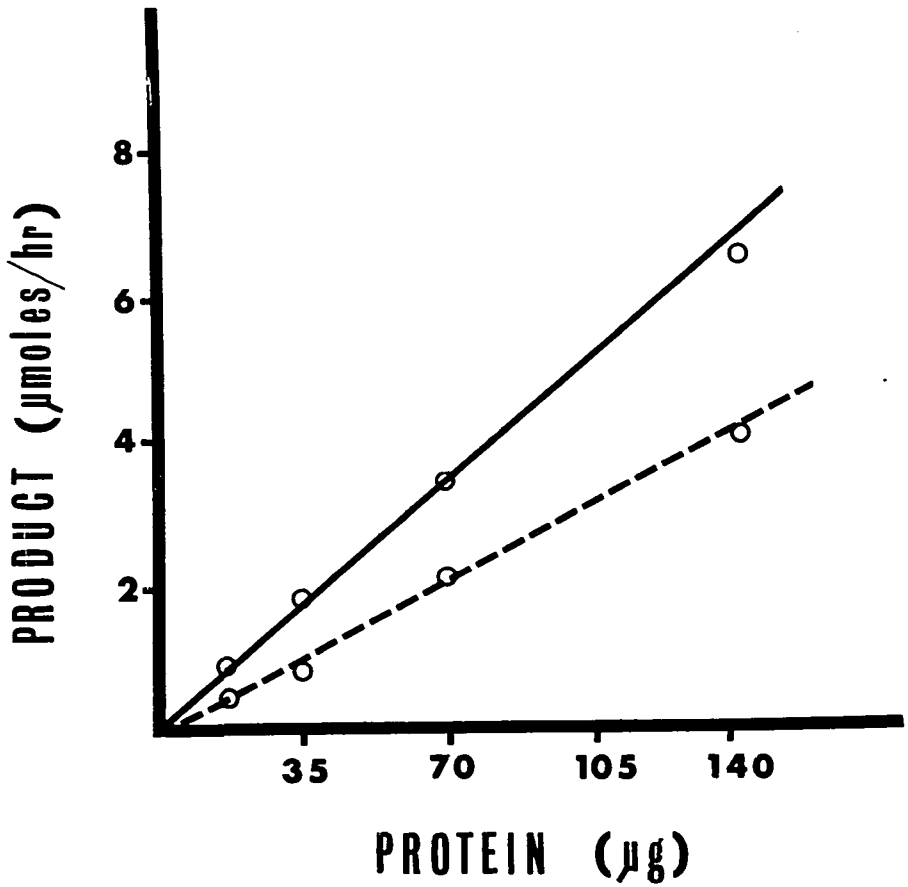

PRODUCT (µmoles/hr)
8
6
4
2
35
70
105
140
PROTEIN (µg)

FIGURE 13

EFFECT OF ENZYME CONCENTRATION ON REACTION RATE OF

M. SODONENSIS NUCLEASE

Diesterase activity O————O

Monoesterase activity O--------O

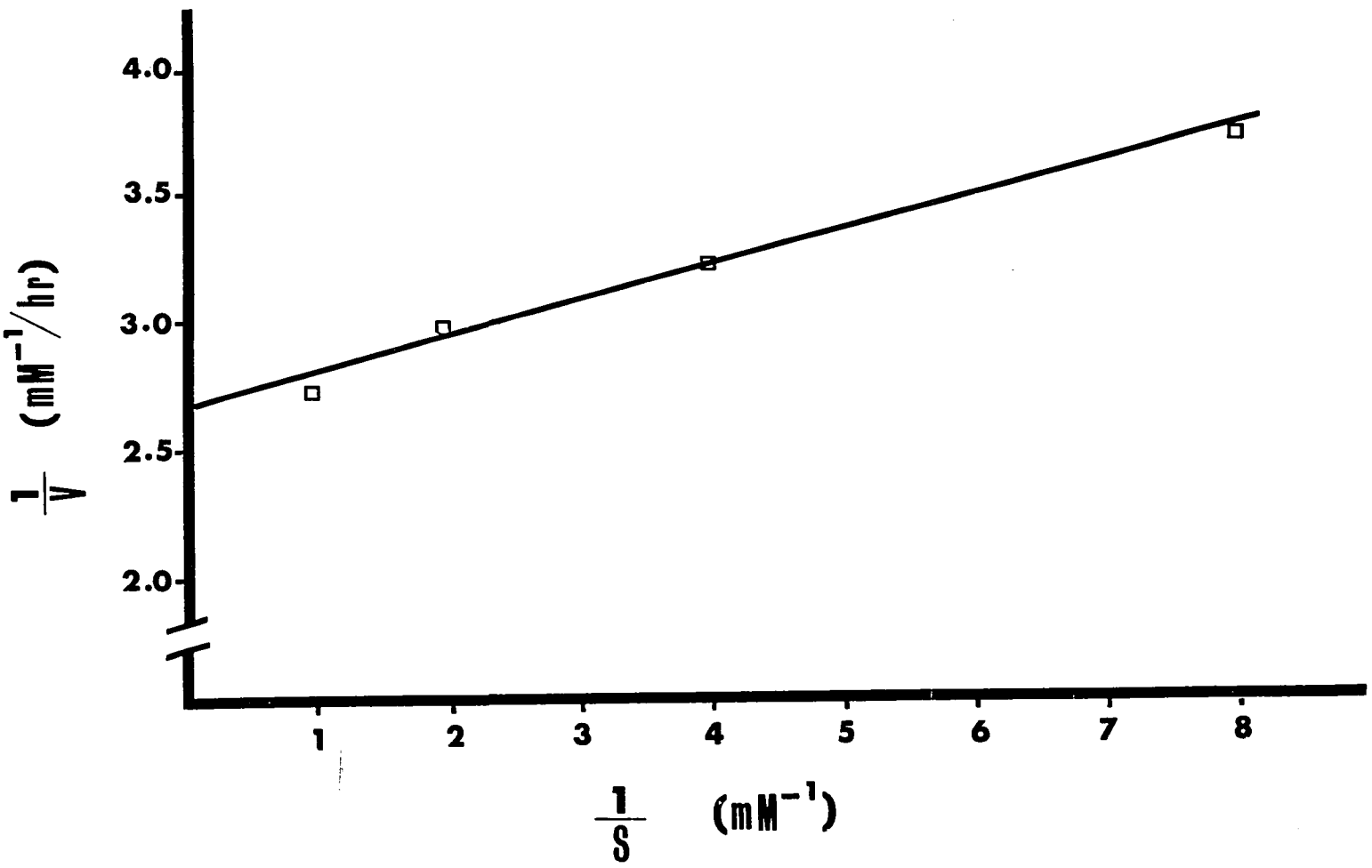

4.0
3.5
3.0
2.5
2.0
1/V (mM⁻¹/hr)
1
2
3
4
5
6
7
8
1/S (mM⁻¹)

FIGURE 14

LINEWEAVER-BURK PLOT OF VELOCITY VS AMP CONCENTRATION

Assays were carried out as described in Methods with varying concentrations of AMP.

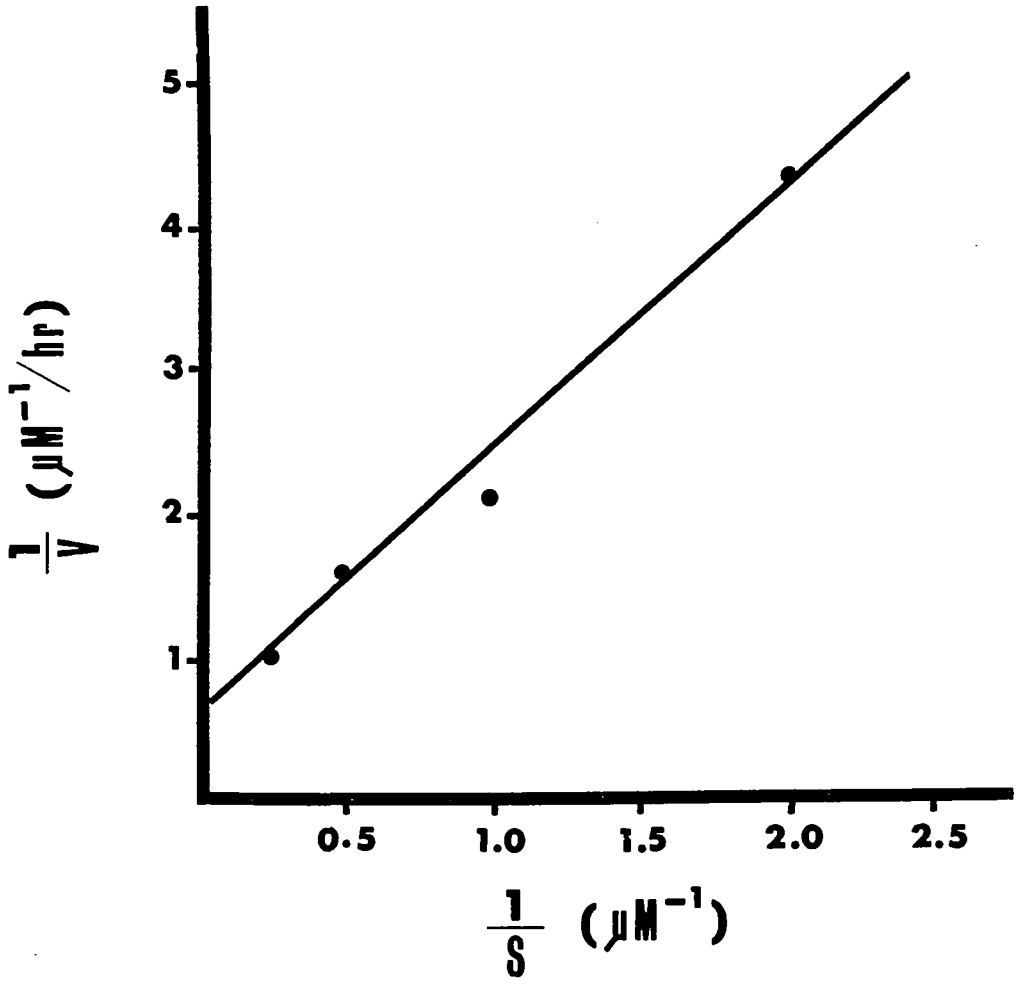

5
4
3
2
1
0.5
1.0
1.5
2.0
2.5
1/V (μM⁻¹/hr)
1/S (μM⁻¹)

FIGURE 15

LINEWEAVER-BURK PLOT OF VELOCITY VS RNA CONCENTRATION

Assays were carried out as described in Methods using varying concentrations of ^{14}C RNA. RNA concentration was based on an assumed molecular weight of 500,000 and the amount of RNA rendered acid-soluble was determined.

FIGURE 15

LINEWEAVER-BURK PLOT OF VELOCITY VS RNA CONCENTRATION

Assays were carried out as described in Methods using varying concentrations of ^{14}C RNA. RNA concentration was based on an assumed molecular weight of 500,000 and the amount of RNA rendered acid-soluble was determined.

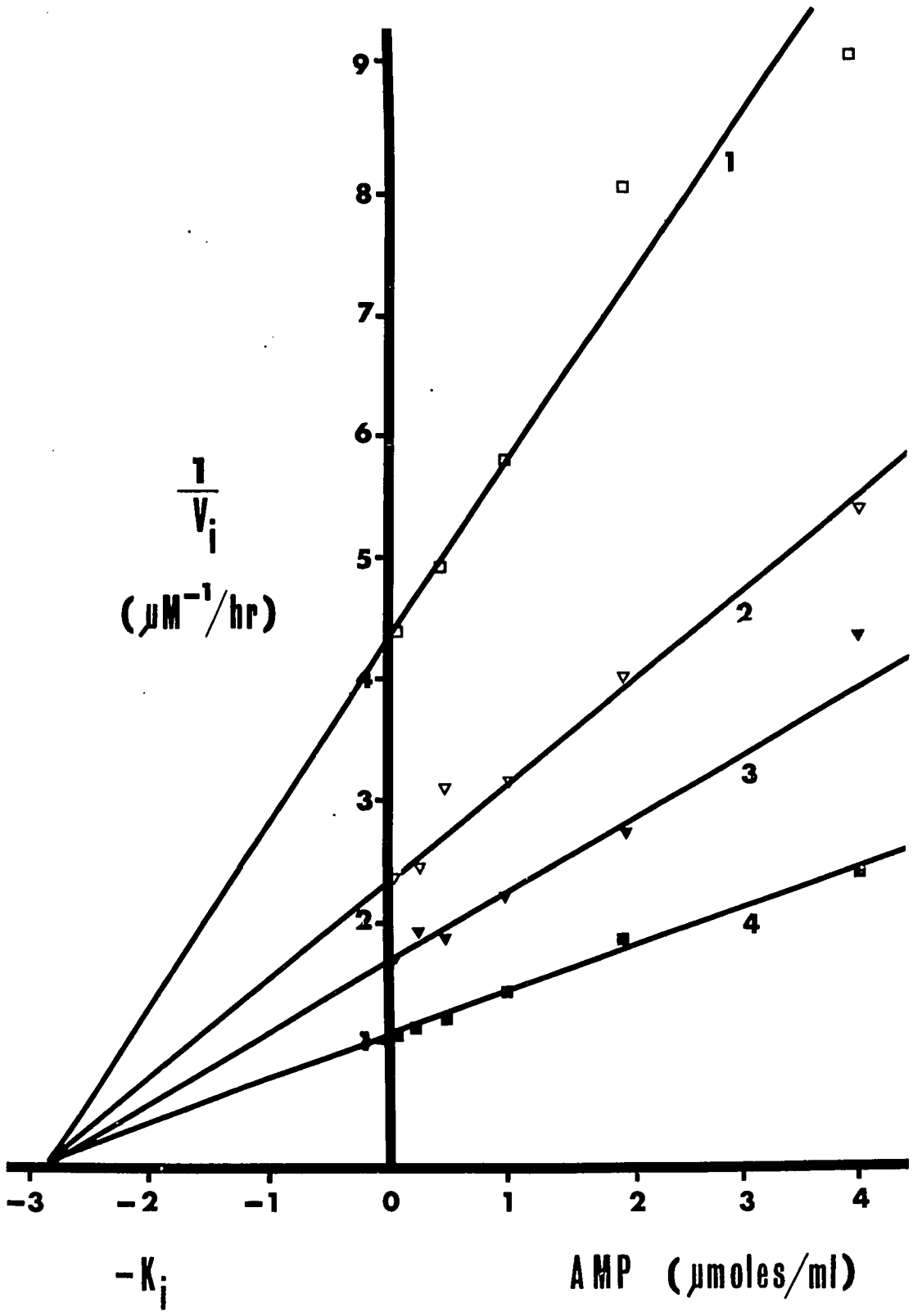

9
8
7
6
5
4
3
2
1
1
V_i
(μM^-1/hr)
1
2
3
4
-3
-2
-1
0
1
2
3
4
-K_i
AMP (μmoles/ml)

FIGURE 16

GRAPHICAL DETERMINATION OF THE INHIBITORY EFFECT OF AMP ON DIESTERASE ACTIVITY OF M. SODONENSIS NUCLEASE

Assays were carried out as described in Methods and the release of UTCA soluble label from varying concentrations of ^{14}C RNA in the presence of varying amounts of AMP was determined. Concentrations of RNA were as follows:

Curve 1 - 0.25 mg/ml

Curve 2 - 0.5 mg/ml

Curve 3 - 1.0 mg/ml

Curve 4 - 2.0 mg/ml

and in a Lineweaver-Burk plot (Fig. 17). A K_i of 2.9×10^{-3}M was obtained from both plots. As Fig. 16 illustrates, the lines meet at a point on the abscissa, this intercept corresponding to $-K_i$. The Lineweaver-Burk plot in Fig. 17 indicates that the V_{max} was altered but the K_m was not affected. In addition a plot of $\frac{V}{V_i}$ versus [i] showed that the slope was independent of the substrate concentration. The plots are typical of non-competitive inhibition in the simplest system where (West and Todd, 1961) an irreversible enzyme-inhibitor complex is formed. The kinetics of the nuclease system, however, are more complex and cannot likely be explained in a simple manner. Possible reasons for this "apparent non-competitive" inhibition are given in the Discussion.

