EFFECT OF POLYACRYLAMIDES ON GROWTH AND CERTAIN ENZYME ACTIVITIES OF SEVERAL *PSEUDOMONAS* STRAINS

By

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CHAPTER I

INTRODUCTION

Polyacrylamide, polymers of acrylamide or copolymers of acrylamide and acrylic acid, with very high molecular weight are water soluble materials producing highly viscous solutions. These solutions are useful in the micellar-polymer process of enhanced oil recovery as mobility control agents. To be useful in enhanced oil recovery, a polymer must maintain its properties, including viscosity, for long periods of time at relatively high temperatures under various conditions of pressure, pH, ionic strength and flow rate (McCormick et al., 1979).

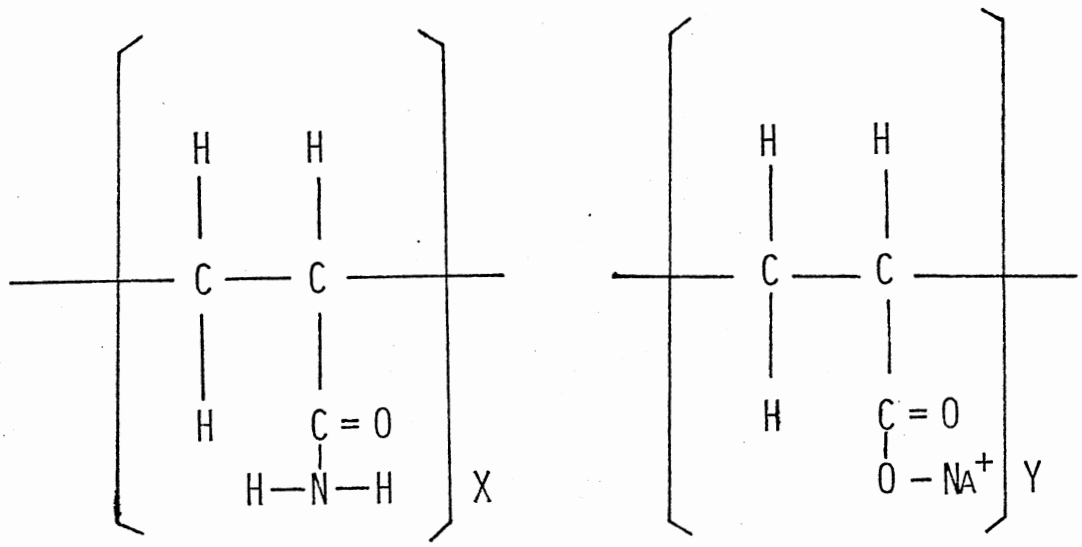
Along with the widespread use of these synthetic chemical compounds in recent years, there has developed a great concern for the deposit and ultimate fate of these chemicals once been used. Most of these synthetic compounds have been considered to be resistant to biodegradation. Many microorganisms are capable of attacking a relatively long hydrocarbon chain; those with 10 to 18 carbon atoms are attacked with greatest rapidity and frequency, usually by means of enzymes termed oxygenases (Klug and Markovetz, 1971). However, extremely high molecular weight compounds consisting of a linear chain of $-CH_2-$ groups, e.g., polyacrylamides are difficult to degrade. For this reason it would be advisable to prevent them from entering any ecosystem to avoid ecological damage. A study has been made on the toxicity of these polymers used in enhanced oil recovery (McCormick et al., 1979). Polyacrylamide polymers used as mobility

control agents can be considered to be essentially non-toxic. However , some health effects may occur due to the physical nature of the compounds used (i.e.,broth or powder). Polyacrylamide powders are implicated as respiratory hazards because exposure to the polymer powders occurs at EOR facilities. All water soluble polyacrylamides are suspected to have local carcinogenic ability when implanted in the skin of laboratory animals. The study of long-term environmental effects has not been made. The Department of Energy has been interested in the biodegradability of these synthetic polymers; this sponsorship has led to this research study.

The structure of polyacrylamide is shown in Figure 1. Acrylamide: $CH_2CHCONH_2$ is the parent compound of a large class of monomers (Thomas, 1964). In the presence of free radicals, acrylamide polymerizes rapidly to the high molecular weight polymers. The polymers may vary in molecular weight (in the range 10^6-10^7 daltons), degree of hydrolysis of amide groups, and in physical properties, such as viscosity of aqueous solutions and stability of solutions under conditions of high temperature, pressure, salinity, or pH.

Pitter (1976) has made studies of biodegradability of a number of organic substances. His means of evaluating degradability was dependent on measuring the decrease in organic carbon in a medium containing the substances being tested as a sole source of carbon. Inocula consisted of adapted activated sludge taken from sewage plant. He was able to measure the per cent removal of a compound obtained when the system reached equilibrium (i.e., no more decrease of COD-chemical oxygen demand occurred). From these data he was able to determine the rate of biodegradation, i.e., mg COD removal $g^{-1}h^{-1}$. Compounds were evaluated as being readily biodegradable, or biologically hard to decompose.

Figure 1. The Structure of Polyacrylamide. A copolymer of acrylic acid and acrylamide.



The Principle of Microbial Infallibility (Alexander, 1965) states that any molecule synthesized by a living organism can be degraded by some microorganism somewhere. It is difficult to prove the truth of this principle. If the range of compounds is extended to include those synthesized only in the laboratory, the probability of its being true is greatly diminished.

Pitter concluded that factors affecting biodegradability of a compound in nature or in the environment, can be divided into three groups: 1) physical-chemical factors (temperature, solubility, degree of dispersion of the compound in the medium, pH, dissolved oxygen); 2) biological factors (history of the microbial culture, its age, manner and time of its adaptation, toxicity of the compound, effect of other substrates); 3) chemical factors (size of molecule, length of chain, kind, number, and position of substituents in the molecule, stereochemistry).

Hains and Alexander (1975) have studied the biodegradability of polyethylene glycols. Early research demonstrated that polyethylene glycols were recalcitrant as shown by BOD and short term activated sludge test, but recent studies using long term adapted biomass and a pure culture haye reyealed that higher molecular weight polyethylene glycols can be degraded. From these studies, we might be tempted to suggest that most recalcitrant polyacrylamide compounds may be degradable if given the appropriate culture conditions.

Since the structural conformation of these polymers are cross-linked in aqueous solution, the breakdown products and the degradation mechanism should be determined and evaluated regarding its effects on biodegradability.

In determining the biodegradability of substances used in enhanced

oil recovery, the most valid approach would be to determine if the soil contained microbial cultures (mixed or pure) capable degrading the synthetic chemical compounds. Payne (1970) suggested that presence of such cultures in a given soil could be determined by serial transfer enrichment technique.

From the biodegradability screening, we observed that a number of these polymers, as a sole source of carbon-energy in enrichment culture, would enhance the growth of soil bacteria, often increasing the viable cell count 100-fold over what it was without the polymer (Grula, 1979). Pure cultures isolated from carbon-energy enrichments were always members of the genus Pseudomonas. Later a pure culture of Pseudomonas aeruginosa was isolated from enrichment media in which the polymer was the sole nitrogen compound (Grula,1979). This ability to enhance growth of microorganisms may have occurred because of spontaneous or enzymatic hydrolysis of the polymer molecule. In either case, ammonia would be produced from the amide groups of the polymer. We plan to determine: first, the degree of growth stimulation by the polymer of various strains of Pseudomonas under various conditions, and second, to determine if resting (non-growing but metabolizing) cells are able to release ammonia from the polymer. Such a release would be tentative evidence for the existence of an extracellular enzyme capable of catalyzing ammonia hydrolysis.

In addition, some experiments were done which related to the loss of viscosity (screen factor) by solutions of the polymer possibly resulting from microbial growth. Loss of screen factor (related to but distinct from true viscosity (Jennings et al., 1976) in the oil reservoir, with resultant loss of mobility, is a problem of considerable practical significance.

CHAPTER II

LITERATURE REVIEW

Most of the studies on microbial amidases have been concerned with enzymes which attack glutamine (McIlwain, 1948), nicotinamide (Halpen and Crossowiz, 1957). Amidases which attack the aliphatic amides formamide and acetamide have been reported in Mycobacteria spp. by Halpen and Crossowiz (1957). It was reported that some Pseudomonas spp. could use aliphatic amides as the sole source of carbon and nitrogen, e.g. Pseudomonas aminovorans grew on propionamide, caproamide, lactamide or succinamide. Kelly and Clarke (1962) reported Pseudomonas aeruginosa produces an aliphatic amidase which is induced by several substrate and non-substrate amides. The inducible aliphatic enzyme produced by wild-type strains of Pseudomonas aeruginosa enables them to grow with acetamide or propionamide as the sole source of carbon and nitrogen. The enzyme is relatively specific and hydrolyzes butyramide at only 2 per cent of the acetamide rate while the higher aliphatic amides are not cleaved to any significant extent.

Mutant strains have been isolated with altered amide growth phenotypes including some which are constitutive for the synthesis of the wild-type amidase and others which are constitutive and produce amidases with altered substrate specificities.

Brammar, Clarke and Skinner (1967) described magno-constitutive mutant C11 which is unable to grow on butyramide and produces a consti-

tutive enzyme indistinguishable from the wild-type amidase. Brown, Brown and Clarke (1969) described mutants able to utilize butyramide and valeramide which produced amidases with altered substrate specificities. Brown and Clarke (1972) isolated mutants which utilized the N-substituted amide acetamide (N-phenylacetamide) as carbon source. The amidases produced by one of these strains differed from the wild-type enzyme by a single amino acid sustitution.

Brown and Clarke (1970) isolated regulatory mutants which were resistant to repression by amide analogues. Betz and Clarke (1971) isolated phenylacetamide-utilizing strains of Pseudomonas aeruginosa and concluded that if mutant strains were required to attack specific amides it might be advantageous to select for them on a medium containing an amide with a larger side-chain than the required substrate.

Kelly and Kornberg (1962) reported the amidase from Pseudomonas aeruginosa is a multi-headed enzyme which catalyzed two enzymic reaction at different sites. First is the hydrolysis of several aliphatic amides as shown below:

$$R\cdot CONH_2 + HOH \longrightarrow R\cdot COOH + NH_3$$

Second is the amide transferase which can catalyze the transfer of the acyl moiety of short-chain aliphatic amides to hydroxylamine as shown below:

$$R\cdot CONH_2 + NH_2OH \longrightarrow R\cdot CONHOH + NH_3$$

Brown et al. (1973) reported that Pseudomonas aeruginosa amidase is an oligomeric protain, molecular weight 200,000, comprising 6 protomers each of molecular weight 33,000.

Hains and Alexander (1975) described a strain of Pseudomonas aeruginosa obtained from enrichment cultures with polyethylene glycols 20,000 (a polymer with an average molecular weight of 20,000) as the carbon source which excreted on extracellular enzyme which converted low and high molecular weight polyethylene glycols to a product utilized by washed Pseudomonas aeruginosa cells.

An important feature of these polymers is their size, and it is known that the very high-molecular-weight polymers are too large to penetrate the bacterial cell. Because of this lack of penetration of the polymer into the cell, it is likely that the degradation and utilization of the polymer is initiated by an extracellular enzyme.

CHAPTER III

MATERIALS AND METHODS

Test Organism

The microorganisms used in this study were isolated from enrichment cultures using soil as an inoculum with different polymers as substrates. These organisms were purified and some of these were identified as Pseudomonas aeruginosa. They are gram-negative, motile, short rods which form smooth, raised colonies on nutrient agar. Pseudomonas aeruginosa exhibits a positive reaction for oxidase, catalase, gelatin hydrolysis, no intracellular accumulation of poly-β-hydroxybutyrate, no production of slime from sucrose, arginine dihydrolase production and ability to grow at 41^0 C. Polar flagellation- a single polar flagellum- is a primary criterion of the genus Pseudomonas. Pseudomonas aeruginosa is placed in the group of fluorescent pseudomonads. These organisms produce a yellow-green water soluble pigment which fluoresences under ultraviolet light. In addition, typical strains of Pseudomonas aeruginosa produce the blue phenozine pigment pyocyanin. Some of our strains produced the yellow-green fluorescent pigment when grown on the basal chemically defined medium; however, none produced pyocynin under our conditions. Aility to grow at 41^0 C would exclude Pseudomonas fluorescens and Pseudomonas putida, other members of this group. Therefore we will refer to our strains as Pseudomonas aeruginosa, recognizing that they may be atypical in some respects.

Stock cultures of organisms isolated were maintained on tryptic soy agar slants and stored at 4^0 C. The properties of the strains we have isolated and used are listed in Table I.

Media and Growth of Cultures

Composition of the chemically defined basal medium used for growth study is given in Table II. The soil extract was prepared by adding 10 g wet weight of the soil into a flask containing 50 ml distilled water. This was autoclaved and cheesecloth filtered and the solution was collected and kept in a sterile flask.

In experiments done in an attempt to restore enzyme activity, two ml of soil extract were added to the complete chemically defined basal medium with polymer J279; a suspension of cells was inoculated; incubation was for 24 hours on 37^0 C shaker. Two ml of the culture was transferred to the second flask with the complete chemically defined basal medium, polymer J279 and soil extract. Culture was serially transferred three times, the organism was reisolated, purified and the enzyme activity was checked.

The inoculum for all growth experiments was prepared by washing the cells of a 24 hour old slant culture with saline twice and resuspending in 5 ml saline and standardizing to 1.0 absorbance at 540 nm. The inoculum used in growth experiments was one drop in 5 ml medium. Erlenmeyer flasks (250 ml) containing 50 ml of the medium were inoculated with the standardized cell suspension and incubated at 37^0 C on a reciprocal shaker (100 oscillations per minute). The degree of growth was determined by measuring the optical density of the bacterial suspensions on Bausch and Lomb Spectronic 20 Spectrophotometer at 540 nm; using cuvettes 10 mm in diameter. The chemically defined basal medium without inoculum was

TABLE I

CHARACTERISTICS OF ORGANISMS ISOLATED
FROM EOR ENRICHMENT CULTURES

	Pseudomonas aeruginosa								*Pseudomonas delafieldii*	*Pseudomonas pseudomallei*
	AA	mAA	2R	Y	F	L-1	D	Q-1	1R	J2
Fluorescent pigment	+	+	+	+	+	+	-	+	-	-
Growth at 41^0 C	+	+	+	+	+	+	+	+	-	-
Slime Production from Sucrose	-	-	-	-	-	-	-	-	-	+
Arginine dihydrolase	+	+	+	+	+	+	+	+	-	-
Ability to grow on DL-β-hydroxybutyrate	-	-	-	-	-	-	-	-	+	-
Oxidase	+	+	+	+	+	+	+	+	+	+
Catalase	+	+	+	+	+	+	+	+	+	+
Denitrification	+	+	+	+	+	+	+	+	-	+
Hydrolysis of:										
Gelatin	+	+	+	+	+	+	+	-	-	+
Starch	-	-	-	-	-	-	-	-	-	+
Lipid	-	-	-	-	-	-	-	-	+	+

TABLE II

COMPOSITION OF BASAL CHEMICALLY DEFINED MEDIUM USED IN ENRICHMENT CULTURES

Substance	Amount per 100 ML
NH_4Cl	400 MG
$MgSO_4 \cdot 7H_2O$	3 MG
KH_2PO_4	136 MG
K_2HPO_4	174 MG
Trace Minerals:	
H_3BO_3	0.5 ug
$CaCO_3$	10.0 ug
$CuSO_4 \cdot 5H_2O$	1.0 ug
$FeSO_4(NH_4)_2SO_4 \cdot 6H_2O$	50.0 ug
KI	2.0 ug
$MnSO_4 \cdot H_2O$	2.0 ug
MoO_3	5.0 ug
$ZnSO_4 \cdot 7H_2O$	5.0 ug

used to set instrument at 0 absorbance (100% T). The pH of the cultures was measured on Orion Research digital ionalyzer 501. For certain experiments, pH values of the culture were adjusted to 7.0 at 6, 9, 12 hour incubation time using 0.1 N sodium hydroxide or hydrochloric acid, Large quantities of cells were needed for isolation of enzyme and these cells were grown in Fernbach flask (2800 ml) containing 500 ml of the growth medium. The cells were harvested by centrifugation (5,000 x g for 10 min) and washed twice with tris-buffer or phosphate buffer for enzyme assay.

Chemicals

Polyacrylamide, in dry form, is a white, free flowing, amorphous solid. Its solid bulk density is 46 lb/cu.ft.. The pH of the 0.5 per cent solution at 20^0 C is 10.1. It is non-toxic, non-corrosive and slightly hygroscopic. It is irritating to the eyes and mucous membranes. It has very low toxicity when ingested. No adverse affects of toxicological significance have been observed when handled by workers in typical applications (McCormic et al., 1979). Polyacrylamides used were obtained from Dowell Division, Dow Chemical Company, Tulsa, Oklahoma. Molecular weights, degrees of hydrolysis, and designations of polymers are given in Table III. The acetamide, propionamide, butyramide were obtained from Aldrich Chemical Company. The Carbowax 6,000 used were obtained from Fisher Scientific Company.