The 1 hr digests containing both RNA and AMP were assayed for P_i as a measure of the total velocity (V_t) and compared with the velocities obtained with each substrate alone. Since the products of both reactions are nucleosides and P_i, the assay of P_i was considered valid for total velocity. Table VII shows the results obtained with digests containing 1 mg/ml and 2 mg/ml of RNA in the presence of 2 μmoles of AMP. As indicated in the table, the total velocity value V_t in the mixture with the 2 substrates falls between the 2 velocities obtained with RNA and AMP separately.

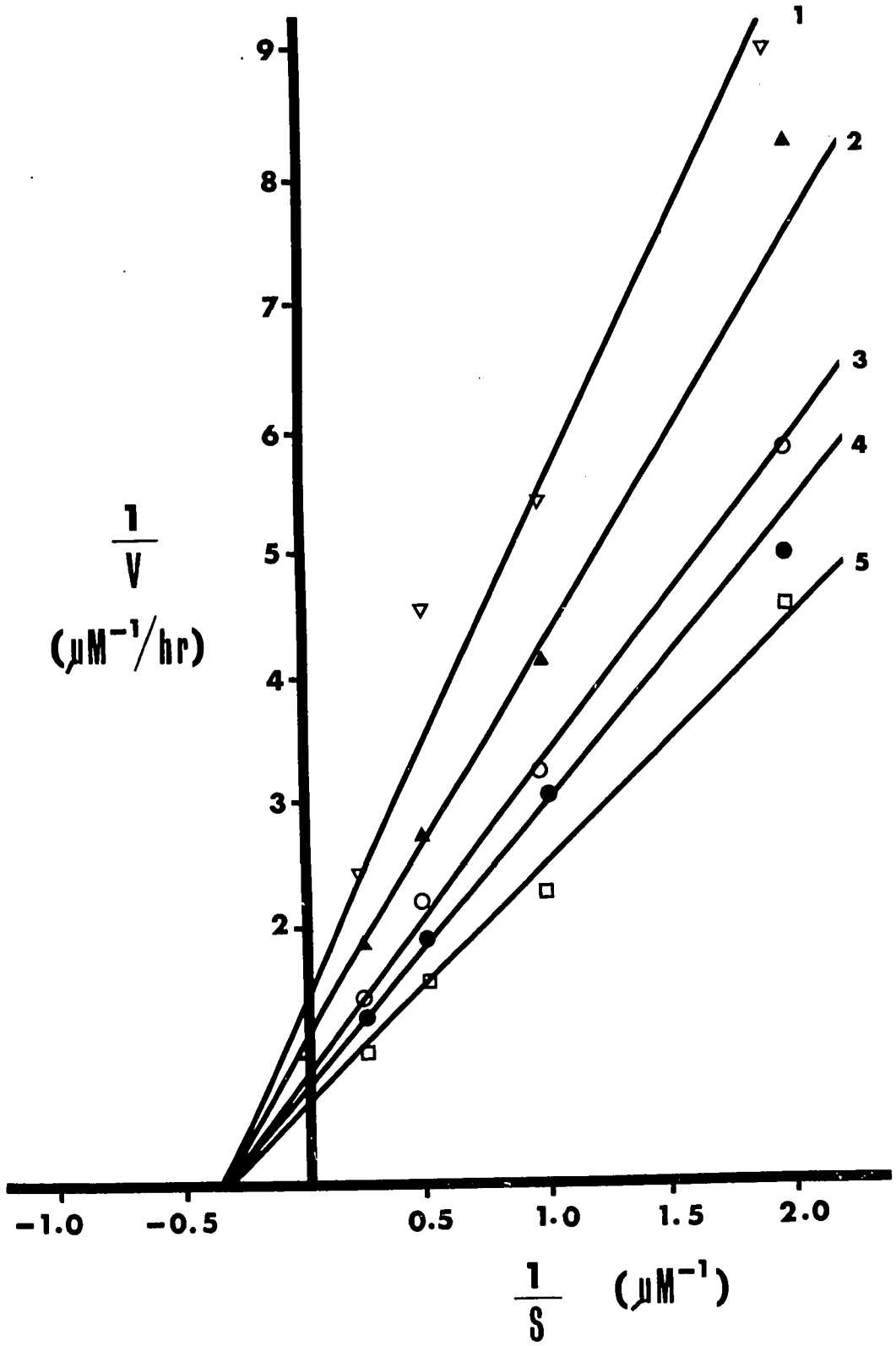

1
2
3
4
5
9
8
7
6
5
4
3
2
1/V
(μM⁻¹/hr)
-1.0
-0.5
0.5
1.0
1.5
2.0
1/S (μM⁻¹)

FIGURE 17

LINEWEAVER-BURK PLOT OF DIESTERASE ACTIVITY OF THE

M. SODONENSIS NUCLEASE IN THE PRESENCE OF AMP

Activity was followed as described in Fig. 16. Concentrations of AMP were as follows:

Curve 1 - 4 µmoles/ml

Curve 2 - 2 µmoles/ml

Curve 3 - 1 µmole/ml

Curve 4 - 0.5 µmole/ml

Curve 5 - Control, no AMP

TABLE VII

COMPARISON OF THE "MIXED SUBSTRATE" VELOCITY

WITH "SINGLE SUBSTRATE" VELOCITIES OF

M. SODONENSIS NUCLEASE

RNA Concentration (mg/ml)	AMP Concentration (μmoles/ml)	Velocity*
2	-	1.455
2	2	1.03
1	-	0.963
1	2	0.87
-	2	0.457

* μmoles of P_i released/hr

5. Role of Cations in Heat Stability

Purified M. sodonensis nuclease was heated at 50° C for 5 mins in the presence and absence of Mg^{++}, Mn^{++}, and Ca^{++}. The ions were present in the concentration required for optimum activity. The activities relative to that of the unheated control are presented in Table VIII. It can be seen from the data that both Mg^{++} and Mn^{++} markedly increased the heat sensitivity. Ca^{++}, however, had a protective effect and this protective action was sufficient to counteract the adverse effect of Mg^{++} and Mn^{++} when all three were present.

6. Enzyme Activation

Several sulfur containing compounds were found to increase enzyme activity when added to the assay mixture at a concentration of 10^{-2}M. These are listed in Table IX. Since mercaptoethanol appeared most effective on both activities it was routinely added to the assay mixtures to yield a final concentration of 10 µmoles/ml.

A marked stimulatory effect was obtained in some enzyme preparations, when either glycine or histidine was added to the assay systems. As shown in Fig. 18, histidine was effective at a much lower level than was glycine. Valine and alanine were also somewhat stimulatory although to a lesser degree than glycine. The effect was probably due to a chelation of toxic ions since these amino acids have been shown to act as chelators. This was supported by the fact that a stimulatory effect

TABLE VIII

EFFECT OF CATIONS ON HEAT STABILITY OF M. SODONENSIS NUCLEASE

Addition	Relative Activity	
	Diesterase	Monoesterase
None	66	88
Mg^{++}	12	39
Mn^{++}	7	30
Ca^{++}	130	102
Ca^{++}, Mg^{++}, Mn^{++}	103	120

Enzyme was heated for 5 mins at 50° C in the presence of the various ions. Activity is expressed relative to an unheated control.

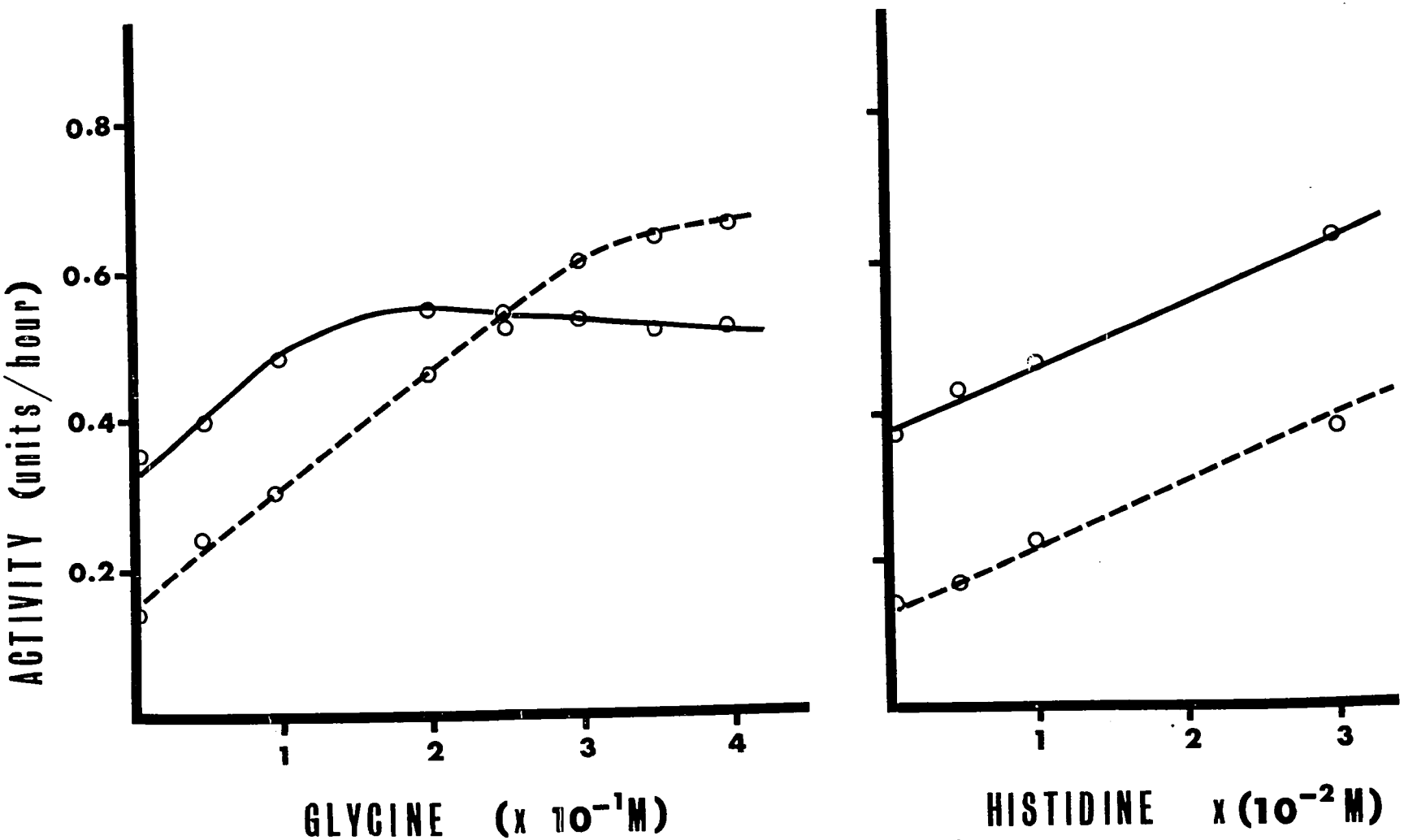
TABLE IX

ACTIVATION OF M. SODONENSIS NUCLEASE BY

BY SULFUR-CONTAINING COMPOUNDS

Additions	Concentrations* (M)	Relative Activity	
		Monoesterase	Diesterase
None	-	100	100
Mercaptoethanol	.01	500	200
Cysteine	.01	230	184
Reduced glutathione	.01	270	146

* Final concentration in assay mixture


ACTIVITY (units/hour)
0.8
0.6
0.4
0.2
1
2
3
4
GLYCINE (x 10⁻¹M)
1
2
3
HISTIDINE x (10⁻²M)

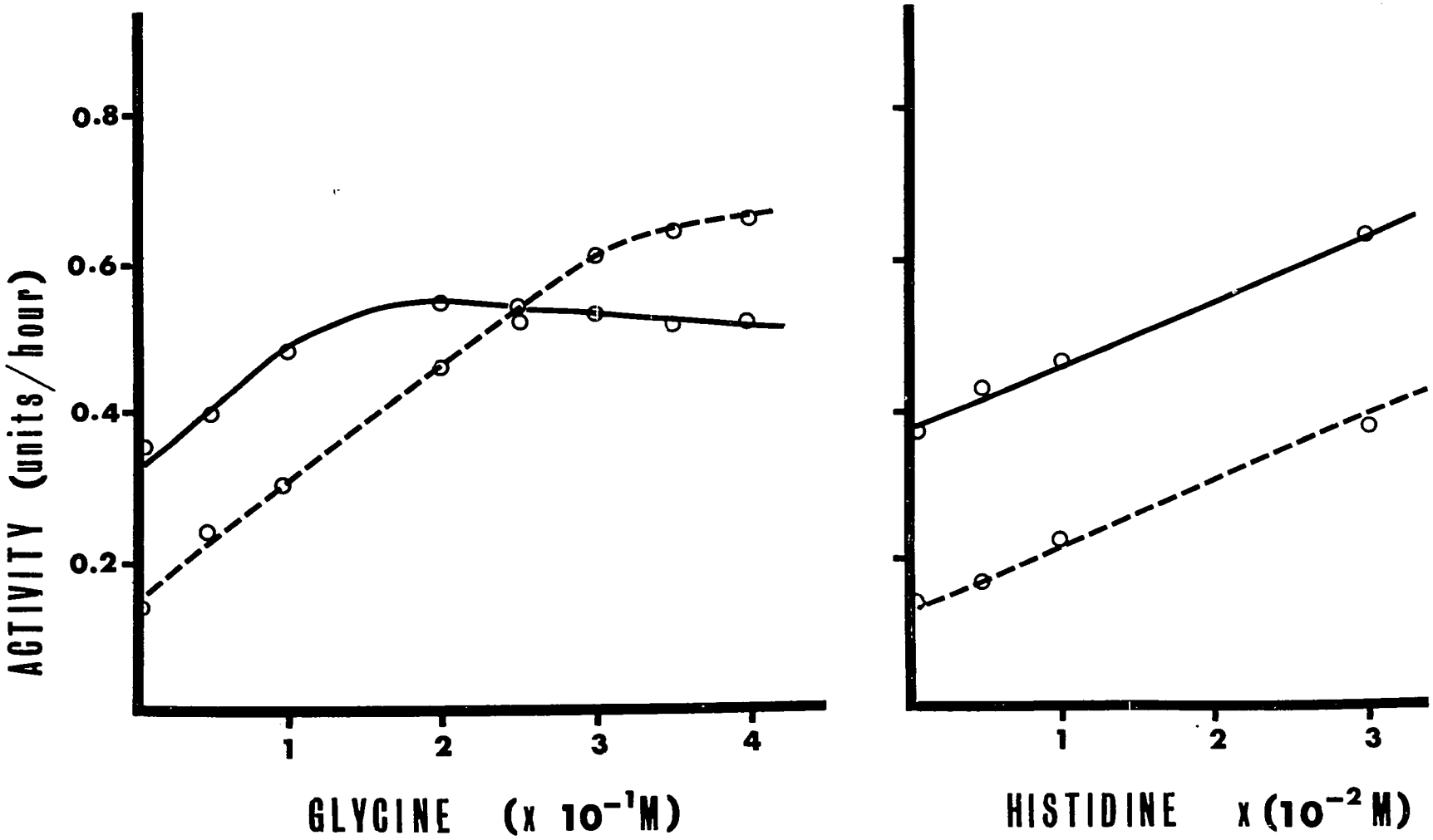

ACTIVITY (units/hour)
0.8
0.6
0.4
0.2
1
2
3
4
GLYCINE (x 10⁻¹M)
1
2
3
HISTIDINE x (10⁻²M)

FIGURE 18

EFFECT OF GLYCINE AND HISTIDINE ON THE NUCLEASE ACTIVITY OF *M. SODONENSIS*

Assays for monoesterase and diesterase activities were carried out as described in Methods. The amino acids were added to the assay mixtures to yield the final concentrations indicated in the figure.