The polymer solutions were prepared as stock solution and stored at 4^0 C. The preparation was made by uniformly sprinkling dry powder into a sterile flask with distilled water and magnetically driven stirrer. A well developed vortex occurs within 60 seconds. A satisfactory dispersion can be obtained with such a stirrer. As soon as all the polymer has

TABLE III

THE PROPERTIES OF POLYACRYLAMIDES USED IN BIODEGRADATION STUDIES

Designation	Molecular Weight	Degree of Hydrolysis (per cent)
J 332	$9\text{-}10 \times 10^6$	25-35
J 333	$6\text{-}\ 7 \times 10^6$	25-35
J 334	$3\text{-}\ 4 \times 10^6$	25-35
J 279	3×10^6	1-4
Polyacrylamide D	4×10^6	8-10
Cationic PAA	4×10^6	(Reacted PAA-none)

been added, the stirrer is slowed to a speed of 60 to 80 rpm to avoid mechanical degradation. After 2 or 3 hours the stirrer should be turned off. The solutions were allowed to stand overnight and stirred about 20 minutes before using (Jennings et al., 1976).

Nessler reagent was prepared by dissolving 50g KI in 50 ml H_2O and adding saturated mercuric chloride solutions until a permanent precipitate just formed. Four hundred milliliters 9N sodium hydroxide was added and solution was diluted to 1 liter. After standing for a few minutes, decant clear liquid and keep in the dark.

Methods of Chemical Analysis

Analysis for Ammonia

The assay for the determination of enzyme activity was based on the amide hydrolysis reaction (Kelly and Clarke, 1962). The nature of the reaction is described as below:

$$R \cdot CONH_2 + HOH \longrightarrow R \cdot COOH + NH_3$$

The ammonia released from the amide group was determined by reacting with Nessler reagent. Spectrophotometric procedure (absorbance at 450 nm) is used to quantitate the ammonia which reacts with Nessler reagent to produce yellow to brown color complexes (Kolthoff and Sandell, 1938).

Preparation of the Standard Curve

A ammonium chloride stock solutions was prepared by dissolving 0.3819 g ammonium chloride in 100 ml distilled water. One ml of this stock solution was diluted to 100 ml to make a standard solution. Graded amounts of standard ammonium chloride solution were added to a final volume 9 ml and

1 ml Nessler reagent was added, tubes were allowed to stand 30 minutes, after which the absorbance was read in a Bausch and Lomb Spectronic 20 Spectrophotometer at 450 nm. A standard curve of ammonium was prepared (Figure 2). Certain polyacrylamides interfered to some extent (above 1%) with color development; a separate standard curve prepared with polymer in the test medium was used when necessary. A final concentration of polymer in the solution below 1.0 per cent does not interfere with color development in the solution (Figure 2).

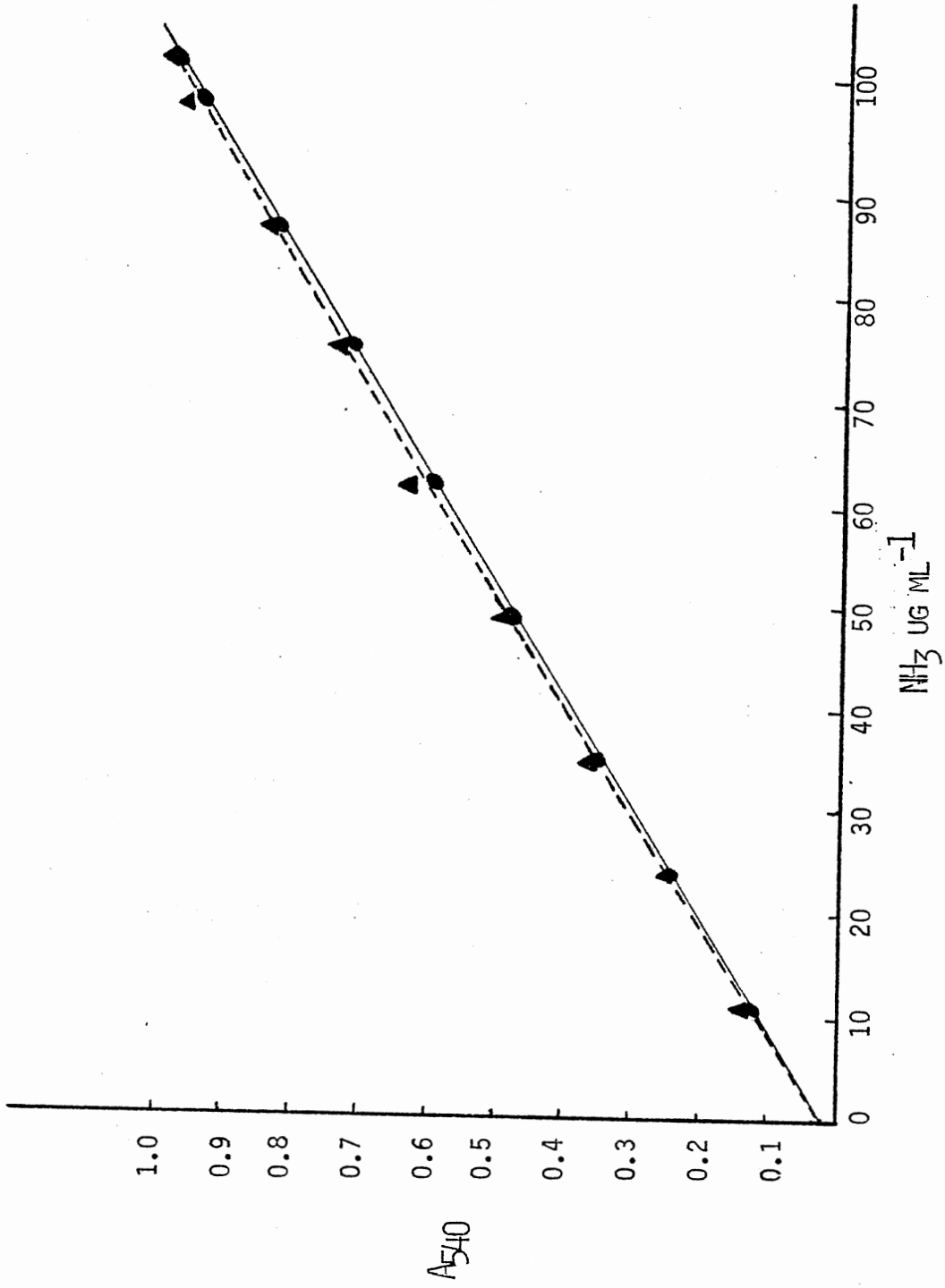
Enzyme Assay

Assays for amidase-type enzymes, capable of releasing NH_3 from polymers or from low molecular weight aliphatic amides, were carried by mixing equal volume of washed cell suspension, substrate, tris-buffer (0.1M, pH7.2), with final 1.0 absorbance at 540 nm at 37^0 C water bath shaker for one hour. All cell suspension used in enzyme assay was standardized to 1.0 absorbance at 540 nm by adding or removing buffer. The enzyme assay was carried out with same amounts of cells and the time running was 60 minutes. Cells were centrifuged down at 5,000 x g for 10 minutes. The supernatant was used for enzyme assay. Eight ml of distilled water and one ml Nessler reagent were added to 1 ml of the supernatant (from enzyme assay mixture) and allowed to stand 30 minutes. The absorbance at 450 nm was measured. Substrate (no cells), endogenous (no substrate) and boiled cells controls were always run.

Measurement of Viscosity

We measured screen factor (Jennings et al., 1976) of our polymer solutions rather than true viscosity because, according to reservoir

Figure 2. The Standard Curve of Ammonium. Used to determine the micrograms of ammonium produced from amide group of polymer by certain enzyme. The dash line represents the standard curve with 0.1 per cent polymer-▲. The solid line is the standard curve without polymer-●.


1.0
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
A540
0
10
20
30
40
50
60
70
80
90
100
NH3 UG ML-1

engineers, the screen factor of a polymer solution is a better indicator of its ability to act as mobility control agent in an oil reservoir. Screen factor was measured using a screen viscometer*. A diagram of a cross section of a screen viscometer is shown in Figure 3. The polymer solution was prepared as described above and filtered by using filter paper under a few inches of gravity head to avoid plugging of the screen. The polymer solution was loaded onto the bulb in the viscometer by using 10" water pressure vacuum to avoid the degradation by pulling the solution through the screens too rapidly.

The time required for the polymer solution to run from the upper decal line to the lower decal line on two bulbs is the flow time. A standard temperature of 25^0 C has been used for studies, The screen factor is described as below:

$$F_s = \frac{t_p}{t_s} F_t$$

$$F_t = 1.00 + 0.017\ (T-25)$$

At 25^oC

$$F_s = \frac{t_p}{t_s}$$

F_s = Screen Factor

F_t = Temperature Correction Factor

t_p = Polymer Solution Flow Time, Seconds

t_s = Solvent Flow Time, Seconds, Solvent (distilled water)

Shear Degradation of Polymer

Polyacrylamides can be degraded by shear stress and result in smaller molecular weight molecule.

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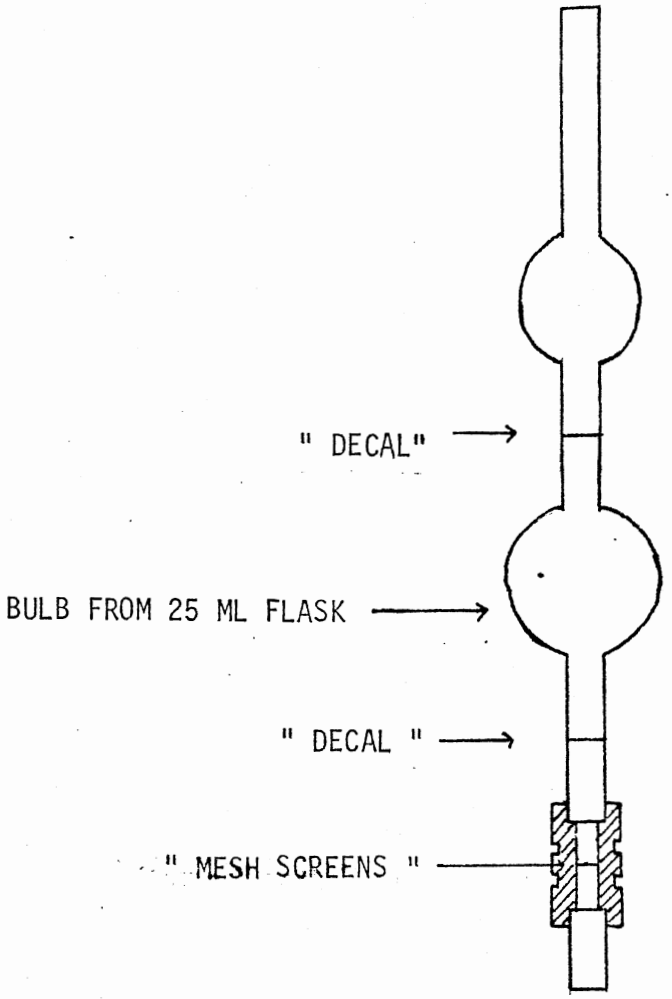
*Courtesy of Dowell Division, Dow Chemical Company

Figure 3. Screen Viscometer.


" DECAL"
BULB FROM 25 ML FLASK
" DECAL "
" MESH SCREENS "

Shear stress is a mechanical stress-any movement of the solvent tends to cause a mechanical stress which breaks the covalent bonds of the long $-CH_2-$ chain of the polymer. This results in low molecular weight smaller fragments. These small fragments result in a low viscosity (screen factor) of the solution. Agitation or violent stirring of a polyacrylamide solution in a Waring Blendor will lower the screen factor of a polyacrylamide solution to close to 1.0 in a short time (5-10 minutes). Even gentle agitation can cause some loss of screen factor, and the possibility of such a loss must always be kept in mind in doing experiments with polyacrylamides.

Concentrations of polymer J279 varying from 0.05 per cent to 0.5 per cent were passed through a screen viscometer before and after being placed for 5 minutes in Waring Blendor. The time in seconds for the specified volume of each solution to pass through the viscometer are given in Table IV.

Effect of Degradation of Polymer on Interference with Nessler Reaction

According to the standard curve of ammonia with polymer, 0.5 ml of 0.1 per cent J279 does not interfere with color development in the Nessler reaction.

The ammonia standard curve with either blendored or unblendored polymer is indistinguishable from the standard curve with ammonia alone. The above was true for a concentration of 0.5 per cent J279. However, with 1.0 per cent J279, unblendored polymer interfered very significantly with Nessler reaction. Blendored (shear-degraded) polymer mixed with standard NH_4Cl solutions yielded strange results in that, at low levels

TABLE IV

THE EFFECT OF DEGRADING VARIOUS CONCENTRATIONS OF POLYMER J279 IN WARING BLENDOR ON SCREEN FACTOR

J279 Concentration per cent (w/v)	Blendored Screen Factor		Unblendored Screen Factor	
0.05	12.2	1.37	176.8	19.86
0.1	13.1	1.47	194.5	21.85
0.2	14.3	1.61	268.9	30.21
0.4	14.5	1.63	365.8	41.10
0.5	14.6	1.64	426.5	47.92

and no added NH_4Cl, $O.D_{450}$ values were unusually high (Table V). This would seem to indicate that shear stress by Waring Blendor releases free ammonia from the molecule; this compensate for interference with color development with higher concentration of NH_4Cl.

Isolation of Enzyme

Growth of Test Strain

Strain modified AA was used. After preliminary experiments showed that amidase activity of harvested cells fell off markedly if the pH of the medium were allowed to drop below 6.9, pH of the medium was adjusted at 6, 9, and 12 hours during growth to optimize condition for enzyme production. Large quantities of cells were prepared and harvested by centrifugation at $4^{o}C$, and 5,000 x g for 10 minutes. Culture supernatants were collected if it was desired to concentrate and analyze them for enzyme activity. The cells were washed twice with tris buffer (0.1 M, pH 7.2) and about 10 g of cells (wet weight) were suspended at $4^{o}C$ in 100 ml tris-buffer for ultrasonic extracts.

Concentrating the Spent Medium

The supernatant was put into dialysis bag (50 ml each) and was dialyzed aginst 40 per cent Carbowax 6,000 at $4^{o}C$ for 12 hours. After dialysis, the outside of the bag was washed three times in distilled water. Then, the concentrated supernatant was washed out by 20 ml tris buffer to make 10-fold concentrate. This concentrated supernatant was tested for enzyme activity on 0.1 per cent polymer J279 and 0.2 M propionamide.

TABLE V

NESSLER REACTION OF STANDARD AMOUNT OF NH_4Cl AND ADDED WITH 0.1%, 0.5%, 1.0% BLENDORED OR UNBLENDORED POLYMER J279

NH_3 ug ml^{-1}	A_{450}						
	Standard NH_4Cl	Added 0.1 per cent J279		Added 0.5 per cent J279		Added 1.0 per cent J279	
		Unblendored	Blendored	Unblendored	Blendored	Unblendored	Blendored
0	0	0.04	0.03	0.13	0.15	0.07	0.23
12.2	0.14	0.20	0.18	0.20	0.25	0.10	0.25
24.4	0.29	0.36	0.34	0.32	0.35	0.10	0.36
36.6	0.43	0.49	0.46	0.47	0.50	0.17	0.43
48.8	0.58	0.63	0.60	0.66	0.68	0.28	0.66
61.0	0.72	0.75	0.72	0.74	0.85	0.32	0.68
73.2	0.84	0.87	0.86	0.85	0.92	0.40	0.80
85.4	0.96	0.98	1.0	0.94	1.10	0.42	0.90
97.6	1.10	1.15	1.1	1.0	1.15	0.52	0.90
109.8	1.20	1.20	1.15	1.1	1.20	0.65	0.95

Preparation of Ultrasonic Extracts

The method of Brown, Brown and Clarke (1969) was used to prepare ultrasonic extracts. Twenty ml portions of cell suspension prepared as described above were exposed for 2 minutes (30 seconds bursts) at $0^{o}C$ to the output of a Branson Model 350 Sonifer at 4.0 A. The suspension of broken cells were combined and cell debris was removed by centrifugation at 25,000 x g for 20 minutes at $4^{o}C$. The supernatant was treated with bovine pancrease deoxyribonuclease and incubated at $25^{o}C$ for 15 minutes and then heated rapidly to $60^{o}C$ for 10 minutes and cooled rapidly at $0^{o}C$ for 5 minutes.

The heat-treated solutions were centrifuged at 11,000 x g for 20 minutes at $4^{o}C$ and the supernatant was fractionated by ammonium sulfate precipitation. The precipitate was collected by centrifugation (11,000 x g for 20 minutes) after 50, 55, 65, 75 per cent saturation with ammonium sulfate. At each stage, the solution was allowed to stand at $0^{o}C$ for 30 minutes before centrifugation. The precipitate of each fraction was dissolved in tris-buffer (0.1 M, pH 7.2) containing 0.15 M potassium chloride and dialyzed against two changes of tris-buffer and centrifuged at 15,000 x g for 10 minutes to remove any precipitate. Then each fraction was tested for enzyme activity on polymer J279 and propionamide.

Sephadex G-200 Column Fractionating
the Spent Medium

G-200 Sephadex column was prepared by weighing 14.3 g G-200 Sephadex and putting into 500 ml 0.1 M tris-buffer in 1000 ml beaker in a $100^{o}C$ water bath for 1 hour with shaking at 10 minutes intervals. After the beads were swollen, they were cooled down to room temperature, buffer

and beads were degassed, and column was poured and packed. Void volume and flow rate were checked using blue dextran 2000 and tryptophan for collection volume.