Diesterase activity O———O

Monoesterase activity O--------O

was also obtained with low concentrations of EDTA as shown in Table X. Inhibition occurs at the higher concentrations due to chelation of essential ions such as Mg^{++}, Mn^{++} and Ca^{++}, etc. At lower concentrations heavy metals such as Fe^{+++}, Hg^{++}, Cu^{++}, etc, would be preferentially bound since they have a greater avidity for EDTA.

7. Enzyme Inhibition

Since physical methods had failed to separate monoesterase and diesterase activities it was hoped that perhaps inhibitor studies might be of value. If two proteins were involved it might be possible to obtain a selective inhibition of one activity and to separate the enzyme-inhibitor complex thus formed. Two types of inhibitors were employed: a) those which act on the active site of the enzyme (eg. p-chloromercuribenzoate) and b) nucleotide analogues which would compete with the substrate.

Table XI shows the results obtained when PCMB was present in the assay mixture at the final concentrations indicated. Inhibition of both activities occurred although monoesterase was affected slightly more than was the diesterase. This correlated with the activation obtained by compounds such as mercaptoethanol which was found to have a greater effect on the monoesterase activity.

Several nucleotide analogues were employed: inosine-5'-chloromethylphosphonate, inosine-5'-fluorophosphonate, adenosine-5'-monoacetate, adenosine-5'-phosphomorpholidate, and 6-azauridine-5'-monophosphate as well as compounds such as R-5-P and R-1-P. As mentioned previously these last two compounds would not serve as substrates, nor did they exhibit

TABLE X

EFFECT OF EDTA ON ACTIVITY OF M. SODONENSIS NUCLEASE

EDTA Concentration* (M)	Relative Activity	
	Monoesterase	Diesterase
0	100	100
10^{-2}	50.8	50
10^{-3}	104	70
10^{-4}	132	80
10^{-5}	140	102
10^{-6}	150	130

* Final concentration in assay mixture

TABLE XI

INHIBITION OF ACTIVITY OF *M. SODONENSIS* NUCLEASE BY PCMB

PCMB Concentration* (M)	Relative Activity	
	Monoesterase	Diesterase
0	100	100
1.25×10^{-4}	87	95.7
2.5×10^{-4}	73.8	82.4
5.0×10^{-4}	58	74.3
1.0×10^{-3}	46	61.5
2.0×10^{-3}	31	48

* **Final concentration in assay mixture.** Experiments were carried out in the absence of mercaptoethanol.

any inhibitory effect on either activity when added to the assay mixtures. The lithium salts of inosine-5'-chloromethylphosphonate and inosine-5'-fluorophosphonate were added to the assay systems to yield the final concentrations indicated in Table XII. IMP (1 µmole/ml) and RNA (1 mg/ml) were the substrates employed. As shown in Table XII, both analogues were inhibitory although there seemed to be no difference in the two concentrations. Table XIII shows the results obtained when the enzyme was incubated overnight with inosine-5'-fluorophosphonate in the absence of substrate. Enzyme plus buffer served as the control and after incubation, the remaining assay ingredients were added to yield the same concentrations of substrate as in the previous experiment. When the results from the two experiments are compared it can be seen that inhibition of both activities occurred but that the inhibition was less in the presence of the substrate. This was particularly noticeable in the case of the diesterase, an expected result in view of the fact that the enzyme has a greater affinity for RNA.

Two 5' analogues of adenosine were tested, adenosine-5'-phosphomorpholidate and adenosine-5'-monoacetate, but neither had any effect on either monoesterase or diesterase activity.

Table XIV shows the effect of 6-azauridine-5'-monophosphate on both activities in the presence of substrate. There appeared to be little effect if any on the diesterase activity and the effect on monoesterase was less than that exerted by the inosine analogues. Again

TABLE XII

EFFECT OF INOSINE ANALOGUES ON ACTIVITY

OF *M. SODONENSIS* NUCLEASE

Addition	Concentration* (μmoles/ml)	Relative Activity	
		Monoesterase	Diesterase
None	-	100	100
Inosine fluoro-phosphonate	2	56	69
	4	56	66
Inosine chloro-methyl phos-phonate	2	54	66
	4	54	53.5

* Final concentration in assay mixture

TABLE XIII

EFFECT OF PREINCUBATION WITH INOSINE FLUOROPHOSPHONATE ON ACTIVITY OF M. SODONENSIS NUCLEASE

Analogue Concentration* (μmoles/ml)	Relative Activity	
	Monoesterase	Diesterase
0	100	100
2	58	33
4	19	10

* Concentration in the final assay mixture

TABLE XIV

EFFECT OF 6 AZAURIDINE-5'-MONOPHOSPHATE ON

ACTIVITY OF M. SODONENSIS NUCLEASE

Concentration* (µmoles/ml)	Relative Activity	
	Monoesterase	Diesterase
0	100	100
0.66	90	98
1.33	74.2	95.9
2.7	62.2	94.29

* Final concentration in assay mixture

this result is not unexpected since the affinity of the enzyme for pyrimidine nucleotides is less than that for purine nucleotides. When UMP was added to the diesterase system at a concentration of 2.7 µmoles/ml, 85% of the original activity was retained whereas AMP (2 µmoles/ml) reduced the activity to 55%.

IV. Control of Enzyme Production

The major routes of incorporation of NH_3 by microorganisms are: 1) the amination of α-keto acids; 2) the amidation of α-amino acids to form amides; 3) the synthesis of carbamyl phosphate which is further involved in pyrimidine synthesis as well as in the citrulline and arginine pathway. NH_3 is also involved in the amination of XMP → GMP and UTP → CTP. The stimulatory effect of NH_3 on the growth of *M. sodonensis* has been described (Campbell *et al* 1961a) and it was proposed that NH_3 was primarily involved in carbamyl phosphate synthesis. NH_3 is also required for nuclease production and, in its absence, no enzyme is formed. The stimulatory effect of NH_3 on nuclease production is probably not due to NH_3 *per se* but rather to some intracellular intermediate whose synthesis is NH_3-dependent. It was therefore of interest to determine what NH_3-dependent systems were present in the cell and what interconversions were taking place in an attempt to explain the NH_3 effect.

1. Intracellular Enzymes

Cell free extracts were prepared and assayed as described in Methods. The following enzymes were demonstrated:

(a) Carbamyl phosphate synthetase: Earlier attempts to demonstrate carbamyl phosphate synthesis in M. sodonensis had been unsuccessful although some evidence had been obtained by Bunch (1966) who measured the reverse reaction, ie. the formation of ATP from carbamyl phosphate and ADP. However by making use of the inhibitory effect of CTP (Gerhart and Pardee, 1962) on aspartic transcarbamylase activity it was possible to accumulate carbamyl phosphate and measure it directly by the release of alkali-labile phosphate. The results of one such experiment are shown in Table XV. Tubes 2, 4 and 6 were non-enzymic controls and the P_i present was due to chemical degradation of ATP and CTP. The measure of carbamyl phosphate is the difference between tubes 1 and 3 in the presence and absence of carbamate. When CTP was not added to the system (tube 5) carbamyl phosphate was not accumulated but was utilized in the pyrimidine pathway.

(b) Aspartic transcarbamylase: It was apparent from the above experiments that aspartic transcarbamylase was present since it could be inhibited by CTP causing accumulation of carbamyl phosphate but it was also demonstrated directly by measuring

TABLE XV

CARBAMYL PHOSPHATE SYNTHETASE ACTIVITY OF M. SODONENSIS

Reaction Mixture	Absorbance	Alkali-labile P_i (μmoles)	μmoles P_i/mg proteins
1. complete	1.595	.216	4.1
2. complete, no enzyme	.290	.039	-
3. minus carbamate	1.045	.14	2.7
4. minus carbamate, no enzyme	.325	.04	-
5. minus CTP	1.000	.134	2.56
6. minus CTP no enzyme	.085	.01	-

the formation of carbamyl aspartate (Gerhart and Pardee, 1962). The levels of activity were very low (.09 µmoles of carbamyl aspartate formed/mg of protein), which is not surprising in such a crude system. As with carbamyl phosphate, an active pyrimidine pathway would not allow accumulation of an intermediate.

(c) Ornithine transcarbamylase: The formation of citrulline from carbamyl phosphate was readily demonstrated. Fig. 19 shows the results of an experiment in which increasing amounts of cell extract were added to the assay system. A linear relationship was obtained with the amount of citrulline formed proportional to the protein concentration.

(d) UTP Aminase: The amination of uridine compounds was demonstrated in extracts of M. sodonensis by the method of Lieberman (1956). A crude extract of E. coli B was employed as a positive control. Table XVI shows the activity of the extracts on uridine substrates with NH_4^+ and glutamine as amino donors. Activity was obtained in both extracts with either NH_4^+ or glutamine as an amino donor and with any of the U compounds tested. In such a crude system so many interconversions take place that it is impossible to determine the level of U → C conversion

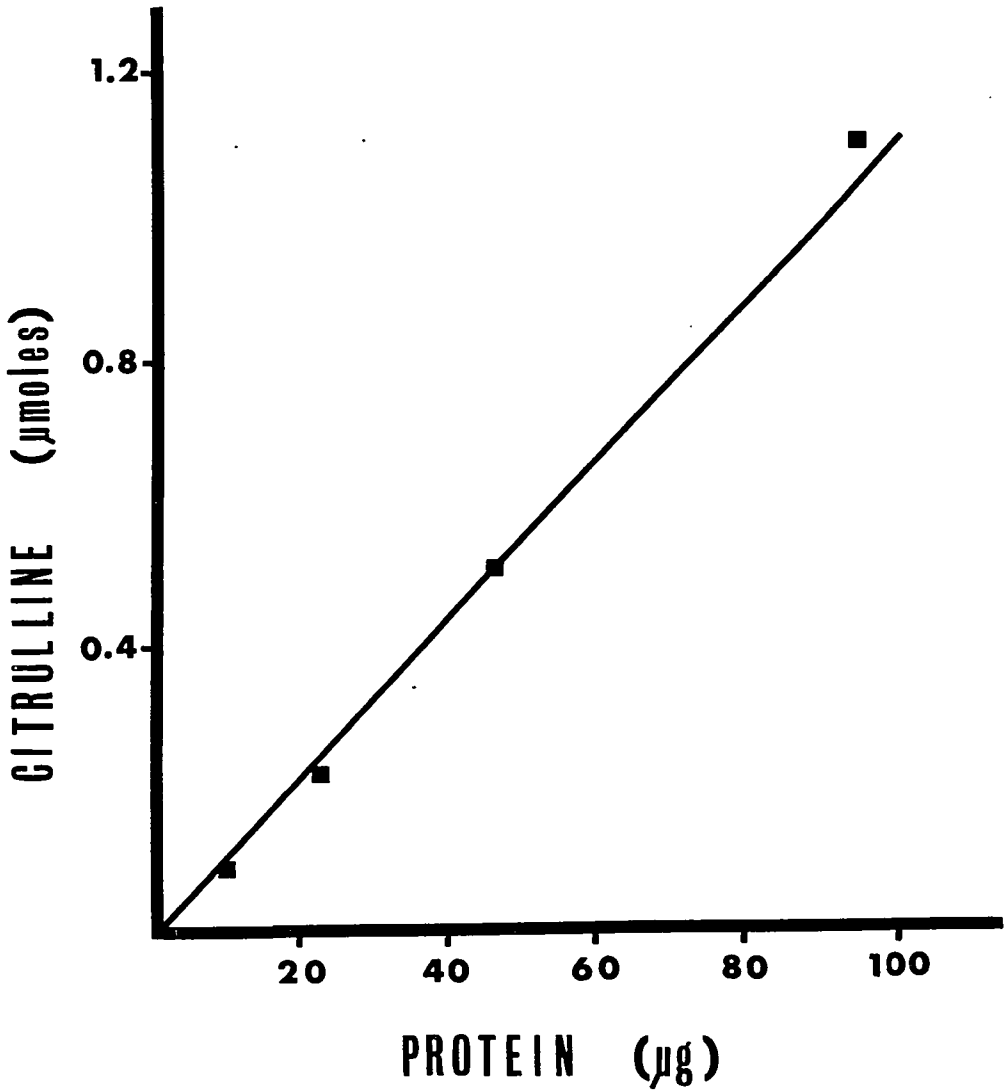

1.2
0.8
0.4
CITRULLINE (µmoles)
20
40
60
80
100
PROTEIN (µg)

FIGURE 19

RELATIONSHIP OF PROTEIN CONCENTRATION TO ORNITHINE TRANSCARBAMYLASE ACTIVITY IN M. SODONENSIS

Varying amounts of cell free extract were added to the assay mixture and citrulline formation was determined as described in Methods.

TABLE XVI

AMINATION OF URIDINE COMPOUNDS BY CELL FREE EXTRACTS

OF M. SODONENSIS

Addition	Experiment #1		Experiment #2	
	E. coli	M. sod.	E. coli	M. sod.
UTP + NH_4^+	3.97	7.8	5.3	4.56
UDP + NH_4^+	-	7.8	-	-
UR + NH_4^+	4.05	4.5	-	-
UTP + GLUTAMINE	4.5	13.3	6.45	4.61
UDP + GLUTAMINE	-	11.1	-	-
UR + GLUTAMINE	3.6	4.8	-	-

Activity is expressed in units/mg of protein. 1 unit is an increase in OD of 0.1 in 30 mins.

without extensive purification of the system. It is likely, however, that amination occurs at the UTP level, analagous to the E. coli system, since the activity on UTP and UDP was much higher than on UR. The activity on glutamine may be due to the presence of a glutaminase. As the results in experiment #2 indicate, partial purification (ie. dialysis) destroys much of the activity of glutamine as an NH_3 donor in the case of M. sodonensis. Aliquots of the UTP digest at time zero and at 30 mins were removed and chromatographed using the ethanol solvent and $(NH_4)_2SO_4$ saturated paper described in Methods. A disappearance of UTP concurrent with the appearance of CDP and C confirmed the amination reaction.

(e) XMP aminase: The formation of GMP from XMP was demonstrated and confirmed earlier results obtained by Campbell (personal communication). The increase in absorption at 290 mμ was determined after 30 mins incubation at 25° C. The level of activity was very low and an increase in absorption of only .052 and .047 was obtained in two separate experiments.

(f) Cytidine deaminase: Conversion of cytidine to uridine was measured by following the decrease in absorption at 290 mμ. Since in the crude system interconversions are continually occurring uridine controls were employed. As indicated by

the results in Table XVII, deamination occurred only at the nucleoside level. Although there was a slight decrease in absorption with CMP it was not significant and could be accounted for by the presence of contaminating CR.

(g) Glutaminase: Both E. coli and M. sodonensis extracts were assayed for glutaminase activity. Conway diffusion plate assays were employed and the activity was determined by measuring the release of NH_3. As shown in Table XVIII, glutaminase was present in both cell extracts and the activity was reduced after dialysis. A total of 9 µmoles of glutamine was present in each reaction mixture and as can be seen from the table, 9 µmoles of NH_3 were released by the undialysed M. sodonensis extract at 2 hrs. The results explain the activity obtained with glutamine in the UTP amination experiments and suggest that this latter reaction is NH_3 dependent. The 2 hr digest was chromatographed using the phenol:H_2O:ammonia solvent system described in Methods. Duplicate sheets were sprayed with ninhydrin and dinitrophenylhydrazine reagents. Only glutamic acid was present indicating that glutamine had been completely deaminated. Since glutamic acid was not deaminated to form α-ketoglutaric acid, the release of NH_3 was considered to be a valid assay for glutaminase.

TABLE XVII

CYTIDINE DEAMINASE ACTIVITY OF CELL FREE EXTRACTS

OF M. SODONENSIS

Substrate	Optical Density at 290 mμ		ΔOD
	0'	90'	
CTP	1.170	1.415	+ .245
CMP	1.240	1.415	+ .175
CR	1.375	1.105	- .270
UTP	.293	.540	+ .247
UR	.282	.510	+ .228

Optical densities were read at time zero and after 90 mins incubation at 37° C.

TABLE XVIII

GLUTAMINASE ACTIVITY IN CELL FREE EXTRACTS

OF *M. SODONENSIS*

Cell Extract	1 hour		2 hours	
	µmoles NH_3 evolved	Activity	µmoles NH_3 evolved	Activity
E. coli				
undialysed	3.4	1.4	6.2	2.6
dialysed	1.8	1.07	4.0	2.4
M. sodonensis				
undialysed	6.8	6	9.0	8
dialysed	3.8	3.9	6.8	7

Activity expressed as µmoles NH_3/mg protein.

2. Whole Cell Studies

Once the intracellular NH_3 dependent systems had been demonstrated, one could then select several potential compounds to test for their effect on growth and nuclease production. Several amino acids such as aspartic acid, ornithine, citrulline and arginine were employed as well as glutamine and asparagine. Each compound was added to the synthetic medium to yield a final concentration of 10 µmoles/ml (equivalent to the NH_4^+ it was replacing). As shown in Fig. 20 citrulline, aspartic acid and ornithine had some stimulatory effect on growth, glutamine completely replaced NH_4^+, and asparagine, although slower in its effect, eventually reached the level of NH_4^+. Glutamine and asparagine, however, were the only compounds having any effect on enzyme production as shown in Table XIX. The effect of glutamine as an amino donor was explained by the presence of glutaminase in the crude extracts. The long lag with asparagine, both in growth and in enzyme production, indicated that asparaginase, in contrast to glutaminase, was inducible and this was confirmed by failure to detect asparaginase in cells grown in the absence of asparagine. Once asparaginase was induced, then asparagine, like glutamine, could function as an NH_3 source.

All the available ribo- and deoxyribo- nucleosides were added in varying concentrations to the synthetic medium. Of all the compounds tested, only cytidine and deoxycytidine stimulated growth and enzyme production over the basal level. Adenosine, deoxyadenosine and inosine were inhibitory at all concentrations tested (the lowest tested was 1.0

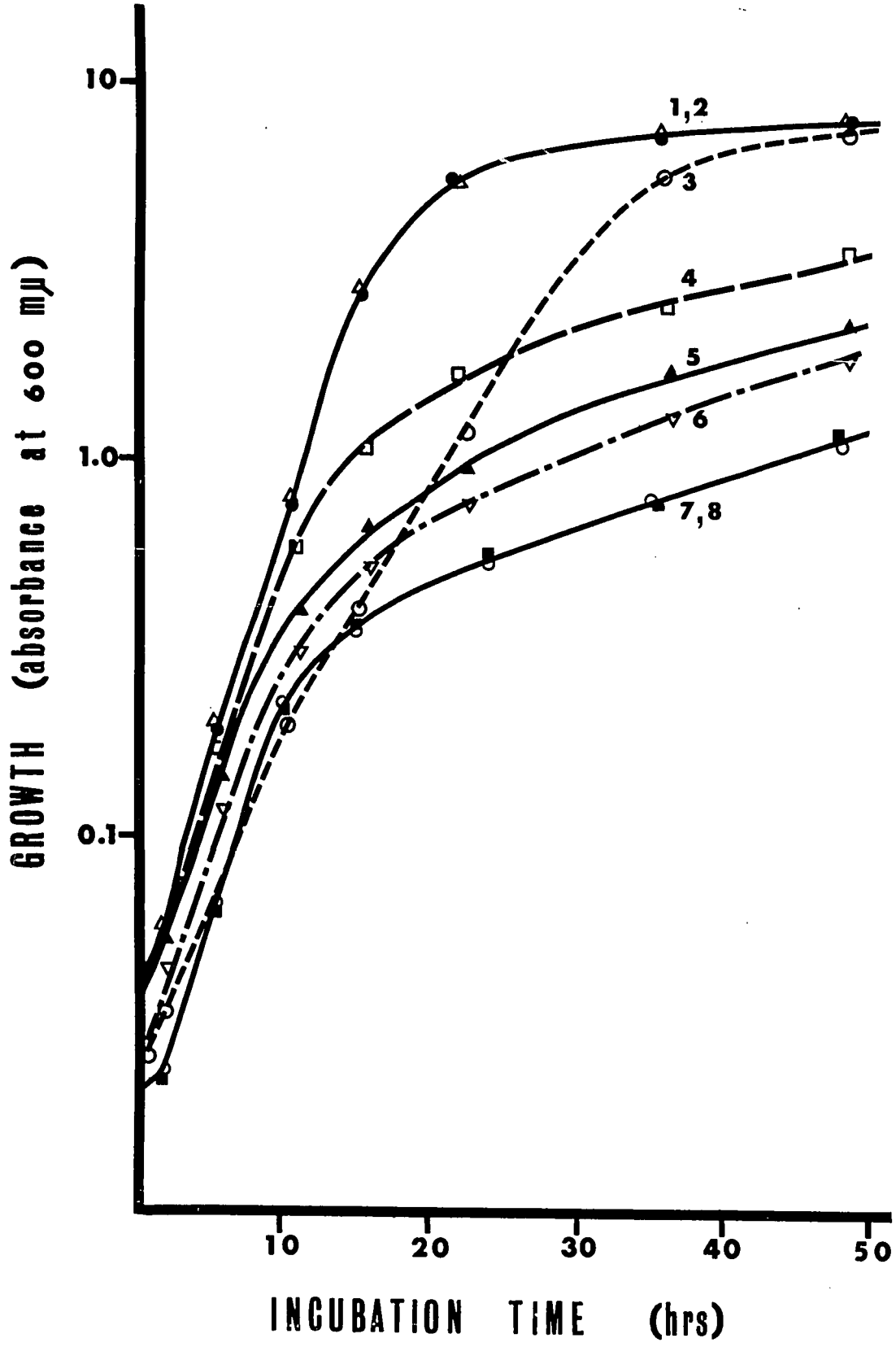

10
1.0
0.1
GROWTH (absorbance at 600 mμ)
1,2
3
4
5
6
7,8
10
20
30
40
50
INCUBATION TIME (hrs)

FIGURE 20

EFFECT OF AMINO ACIDS AND AMIDES ON THE GROWTH OF

M. SODONENSIS

The individual compounds were added to the synthetic medium to yield a final concentration of 10 µmoles/ml.

Curve 1 - Synthetic medium + NH_4^+

Curve 2 - Synthetic medium + glutamine

Curve 3 - Synthetic medium + asparagine

Curve 4 - Synthetic medium + citrulline

Curve 5 - Synthetic medium + aspartic acid

Curve 6 - Synthetic medium + ornithine

Curve 7 - Synthetic medium

Curve 8 - Synthetic medium + arginine

TABLE XIX

EFFECT OF AMINO ACIDS AND AMIDES ON NUCLEASE PRODUCTION BY M. SODONENSIS

Medium	Diesterase Activity				
	15 hr	24 hr	30 hr	36 hr	48 hr
Synthetic	-	-	-	-	-
Synthetic + citrulline	-	-	-	-	-
Synthetic + ornithine	-	-	-	-	-
Synthetic + arginine	-	-	-	-	-
Synthetic + Aspartic acid	-	-	-	-	-
Synthetic + NH_4^+	.425	4.104	4.237	4.148	1.933
Synthetic + glutamine	.2	2.1612	4.2046	4.68	1.6
Synthetic + asparagine	-	-	-	.944	1.48

Activity is expressed in units/mg dry cell wt.

µmoles/ml), and the inhibition could not be reversed by the addition of NH_4^+.

The effect of cytidine on growth and nuclease production was further investigated and compared with that of NH_4^+. Media were prepared and inoculated with a standard inoculum and aliquots were removed at intervals for estimation of cell growth and nuclease production. Fig. 21 shows the growth curves obtained in media containing cytidine or NH_4^+ at concentrations of 0.2, 1.0 and 10 µmoles/ml. Levels of activity were very low in some of the samples and could not be detected by the quantitative procedure. The more sensitive plate assays were therefore employed in addition to the tube assay for diesterase activity. The results of the qualitative procedure are given in Table XX. Table XXI and XXII show the results of the quantitative assays for diesterase and monoesterase activity in culture supernatants at various stages of growth. Both activities were affected in the same manner.

The culture supernatants were tested to determine the fate of cytidine. Aliquots were diluted in 0.2N HCl and the absorption at 290 mµ and 267 mµ was measured. The latter wavelength is the isosbestic point of uridine and cytidine and any decrease in absorption would indicate a loss of base. Fig. 22 shows the results obtained with the supernatant from the 1 µmole/ml concentration. It is apparent that in addition to the deamination of cytidine there was also an uptake of nucleoside by the cell. The same situation occurred in the 0.2 and

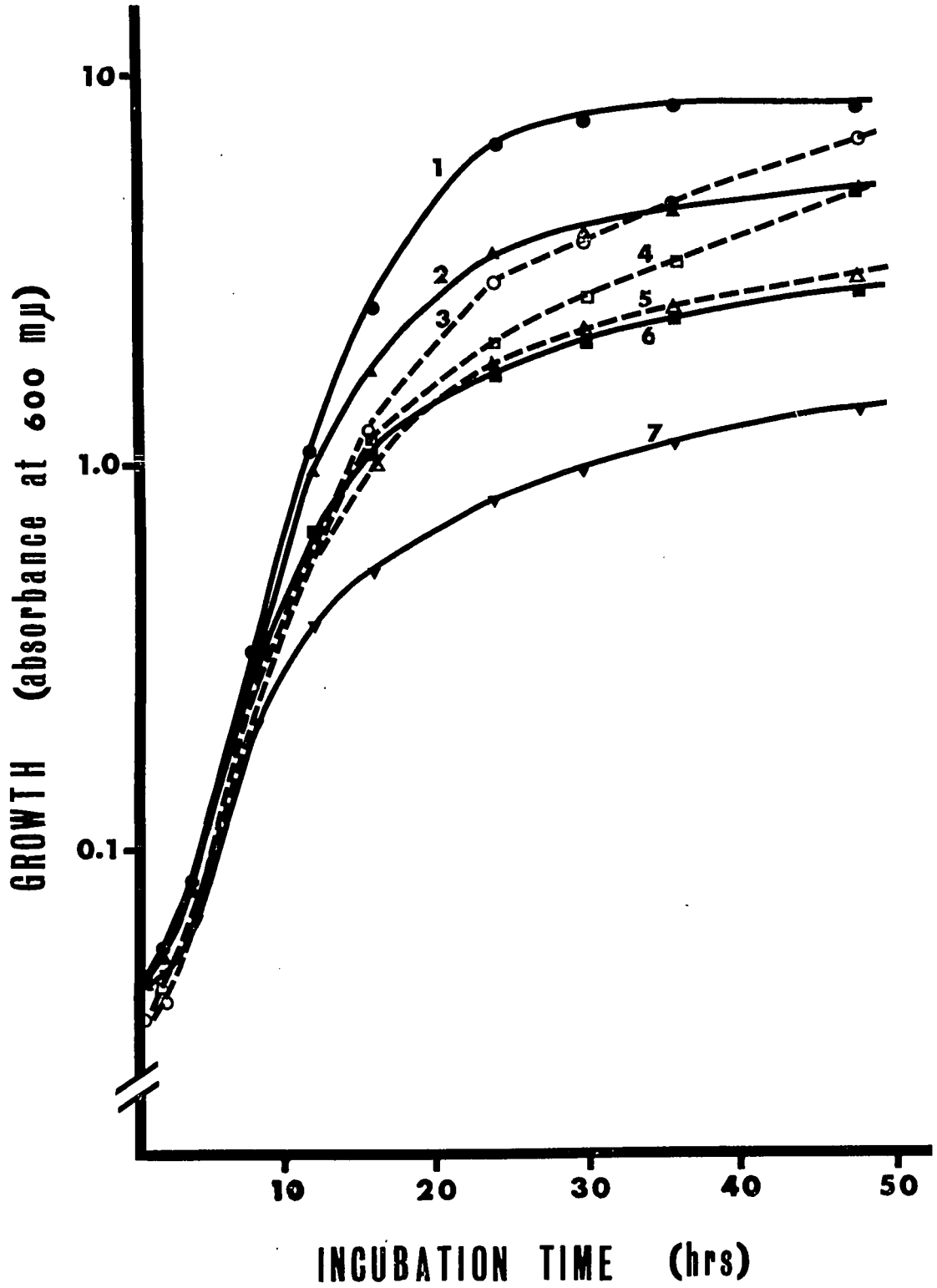

10
1.0
0.1
GROWTH (absorbance at 600 mμ)
1
2
3
4
5
6
7
10
20
30
40
50
INCUBATION TIME (hrs)

FIGURE 21

GROWTH RESPONSE OF M. SODONENSIS IN THE PRESENCE OF VARYING AMOUNTS OF CYTIDINE AND NH_4^+

Cytidine and NH_4Cl were added to the synthetic medium to yield the final concentrations designated in the figure.