The concentrated spent medium of a culture of strain mAA was lyophilized. The resultant powder was resuspended in 3 ml tris-buffer and the suspension was loaded onto the G-200 Sephadex column. The reservoir, the UV monitor and the chart recorder were connected. Void volume was collected and 5 ml of each fraction was collected with total volume of 220 ml. Each fraction was tested for enzyme activity on polymer J279 and propionamide.

Analysis of Protein Content

The protein content of each fraction was determined using the Bio-Rad protein assay method.

CHAPTER IV

EXPERIMENTAL

The first experimental approach adopted in the attempt to find an explanation for the ability of polyacrylamide polymers to stimulate growth of soil microbes was to determine what nutritional role, if any, these polymers played in the growth of soil microbes.

Effects of Polymers on Growth of Various Strains

For initial nutritional tests, certain pseudomonads, strain 1R, 2R and AA, isolated from polyacrylamide soil enrichment cultures were chosen. I first attempted to find out if these strains could grow on the basal defined medium. Suspensions of washed cells from a 24 hour old nutrient agar slant were used as an inoculum. Primary tests showed that growth, as measured by turbidity at 24 hours, varied significantly with the carbon-energy source (glucose, xylose, and acetate were usually used in these tests) and with the inoculum size.

As is often the case with defined media, a small inoculum will distinguish different responses more clearly; thus we adopted one drop in 5 ml medium of a cell suspension of standardized turbidity as an inoculum in all experiments. Under these conditions, strain AA, 1R and 2R could grow on the defined medium abundantly with glucose as carbon source, and less abundantly with xylose or acetate.

Adding polymer J279 (at a final concentration of 0.2% w/v, or 2000 ppm) was not toxic for the strains, as shown by data in Table VI. Polymers as a sole source of nitrogen (NH_4Cl omitted from medium) supported a lower level of growth as shown in Table VII. Strain AA showed a significantly higher level of growth with all these carbon source than either strain 1R or 2R.

We next tested whether strain AA could continue to grow on serial transfer in the defined medium with polymer J279 as a sole nitrogen source. Transfers were made at 48 hour intervals, using one drop of inoculum. Results are given in Figure 4. It was found that J279 could sustain a certain level of growth on serial transfer, but less than the same medium with ammonium chloride. The ratio of growth attained with only J279 to that with J279 plus NH_4Cl varied with the substrate; this ratio was highest with acetate.

Using a similar test with strain 1R and 2R, it was found that they would grow on serial transfer with J279 as a sole nitrogen source, but only minimally.

In order to determine if other acrylamide polymers could support growth as a sole nitrogen source, we tried, using strain AA, 1R and 2R, polyacrylamide D (8-10% hydrolyzed), and a cationic polyacrylamide (Alkyl group substituted for H on amide). The cationic polyacrylamide did not support growth of any of the strains; this is not unexpected since the amide group is covered and there is no way ammonia can be released to support growth. The cationic polyacrylamide was not toxic for strain AA in the presence of NH_4Cl and glucose as a carbon source.

Polyacrylamide D supported a moderate amount of growth with all strains, and each of the three substrates on serial transfer. It was not

TABLE VI

EFFECTS OF 0.2% POLYACRYLAMIDE J279 IN COMPLETE MEDIUM ON GROWTH OF VARIOUS STRAIN

Strain	A_{540} Carbon Source		
	Glucose	Xylose	Acetate
AA	0.95	0.87	0.43
1R	1.40	0.70	0.41
2R	0.96	0.81	0.32

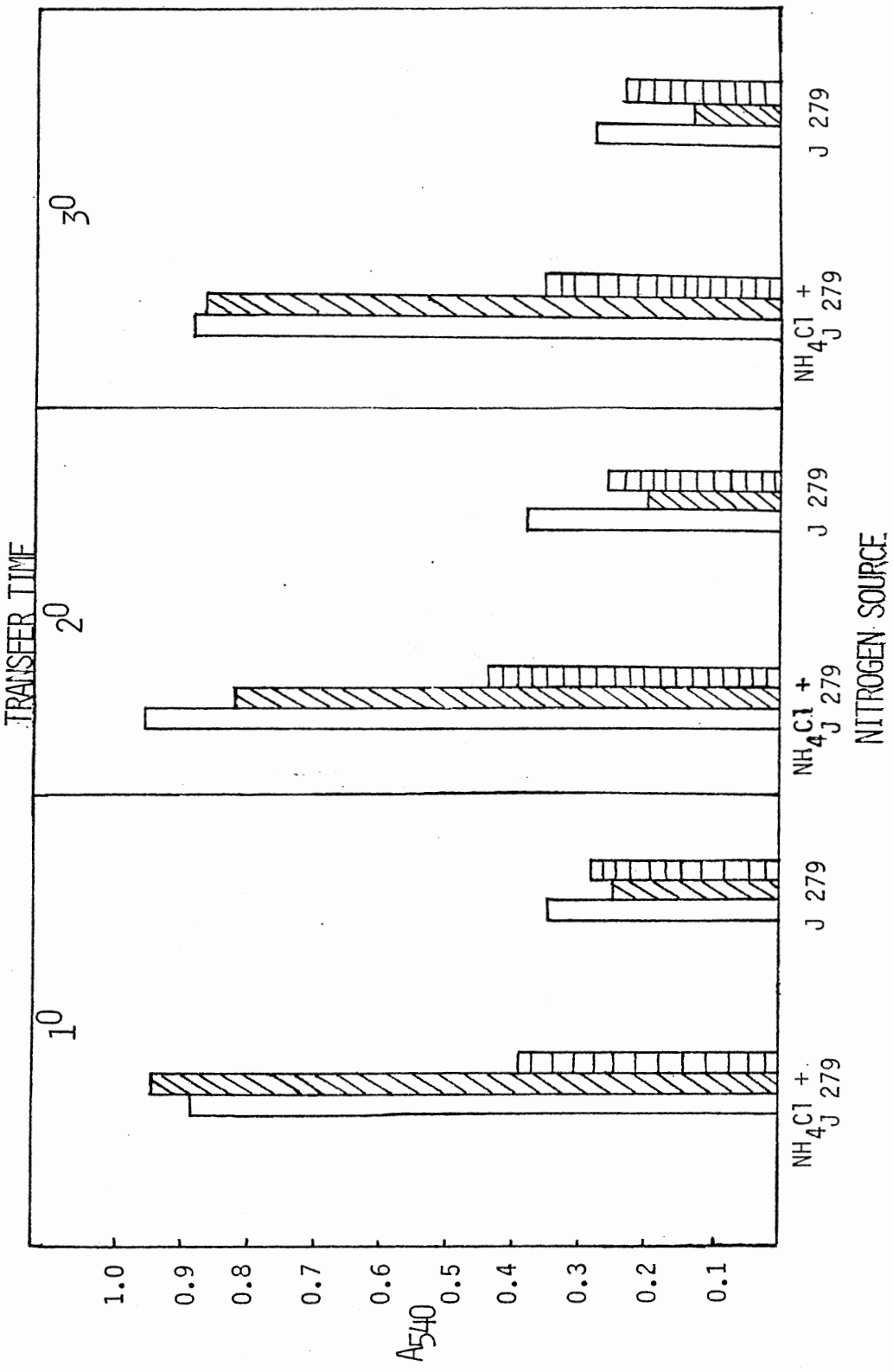
* Besides NH_4Cl, 0.2% J279 was added in each medium as a nitrogen source.

TABLE VII

GROWTH OF VARIOUS STRAIN WITH 0.2% POLYACRYLAMIDE J279 AS SOLE NITROGEN

Strain	A_{540} Carbon Source		
	Glucose	Xylose	Acetate
AA	0.29	0.25	0.27
1R	0.19	0.10	0.15
2R	0.15	0.13	0.19

Figure 4. Growth of Strain AA on Serial Transfer with Polymer J279 as Sole Nitrogen Source or with NH_4Cl and J279 as Nitrogen Source in the Medium. Glucose-□, Xylose-▨, Acetate-目.


TRANSFER TIME
1⁰
2⁰
3⁰
A540
1.0
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
NH4Cl + J 279
J 279
NH4Cl + J 279
J 279
NH4Cl + J 279
J 279
NITROGEN SOURCE

toxic under any of the conditions used. However, growth in none of the cultures was as high as it had been with polymer J279 and strain AA (Table VIII).

Growth as a Function of Polyacrylamide Concentration

Previous experiments have used a polymer concentration of 0.2%. Since an increased growth with increasing levels of polymer would tend to confirm its ability to serve as a sole nitrogen source for the test strains of Pseudomonas, an experiment to determine growth as a function of polymer concentration from 0.05% to 0.5% was done, using strain AA and three carbon source glucose, xylose and acetate (Table IX). Growth was a function of polymer concentration up to 0.5% with all three substrates. At this concentration, growth (Absorbance at 48 hours of serial culture) was more than half of the control (NH_4Cl added), with glucose and acetate, and almost half with xylose. Figure 5 illustrates growth of strain AA in the defined medium as a function of polymer concentration. Polyacrylamide D, tested in the same manner, showed essentially the same results. Polyacrylamide J333 (25%-35% hydrolyzed) was also tested in this manner (Figure 6). Results were the same, except that xylose was unable to serve as a carbon source, as it did in the case of J279 and D.

This was true even though NH_4Cl was added to the medium. Apparently xylose was toxic in the presence of J333. This was one of a number of observations we made that are hard to explain on the basis that the polyacrylamide acts only because of NH_3 released by spontaneous hydrolysis.

From the results of these tests we have concluded that certain polyacrylamides (as they are available commercially-no special purification)

TABLE VIII

GROWTH OF VARIOUS STRAIN WITH 0.2% POLYACRYLAMIDE D AS SOLE NITROGEN SOURCE OR WITH BOTH PAA D AND NH_4Cl AS NITROGEN SOURCE

Strain	A_{540}					
	Glucose		Xylose		Acetate	
	D	D+ NH_4Cl	D	D+ NH_4Cl	D	D+ NH_4Cl
AA	0.23	0.85	0.12	p.75	0.21	0.39
1R	0.20	1.10	0.13	0.75	0.16	0.37
2R	0.18	0.92	0.16	0.85	0.20	0.29

TABLE IX

GROWTH OF STRAIN AA ON DIFFERENT CONCENTRATION
OF POLYACRYLAMIDE J279 (0.05%-0.5%)
WITH THREE CARBON SOURCE

Polymer Concentration	Carbon Source		
per cent (w/v)	Glucose	Xylose	Acetate
0.05	0.07	0.06	0.07
0.1	0.16	0.10	0.12
0.2	0.29	0.18	0.21
0.4	0.43	0.20	0.34
0.5	0.47	0.28	0.36

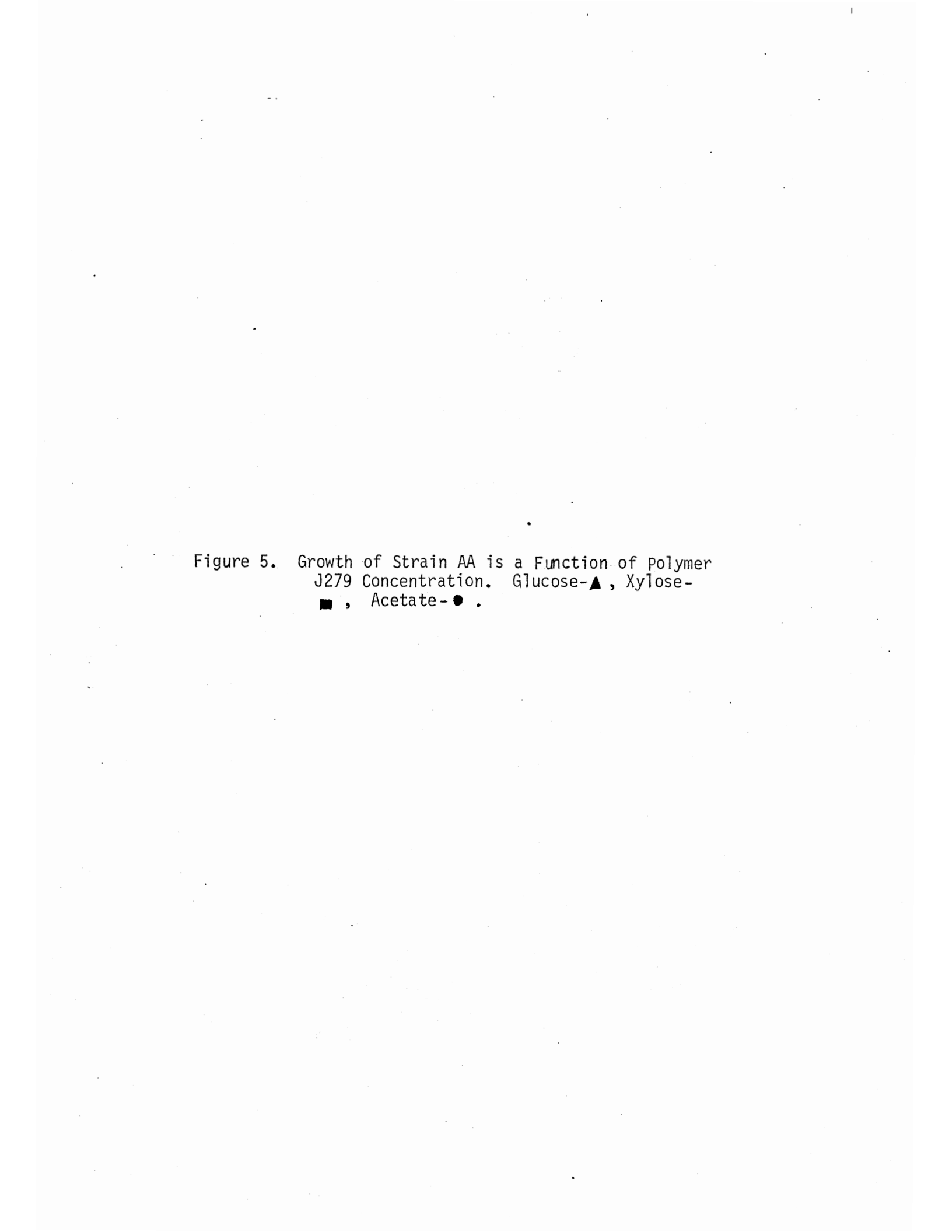


Figure 5. Growth of Strain AA is a Function of Polymer J279 Concentration. Glucose-▲, Xylose-■, Acetate-●.

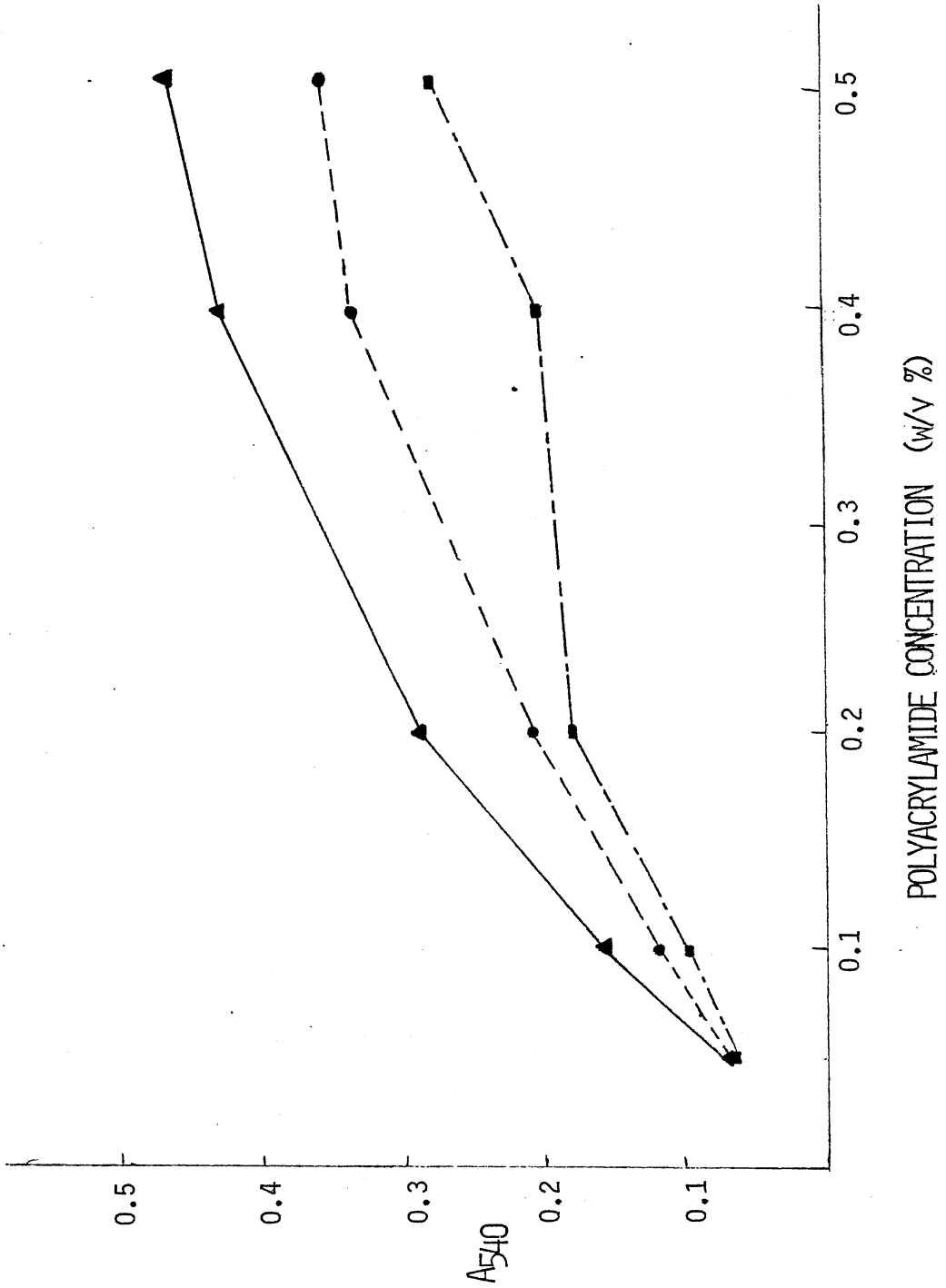
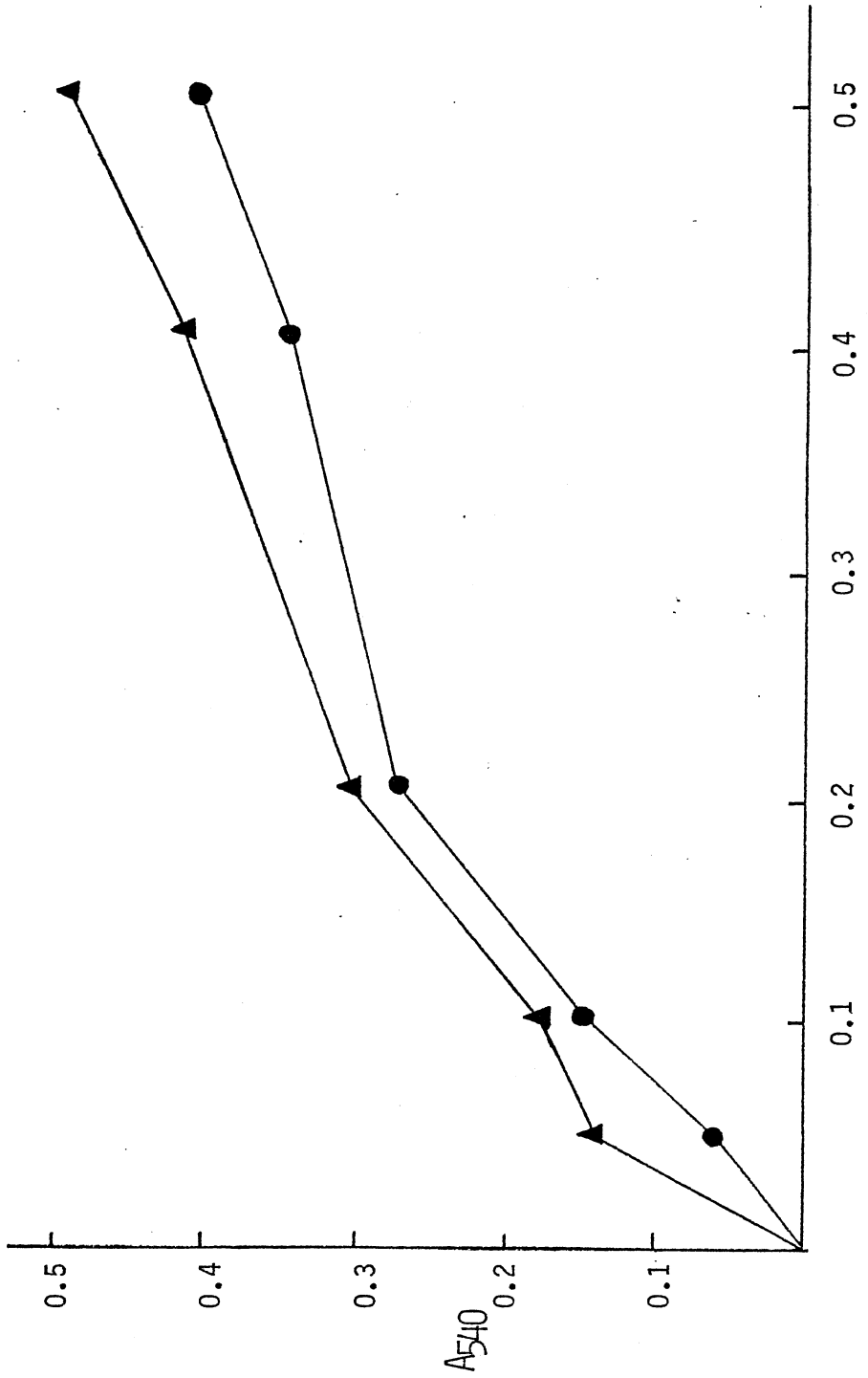

0.5
0.4
0.3
0.2
0.1
A540
0.1
0.2
0.3
0.4
0.5
POLYACRYLAMIDE CONCENTRATION (w/v %)

Figure 6. The Growth of Strain AA as A Function of Polymer J333 and D Concentration. Glucose was used as carbon source. J333-● , D-▲ .


0.5
0.4
0.3
0.2
0.1
A540
0.1
0.2
0.3
0.4
0.5

can indeed serve as a source of nitrogen for at least some strains of Pseudomonas. This could occur by either or both of two processes: 1) ammonia released by spontaneous hydrolysis of the amide groups of the polymer could be assimilated by the microorganisms, and the removal of the NH_4^+ groups would bring about more hydrolysis through the Law of Mass Action; or 2) an extracellular enzyme could catalyze hydrolysis of NH_4^+ which would be available for assimilation by the microorganisms. This hypothetical enzyme would have as a natural substrate some molecule other than the polyacrylamides. It would necessarily be extracellular because it is not possible for the high molecular weight polyacrylamides (molecular weight 10^6-10^7 daltons) to traverse the cell membrane or cell wall and get inside the cell.

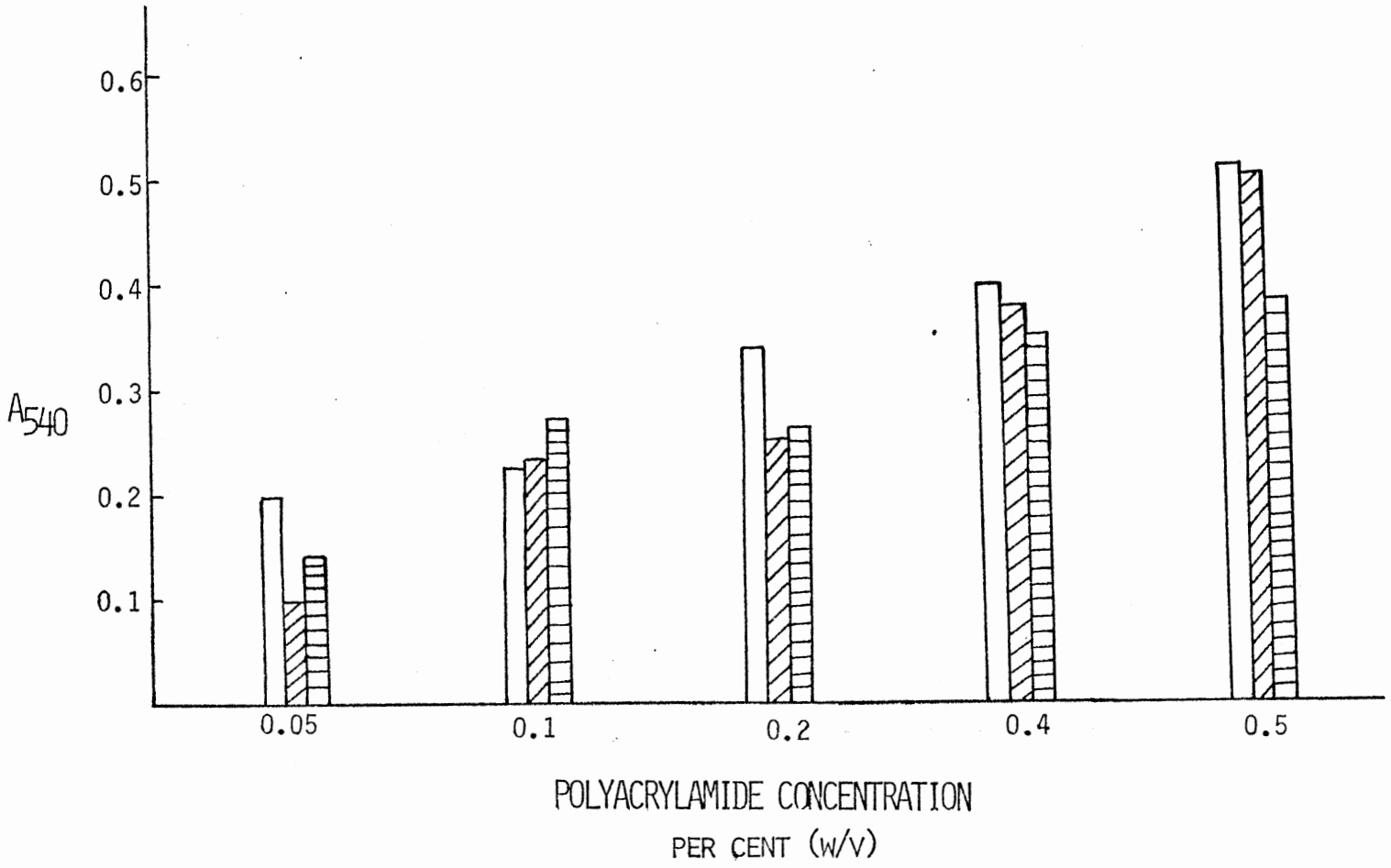
Attempts to Obtain Evidence for the Existence of an Enzyme Catalyzing Hydrolysis of NH_3 from Polyacrylamides

We first determined that polyacrylamide J279 at 0.5% in the uninoculated medium did not release a measurable amount of NH_3 when incubated for 48 hours at 37^0 C. This was true in the presence or in the absence of added NH_4Cl.

The fact that the level of growth attained was not affected by the stated degree of hydrolysis of the polymer (Figure 7) indicates either that an enzyme is involved or that assimilation of the NH_3 spontaneously released quickly stimulates the release of more NH_3. It cannot be concluded on this basis that such an enzyme exist.

We sought to determine if washed cells of a bacterial strain capable of growing on polyacrylamide could stimulate the release of ammonia from

Figure 7. The Growth of Strain AA is a Function of Polymer Concentration with Glucose as Carbon Source Polymer J279-□ , D-▨ , J333-目 .


0.6
0.5
0.4
0.3
0.2
0.1
A540
0.05
0.1
0.2
0.4
0.5
POLYACRYLAMIDE CONCENTRATION
PER CENT (W/V)

a polymer solution. Strain AA was chosen because of its relatively good growth on polyacrylamide medium. It was grown in the complete basal medium at 37° C for 24 hours. Harvested and washed cells (5 ml) were mixed with equal amount of 0.1% polyacrylamide J279 (only 1-4% hydrolyzed). No added buffer was used. Analysis of the supernatant after incubating 1 hour at 37°C with Nessler reagent yielded negative results.

To check the possibility that hypothetical enzyme was inducible. we next grew cells of strain AA in the complete basal medium containing 0.05% J279. In the enzyme assay of harvested cells, it was observed that cells had a very high endogenous activity.

A repeated test on uninoculated medium, using J334 and J279 in the medium at 0.5%, showed that in 48 hours at 37°C shaker, no detectable ammonia was released. It may be concluded that, in the absence of microbial cells, spontaneous hydrolysis of the amide linkage in these polymers is negligible in the time frame with which we are concerned,

A further test was done in the same manner as the previous one, except that the enzyme assay was done in a small flask instead of in a tube. This resulted in the production of a significant amount of ammonia by active cells in the presence of the polymer (Table X). Active cells resulted in production of 4.88 µg ammonia per ml; boiled cells did not produce a significant amount of ammonia. A second trial resulted in the release of 5.60 ug ammonia per ml, as compared to 1.22 for the endogenous control.

Using strain 2R instead of AA resulted in no detectable release of ammonia. This is important because it provides good evidence that the mere presence of bacterial cells does not necessarily cause release of ammonia from the polymer. Strain 1R also proved to be completely

TABLE X

THE AMMONIA RELEASING ACTIVITY OF STRAIN AA ON POLYACRYLAMIDE J279

Assay Mixture	NH_3 Released ug ml^{-1}
Cells + PAA	4.88
Cells + Saline	1.22
PAA + Saline	0
Boiled Cells + PAA	0
Boiled Cells + Saline	0

negative. Previous tests of the ability of strains 1R and 2R to grow on polymer as a nitrogen source had shown significantly less growth as J279, but about the same level of growth on polyacrylamide D (8-10% hydrolyzed) It is possible that differences between strain AA and strain 1R and 2R in growth on J279, and differences in ability to release ammonia from J279, may be related.

We next set out to determine activity (ammonia-releasing) of cells as a function of pH. To control pH, we added 0.1M phosphate buffer to the system, adjusted to the desired pH. A preliminary test indicated approximately fourfold higher activity at an initial pH of 7.0 than at 5.5 (it should be noted that in this trial, active cells plus buffer without polymer resulted in a relatively high $O.D_{450}$, indicating a high endogenous activity). Heat-killed cells plus polymer resulted in a relatively high $O.D_{540}$. This may indicate that boiled cells were not fully heat-inactivated. Extending the pH range covered, we found that use of buffers at pH 5.0, 8.0 or 8.5 resulted in essentially no activity. Apparent endogenous release of NH_3 was approximately the same at all pH values from 5.0 to 8.5.

Ammonia releasing activity of washed cells of strain AA on polymer J279 as a function of pH is shown in Figure 8. Amount of growth of strain AA in the defined medium as a function of initial pH of the medium is shown, using either J279 or NH_4Cl as a sole nitrogen source, in Figure 9. Growth is much heavier with NH_4Cl (as was expected); in both cases the optimum pH, in terms of O.D of culture at 48 hours, was 7.0.

At this point we have interpreted our results to mean that certain polyacrylamides can, in a chemically defined medium, serve as a sole nitrogen source for one strain of Pseudomonas. The polymer can

Figure 8. The pH-Activity Curve. The Ammonia-Releasing Activity of strain AA is a function of pH.