Curve 1 - Synthetic medium + NH_4^+ (10 µmoles/ml)

Curve 2 - Synthetic medium + NH_4^+ (1 µmole/ml)

Curve 3 - Synthetic medium + CR (10 µmoles/ml)

Curve 4 - Synthetic medium + CR (1 µmole/ml)

Curve 5 - Synthetic medium + CR (0.2 µmoles/ml)

Curve 6 - Synthetic medium + NH_4^+ (0.2 µmoles/ml)

Curve 7 - Synthetic medium

TABLE XX

QUALITATIVE ASSAY OF DIESTERASE ACTIVITY IN M. SODONENSIS CULTURE SUPERNATANTS

Medium	Age of Culture (hrs)					
	12	16	24	30	36	48
Synthetic	-	-	-	-	-	-
Synthetic + NH_4^+						
10 µmoles/ml	±	++	+++	+++	+++	+++
1.0 µmoles/ml	-	-	±	±	+	+
0.2 µmoles/ml	-	-	-	-	-	-
Synthetic + Cytidine						
10 µmoles/ml	-	-	-	±	+	+++
1.0 µmoles/ml	-	-	-	-	-	+
0.2 µmoles/ml	-	-	-	-	-	-

TABLE XXI

EFFECT OF CYTIDINE AND NH_4^+ ON DIESTERASE ACTIVITY OF *M. SODONENSIS* CULTURE SUPERNATANTS

Medium	Activity					
	12 hr	16 hr	24 hr	30 hr	36 hr	48 hr
Synthetic	-	-	-	-	-	-
Synthetic + NH_4^+						
10 µmoles/ml	-	.171	.88	.835	.76	.72
1.0 µmoles/ml	-	-	-	-	-	.3
0.2 µmoles/ml	-	-	-	-	-	-
Synthetic + cytidine						
10 µmoles/ml	-	-	-	-	.37	.445
1.0 µmoles/ml	-	-	-	-	-	.207
0.2 µmoles/ml	-	-	-	-	-	-

Activity is expressed in units/mg dry cell wt/

TABLE XXII

EFFECT OF CYTIDINE AND NH_4^+ ON MONOESTERASE ACTIVITY OF

M. SODONENSIS CULTURE SUPERNATANTS

Medium	Activity					
	12 hr	16 hr	24 hr	30 hr	36 hr	48 hr
Synthetic	-	-	-	-	-	-
Synthetic + NH_4^+						
10 µmoles/ml	-	.58	1.565	1.234	.791	.628
1.0 µmoles/ml	-	-	-	-	.314	.543
0.2 µmoles/ml	-	-	-	-	-	-
Synthetic + cytidine						
10 µmoles/ml	-	-	-	.311	.752	1.55
1.0 µmoles/ml	-	-	-	-	-	.614
0.2 µmoles/ml	-	-	-	-	-	-

Activity is expressed in units/mg dry cell wt.

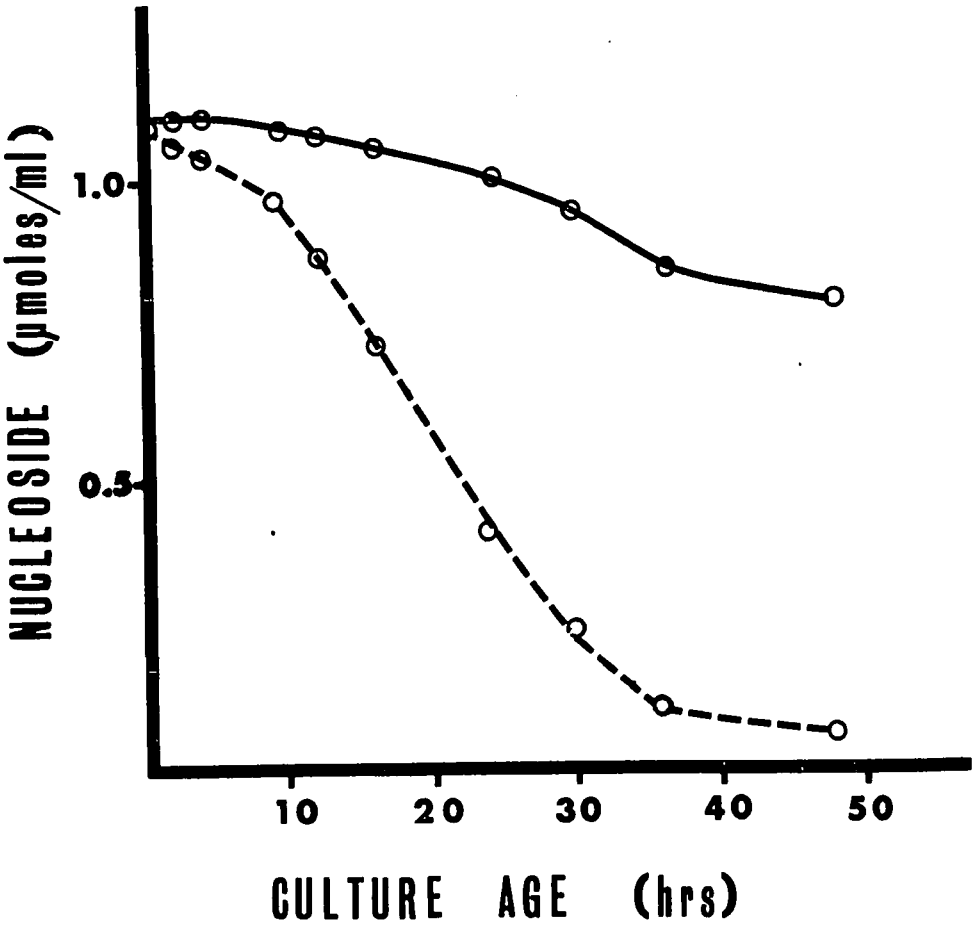

NUCLEOSIDE (μmoles/ml)
1.0
0.5
10
20
30
40
50
CULTURE AGE (hrs)

FIGURE 22

INCORPORATION OF EXOGENOUS CYTIDINE BY M. SODONENSIS

M. sodonensis was grown in synthetic medium containing 1 μmole/ml of cytidine. Aliquots were removed and centrifuged and the supernatant diluted in 0.2N HCl. Loss of cytidine was determined by measuring absorption at 290 mμ. Loss of nucleoside was followed by loss of absorption at 267 mμ.

Nucleoside O———O

Cytidine O--------O

the 10 μmole/ml concentrations. Fig. 23 shows the absorption spectra of the acidified supernatant of one of these concentrations(1 μmole/ml) at various time intervals. There was a shift in the absorption maximum from 280 mμ to 260 mμ with time confirming the conversion of cytidine to uridine.

The stimulatory effect of cytidine could have been due to either cytidine itself or to the NH_3 which was released by the deaminase and then used in the synthesis of some other compound. In an attempt to determine what was occurring, an experiment was conducted using uridine which by itself had no stimulatory effect on either growth or enzyme production. If the pyrimidine was important then one should be able to see an additive effect of uridine in the presence of sub-optimal levels of NH_4^+. M. sodonensis was grown in synthetic medium containing NH_4^+ or uridine alone at the above concentrations. The growth response and enzyme production in each medium was compared. Since low levels of diesterase activity are difficult to detect by the standard technique, particularly in the presence of a UV absorbing compound such as uridine, the radioactive assay was employed and digests were incubated for 16 hrs. Uridine alone did not increase the cell yield over the basal level, nor was any enzyme produced. However, when uridine was present in addition to NH_4^+, there was a slight increase in growth and a significant increase in both monoesterase and diesterase activity per unit cell. The growth curves in the two media are shown in Fig. 24. As can be seen in Fig. 25, the addition of uridine in the presence of NH_4^+

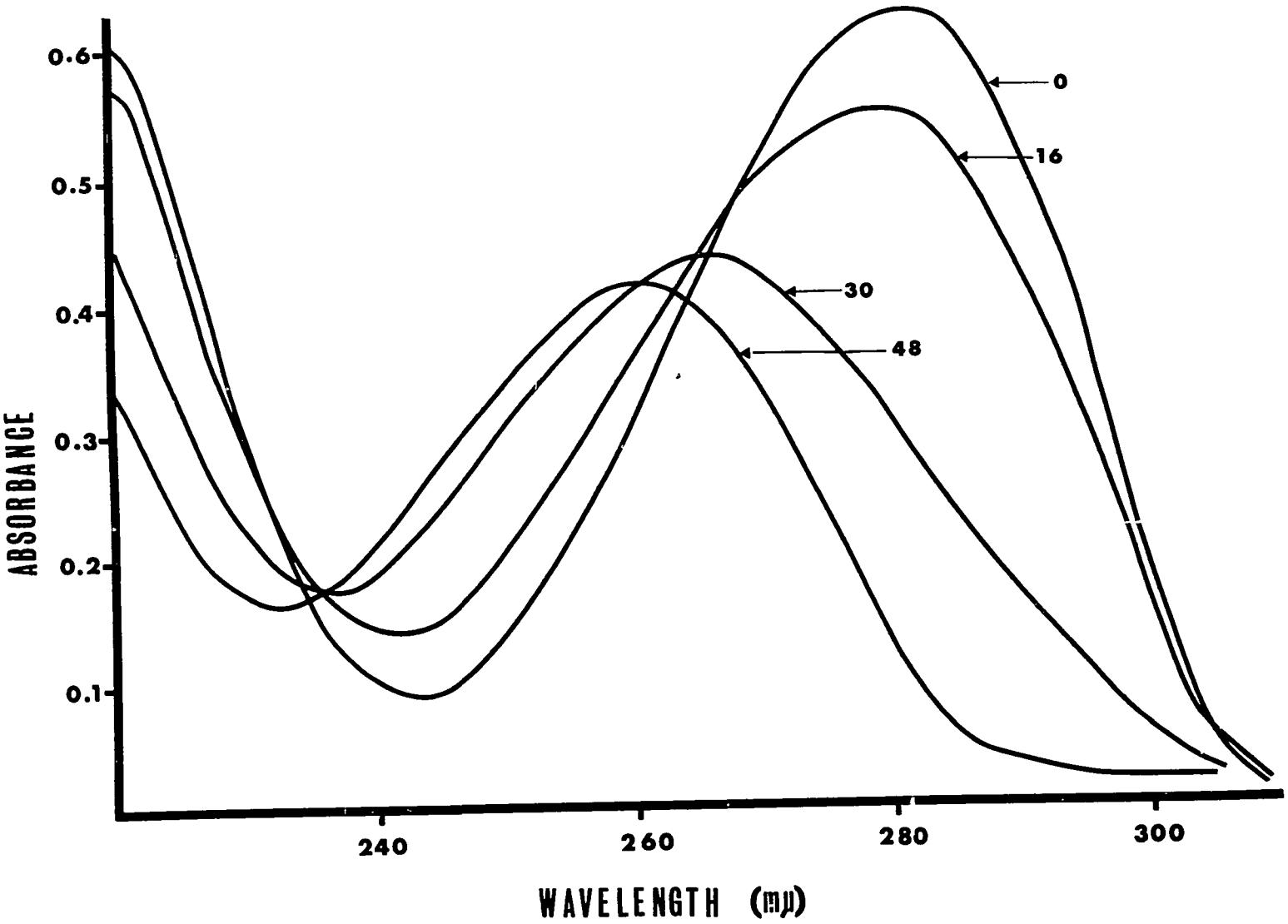

0.6
0.5
0.4
0.3
0.2
0.1
ABSORBANCE
0
16
30
48
240
260
280
300
WAVELENGTH (mμ)

FIGURE 23

CHANGE IN ABSORPTION SPECTRA OF M. SODONENSIS

CULTURE SUPERNATANTS

Aliquots of the supernatant from synthetic medium containing cytidine (1 µmole/ml) were removed at 0, 16, 30 and 48 hrs and diluted in 0.2N HCl.

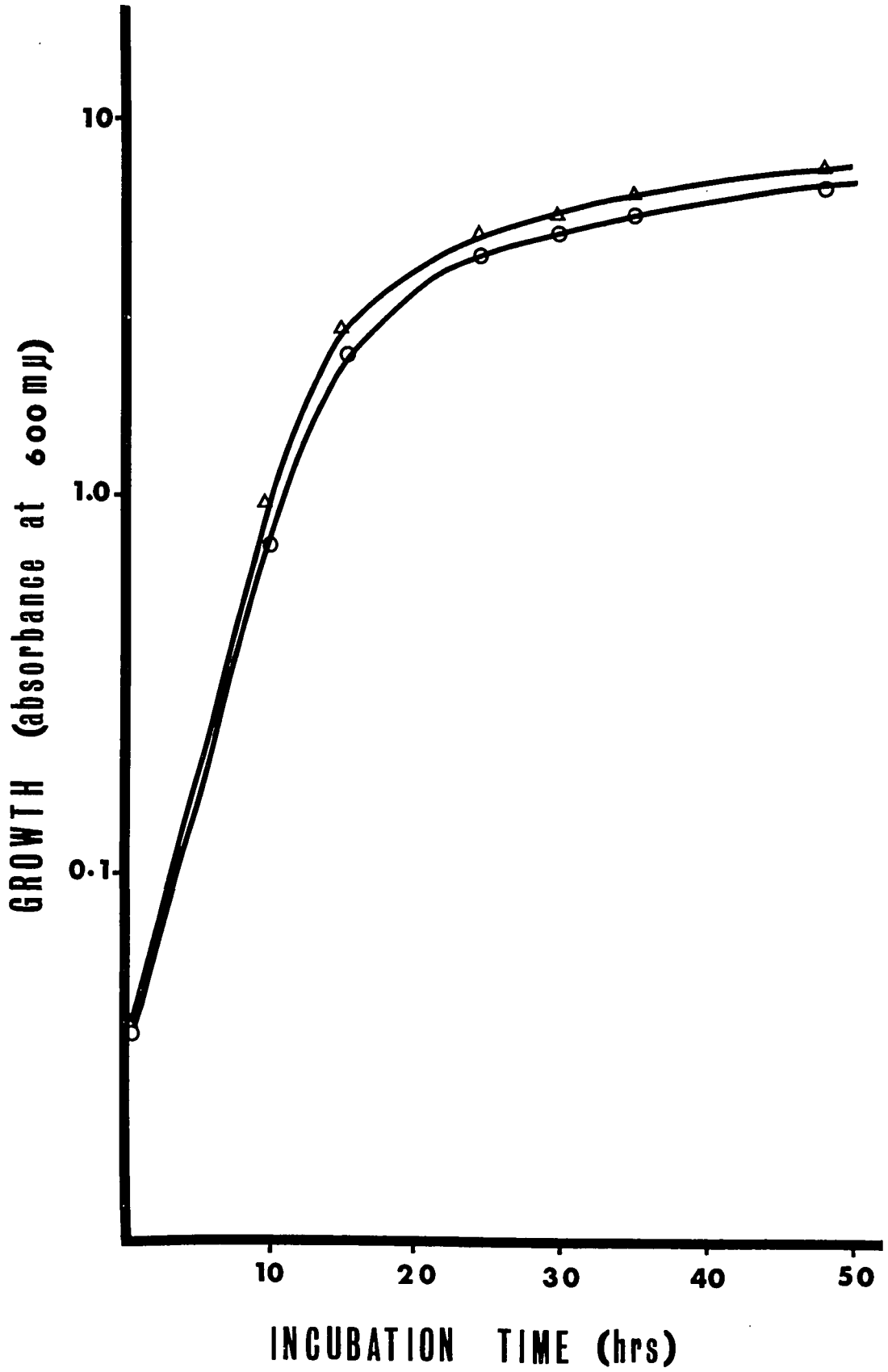

10
1.0
0.1
GROWTH (absorbance at 600 mμ)
10
20
30
40
50
INCUBATION TIME (hrs)

FIGURE 24

GROWTH RESPONSE OF M. SODONENSIS IN MEDIA CONTAINING URIDINE AND SUB-OPTIMAL LEVELS OF NH_4^+

NH_4^+ and uridine were added to the synthetic medium to yield a final concentration of 1 µmole/ml and 10 µmoles/ml respectively.