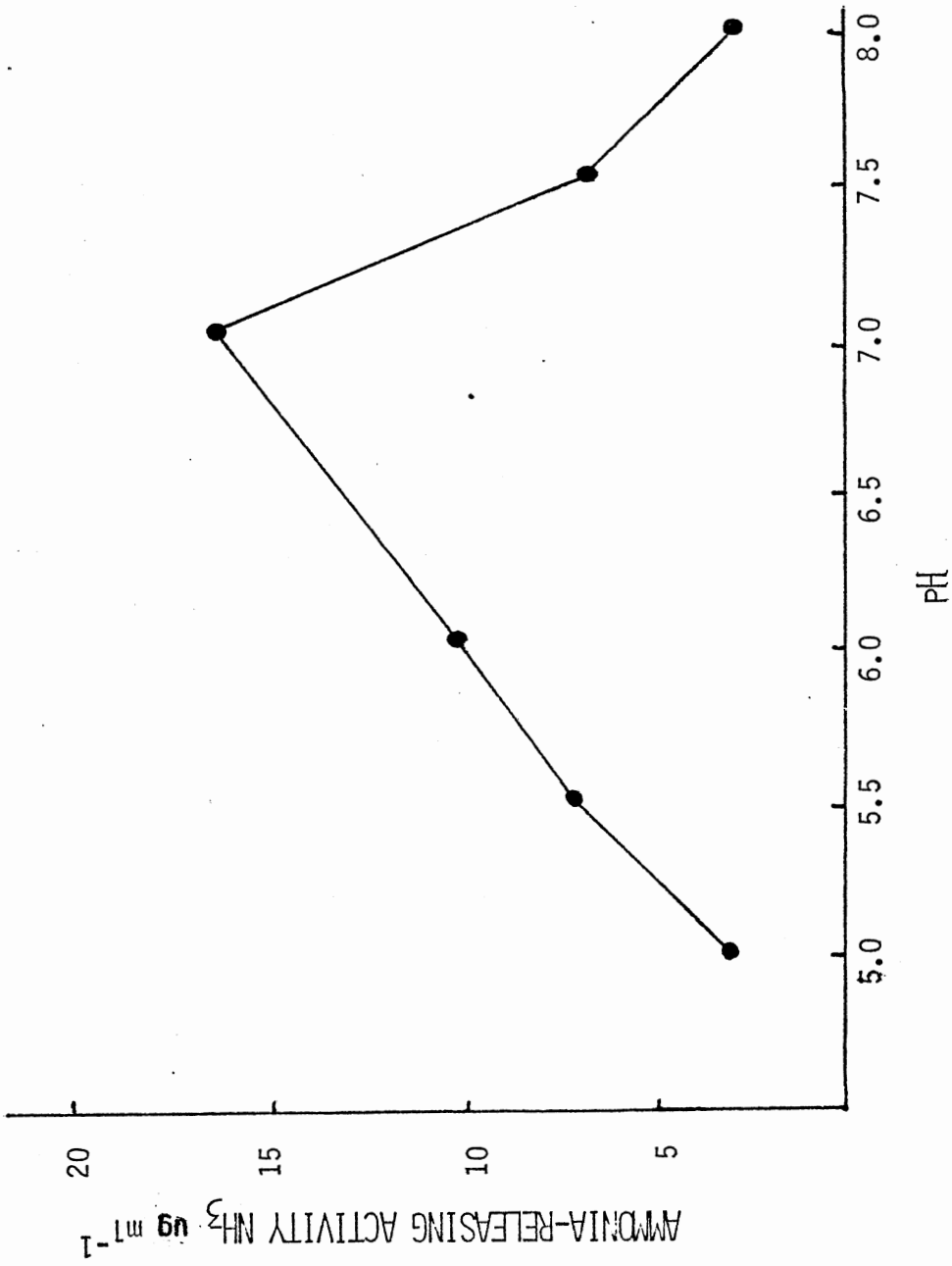
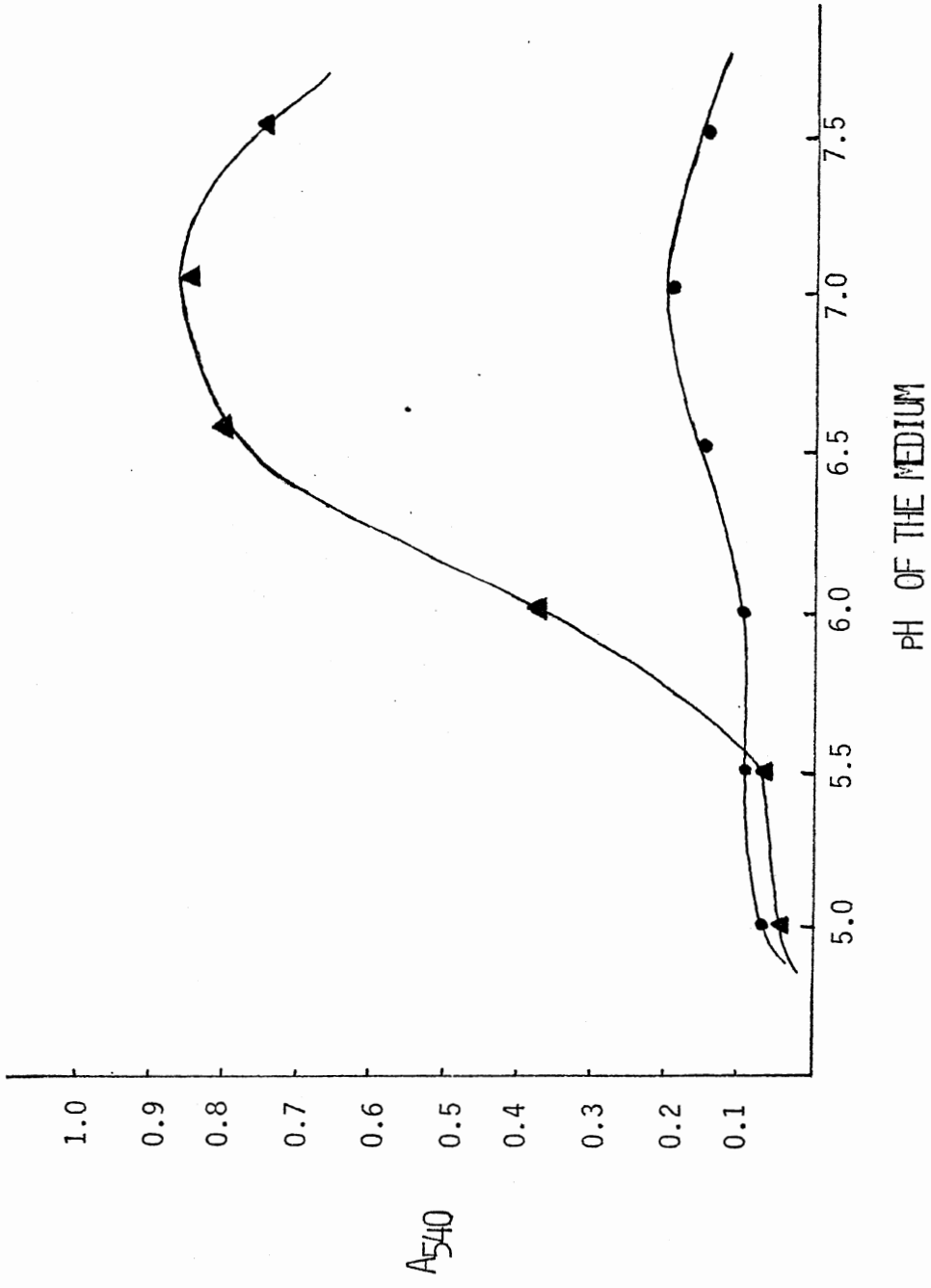

AMMONIA-RELEASING ACTIVITY NH_3 µg ml^{-1}
20
15
10
5
5.0
5.5
6.0
6.5
7.0
7.5
8.0
pH

Figure 9. Growth of Strain AA as a Function of pH of the Medium. Polymer or NH_4Cl was used as sole nitrogen source. Polymer J279-●, NH_4Cl -▲.


1.0
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
A540
5.0
5.5
6.0
6.5
7.0
7.5
PH OF THE MEDIUM

serve as a sole nitrogen source because the pseudomonad used can (at least to a small extent) enzymatically hydrolyze enough NH_4^+ from a polymer molecule to sustain a limited level of growth. This occurs with glucose, xylose, or acetate as carbon source (J279 as nitrogen source), or with glucose and acetate (J333 as nitrogen source). We felt it was of interest to determine if J279 could serve as a carbon source and nitrogen source in the defined medium; also, we tested the ability of xylose to serve as sole carbon source with NH_4Cl, or 0.1% J279, or both, in the medium.

Results from a three serial culture test showed that J279 could not support growth as a carbon and nitrogen source. With xylose as carbon source, NH_4Cl plus polymer supported good growth, polymer alone supported moderate growth, but NH_4Cl alone did not support growth. Data are given in Table XI.

This observation was repeated twice. At present we have no explanation. Xylose is adequate as sole carbon source (polymer cannot fill this role) if conditions are satisfactory, but with NH_4Cl as sole nitrogen source, they evidently are not satisfactory. These observation have not been satisfactorily explained, but they do seem to indicate that polyacrylamide does not support growth as a sole nitrogen source simply because of spontaneous hydrolysis or because of the presence of ammonia as an impurity in the polymer solutions.

Possibility of the Existence of an Enzyme Capable of Catalyzing the Release of Ammonia from the Polymer Molecule

We believe that we have established clearly that polyacrylamide

TABLE XI

THE EFFECTS OF CARBON SOURCE AND NITROGEN SOURCE ON GROWTH OF STRAIN AA

Carbon Source	Nirogen Source	A_{540}
Xylose	J 279	0.23
Xylose	NH_4Cl	0.03
Xylose	J 279 + NH_4Cl	0.91
—	J 279	0.04

solution support and stimulate growth of aerobic pseudomonads because they supply ammonia, assimilable nitrogen source for these microorganisms. These question remains, however, whether the ammonia (probably in form of NH_4^+ ion) is derived solely from spontaneous hydrolysis of the polymer, or if some extracellular enzyme produced by the microorganism catalyzes release of NH_4^+ to a greater extent than would occur by chemical action alone. The natural substrate of this hypothetical enzyme would not be polyacrylamide, since these polymers are strictly Xenobiotic, and do not have any role in life processes. We are suggesting that the amide linkage in polyacrylamides is similar enough to that in protein, or in low molecular weight amides (acetamide, propionamide, etc.) to be subject to hydrolysis to a certain degree. The monomer acrylamide is not known to be a substrate for any low molecular weight aliphatic amidase. This postulated enzyme would of necessity be extracellular, since there is no known way in which the large polyacrylamide molecule (molecular weight 10^6-10^7 daltons) can get inside a bacterial cell.

Evidence pointing to the existence of such an enzyme is circumstantial. It may be summed up as follows: 1) Different strains, all equally capable of utilizing ammonia as a sole nitrogen source, grow to different extents on the same polymer; 2) Extent of growth of a given strain is not dependent on the degree of hydrolysis of the polymer; 3) Ammonia releasing activity of active cells is heat-labile and is a function of pH over the range 6.0-8.0; 4) In one instance, a polymer with a high degree of hydrolysis (J333) would not support growth with xylose as a carbon source, whereas it did with glucose, or acetate, as a carbon source; 5) Strain mAA, which can utilize NH_4Cl as a sole nitrogen source with glucose as a carbon source, and which grows very well with xylose as carbon

source and polymer plus NH_4Cl as nitrogen source, cannot grow with xylose and NH_4Cl as sole carbon and nitrogen sources. It does grow moderately with xylose plus polymer (J279) as sole nitrogen source. If the polymer only functioned as a chemical reservoir of ammonia, such a situation would no exist; and 6) One of the most active bacterial strains in terms of growth on polyacrylamides and ability to release ammonia from J279 lost the latter activity after some months of culture on artificial media. This ability was restored (twice) by serial culture in the presence of soil extract.

On the other hand, the ability of a polymer solution within a dialysis bag suspended in the basal medium without any other nitrogen source to support growth* in the medium outside the bag, and the failure of growth of strain mAA in a medium prepared using dialyzed polymer solution (dialysis removes available ammonia) indicate that chemical or spontaneous hydrolysis is the means by which ammonia is released from polymer. It should be noted that while, both of these observations prove that spontaneous hydrolysis can be a means whereby ammonia is made available to the microorganism, they do not rule out the existence of an enzyme catalyzing such hydrolysis. We have already shown that an uninoculated solution of polymer does release a detectable amount of ammonia in 48 hours at 37^{o} C, with shaking (shaking provide a certain amount of shear stress, resulting in a lowered viscosity of the solution). Apparently the stated degree of hydrolysis is a stable characteristic of the polymer molecule. Only if ammonia in solution is being removed, as would occur if microbial assimilation were taking place, would the polymer hydrolyze further.

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*Experiment done by B.P. Crider, Oklahoma State University, Stillwater, Oklahoma, November 1979.

Many of the experiments to be described in the remainder of this thesis were designed to help decide between these alternatives.

Loss of the Ability of Strain AA to Release Ammonia from J279 Solution

This loss is summarized in data in the following table (Table XII). In each of these cases, cells were grown in the same way, cell suspensions were prepared in the same way, and were standardized such that about the same number of cells were used in each experiment.

We attempted to restore activity of AA cells by serial transfer in the basal medium containing glucose, J279 0.1%, soil extract (2 ml/50 ml medium) and NH_4Cl. NH_4Cl concentration was lowered to 0.02%, and a high level of polymer J279 was added in order to facilitate the selection of strains most able to benefit from the presence of the polymer, i.e., strains with an enzyme capable of catalyzing the release of ammonia from the polymer. Culture was transferred three times at 24 hours intervals; incubation was on a shaker at 37^{o} C. The first test proved to be negative; cells from the fourth serial culture failed to release a significant amount of ammonia. A test showed that strain AA could still grow on polymer J279 as a sole nitrogen source, with extent of growth still a function of polymer concentration.

Experiment was repeated later; again, strain AA was used. This time, cells harvested from the fourth serial culture produced a significant amount of ammonia from J279 (Table XIII). Again, a large amount of endogenous ammonia was released-a property absent from cells which had apparently lost ability to act on polymer.

The strain derived from this experiment will thereafter be referred

TABLE XII

THE LOSS OF AMMONIA RELEASING ABILITY OF STRAIN AA ON POLYACRYLAMIDE J279

Strain	NH_3 Released $\mu g\ ml^{-1}$	Date
AA	5.6	12-27-79
AA	4.27	1-10-80
AA	1.05	1-21-80
AA	0	1-30-80

TABLE XIII

THE AMMONIA RELEASING ACTIVITY OF STRAIN MODIFIED AA ON POLYMER J279

Assay Mixture	NH_3 Released ug ml^{-1}
Cells + PAA	4.27
Cells + Buffer	3.05
PAA + Buffer	0
Boiled Cells + PAA	0
Boiled Cells + Buffer	0

to as mAA (modified AA).

Properties of Strain AA and mAA as Compared at this Time

Strain AA although not producing cells able to release ammonia from J279 when grown in a glucose medium, did show some activity when cells were grown in the presence of xylose as a sole carbon source (Table IX). In this case endogenous activity was not extremely high.

In comparing NH_3 released by glucose-grown AA and mAA cells, the first test showed a significantly greater production of ammonia from polymer by strain mAA- an $O.D_{450}$ in the Nessler reaction of 0.08 as compared to 0.04 for the unmodified strain AA.

Experiments Relating Polymer Properties and Modificatios Thereof to Growth and Ammonia Releasing Activities of Microbial Strains

Dialysis of Polymer

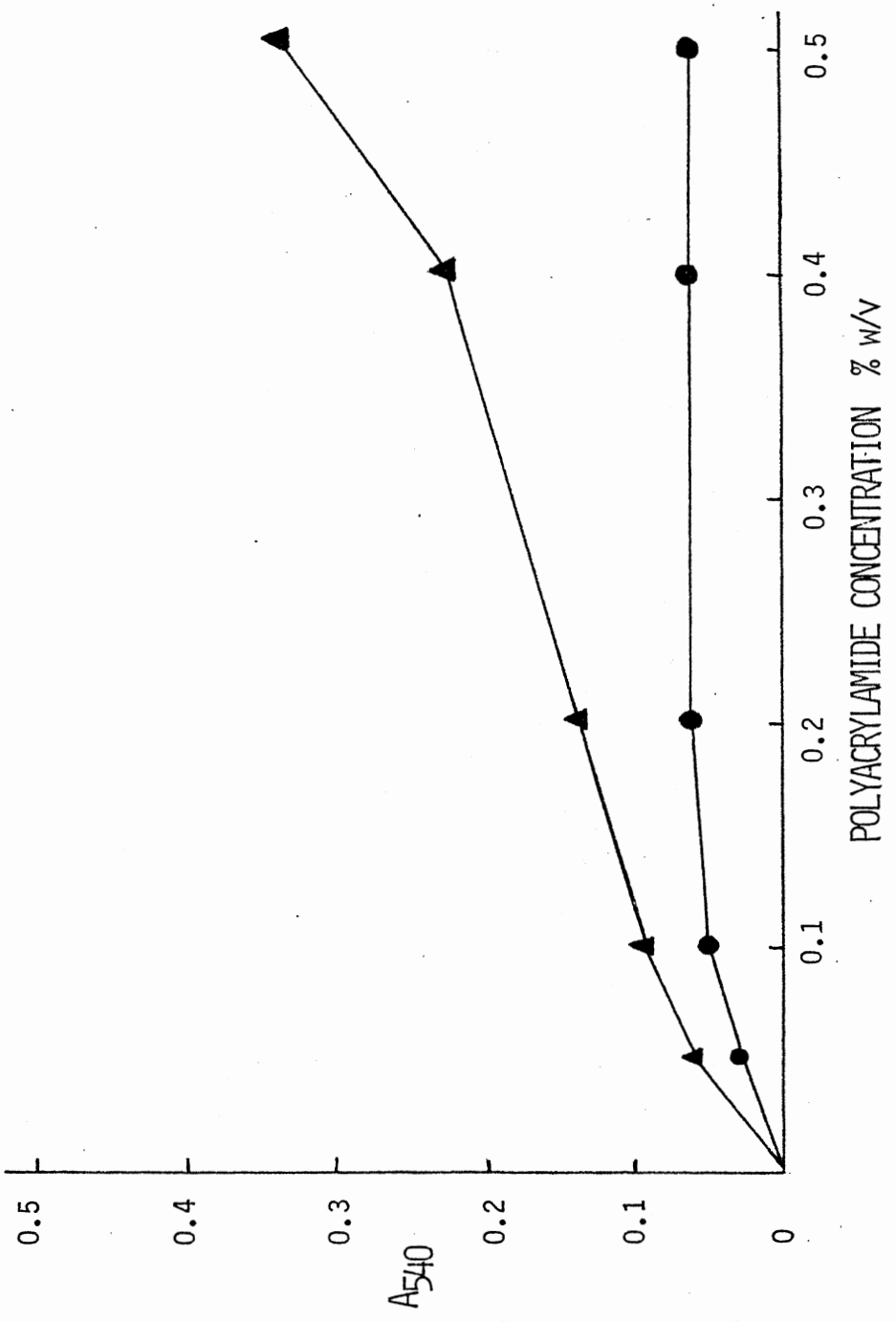
It is possible to remove free ammonia (or ammonium ion) from polyacrylamide solutions by dialysis. In order to get information on the extent to which free ammonia in the medium with polymer contributes to growth of microorganism, we dialyzed solutions of J279 at concentrations of 0.05%-0.5% against phosphate buffer pH 7.0 for 48 hours, changing phosphate buffer every 12 hours. The growth of strain AA in media made up with each of the various concentrations of polymer is shown in Figure 10. In the second serial culture even less growth occurred in cultures with dialyzed polyacrylamide. This indicates that free ammonia in the polymer

TABLE XIV

THE AMMONIA RELEASING ACTIVITY OF STRAIN AA GROWN ON XYLOSE-CARBON SOURCE AND POLYMER J279-NITROGEN SOURCE MEDIUM

Assay Mixture	NH_3 Released ug ml^{-1}
Cells + PAA	3.10
Cells + Buffer	0
PAA + Buffer	0
Boiled Cells + PAA	0
Boiled Cells + Buffer	0

Figure 10. The Effect of Dialysis of Polyacrylamide on Growth of Strain AA. Dialyzed-J279-● , Undialyzed-J279-▲ .


0.5
0.4
0.3
0.2
0.1
0
A540
0.1
0.2
0.3
0.4
0.5
POLYACRYLAMIDE CONCENTRATION % w/v

solution plays a major role in microbial growth based on polyacrylamide as a sole nitrogen source. However, this is not the only interpretation. Polymer J279 is hydrolyzed only to the extent of 1.0%-4.0%. Removal of small amount of free ammonia by dialysis would shift the equilibrium in the direction of hydrolysis, and in 48 hours might remove most of the amide groups on the molecule. It should be possible to determine if this is the case by direct chemical analysis.

We attempted this by dialyzing solutions of J279 of concentrations 0.05, 0.10, 0.20, 0.40, and 0.50 per cent, and comparing the Nessler reactions of these with undialyzed solutions. Results are given in Table XV, 3/4 to 3/5 of ammonia was removed by the dialysis procedure used here. The amount of growth obtained with dialyzed polymer (Figure 10) is less than would be expected from these figures.

Shear Degradation of Polymer

It is possible that a polymer of lower molecular weight, i.e., a shorter carbon chain, could be attacked more easily by the postulated microbial enzyme releasing ammonia from the amide groups on the molecule. Accordingly, we compared the abilities of washed cells of strain AA and mAA to grow on both undegraded and degraded polymer. Solutions of polymer J279 ranging in concentration from 0.05 per cent to 0.5 per cent were prepared, and a portion of each was subjected to shear stress in Waring Blendor for 5 minutes. This is sufficient to reduce the screen factor to values in the range 1.2 to 1.5 as compared with approximately 20 for 0.05 per cent solution, and approximately 100 for a 0.10 per cent solution. Extent of reduction depends on concentration of polymer (Table IV). Cultures were grown in tubes for 48 hours at 37^{o} C. One serial transfer was

TABLE XV

THE NESSLER REACTION OF DIFFERENT CONCENTRATIONS OF POLYMER BOTH DIALYZED AND UNDIALYZED SOLUTION

J279 Concentration per cent (w/v)	Polymer	NH_3 Released ug ml^{-1}
0.05	Un-dialyzed	0
0.1	Un-dialyzed	2
0.2	Un-dialyzed	7
0.4	Un-dialyzed	12
0.5	Un-dialyzed	17
0.05	Dialyzed	0
0.1	Dialyzed	1.5
0.2	Dialyzed	4
0.4	Dialyzed	8
0.5	Dialyzed	10

done. Results are given in Table XVI. No difference in growth of the two strains was observed between media with degraded, as compared to the undegraded polymer. Both strains grew to the same extent.

An experiment was done to compare the abilities of strain AA and mAA to release ammonia from undegraded J279 as compared to degraded J279. Growth medium was prepared with 0.05% blendored J279 as a sole nitrogen source. This material was chosen as sole nitrogen source because we thought that possible effects of enzyme induction would be greatest, and therefore any difference in enzyme activity would be most noticeable.

Growth was not heavy, but it was sufficient to prepare a heavy washed cell suspension of each strain. Results of enzyme assay are given in Table XVII. Amounts of ammonia released are small, and the major portion seems to come from the polymer itself. Unlike previous experiments, the endogenous release of ammonia was low. If one subtracts the $O.D_{450}$ obtained in the presence of cells, it is found that release from the degraded polymer is significantly higher.

Attempt to Select Strains from Soil Using Serial Transfer Enrichment Cultures in Which Polymer was Sole Nitrogen Source

The strains we used previously were isolated from soil enrichment cultures in which a polyacrylamide had been added as sole carbon source. Because of this, there was no positive selection pressure for the strains to derive nitrogen (as ammonia) from polyacrylamides. Accordingly, we attempted to isolate such strains from soil by setting up experiment cultures in which the polymer was a sole nitrogen source. Plate counts in fourth serial cultures are given in Table XVIII.

TABLE XVI

THE EFFECTS OF SHEAR STRESS OF POLYMER ON GROWTH OF STRAIN AA MODIFIED STRAIN AA

J279 Concentration percent w/v	A_{540} Strain AA		Strain mAA	
	Degraded J 279	Undegraded	Degraded J279	Undegraded
0.05	0.09	0.10	0.11	0.10
0.1	0.22	0.22	0.25	0.26
0.2	0.26	0.25	0.29	0.28
0.4	0.31	0.31	0.32	0.30
0.5	0.39	0.40	0.41	0.40

TABLE XVII

THE AMMONIA RELEASING ACTIVITY OF STRAIN AA AND mAA ON DEGRADED AND UNDEGRADED POLYMER

Strain	Polymer	Assay Mixture	Nessler Reaction A_{450}
AA	Undegraded	Cells + PAA	0.10
AA	Degraded	Cells + PAA	0.12
AA	—	Cells + Buffer	0.01
mAA	Undegraded	Cells + PAA	0.11
mAA	Degraded	Cells + PAA	0.13
mAA	—	Cells + Buffer	0.01
—	Undegraded	PAA + Buffer	0.06
—	Degraded	PAA + Buffer	0.04

TABLE XVIII

THE ABILITY OF POLYMER J279 and J334 TO STIMULATE GROWTH OF MICROORGANISMS IN TWO SOIL SAMPLES

Incubation Temperature ^{0}C	Soil Sample	pH of Fourth Flask	Polymer Added	Numbers of Cells per ml	Ratio of Cell Number in Culture with Polymer to Number in Culture Without Polymer
25	1	6.5	—	1.8×10^{7}	—
25	2	6.6	—	3.2×10^{7}	—
37	1	6.6	—	1.3×10^{8}	—
37	2	6.7	—	2.0×10^{6}	—
25	1	4.3	J279	1.8×10^{9}	1.0×10^{2}
25	2	4.6	J279	6.1×10^{8}	2.0×10^{1}
37	1	4.2	J279	5.9×10^{8}	4.5×10^{0}
37	2	4.5	J279	5.3×10^{7}	2.1×10^{1}
25	1	7.1	J334	2.7×10^{8}	1.5×10^{1}
25	2	4.4	J334	6.0×10^{8}	2.0×10^{1}
37	1	7.3	J334	2.0×10^{8}	1.5×10^{0}
37	2	7.0	J334	1.3×10^{9}	6.5×10^{2}

From these results, it can be seen that both polymers significantly enhanced the growth of soil microbes. Only in one case (soil sample 1, with J334, at 37°C) was the polulation not significantly higher than in the control. Reason for marked alteration in pH of the medium (2 units lower than control with J279, and somewhat higher than control with J333) is unknown, but it is related to the chemistry of the polymer molecule. J279 is 1-4% hydrolyzed, whereas J334 is 25-35% hydrolyzed.

Two strains were isolated from the J279 enrichment cultures, one from each soil sample. Cell suspensions of these strains were prepared by growing cultures in complete basal medium containing 0.05% J279 at 37°C for 24 hours. Enzyme assays were done using phosphate buffer at pH 7.0. In neither case was much ammonia released. Heat-inactivated cells (boiled 10 minutes) released nearly as much as active cells. However, neither active nor inactivated cells showed a high endogenous activity. If these strains have any activity, it is very low. The selection pressure we hoped to establish did not operate effectively under the condition used.

Experiments Using Low Molecular Weight Amides as Substrates and Comparison of Results on Known Amidases of Various Strains With Results Obtained with These Same Strains on Polymers

As we indicated previously, polyacrylamides cannot be the natural substrate of any microbial enzyme (hypothetical at this point) catalyzing the release of ammonia from these polymers. It seemed reasonable, since the proposed enzyme is attacking an amide linkage, that this enzyme would

be an amidase, capable of hydrolyzing low molecular weight aliphatic amides and releasing ammonia from them.

Wild-type strains of Pseudomonas aeruginosa produce an inducible aliphatic amidase which enables them to grow with acetamide or propionamide as sole source of carbon and nitrogen (Brown et al., 1973). A preliminary test was done, using strain AA, to determine if it could use butyramide as a sole nitrogen source, and if washed cells could release ammonia from butyramide. Growth was poor and with $O.D_{540}$ 0.08 and 0.075 in duplicate cultures. However, the washed cells released large amounts of ammonia from butyramide (42.6 NH_3 ug/ml by a standardized suspension).

Tests for activity on butyramide comparing strain AA with strain mAA showed more release of ammonia by strain mAA, 2.84 ug/ml, as compared to 1.42 ug/ml for the unmodified strain. In order to confirm the activities of these strains on butyramide, cultures of each were grown in the defined medium containing 0.05% butyramide in addition to NH_4Cl.

Washed cells harvested at 24 hours were tested for activity in releasing ammonia from butyramide. Samples were taken from assay mixture at 10 minutes interval for one hour. Releases of ammonia by each stain were approximately equal. The major part (80% to 90%) of the ammonia was released in the first five minutes. Activity was not high. Growth of the strains in defined medium wihtout NH_4Cl, but with 0.05% butyramide did not result in cells with a high specific activity (it was thought that NH_4Cl in the medium might have repressed the formation of amidase) in the case of cells of strain AA. The modified strain AA showed about 20 per cent higher activity when grown with NH_4Cl in medium, even though growth was poor. Also, activity on butyramide was much less than we

obtained from strain AA on the first experiment. The only apparent difference between the two tests is that in the early experiment, where the cells had high activity, the butyramide concentration was 0.1%, whereas in the later experiment it was 0.05%.

Comparative Activity on Acetamide, Propionamide and Butyramide

According to Clarke and coworkers (P.R. Brown et al., 1973), the inducible aliphatic amidase produced by wild-type strains of Pseudomonas aeruginosa enables them to grow with acetamide or propionamide as the sole source of carbon and nitrogen. The enzyme is relatively specific and hydrolyzes butyramide at only 2% of the acetamide rate, while the higher aliphatic amides are not cleaved to any significant extent. In view of the fact that enzyme described above is probably the most prevalent amidase among wild-type Pseudomonas aeruginosa, we compared the activity of cells of strain mAA on each of these three substrate.

The growth medium was the complete defined medium with NH_4Cl, 0.05% of one of the three amides, and 0.075% glucose. Cells were harvested at 24 hours. Extent of growth, and pH of the medium at this time were as follows:

Substrate	A_{540}	pH
Acetamide	0.21	6.8
Propionamide	0.97	7.2
Butyramide	0.93	7.0

Apparently the amidase enzymes of our strain mAA are different from those of most wild-type strains of Pseudomonas aeruginosa. Cells from each medium were harvested, washed, and adjusted to equivalent

concentrations. In the enzyme assay, each type of cell was tested only against its homologous substrate. Results are given in Table XIX. Cells grown in a medium containing propionamide showed the greatest activity toward the homologous substrate, and also a significantly greater endogenous activity, than the other two type of cells.

Effects of Various Environmental Parameters on Activity of Cells of Strain mAA on Propionamide

The first component of the defined medium we tested was glucose. The complete medium (including NH_4Cl) was prepared with three concentration of glucose, 0.15%, 0.10%, and 0.075%. Propionamide at 0.05% was added since the enzyme is inducible. Growth of strain mAA in these media after 24 hours at 37°C is given in Table XX.

Enzyme assay, using washed cells, tris-buffer at pH 7.2 and propionamide , yielded results as given in Table XXI. Clearly, cells grown with the intermediate level of glucose (0.10 %) had highest propionamidase specific activity. It is possible that the final pH of the medium influenced activity independently of glucose.

Ammonium chloride was next tested in the complete defined medium with 0.05% propionamide. Concentrations used were 0.08%, 0.04%, and 0.004%. Growth of strain mAA is given in Table XXII.

Results of enzyme assay are given in Table XXIII. It is interesting that cells grown with the highest level of NH_4Cl (0.08%) demonstrated amidase activity 62 per cent greater than cells grown at 0.004% NH_4Cl, even though extent of growth and final pH of the medium were nearly the same.

TABLE XIX

THE AMIDASE ACTIVITY OF STRAIN mAA ON SUBSTRATE ACETAMIDE, PROPIONAMIDE AND BUTYRAMIDE

Cells Grown on Medium with	Substrate	Assay Mixture	NH_3 Released ug ml^{-1}
Acetamide	Acetamide	Cells + Acetamide	3.16
Acetamide	——	Cells + Buffer	0.6
——	Acetamide	Acetamide + Buffer	1.0
Propionamide	Propionamide	Cells + Propionamide	93.6
Propionamide	——	Cells + Buffer	9.06
——	Propionamide	Propionamide + Buffer	1.42
Butyramide	Butyramide	Cells + Butyramide	3.06
Butyramide	——	Cells + Buffer	0.5
——	Butyramide	Butyramide + Buffer	1.92

TABLE XX

THE GROWTH OF STRAIN mAA IN THE MEDIUM WITH PROPIONAMIDE AND DIFFERENT CONCENTRATION OF GLUCOSE

Glucose Concentration per cent (w/v)	A_{540}	Final pH in the Medium
0.15	1.10	6.7
0.10	0.86	7.0
0.075	0.60	7.2

TABLE XXI

THE AMIDASE ACTIVITY OF STRAIN mAA GROWN WITH DIFFERENT CONCENTRATION OF GLUCOSE ON SUBSTRATE PROPIONAMIDE

Glucose Concentration per cent (w/v)	Assay Mixture	NH_3 Released $\mu g\ ml^{-1}$
0.15	Cells + Propionamde	10.2
0.15	Cells + Buffer	0.5
0.10	Cells + Propionamide	65.0
0.10	Cells + Buffer	0.5
0.075	Cells + Propionamide	52.8
0.075	Cells + Buffer	0.5
——	Propionamide + Buffer	1.02

TABLE XXII

THE GROWTH OF STRAIN mAA IN THE MEDIUM WITH PROPIONAMIDE AND DIFFERENT CONCENTRATION OF NH_4Cl

Ammonium chloride Concentration per cent (w/v)	A_{540}	Final pH in the Medium
0.0	0.94	6.8
0.04	0.93	6.9
0.004	0.42	6.9

TABLE XXIII

THE AMIDASE ACTIVITY OF STRAIN mAA GROWN WITH DIFFERENT CONCENTRATIONS OF NH_4Cl ON SUBSTRATE PROPIONAMIDE

NH_4Cl Concentration with Which Cells Grown per cent (w/v)	Assay Mixture	NH_3 Released ug ml^{-1}
0.08	Cells + Propionamide	97.6
0.08	Cells + Buffer	0.6
0.04	Cells + Propionamide	56.8
0.04	Cells + Buffer	0.5
0.004	Cells + Propionamide	32.6
0.004	Cells + Buffer	0.5
———	Propionamide + Buffer	1.2

The next experiment was done to elucidate the effect of final pH of the medium on amidase activity, and the effect of NH_4Cl concentration in the presence of limiting glucose (0.075%). Results in the growth media are given in Table XXIV.

With NH_4Cl concentration limiting, pH of the cultures with three concentrations of glucose was controlled by limiting growth. With glucose limiting, pH and growth were very similar with each of the three NH_4Cl concentration.

Results of amidase assay using cells harvested from six media are given in Table XXV. Glucose concentration over the range 0.075 to 0.15 per cent does not affect activity if pH of the medium is controlled. In addition, if growth is limited by glucose concentration, NH_4Cl concentration does not affect amidase specific activity of cells.

An experiment to provide further information on pH of the culture as a factor controlling amidase activity was done. Cells were grown in the defined medium with 0.05% propionamide, and 0.08% NH_4Cl. Two flasks containing 0.15% glucose were prepared; in one of these the pH was controlled by adjusting at 6 and 12 hour to 7.1-7.2, and in the other, no adjustments were made. A third flask was prepared with 0.10% glucose. In the pH-adjusted flask pH had fallen to 6.8 at 6 hour, when it was adjusted to 7.2. At 12 hour pH had fallen to 6.6, and was readjusted to 7.1. Therefore it remained at 7.2 or higher. Cells were harvested at 24 hour. Results are given in Table XXVI.

Even though more growth occurred in the culture in which pH was not adjusted, specific amidase activity was twice as high in the culture in which pH was not allowed to stay below 7.0 from 12 hr. to 24 hr. The pH in the medium is a major factor affecting the enzyme activity.

TABLE XXIV

THE GROWTH OF STRAIN mAA IN THE MEDIUM WITH PROPIONAMIDE AND LIMITING CONCENTRATION OF NH_4Cl AND GLUCOSE

NH_4Cl Concentration per cent (w/v)	Glucose Concentration per cent (w/v)	A_{540}	pH
0.04	0.15	0.61	7.0
0.04	0.10	0.53	7.2
0.04	0.075	0.39	7.2
0.08	0.075	0.45	7.3
0.04	0.075	0.40	7.3
0.004	0.075	0.39	7.2

TABLE XXV

THE AMIDASE ACTIVITY OF STRAIN mAA GROWN IN MEDIUM WITH PROPIONAMIDE AND LIMITING CONCENTRATIONS OF NH_4Cl AND GLUCOSE

NH_4Cl Concentration with Which Cells Were Grown per cent per cent (w/v)	Glucose concentration with Which Cells Were Grown per cent (w/v)	Assay Mixture	NH_3 Released $\mu g\ ml^{-1}$
0.04	0.15	Cells + Propionamide	33.6
0.04	0.15	Cells + Buffer	0.5
0.04	0.10	Cells + Propionamide	30.6
0.04	0.10	Cells + Buffer	0.5
0.04	0.075	Cells + Propionamide	32.6
0.04	0.075	Cells + Buffer	0.5
0.08	0.075	Cells + Propionamide	31.6
0.08	0.075	Cells + Buffer	0.5
0.04	0.075	Cells + Propionamide	33.6
0.04	0.075	Cells + Buffer	0.5
0.004	0.075	Cells + Propionamide	32.6
0.004	0.075	Cells + Buffer	0.5
—	—	Propionamide + Buffer	1.32

TABLE XXVI

THE AMIDASE ACTIVITY OF STRAIN mAA IN THE MEDIUM WITH pH ADJUSTED AND DIFFERENT CONCENTRATIONS OF GLUCOSE

Glucose Concentration per cent (w/v)	pH in the Medium	NH_3 Released ug ml^{-1}
0.15	6.7	35.6
0.15	Adjusted to 7.2	74.2
0.10	7.2	63.0

Effect of Substrate Concentration

in the Medium

Three levels of propionamide, 0.01, 0.05 and 0.10 per cent , were added to the basal medium with 0.08% NH_4Cl and 0.1% glucose. pH and absorbance values of cultures are given in Table XXVII. Propionamide significantly enhance growth, even in the presence of excess NH_4Cl. The two higher levels of propionamide resulted in a significantly higher final pH in the medium. Therefore, the higher specific propionamidase activities (Table XXVIII) of cells grown at the higher levels of propionamide cannot be unequivocally attributes to the higher propionamide concentration.