Synthetic medium + NH_4^+ O——————O

Synthetic medium + NH_4^+ + uridine Δ ——————Δ

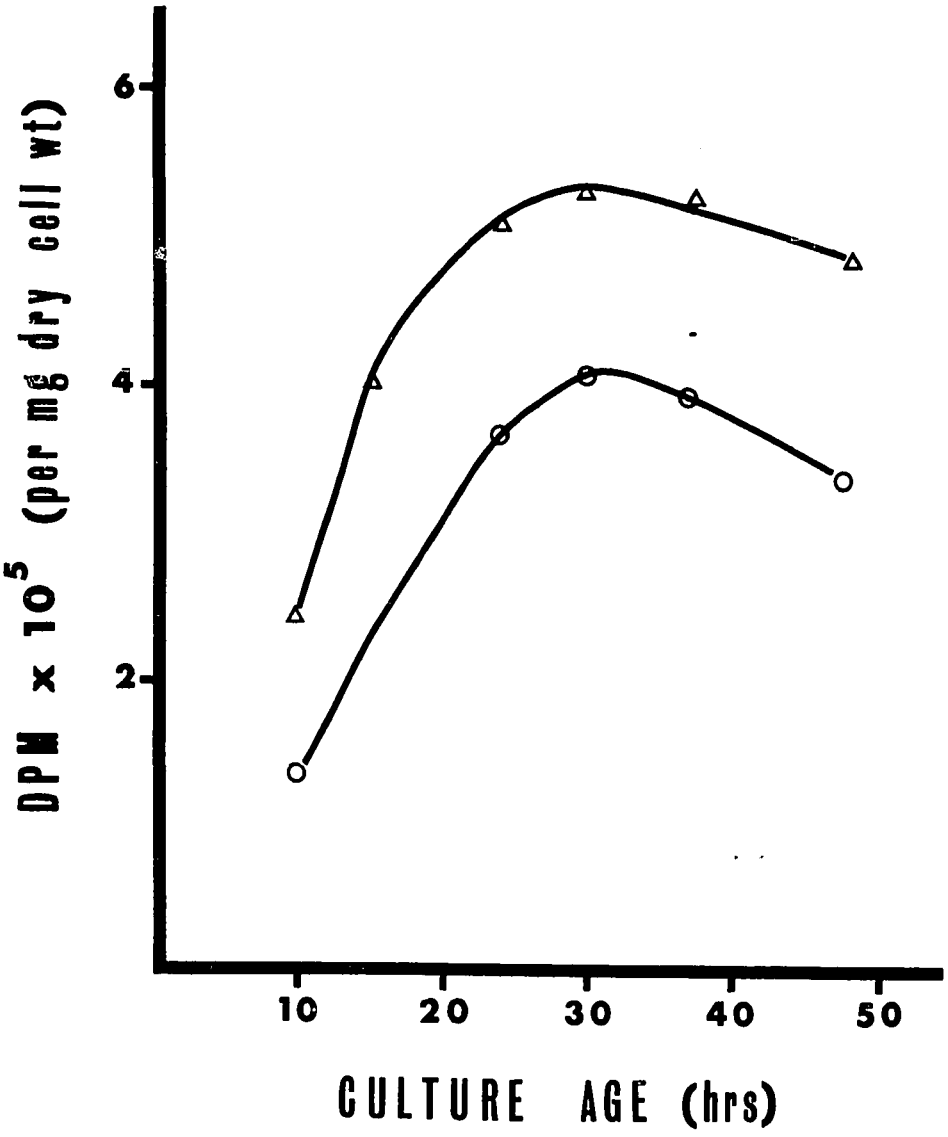

DPM x 10^5 (per mg dry cell wt)
6
4
2
10
20
30
40
50
CULTURE AGE (hrs)

FIGURE 25

EFFECT OF URIDINE AND NH_4^+ ON DIESTERASE ACTIVITY OF

M. SODONENSIS CULTURE SUPERNATANTS

Activity was followed by the release of UTCA-soluble label from ^{14}C RNA.

Synthetic medium + NH_4^+ O——————O

Synthetic medium + NH_4^+ + uridine Δ——————Δ

markedly increased the diesterase activity and the similar effect on monoesterase activity is shown in Table XXIII.

As well as inducing nuclease production, it was possible to repress the synthesis by increasing the phosphate concentration in the medium. The phosphate concentration of the normal synthetic medium is 3.17×10^{-4} M which is in excess of that required for optimum growth. Increasing the level of phosphate had no effect on the growth curve, however it had a marked effect on nuclease production. Table XXIV shows the results obtained. Cells were harvested at 24 hrs and the supernatants assayed for monoesterase and diesterase activity. Both activities were repressed to the same extent and, at a concentration of 20 times that required for optimal growth, no activity could be detected although the cell yield is essentially identical.

V. Role of the Cell Wall in Enzyme Production

Nuclease activity could be demonstrated only in culture supernatants of actively growing cells and never at any time in the soluble intracellular fraction. It seemed highly unlikely, therefore, that the appearance of activity in the supernatant was simply due to leakage of an intracellular enzyme. Attempts were made to determine whether the enzyme or its precursors could be detected in the cell envelope. Two methods were employed: a) demonstration of activity and b) immunological cross reactivity of nuclease and cell wall. M. sodonensis was grown in TCS broth and the cells were harvested and

TABLE XXIII

EFFECT OF URIDINE AND NH_4^+ ON MONOESTERASE ACTIVITY OF *M. SODONENSIS* CULTURE SUPERNATANTS

Additions	Activity				
	15 hr	24 hr	30 hr	36 hr	48 hr
None	-	-	-	-	-
NH_4^+ (1 µmole/ml)	-	.19	.416	.352	.395
UR (10 µmole/ml)	-	-	-	-	-
NH_4^+ (1 µmole/ml) + UR (10 µmoles/ml)	.406	.566	.602	.68	.86

Activity is expressed in units/mg dry cell wt.

TABLE XXIV

EFFECT OF PHOSPHATE ON NUCLEASE PRODUCTION BY M. SODONENSIS

Phosphate Concentration (M)	Relative Activity	
	Diesterase	Monoesterase
3.17×10^{-4}	100	100
6.34×10^{-4}	44.9	43.9
1.58×10^{-3}	15	12.6
3.17×10^{-3}	6	5
6.34×10^{-3}	0	0

TABLE XXIV

EFFECT OF PHOSPHATE ON NUCLEASE PRODUCTION BY M. SODONENSIS

Phosphate Concentration (M)	Relative Activity	
	Diesterase	Monoesterase
3.17×10^{-4}	100	100
6.34×10^{-4}	44.9	43.9
1.58×10^{-3}	15	12.6
3.17×10^{-3}	6	5
6.34×10^{-3}	0	0

washed 3X in distilled H_2O. Purified cell walls were prepared according to the following procedure.

Washed Whole Cells
↓ - disrupted and washed 3X in 0.5M NaCl
"Crude walls"
↓ - 30 mins at 90° C in 0.5M NaCl
- washed 4X in 1M NaCl
"Heated crude walls"
↓ - continuous dialysis - 24 hrs in H_2O
- 4 phenol extractions at 70° C
- continuous dialysis to remove phenol
- washed 4X in 1M NaCl
- sonicated for 1 min after each washing
"Phenol treated walls"
↓ - trypsin - 14 hrs
- washed 2X in .01M TRIS pH 8.5
- sonicated for 1 min after each washing
- washed 2X in 1M NaCl
- dialysed 48 hrs in H_2O
- lyophilized
"Pure walls"

1. Activity in Walls

Samples of "crude walls" were prepared from 12, 18 and 24 hr cultures and assayed for diesterase activity by measuring the release of UTCA-soluble label from ^{14}C RNA. The level of activity was very low and accurate quantitation had awaited the development of this more sensitive isotope technique. Table XXV shows the relationship of "bound", to "free" activity in cells of different ages. "Heated crude," "phenol treated" and "pure" walls were also assayed for

TABLE XXV

DISTRIBUTION OF "BOUND" VS "FREE" NUCLEASE IN A GROWING CULTURE OF *M. SODONENSIS*

Age of cells (hrs)	Activity	
	"Crude" cell walls	Culture Supernatants
12	1.31	307
18	0.84	248
24	0.56	241

Activity is expressed in units/mg dry whole cells.
1 unit = 1 mμmole of nucleoside released/hr.

activity. Table XXVI shows the activity per mg dry weight of each fraction. Although the quantitation of crude and phenol treated walls is not too precise, several conclusions may be drawn: i) enzyme activity is present in the cell envelope in detectable quantities, ii) activity is retained throughout the purification procedure until the final trypsin treatment which completely destroys all activity and iii) the enzyme must be very firmly bound to the wall since it withstands such vigorous treatment as high salt concentration, heating, sonication and phenol treatment. The free enzyme is normally very heat labile and diesterase activity can be destroyed by 5 mins heating at 55° C. It is therefore surprising that any activity persists after 30 mins at 90° C. The wall bound enzyme must be firmly held in a stable configuration.

2. Immunological Studies

Anti-cell wall and anti-enzyme sera were prepared by injection of the purified antigens into rabbits weighing about 2 kg. The rabbits were subjected to a series of injections 3 times a week for 6 weeks at which time they were bled and their sera retained for the serological tests. A titre of $\frac{1}{320}$ was obtained for the anti-cell wall serum as measured by agglutination of purified M. sodonensis cell walls. As shown in Table XXVII a cross reaction was obtained between enzyme and anti-cell wall serum with both the ring test and the qualitative tube precipitation test. A positive test was also obtained by the

TABLE XXVI

DIESTERASE ACTIVITY IN ISOLATED CELL WALL

FRACTIONS OF M. SODONENSIS

Fraction	Activity
"Crude"	.0438
"Heated Crude"	.0037
"Phenol Extracted"	.015
"Pure Walls"	-

Activity is expressed in units/mg dry weight of fraction.
1 unit = 1 μmole acid-soluble nucleoside/hr.

TABLE XXVII

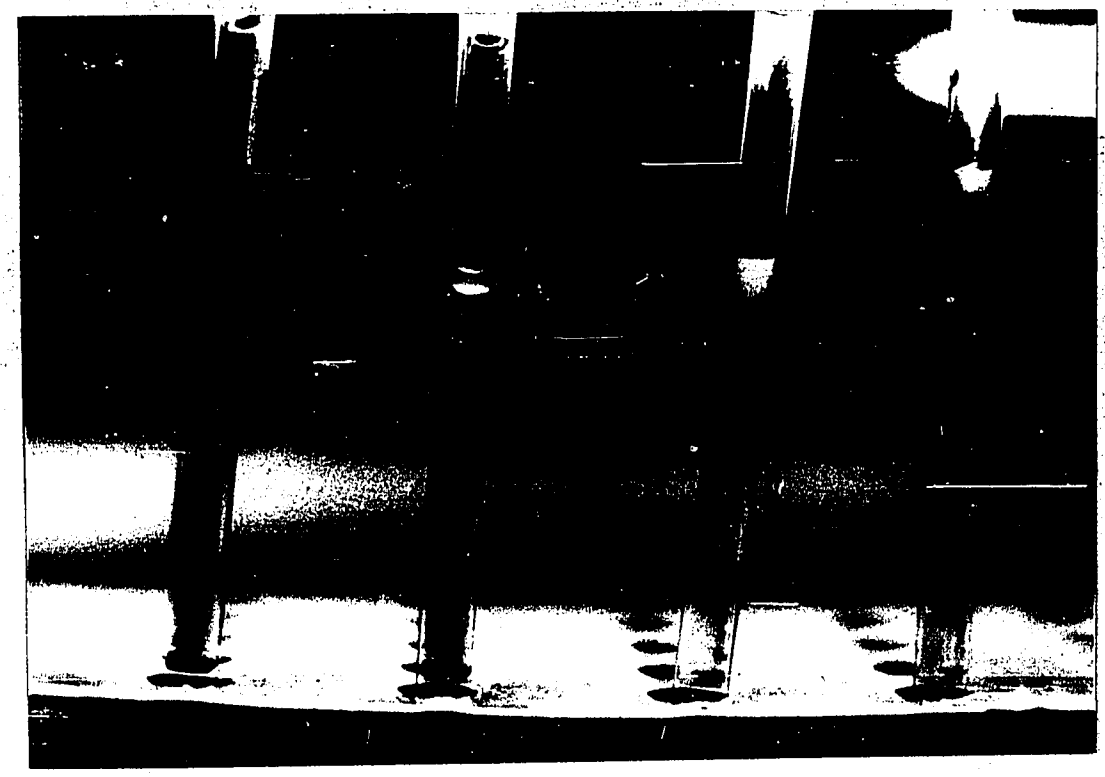
CROSS REACTION BETWEEN PURIFIED M. SODONENSIS NUCLEASE AND ANTI-CELL WALL SERUM

Test Mixture	Ring Test	Tube Precipitin
Anti-cell wall serum + enzyme	+	+
Anti-cell wall serum + buffered saline	-	-
Control serum + enzyme	-	-
Control serum + buffered saline	-	-

Oudin single gel diffusion technique. As shown in Fig. 26, a single band of precipitation appeared in the tube containing enzyme and anti-cell wall serum.

Since the supply of pure enzyme was limited and extremely valuable, only one rabbit was used for the production of antiserum. Unfortunately this rabbit died before the course of injections was completed and as a result the titre of the anti-enzyme serum was very low. Agglutination tests were carried out with M. sodonensis cell walls but because of the low titre of the serum these results were not considered valid for precise quantitative interpretation. The effect of anti-sera on enzyme activity is shown in Table XXVIII. Two experiments were conducted, one in which the enzyme was preincubated with the serum at 37° C for 1 hr and the second in which the antisera were added to the assay mixture at zero time. Although a visible cross reaction was obtained between the enzyme and the anti-cell wall serum, there was little or no effect on enzyme activity. This suggests that the complex was formed with the carbohydrate moiety of the enzyme and not with the protein since presumably this was destroyed with trypsin treatment of the walls. Since the amount of enzyme activity contained in the walls is very low it can be concluded that more carbohydrate than protein is present.