Time of Harvest

We compared the specific amidase activity of cells of strain mAA harvested at 18 hour with that of cells harvested at 24 hour. Previously all our tests had been done with 24 hour cells. Medium used was the usual defined medium with 0.15% glucose, 0.10% propionamide and 0.08% NH_4Cl.

The medium pH was adjusted at 3 hour intervals to 7.0-7.1. One-half the culure was removed at 18 hour, and the cells were harvested. At this this time, O.D of the culture was 0.94, and pH, 7.3. At 24 hour (O.D 1.05 and pH 7.6) cells from the other half of the culture were harvested. As in all cases, both cell suspensions were adjusted to the same density. Results of the enzyme assay are given in Table XXIX. Since 18 hour cells showed a significantly higher specific amidase activity than 24 hour cells, time of harvest of cells in future experiment will be adjusted accordingly.

TABLE XXVII

THE GROWTH OF STRAIN mAA IN DIFFERENT CONCENTRATIONS OF PROPIONAMIDE

Propionamide Concentration per cent (w/v)	A_{540}	pH in the Medium
0.01	0.77	6.6
0.05	0.82	7.1
0.10	1.05	7.3

TABLE XXVIII

THE AMIDASE ACTIVITY OF STRAIN mAA GROWN WITH DIFFERENT CONCENTRATION ON PROPIONAMIDE

Propionamide Concentration per cent (w/v)	NH_3 Released ug ml^{-1}
0.01	35.6
0.05	63.7
0.10	67.0

TABLE XXIX

THE EFFECT OF HARVEST TIME ON AMIDASE ACTIVITY OF STRAIN mAA

Harvest Time Hour	Assay Mixture	NH_3 Released ug ml^{-1}
24	Cells + Propionamide	57.8
24	Cells + Buffer	0.5
18	Cells + Propionamide	72.2
18	Cells + Buffer	0.5
—	Propionamide + Buffer	1.22

Comparison of Strains

Strain mAA, resulting from a serial transfer with soil extract, has shown in past tests a measurably greater ability to release ammonia from polyacrylamide than the strain from which it was derived, strain AA. If strain mAA showed higher propionamidase activity than its parent strain, this would be compatible with the hypothesis that the enzyme releasing ammonia from the polymer is this amidase.

Cells of each strain were grown in the defined medium containing 0.1 % glucose, 0.08% NH_4Cl, and 1.0% propionamide. The latter concentration is 20X that used in previous experiments (0.05%). Final O.D and pH values of the cultures were: mAA, 0.8- and 7.0; AA, 0.68 and 6.8. Results of enzyme assay are given in Table XXX. Specific activity of strain mAA cells was 81% greater than that of the AA cells. This indicates a possibility that the propionamidase may be attacking the $C(=O)NH_2$ linkage of the polymer. More evidence will be needed to arrive at a definite conclusion.

Ability of Various Pseudomonas Strains to Grow on Either Acetamide, Propionamide, or Butyramide as a Sole Carbon and Nitrogen Source

Various strains of Pseudomonas (often the species Pseudomonas aeruginosa) are able to grow on a minimal medium with either acetamide or propionamide as a sole carbon and nitrogen source. Mutants of one strain are able to utilize butyramide (Brown, Brown and Clarke, 1969).

We have a number of pseudomonads isolated from polyacrylamide-containing soil enrichment cultures. It seemed to be worthwhile to screen

TABLE XXX

THE AMIDASE ACTIVITY OF STRAIN AA AND mAA ON PROPIONAMIDE

Strain	Assay Mixture	NH_3 Released ug ml^{-1}
mAA	Cells + Propionamide	65.0
mAA	Cells + Buffer	0.4
AA	Cells + Propionamide	34.6
AA	Cells + Buffer	0.3
—	Propionamide + Buffer	1.02

these cultures for ability to grow in a minimal (defined) medium with one of the three amides (acetamide, propionamide and butyramide) as sole carbon and nitrogen source. We could then check those strains which grow well (meaning that they possess the amidase for that particular amide) for ability to release ammonia from polyacrylamide.

Cells of seven strains (AA, mAA and five we had not used before which are listed in Table XXXI) were grown in the defined medium with either acetamide (0.10M), propionamide (0.125M) or butyramide (0.15M) as carbon-nitrogen source. Results, in terms of optical density ($O.D_{540}$) and pH values of cultures, are given in Table XXXII.

In accord with the findings of Clarke and coworkers (1969) few wild-type pseudomonads are able to hydrolyze butyramide. Growth of all strains including AA and mAA, is considerably better on acetamide than propion-amide, an observation somewhat puzzling in view of our earlier results.

Extent of growth on propionamide varied, with strain D and mAA show-ing the most growth.

In addition to determining growth, cells from acetamide were harvest-ed , and amidase activity on the growth substrates acetamide and propion-amide was determined. Results are given in Table XXXIII.

In spite of the much better growth of all strains on acetamide than propionamide, the specific amidase activity of all strains except D was significantly greater with propionamide than with acetamide as a substrate. This may be related to the possibility that the strains involved find the acetyl ($CH_3C\overset{=O}{\diagdown}$) more satisfactory as a source of carbon than the propionyl ($CH_3CH_2C\overset{=O}{\diagdown}$) group, and in either case the amount of ammonia released is not limiting.

It is impossible to correlate the amount of growth of each strain on

TABLE XXXI

SEVERAL PSEUDOMONAS STRAINS ISOLATED
FROM ENRICHMENT CULTURE

Strain	Genus	Compound added in Enrichment Culture
YY	Pseudomonas aeruginosa	Whitco 1250
O	Pseudomonas aeruginosa	Marathon
M	Pseudomonas aeruginosa	Marathon
Y	Pseudomonas aeruginosa	Marathon
D	Pseudomonas aeruginosa	Compound A
AA	Pseudomonas aeruginosa	Polyacrylamide J334

TABLE XXXII

GROWTH AND pH OF CULTURES OF DIFFERENT STRAINS OF *PSEUDOMONAS* GROWN IN MEDIA WITH EITHER ACETAMIDE , PROPIONAMIDE OR BUTYRAMIDE AS SOLE CARBON-NITROGEN SOURCE

Strain	Substrate					
	Acetamide		Propionamide		Butyramide	
	A_{540}	pH	A_{540}	pH	A_{540}	pH
YY	1.05	8.5	0.21	7.2	0.01	7.0
O	1.0	8.6	0.23	7.4	0.01	7.0
M	1.15	8.5	0.23	7.4	0.03	7.0
Y	1.0	8.5	0.11	7.2	0.02	7.0
D	1.1	8.6	0.42	7.6	0.02	7.0
AA	1.05	8.4	0.33	7.6	0.01	7.0
mAA	1.15	8.5	0.41	7.6	0.02	7.0

TABLE XXXIII

THE AMIDASE ACTIVITY OF DIFFERENT STRAIN GROWN WITH ACETAMIDE ON SUBSTRATE ACETAMIDE AND PROPIONAMIDE

Strain	Substrate Acetamide NH_3 Released ug ml^{-1}	Substrate Propionamide NH_3 Released ug ml^{-1}
YY	23.4	38.6
O	26.4	37.6
M	19.4	46.8
Y	34.6	63.0
D	28.4	17.2
AA	23.4	36.6
mAA	35.6	67.0

* All cell suspensions were adjusted to the same optical density

propionamide with the specific amidase activity of washed cells on substrate. Again, efficiency in utilizing the substrate as a carbon source may be a major factor in determining the amount of growth on that substrate. It may be of interest that strains mAA and D had the highest specific activities on acetamide, and strain Y and mAA the highest activity on propionamide.

We next attempted to determine if there was any correlation between degree of amidase production and ability to grow on J279, and further tested growth as a function of J279 concentration. Results are given in Table XXXIV. In this test, strain D showed significantly more growth (approximately twice the O.D values) at each concentration of J279. Regarding specific activity on acetamide and propionamide, strain D showed low activity on propionamide, and lower activity than either mAA or Y on acetamide. This is not good correlation of amidase activity with ability to grow on polymer.

Xylose as Carbon Source

Strain AA grew poorly in the defined medium with xylose as a carbon source and NH_4Cl as sole nitrogen source, although it would grow moderately with xylose and J279. This effect could not be explained; however, we thought it would be of interest to try strain m AA, Y and D, and to check the pH of the medium. Results of this test are shown in Table XXXV. Strains D and mAA showed the same effect as strain AA had- considerably better growth occurred with the polymer than with NH_4Cl. Strain Y, however, grew poorly under both conditions. The pH of the medium was not a factor in growth.

TABLE XXXIV

THE GROWTH OF STRAIN mAA, Y , D ON DIFFERENT CONCENTRATION ON POLYMER J279

J279 Concentration per cent (w/v)	Strain mAA	Strain Y A_{540}	Strain D
0.05	0.10	0.05	0.10
0.10	0.12	0.09	0.16
0.20	0.18	0.13	0.21
0.40	0.24	0.19	0.38
0.50	0.32	0.21	0.41
0.08% NH_4Cl	0.98	0.95	0.99

TABLE XXXV

THE GROWTH OF STRAIN mAA, Y, D WITH XYLOSE AS CARBON SOURCE AND J279, NH_4Cl AS NITROGEN SOURCE

Nitrogen Source	Carbon Source	Strain					
		mAA		Y		D	
		A_{540}	pH	A_{540}	pH	A_{540}	pH
0.08% NH_4Cl	Xylose	0.04	6.9	0.03	6.9	0.04	6.9
0.5% J279	Xylose	0.20	6.6	0.09	6.8	0.15	6.8

Comparison of Ability of Strain mAA, Y, and D to Release Ammonia from Polymer J279

Cells of these strains were grown in the defined medium with 0.05% J279, 0.08% NH_4Cl, and 0.15% glucose. Duplicate cultures for each were prepared, one of which was pH controlled by adjustment to 7.0 every three hours. Final pH and O.D values of cultures are given in Table XXXVI, and results of enzyme assay in Table XXXVII. The differences between cells harvested from pH-controlled cultures and non-pH-controlled cultures is very great; however, endogenous (no substrate) release of ammonia is also higher with cells from pH-controlled cultures. With all these strains, $O.D_{450}$ values was 0.02 units higher with polymers as substrate than without substrate. It is hard to assess the significance of this small value; yet with all these strains, amount of ammonia released is correlated with the extent of growth on polymer J279.

The experiment was repeated in the same way, except that cells were harvested at 18 hour instead of 24 hour. Growth results are given in Table XXXVIII, and results of enzyme assay in Table XXXIX. Strain Y, which showed far less activity on propionamide than strain mAA or D, did not release any measurable amount of ammonia from the polymer.

Ability of Various Strains to Grow on Defined Medium with Acetamide, Propionamide, or Butyramide as Sole Nitrogen Source

Three different low molecular weight amides were used to check the ability of various strains to grow on them.

TABLE XXXVI

THE GROWTH OF STRAIN mAA, Y, D ON COMPLETE BASAL MEDIUM WITH pH-CONTROLLED OR UN-CONTROLLED

Strain	pH in the Medium	A_{540}	Final pH in the Medium
mAA	Controlled	1.12	7.2
mAA	Un-controlled	1.15	4.5
Y'	Controlled	1.12	7.2
Y	Un-controlled	1.19	4.3
D	Controlled	1.10	7.2
D	Un-controlled	1.20	4.6

TABLE XXXVII

THE AMMONIA RELEASING ACTIVITY OF STRAIN mAA, Y, D ON J279 WITH MEDIUM pH CONTROLLED OR UN-CONTROLLED

Strain	pH in the Medium	Assay Mixture	NH_3 Released ug ml^{-1}
mAA	Controlled	Cells + J279	2.0
mAA	Controlled	Cells + Buffer	0.8
mAA	Un-controlled	Cells + J279	0
mAA	Un-controlled	Cells + Buffer	0
Y	Controlled	Cells + J279	0.4
Y	Controlled	Cells + Buffer	0
Y	Un-controlled	Cells + J279	0
Y	Un-controlled	Cells + Buffer	0
D	Controlled	Cells + J279	3.6
D	Controlled	Cells + Buffer	1.22
D	Un-controlled	Cells + J279	0
D	Un-controlled	Cells + Buffer	0
—	—	J279 + Buffer	0

TABLE XXXVIII

THE GROWTH OF STRAIN mAA, Y, and D OF 18 HOUR INCUBATION WITH MEDIUM pH UNCONTROLLED OR CONTROLLED

Strain	Medium pH	A_{540}	Final pH in the Medium
mAA	Control	0.86	7.2
mAA	Un-controlled	1.0	6.3
Y	Controlled	0.96	7.2
Y	Un-controlled	1.10	6.2
D	Controlled	0.76	7.4
D	Un-controlled	0.96	6.3

TABLE XXXIX

THE AMMONIA RELEASING ACTIVITY OF STRAIN mAA, Y, D OF 18 HOUR INCUBATION AND MEDIUM pH CONTROLLED, UNCONTROLLED

Strain	Medium pH	Assay Mixture	NH_3 Released ug ml^{-1}
mAA	Controlled	Cells + J279	0.3
mAA	Controlled	Cells + Buffer	0
mAA	Controlled	Cells + Propionamide	36.6
mAA	Un-controlled	Cells + J279	0
mAA	Un-controlled	Cells + Buffer	0
Y	Controlled	Cells + J279	0
Y	Controlled	Cells + Buffer	0
Y	Controlled	Cells + Propionamide	5.9
Y	Un-controlled	Cells + J279	0
Y	Un-controlled	Cells + Buffer	0
D	Controlled	Cells + J279	0.6
D	Controlled	Cells + Buffer	0
D	Controlled	Cells + Propionamide	23.4
D	Un-controlled	Cells + J279	0
D	Un-controlled	Cells + Buffer	0
—	—	J279 + Buffer	0
—	—	Propionamide + Buffer	1.02

The ability to grow with any of these low molecular weight amides as sole carbon-nitrogen source is dependent on the ability of the strain to hydrolyze the amide, and on its ability to metabolize the remaining carbon skeleton. It was thought to be of interest to test as many strains as possible for ability to grow on acetamide, propionamide, and butyramide (some wild-type strains utilizing acetamide and propionamide cannot utilize butyramide), and to correlate this ability with the ability to release ammonia from a polyacrylamide.

The first two pseudomonads tested were strain LL (isolated from a n-dodecylbenzene enrichment culture) and strain L-1 (isolated from activated sludge). Both strains grew very well on acetamide, moderately on propionamide, and not at all on butyramide. Regarding amidase activity of harvested cells, with both strains, growth on acetamide yielded cells equally active on acetamide and propionamide whereas cells grown on propionamide showed poor activity on acetamide, and activity on propionamide about equal to that of acetamide-grown cells.

To check activity on polyacrylamide, cells of each strain were grown in defined medium with 0.1M acetamide and 0.05% J179. Results with harvested cells are given in Table XL, in order to compare with values obtained using other strains. It can be seen that LL and L-1, have potent activity on acetamide, and minimal activity on J279.

Tests of growth on the three substrates, and ammonia-releasing activity on polymer, were carried out with strain Q-1, DD and F. Again, all strains grew well on acetamide, less well on propionamide and not at all on butyramide. Enzyme activities of washed cells on acetamide and propionamide followed a pattern similar to that of the last experiment, except that strain F showed very poor activity on acetamide, whether grown

TABLE XL

THE AMMONIA RELEASING ACTIVITY OF STRAIN LL AND L-1 ON SUBSTRATE POLYACRYLAMIDE J279 AND ACETAMIDE

Strain	Assay Mixture	NH_3 Released ug ml^{-1}
LL	Cells + J279	3.66
LL	Cells + Acetamide	116.8
LL	Cells + Buffer	0.82
L-1	Cells + J279	4.88
L-1	Cells + Acetamide	111.8
L-1	Cells + Buffer	2.44
—	J279 + Buffer	0
—	Acetamide + Buffer	5.48

on acetamide or propionamide.

Enzyme activities on the polymer (J279) revealed a different pattern; activity of strain Q-1, which was very active on both acetamide and propionamide, showed little or no activity on the polymer, whereas strain DD released a moderate amount of ammonia, and strain F, a somewhat larger amount (Table XLI).