The results of the immunological studies confirm the presence of enzyme in pure cell walls.



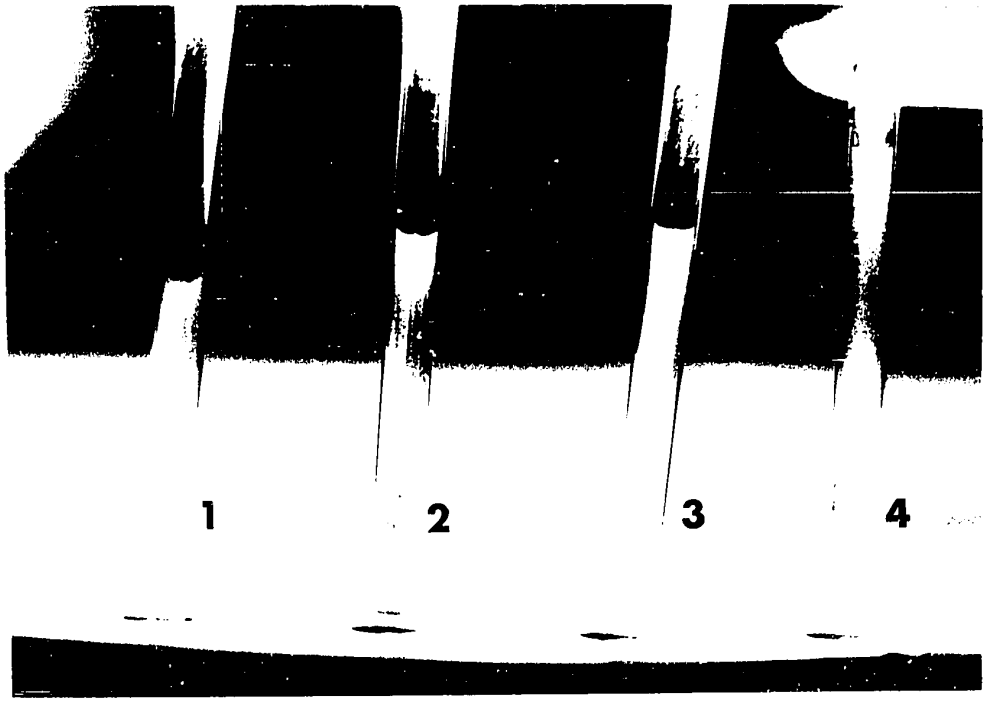

1
2
3
4

FIGURE 26

OUDIN SINGLE GEL DIFFUSION TEST FOR CROSS REACTIVITY

OF NUCLEASE AND PURIFIED CELL WALL OF M. SODONENSIS

Antisera were incorporated into the agar and the solidified columns overlaid with antigen or buffer.

Tube #1 - anti-cell wall serum + buffered saline

Tube #2 - anti-cell wall serum + nuclease

Tube #3 - control serum + nuclease

Tube #4 - control serum + buffered saline

TABLE XXVIII

INACTIVATION OF DIESTERASE ACTIVITY OF M. SODONENSIS NUCLEASE BY ANTI-SERA

Serum added	Relative Activity	
	Preincubation	No preincubation
None	100	100
Anti-Cell wall	93.8	90
Anti-enzyme	52.0	56.8

DISCUSSION

The designation of M. sodonensis nuclease as a true extracellular enzyme (i.e. secreted) rather than an intracellular one released upon lysis is based upon the following observations: a) Nuclease production per unit cell is maximal in logarithmically growing cells at a time when no demonstratable autolysis is occurring. b) When cells reach the stationary phase, i.e. when the number of viable cells is constant, a constant rate of enzyme production is attained which does not increase with cell aging. c) When cells are held under conditions in which autolysis can be demonstrated, no further enzyme release is detectable. d) Production can be repressed and derepressed in actively growing cultures. e) At no time can activity be demonstrated in the intracellular fraction. f) The amount of activity bound to the cell wall in a 12 culture represents only 0.4% of that found in the supernatant. As the cells age, the amount of cell bound activity decreases (0.34% at 18 hrs and 0.23% at 24 hrs) as does the activity in the supernatant indicating no further synthesis in the stationary phase. According to the criteria set forth by Pollock (1962), M. sodonensis nuclease is thus established as extracellular rather than intracellular or surface-bound.

In mammalian tissue, extracellular proteins, eg. digestive enzymes, are accumulated in the cell membrane as zymogen granules and released. This release requires energy plus the addition of a specific inducer (Schramm, 1967). Little is known about the mechanism of release of microbial exoenzymes. If they are formed outside the membrane then

presumably the only barrier is the cell wall which would not appear to offer much resistance. However Mitchell and Moyle (1959) showed that Staphylococcal cell walls were impermeable to dextran (M.W. 10,000) Nomura et al (1958) suggested that the release of α-amylase from Bacillus subtilis might be due to a cell bound autolysin which began to appear in the walls at about the same time that α-amylase appeared in the supernatant. However there was negligible cell-bound α-amylase either before or during release of the enzyme into the supernatant and Pollock (1962) suggested that autolysis could only be involved if liberation automatically involves formation. He postulated that the last step in formation was an activation of a preformed precursor brought about by its detachment from a site on the surface of the cell. Since only a small amount of nuclease activity is present in the walls of M. sodonensis it is possible that an analagous situation exists.

The studies on the production of M. sodonensis nuclease have shown that in a complex medium the enzyme appears in the late logarithmic growth phase and that the amount of enzyme per unit cell remains relatively constant throughout the duration of the experiment although it appears to decrease slightly after the cells enter the stationary phase. In a defined medium the situation differs somewhat. Again enzyme is detected when the cells are in the mid-to-late logarithmic

phase but the amount of enzyme per unit cell increases with time until a maximum is reached early in the stationary phase, followed by a decrease. The production of the nuclease in the complex medium would seem to be a reflection of protein synthesis, i.e. all cellular proteins are being synthesized at the same rate, whereas in the defined medium this is not true. In this case there appears to be a preferential production of nuclease relative to cell growth. This increased synthesis may be in response to an accumulation of some intracellular intermediate or perhaps to a depletion of some constituent which is inhibitory to production. Whatever the reasons, the situation in M. sodonensis differs markedly from that found in Bacillus subtilis. Coleman (1967) studied the production of 3 extracellular enzymes of B. subtilis; α-amylase, proteinase and RNase. He found that the production of these enzymes proceeded at a very low rate until the end of the logarithmic phase. The rate of secretion then increased to a high linear value which persisted well into the stationary phase when cell growth had decreased or ceased. The results were identical in both complex and defined media. Coleman suggests that during cell growth the nucleic acid precursor pool is depleted and hence exoenzyme mRNA formation is limited. When cell growth ceases then the limitation is removed and exoenzymic mRNA can be formed at the maximum rate. However it would appear equally possible that the enzymes are leaking from the old cells and may not, therefore, be truly extracellular. The fact that enzyme production by M. sodonensis decreases during the stationary phase

adds support to its extracellular nature and production by actively growing cells.

The nuclease was recovered from supernatants of M. sodonensis and the purified protein was used for physical and chemical analysis. From the ultracentrifugal data it is apparent that M. sodonensis nuclease is an asymmetric molecule. This asymmetry is indicated by a sharpening of the peak in the sedimentation velocity experiment as well as a discrepancy between the $S^{o}_{20,w}$ value and the molecular weight. Myoglobin, a globular protein, has an $S_{20,w}$ of 2.0 and a molecular weight of 16,900 (Alexander and Johnson, p. 288, 1949), but the $S^{o}_{20,w}$ of 2.1 and molecular weight of 500,000 obtained for M. sodonensis nuclease resemble the results obtained for myosin which is an asymmetric molecule. Kielley and Harrington (1960) found that the $S_{20,w}$ and the molecular weight of myosin varied with the concentration. An $S^{o}_{20,w}$ of 6.4 and a molecular weight of 619,000 was obtained by calculating these values at several concentrations and extrapolating to infinite dilution. The same concentration dependence is noted with M. sodonensis nuclease and is highly indicative of asymmetry. The nuclease appeared to be very viscous and at higher concentrations did not readily leave the meniscus during ultracentrifugation, probably because of interaction between asymmetric molecules. Further viscometric studies, which theoretically could have yielded precise data concerning the molecular dimensions of the protein, were considered not worthwhile until more information was obtained concerning the size

of the carbohydrate and its mode of attachment to the protein.

With the discovery of the glycoprotein nature of the nuclease, the physical data is readily understandable and the asymmetry of the molecule is not surprising. Glycoproteins have certain properties which distinguish them biophysically from the simple proteins. These include a high molecular charge density, high intrinsic viscosity, polydispersity and a high degree of asymmetry (Gottschalk, 1966). Although small protein-like glycoproteins with a low carbohydrate content present no special problems, difficulties in physico-chemical analysis can arise with those of high molecular weight and viscosity. These molecules are generally rich in carbohydrate.

The high molecular weight of M. sodonensis nuclease distinguishes it from other microbial exoenzymes which, as mentioned in the Introduction, may vary from 10,000 - 90,000. The second distinguishing feature is its glycoprotein nature. Although the majority of mammalian and plant extracellular proteins contain carbohydrate (Eylar, 1965) none of the microbial exoenzymes thus far described have been designated as glycoproteins. Taniuchi and Anfinsen (1966) reported the presence of carbohydrate in the nuclease of S. aureus but no detailed analysis was carried out and no suggestion of a glycoprotein was made. More recently, Jackson and Wolfe (1968) found that a protease produced by Myxobacter, strain AL-1, contained 1 mole of hexose per mole of protease but the hexose was not identified.

As mentioned in the Introduction, relatively few species of sugars are found in glycoproteins. In mammalian systems, amino sugars are always present and the linkage of the carbohydrate to the peptide is invariably through an amino sugar. The carbohydrate content of M. sodonensis nuclease varied slightly between preparations, which is often the case when dealing with glycoproteins, but in all cases the hexosamine:neutral sugar ratio remained the same. As the data in the Results section indicates, carbohydrate comprises 21.5% of the enzyme with a hexosamine:glucose:galactose:rhamnose ratio of 4:2:1:1.

Four types of carbohydrate-peptide linkages have been reported: a) an N-acyl-glycosylamine linkage involving the amide-N of asparagine; b) O-glycosidic linkages involving the hydroxyl groups of serine and threonine; c) a glycosidic ester bond between N-acetyl hexosamine and the β or γ carboxyl groups of aspartic or glutamic acids; d) an amide linkage between the carboxyl group of N-acetylmuramic acid and the α-amino group of L-alanine. The data obtained in the analysis of M. sodonensis nuclease clearly indicated the presence of a glucosamine-serine linkage as the major bond between carbohydrate and protein. The release of amino sugar (identified as glucosamine) proceeds very slowly with acid hydrolysis and at 12 hrs the release was not yet complete (Fig. 7). The amino acid-sugar complex was present in the glutamic acid serine region and upon longer incubation time in acid was hydrolyzed with the release of free glucosamine (tryptophane region) plus serine and glutamic acid, as evidenced by the resolution of the peaks in that

region. Glucosamine is thus established as the sugar involved and the data suggests the involvement of serine and/or glutamic acid. Further support for a serine linkage was given by the alkaline hydrolysis data which indicated that β elimination had occurred with the formation of the aminoacrylic acid derivative (Carubelli, 1965). Since at least 80% of the serine residues (440 mμmoles/mg of protein) are involved, this accounts for 65% of the total glucosamine residues. It is not established that all of the glucosamine is involved so it is only possible to state that a serine-glucosamine linkage represents a major portion of the binding. Other linkages may also exist such as a linkage through the γ carboxyl of glutamic acid. Gottschalk and Murphy (1961) produced evidence for the coexistence of both types of linkage in ovine submaxillary gland protein. Conclusive evidence however must await the isolation of sufficient amounts of the amino acid-hexosamine fraction which could then be analysed more precisely for its amino acid and sugar composition.

Although the configuration of the carbohydrate cannot be finally determined from the results presented, some idea can be obtained from the reducing power data. Firstly, the carbohydrate must be highly polymerized since few reducing groups are available in the unhydrolyzed sample. Secondly, glucosamine is not maximally released until 12 hrs of hydrolysis and yet all the reducing power is released by 1 hr. It appears, therefore, that glucosamine is not linked to the peptide in a C-1 position. And finally, a minimum size of 56 monosaccharide units and a minimum mole-

cular weight of about 11,000 can be assigned to the carbohydrate on the basis of the reducing power vs total carbohydrate data. Since the enzyme is about 20% carbohydrate one can then calculate a minimum molecular weight of 44,000 for the peptide. On the basis of the amino acid analysis and assuming 1 cysteic acid residue per peptide, a minimum molecular weight of 10,817 is obtained for the peptide, however if there were 4 cysteic acid residues this value would be 43,268 which is in surprisingly close agreement with figures obtained from the carbohydrate calculations. It is emphasized that the calculations are based on reducing power which is expressed relative to glucose and that this value may vary a bit depending upon which sugar is in fact at the reducing end of the polysaccharide.

The role of the carbohydrate in the activity of the enzyme is not known since it has not yet been possible to remove the carbohydrate without simultaneously inactivating the protein. Treatment with α- and β-amylase and lysozyme had no effect on activity but neither could a release of carbohydrate be demonstrated. The cross reaction of the nuclease with anti-cell wall serum probably occurs chiefly with the carbohydrate moiety (as will be discussed later) and since no inactivation occurs one might assume that the carbohydrate has no functional role. Eylar (1965) suggested that the role of carbohydrate in glycoprotein is a structural rather than a functional one and that it is a chemical label which aids in the transport of the protein through the cell membrane. However since microbial exoenzymes are

thought to be formed outside the membrane (Pollock, 1962) the transport theory does not apply. Although small molecular weight precursors may be transported through the membrane it is possible that the carrier may be lipid rather than carbohydrate, analagous to the situation which has been demonstrated in the biosynthesis of bacterial cell wall murein. Lipid-phosphoacetylmuramyl-pentapeptide and lipid-phosphodisaccharide-pentapeptide have been isolated in S. aureus (Anderson et al, 1965; Matsuhashi et al, 1965) and have been postulated to be membrane transport intermediates. These workers suggested that the nucleotide-glycopeptide precursors were attached to the lipid for transport through the membrane to an incomplete glycopeptide acceptor in the formation of cell wall. It would seem more reasonable to postulate a lipid carrier for the nuclease as well. The role of the carbohydrate then might merely be to impart stability to an extracellular enzyme which must be able to survive variations in environment.

Aside from the anomalous results which were later explained by the presence of amino sugar, the amino acid composition does not appear unusual. Cysteine and methionine are low but Pollock (1962) suggests that a characteristic of exoenzymes is their lack of cysteine. Micrococcal nuclease contains only methionine (Taniuchi and Anfinsen, 1966) while B. subtilis RNase lacks both methionine and cysteine (Lees and Hartley, 1966). The protease of Myxobacter AL-1, however, (Jackson and Wolfe, 1968) was found to contain 2 moles of cysteine per mole of enzyme, so that the complete absence of cysteine is not universal amongst exoenzymes. These workers also reported high levels of serine and glycine and these 2 amino acids, plus aspartic acid, accounted for nearly half

of the total number of amino acid residues. Hashimoto and Pigman (1962) found that the protein moieties of several mammalian glycoproteins contained a high number of serine, threonine and glycine residues, and it is interesting to note that a relatively high level of these amino acids is present in M. sodonensis nuclease as well.

The determination of total protein in a glycoprotein is not straightforward and many difficulties exist which limit the accuracy of the different methods. It will be noted from the amino acid data that the recovery of the amino acids was not quantitative. This was thought to be due to the unreliability of the Lowry protein determination since the color intensity with this method varies for different proteins. It was subsequently demonstrated in this laboratory that glucosamine can give a positive Lowry reaction and therefore in a glycoprotein might contribute significantly to any value obtained by this method.

One of the interesting aspects of this problem has been the existence of both phosphodiesterase and 5'-nucleotidase activities. The diesterase is exonucleolytic and will attack RNA and DNA as well as synthetic polynucleotides such as Poly A, or dinucleotides such as ApA and pTpT. It will not, however, use the synthetic substrates such as bis-(p-nitrophenyl) phosphate or p-nitrophenyl-thymidine-5'-phosphate. It thus differs from the more non-specific phosphodiesterases such as that of snake venom. The 5'-nucleotidase will act on all 5' ribo- and deoxyribonucleotides but in contrast to the 5'-nucleotidase of E. coli (Neu, 1967a) will not attack ATP, ADP nor bis-(p-nitrophenyl) phosphate.

These two activities have been postulated to be associated with one protein (Berry and Campbell, 1967b) and, while the possibility of the existence of two proteins with very similar properties was not discounted, it has so far been impossible to separate the two activities by any technique whatsoever.

The enzyme molecule appears to be homogeneous by all the techniques employed and at no time is diesterase activity found without an associated nucleotidase activity. The data obtained in the present study gives additional support to the "one enzyme-two activity" hypothesis.

The original suggestion was that at least two sites were required for binding of the polymer, one of which was Mg^{++} dependent and more heat labile and the second which was Mn^{++} dependent and involved also in binding of the mononucleotide. The results presented appear to support rather than dispute this theory.

Both activities are stabilized by Ca^{++} and the heat sensitivity of both is increased by Mn^{++} and Mg^{++}. The effect of Mn^{++} and Mg^{++} is more pronounced on the diesterase activity which would be expected if the above hypothesis were true. Both activities are stimulated by the addition of -SH containing compounds such as mercaptoethanol, cysteine and reduced glutathione, and are inhibited by PCMB. The activation and inhibition are more pronounced in the case of the monoesterase and it would seem reasonable to conclude that the common site involved in binding of the polymer and the nucleotide has an -SH group either at

the site or very close to it. If the polymer is bound to 2 sites and the nucleotide at only one, the effect would naturally be greater on the monoesterase activity.

Chelating agents are stimulatory to both activities and presumably this stimulation is due to the removal of toxic heavy metals which are inhibitory to the enzyme. Most chelators have the following order of preference: Fe^{+++}, Hg^{++}>Cu^{++}, Al^{+++}>Ni^{++}, Pa^{++}>Ca^{++}, Zn^{++}>Fe^{++}, Cd^{++}>Mn^{++}>Mg^{++}, Ca^{++}, (Albert, 1961) and would therefore combine with the toxic heavy metals before those such as Mg^{++}, Mn^{++} and Ca^{++} which are required for activity.

Kinetic studies have shown that the enzyme has a greater affinity for RNA than for AMP. It would be expected therefore that in RNA digests a mixture of nucleotides and nucleosides would be found if two enzymes were involved or even two separate and independent sites on the same protein. However the only reaction products identified were nucleosides and P_i and the amounts of P_i and of nucleosides released were equivalent.

When a single enzyme is active on two substrates A and B, and these substrates are both present in a reaction mixture, then A behaves as a competitive inhibitor of B and vice versa. In a mixture containing both A and B, the total velocity (V_t) of the reaction will lie between the two velocities which would be obtained with the same concentrations of A and B alone (Dixon and Webb, 1965). If two proteins or two independent sites are involved, then the total velocity V_t should be equal to the sum of the two velocities. Sturge and Whittaker (1950) found with

horse serum cholinesterase that the rate of hydrolysis of benzoyl choline was 440 µl/hr, that of isoamyl acetate was 131 µl/hr and a mixture of the two was 314 µl/hr (determined manometrically). They concluded from the data that the hydrolysis of the choline and non-choline esters was due to the same enzyme. The results obtained for M. sodonensis nuclease indicate a similar situation. The total velocity of the reaction in the presence of both substrates falls between the individual velocities which adds support to the hypothesis that the two reactions are catalysed by one protein.

RNA and AMP are both substrates for the enzyme and therefore each will behave as a competitive inhibitor for the other activity when both are present in an assay mixture. With the higher affinity for the polymer however, one might expect that the kinetics would vary from the typical competitive type, analagous to the situatinn found in cholinesterase. Myers (1952) found with an inhibitor of cholinesterase (Nu-683) which had a very high affinity for the enzyme (i.e. the displacement of the inhibitor from the enzyme by the substrate was very slow) that although the inhibitor was in fact competitive, the Lineweaver-Burk curve expressed non-competitive kinetics. It has been demonstrated that the affinity of M. sodonensis nuclease is greater for RNA than for AMP and therefore the apparent non-competitive plot was not surprising. Furthermore, one might expect that this "non-competitive" situation would continue for a longer period of time in the nuclease system than in the cholinesterase since, because

of the exonucleolytic mode of action of the diesterase, the effective RNA concentration would not be decreased until the polymer was degraded to the dinucleotide level.

Studies with various analogues and substrates have yielded valuable information concerning the sites of attachment involved in enzyme activity. a) although 5'-nucleotides are substrates for the enzyme, ribose-5'-phosphate alone is not attacked nor will it cause any inhibition of either activity. It is apparent therefore that a purine or pyrimidine base is essential for the binding of the enzyme. b) there is no requirement for a 2' hydroxyl group since DNA and deoxyribonucleotides will serve as substrates. c) a free 3' hydroxyl is essential and if it is blocked in any way the activity is inhibited. Although pTpT will serve as a substrate pTp will not, nor will a 3',5'-cyclic nucleotide. It was also shown that borate, which forms a complex between the 2' and 3' hydroxyl groups, inhibited activity. d) the 5' monophosphate is essential for activity and neither ADP nor ATP are attacked. An ionizable group appears to be essential at this position. Adenosine-5'-monoacetate will not function either as a substrate or as an inhibitor, however inosine fluorophosphonate and inosine chloromethylphosphonate, which have an ionizable group available for binding, will function as inhibitors for both monoesterase and diesterase activity. No inhibition occurs with adenosine-5'-phosphomorpholidate although it has an available ionizable group. Since the morpholine group is large it is likely that steric

hindrance prevents binding. It can be concluded that at least 3 sites on the substrate molecule are important for the binding of the enzyme. A purine or pyrimidine base, a free 3' hydroxyl and an ionizable 5' group.

Although both activities are inhibited by the same compounds, the effect is generally greater on the monoesterase than the diesterase activity. This is not surprising in view of the higher affinity of the enzyme for RNA, i.e. the inhibitor has greater difficulty in displacing the substrate, and adds support to the hypothesis of the shared site for the nucleotide and polymer with an additional site for the polymer alone.

An absolute requirement for NH_3 for the growth of M. sodonensis has been demonstrated previously (Campbell et al, 1961a) and its role in carbamyl phosphate and hence pyrimidine biosynthesis was postulated. NH_3 was also shown to derepress enzyme synthesis. The control is undoubtedly not exerted by NH_3 per se but rather by some metabolic product whose synthesis is NH_3 dependent.

The addition of sub-optimal levels of NH_4^+ to culture media delayed enzyme production and activity could not be demonstrated until the cells were entering the stationary phase. The cell yield at the time of the appearance of enzyme is considerably higher than that in the medium containing optimum NH_4^+. This would seem to support the idea that an accumulation of intracellular "inducer", rather than a depletion of nutrients, is responsible for the initiation of enzyme

synthesis. In the optimum medium ample NH_4^+ is present for all NH_3 dependent systems and the intracellular level of "inducer" could build up rapidly. When NH_4^+ is limiting, however, more time is required to bring the "inducer" to a level at which it will initiate enzyme synthesis.

Several NH_3 dependent systems were demonstrated in M. sodonensis as well as interconversions which utilize or yield NH_3. Although certain of the reactions (eg. UTP aminase) appear to utilize glutamine as well as NH_4^+, it is clear that the enzymes are in fact NH_3 dependent since the glutamine dependence decreases with partial purification. Lieberman (1956) demonstrated with E. coli that the glutamine requirement disappeared with purification. Since the present studies have demonstrated glutaminase activity in both crude systems, the apparent glutamine dependence can be readily explained.

The demonstration of an NH_3 dependent carbamyl phosphate synthetase strongly suggested that a pyrimidine nucleotide was implicated as the active derepressor and this was confirmed by the whole cell studies. With the addition of cytidine (10 μmoles/ml) to the medium, the growth eventually reached the level of that in the normal synthetic medium + NH_4^+. The effect of cytidine was two-fold; an uptake of pyrimidine, and a deamination of cytidine to yield NH_3. As with sub-optimal levels of NH_4^+, the appearance of enzyme was delayed, again indicating the requirement of sufficient levels of intracellular "inducer". Although sufficient NH_3 is ultimately available, the

deamination of cytidine is the rate limiting step. The role of cytidine as the derepressor was further supported by the experiments with uridine. Although uridine alone had no effect on either growth or enzyme production, when it was added in addition to sub-optimal levels of NH_4^+, there was a significant increase in both monoesterase and diesterase activity per unit cell. The amination of uridine compounds has been demonstrated intracellularly and it is, therefore, apparent that cytidine, or more likely a cytidine nucleotide since the intracellular pool is normally at the nucleotide level, is the active derepressor. Additional evidence for the involvement of a nucleotide in the control of enzyme synthesis was obtained from the experiments with phosphate. Synthesis was repressed by high levels of phosphate which would be expected to cause an alteration or imbalance of the nucleotide pool.

Confirmation of the repressor and derepressor must await the analysis of the nucleotide pool under the different conditions. This is the subject of an independent investigation underway in this laboratory.

The induction of exoenzymes by their products is not unusual. Pollock (1962) found that N-acetylglucosamine would induce Streptococcal hyaluronidase, while the enzymes from B. palustris, which depolymerize Pneumococcus Type III and VIII polysaccharides, were inducible by their hydrolytic products (Torriani and Pappenheimer, 1962).

The control studies have shown that diesterase and monoesterase activities are induced or repressed simultaneously by the same compounds. The evidence for a single protein has already been discussed and the control data thus lends further support to the hypothesis.

The synthesis of the enzyme, or the last stages of its formation or activation appears to occur at the cell surface. No activity could be demonstrated in the cell free extract but very low levels could be detected in cell walls at various stages of purification. Many enzymes have been reported to be associated with the envelope fraction of the bacterial cell. ATPase has been found in membranes (Weibull et al, 1962; Ishikawa and Lehninger, 1962; Munoz et al, 1968) and Neu and Heppel (1965) found that a number of degradative surface bound enzymes of E. coli could be released into solution simply by washing in a low salt concentration. The 5'-nucleotidase of E. coli is located in the periplasmic space (Neu, 1967b) and is released by the formation of spheroplasts. Several enzymes have been found associated with cell walls (Young, 1966; Tipper, 1968; Brown et al, 1968) and these, in contrast to membrane associated enzymes appear to be firmly bound. Brown et al (1968) found that the N-acyl muramyl-L-alanine amidase of B. subtilis was covalently bound to teichoic acid. The wall associated enzymes thus far described have all been autolytic enzymes whose function is to expand the peptidoglycan to allow insertion of another segment of wall. The discovery of the nuclease associated with cell walls is the first example of an enzyme not involved with wall bio-

synthesis and suggests that the wall itself has heretofore unsuspected functions. M. sodonensis nuclease appears to be very firmly bound to the cell wall since activity is retained throughout rigorous purification techniques such as drastic alterations in salt concentration, heating and phenol extraction. As was pointed out in Results, it is surprising that any activity is retained after heating at 90° C since the free enzyme is destroyed after 5 mins at 55° C. The extractions with phenol also employ heating to 70° C and yet the specific activity at this step increased. The enzyme which remains, therefore, must be bound in a stable configuration to the wall and, although activity is lost with trypsin treatment, antigenicity is retained.

The immunological studies showed a definite relationship between purified cell walls and enzyme. A good visible cross reaction was obtained with the anti-cell wall serum although there was no inhibitory effect on enzyme activity. This is not surprising since the serum was prepared from trypsin-treated walls which contained no active enzyme and in which the protein was presumably destroyed. The cross reaction must therefore be with the carbohydrate moiety of the enzyme. Although there is a strong cross reaction, there is a very low level of enzyme present in the wall, of which only 20% is carbohydrate. This strongly suggests that the wall contains a large amount of carbohydrate identical to that of the enzyme but which is not attached to the protein moiety. This is supported by the carbohydrate analysis of the enzyme in which 4 sugars were identified, all of which have also been found in pure M. sodonensis cell wall hydrolysates (Johnson, personal communication).

The anti-enzyme serum inactivated the enzyme but because of the low titre of the serum the cross reaction with pure cell walls was not conclusive. It is likely that the carbohydrate moiety, while being an antigenic determinant when bound to the wall, loses much of its antigenicity in the free state (i.e. bound to free enzyme). Therefore in a low titre serum only the anti-protein would be apparent which would react with enzyme protein but not trypsin treated walls. This is supported by the fact that the anti-enzyme serum is effective in inactivating free enzyme.

It can be concluded that there is a relationship between the nuclease and the cell wall but the nature of the relationship cannot be determined at this time. Several possibilities exist:

a) The enzyme may be completely synthesized at the cell surface and released;

b) Inactive precursors may be transported through the membrane, assembled at the surface and released as active molecules;

c) The protein moiety may be synthesized elsewhere and simply attach to a carbohydrate portion at the cell surface.

Whatever the mechanism it is apparent that there is no accumulation of the active molecule but rather a formation (or activation) and an almost immediate release, since the amount liberated per unit cell far exceeds that contained in the walls themselves. The concept of the wall as an active biological entity is a unique one and merits further study. Studies with labelled antibody could yield valuable information concerning the exact location of the enzyme in the wall.

Although much has been learned from these investigations, there are many more problems to be resolved. Indeed the study has perhaps left more questions asked than it has answered. The structure and linkage of the carbohydrate moiety and its function, the involvement of the wall in enzyme formation and release, and the active sites of the enzyme are some of the more interesting aspects and are worthy of further investigation.

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