It will be recalled that strain Q-1 grew very poorly on polymer J279 as a sole nitrogen source.

The results of this experiment were the first to suggest to us that the hypothetical enzyme attacking the polymer J279 was not the same as the amidase hydrolyzing acetamide or propionamide.

Enzyme assays of washed cells were carried out on acetamide, propionamide, butyramide, and J279 as substrate. Results will be summarized to include net ammonia release (endogenous value subtracted) using J279, acetamide, propionamide, and butyramide by cells grown with acetamide.

This experiment was also performed with strains mAA, Y, and D. In this case, pH values of medium were checked at 6, 9, 12 and 18 hour. It was believed of interest to include these data, along with the final level of growth (O.D) obtained. Results are given in Table XLII.

All three strains grew very well on acetamide. strain mAA and D grew moderately on propionamide, whereas strain Y grew poorly.

It is of interest that mAA cells had nearly the same activity on J279 whether grown on acetamide or propionamide; whereas cells grown with propionamide had only about half the specific activity on acetamide of acetamide grown cells (Table XLIII).

TABLE XLI

THE GROWTH OF VARIOUS STRAINS ON ACETAMIDE, AND PROPIONAMIDE, AND AMMONIA RELEASING ACTIVITY ON ACETAMIDE, PROPIONAMIDE AND J279

Strain	A_{540}		NH_3 Released ug ml^{-1} Substrate		
	Acetamide	Propionamide	Acetamide	Propionamide	J279
LL	1.1	0.72	26.4	27.4	2.84
L-1	0.96	0.55	22.4	28.4	2.44
Q-1	0.84	0.44	85.4	97.6	0
DD	0.83	0.41	74.2	88.4	0.82
F	0.80	0.34	18.2	52.8	1.14
Y	0.95	0.09	50.6	78.9	0.63
mAA	1.1	0.40	71.9	96.2	1.02

TABLE XLII

THE GROWTH OF STRAIN mAA, Y AND D GROWN IN THE MEDIUM WITH ACETAMIDE OR PROPIONAMIDE

Strain	A_{540}	
	Acetamide	Propionamide
mAA	1.1	0.40
Y	0.95	0.09
D	1.05	0.40

TABLE XLIII

THE EFFECT OF CARBON-NITROGEN SOURCE ON SPECIFIC ACTIVITY OF STRAIN mAA CELLS ON ACETAMIDE AND J279

Growth Substrate	NH_3 Released ug ml^{-1}	
	Acetamide	J 279
Acetamide	72.0	1.04
Propionamide	37.4	0.95

Correlation of Ability to Grow on Polyacrylamide as Sole Nitrogen Source with Release of Ammonia from J279 by Washed Cells

It is important to determine unequivocally, if possible, whether a relationship exists between ability to grow on the polymer as a sole nitrogen source, and release of ammonia (enzymatic) from the polymer, upon which the extent of growth would depend. Results of this type are given in Table XLIV.

These values probably represent as satisfactory data as can be obtained in this area. Growth is not really abundant in any case, and release of ammonia from the polymer is minimal. Evidence that an enzyme may be involved is the fact that boiled cells released no ammonia from the polymer, or endogenously. Strain Q-1 released no ammonia from polymer, but had the highest specific activity on propionamide. This indicate that the enzyme (amidase) attacking propionamide is not the one releasing ammonia from the polymer.

There is a rough correlation between extent of growth on the polymer (note that growth is a function of concentration up to 0.5% on J279) and amount of ammonia released from the polymer; however, the latter is small in all cases. These results are not imcompatible with the idea that growth with polymer as sole nitrogen source occurs because of a small but significant enzymatic release of ammonia from the polymer.

A further check on the relationship between ability to release ammonia from the same polymer as substrate, was done using strains mAA, L-1. Y, and Q-1. Results are given in Table XLV. A significant positive

TABLE XLIV

THE CORRELATION BETWEEN AMMONIA RELEASING ACTIVITY ON PROPIONAMIDE, POLYACRYLAMIDE AND ABILITY TO GROW ON POLYACRYLAMIDE OF STRAINS OF PSEUDOMONAS

Strain	Polyacrylamide Concentration (w/v)		Ammonia Releasing Activity NH_3 ug ml^{-1}	
	0.05%	0.5%	Substrate	
	A_{540}		J279	Propionamide
Q-1	0.03	0.06	0	97.6
Y	0.05	0.24	0.63	78.9
F	0.09	0.28	1.14	52.8
mAA	0.10	0.34	1.02	96.3
L-1	0.10	0.42	1.02	28.4

TABLE XLV

THE GROWTH OF STRAIN mAA, L-1, Y , Q-1 ON DIFFERENT CONCENTRATION OF J279 AND THE AMMONIA RELEASING ACTIVITY ON J279

Polymer Concentration	A_{540} Strain			
per cent (w/v)	mAA	L-1	Y	Q-1
0.05	0.10	0.10	0.05	0.03
0.1	0.13	0.15	0.09	0.03
0.2	0.18	0.23	0.13	0.05
0.4	0.25	0.35	0.19	0.06
0.5	0.34	0.42	0.24	0.06
NH_3 Released ug ml^{-1} on J279	1.02	1.02	0.63	0

correlation exists between growth and ammonia release, particularly in the case of strain Q-1, which did not grow, and did not release ammonia.

Attempts to Concentrate and Purify Enzyme

Dialysis of Spent Culture Fluid

It seemed reasonable that enzyme (if any) attacking the amide linkage of the polyacrylamides could be concentrated and more clearly demonstrated if the culture supernatant liquid could be concentrated without subjecting the liquid to a high temperature. Dialysis of the spent medium in 40 % Carbowax 6,000 was chosen for the process.

Cells were grown in a medium designed to yield high activity. After centrifugation at 24 hour, the supernatant was dialyzed overnight (in refrigerator). The following day the bag was washed but with a very small volume of water; the latter was assayed for enzyme activity. Initial tests showed that something was interfering with color development in the Nessler reaction. In addition, there seemed to be a turbidity in the blank tube of the enzyme assay resulting from the presence of Carbowax.

Another test of the use of dialysis against Carbowax to concentrate spent medium was done. In this case a preliminary dialysis against distilled water, to remove small molecules present in the medium, was done. After this dialysis, the bags were placed in 40% Carbowax and again dialyzed overnight; in this case, water would be removed and the macromolecules (enzyme) present would be concentrated. Results (with concentrate obtained after second dialysis) are given in Table XLVI.

In an effort to determine the source of interference with the Nessler reaction occurring in Carbowax dialysis, we prepared a standard

TABLE XLVI

THE AMMONIA RELEASING ACTIVITY OF CONCENTRATE OF SPENT MEDIUM OF STRAIN mAA CULTURE ON DIFFERENT SUBSTRATE

Assay Mixture	NH_3 Released ug ml^{-1}
Active Concentrate + J279	14.2
Active Concentrate + Propionamide	30.6
Uninoculated Medium + J279	10.2
Uninoculated Medium + Propionamide	8.2
Undialyzed Distilled H_2O + J279	0
Undialyzed Distilled H_2O + Propionamide	1.2

NH_4Cl solution and dialyzed it, and deionized water, against 40% Carbowax overnight. A third dialysis was prepared in which the NH_4Cl solution was dialyzed against H_2O.

Samples were removed from each bag after overnight dialysis, and tested with Nessler reagent. Cloudiness occurred in both tests prepared from bags that had been in Carbowax solution. No cloudiness appeared in the other. Evidently the Carbowax solution- even though it has not been in contact with the inside of the bag- is responsible for the development of the cloudiness in the Nessler reaction.

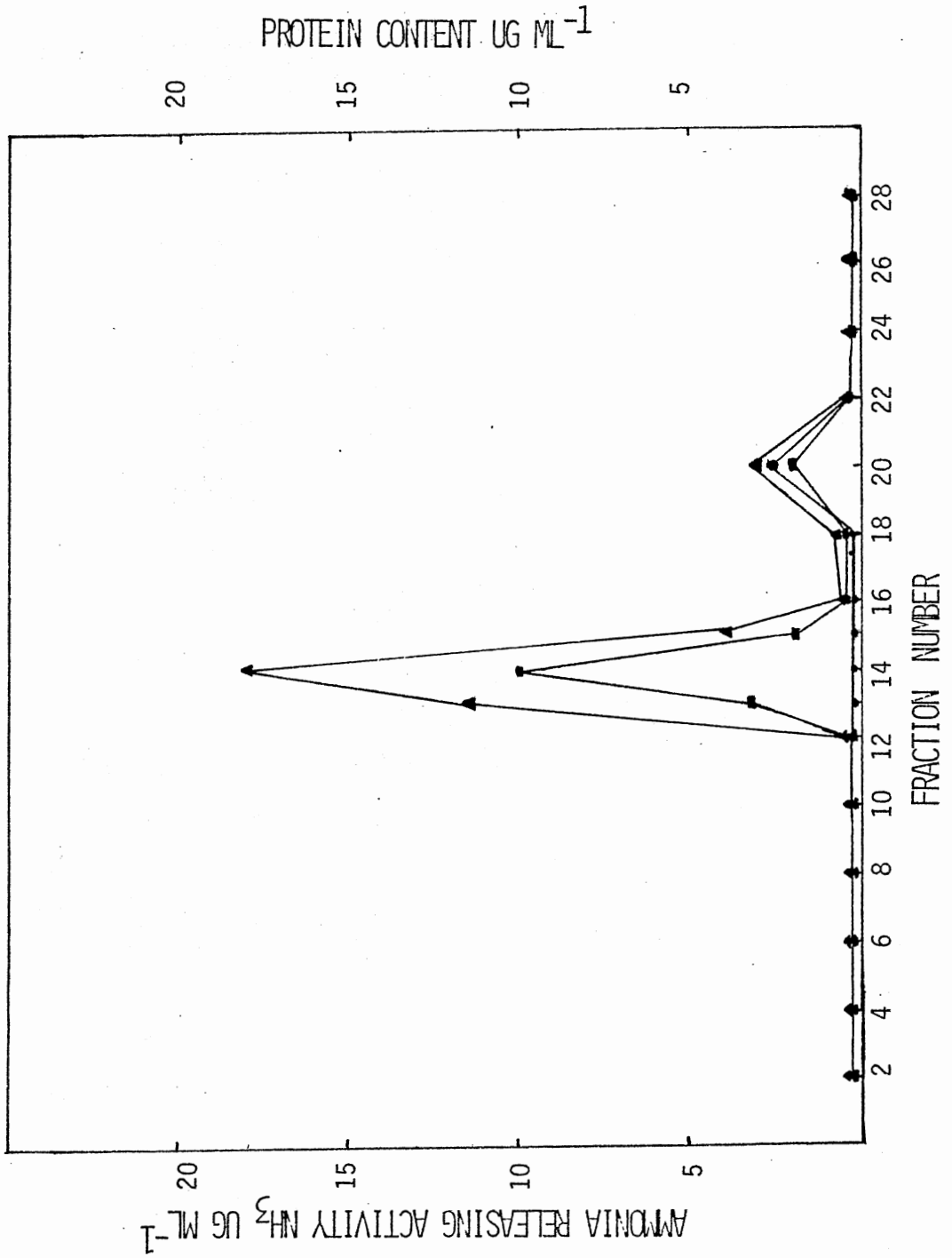
Concentration of the spent medium by lyophilization was attempted. Culture of Strain mAA was grown in a manner to maximize activity. Six hundred ml of spent medium were concentrated 50 fold by suspending powder resulting from the lyophilization in 12 ml buffer.

This preparation was assayed for enzyme activity on both propionamide and J279. It showed activity on both substrates. Endogenous activity, however, was very high. The amidase activity showed a high degree of heat resistance (survived boiling for 10 minutes).

This method of concentration may show promise.

Fractionation via a Sephadex G-200 column of the spent medium, concentrated by dialysis against 40 per cent Carbowax followed by lyophilization, was attempted. Results are shown in Figure 11. While these results must be regarded as tentative, it is clear that peak of protein content and activity on propionamide (fraction 13 and 14) does not coincide with ammonia releasing activity on J279 (fraction 20). If the Carbowax and the possibly the fluorescent pigment with the Nessler reaction could be overcome, clearer results might be obtained. The ammonia electrode should have promise for further work in this area.

Figure 11. The Enzyme Activity of Each Fraction of Strain mAA Culture Spent Medium on Propionamide-▲, Polyacrylamide-●, and the Protein Content-■.


PROTEIN CONTENT UG ML-1
20
15
10
5
AMMONIA RELEASING ACTIVITY NH3 UG ML-1
20
15
10
5
2
4
6
8
10
12
14
16
18
20
22
24
26
28
FRACTION NUMBER

A similar experiment was done; in this case filtration was done in cold room. Only 25 fractions were collected. In this case amidase activity and protein content were spread over fraction 11-19. No really significant activity on J279 occurred, although fraction 11-19 showed a somewhat higher $O.D_{450}$ than the others.

Cell free extracts were prepared and subjected to ammonium sulfate fractionation. A good separation of the amidase was obtained in that 50 per cent ammonium sulfate precipitate, but not the others, was quite active. Results are given in Table XLVII. No significant activity on J279 occurred. This is compatible with the conclusion that amidase and the enzyme attacking the amide linkage in polymer J279 are not the same.

At this time a test of the ability of washed cells of strain mAA to release ammonia from the polymer was done. These cells retained their ability to hydrolyze propionamide, but had no significant activity on J279. This is further evidence that the enzymes are not the same.

Because of the apparent loss of activity, another restoration experiment was attempted. Strain mAA was passed through three serial transfer in the basal medium containing soil extract, NH_4Cl, J279 and glucose. In this case, cells were transferred immediately from the fourth serial culture into the medium for producing enzymatically active cells. These cells showed relatively high activity on J279, very high activity on propionamide, and low endogenous activity. Restoration of activity was successful. The strain restored from this experiment is referred to as m2AA. Using m2AA, an attempt was made to determine the effect of propionamide on ammonia releasing activity on J279. Propionamide in the medium increased growth, and increased specific activity on propionamide, but had no effect on activity on J279. The latter was very low or doubtful.

TABLE XLVII

THE ENZYME ACTIVITY ON POLYACRYLAMIDE J279 AND PROPIONAMIDE AND THE PROTEIN CONTENT OF AMMONIUM SULFATE FRACTIONS OF ULTRASONIC PREPARATION OF MODIFIED STRAIN AA

$(NH_4)_2SO_4$ * Concentration (w/v)	Substrate	NH_3 Released ug ml^{-1}	Protein Content ug ml^{-1}
50%	Polyacrylamide J279	0	540
50%	Propionamide	90	540
55%	Polyacrylamide J279	0	55
55%	Propionamide	4	55
65%	Polyacrylamide J279	0	10
65%	Propionamide	2	10
75%	Polyacrylamide J279	0	0
75%	Propionamide	0	0

* $(NH_4)_2SO_4$ were added as dry powder into each fraction volume. Table for mg of solid ammonium sulfate added to each fraction volume was corrected at 4°C. pH of each fraction was not checked

Since so much evidence has accumulated that the postulated enzyme releasing ammonia from polyacrylamide is not the low molecular weight aliphatic amidase, we considered the possibility that the enzyme we were looking for was an extracellular protease. Many, in fact most, strains of Pseudomonas possess such an enzyme.

In view of this, we thought it would be of interest to attempt to correlate the ability to liquify gelatin, and roughly the degree of liquification, with the ability to release ammonia from polyacrylamide. Six strains were tested, m2AA, m3AA, L-1, F, Q-1, and 1R. Each strain was streaked, in a manner to give isolated colonies, on nutrient agar containing 2 per cent gelatin. After 24 hours, a saturated solution of $(NH_4)_2SO_4$ was added to plates and size of clear area surrounding colonies was noted. Results were suggestive but not conclusive. Strain F showed highest gelatinase activity; strain m2AA (probably had lost activity on polymer) and Q-1 (very poor or no activity on polymer) showed low gelatinase activity. Further tests should be done.

CHAPTER V

CONCLUSIONS

The interactions of polyacrylamide polymers with microorganisms are complex, and to a large extent the nature of these interactions is not understood. Although we have shown that polyacrylamides enhance the growth of soil bacteria, it has not been established that they are biodegradable in the usual sense (able to serve as a carbon-energy source, with the polymer backbone $-CH_2-$ chain being degraded, and assimilated), or that they are cometabolized.

The mechanism of growth enhancement could be via a modification of physico-chemical environmental parameters, or it could be because of an ability to supply certain nutrients.

We believe that in the case of pseudomonads isolated from polyacrylamide enrichment cultures the means of growth stimulation is the ammonia content of polymer solutions. Release of ammonia in addition to that present from spontaneous hydrolysis by enzymatic action is a distinct possibility, but we have not been able to prove conclusively that it occurs.

Several lines of indirect evidence of such an enzyme may be summarized as follows: 1) correlation of ability to grow with polymer as a sole nitrogen source with amount of ammonia released from polymer by resting cells; 2) variation among strains of ability to grow on polymer, although all are able to utilize NH_4Cl with equal facility; 3) failure of

strains tested to grow on xylose plus NH_4Cl, although they grow with xylose plus polymer, or glucose plus NH_4Cl; and 4) enzyme-like properties of ammonia release, such as heat lability and optimum pH.

On the other hand, the fact that polymer placed in a dialysis bag, which is suspended in an ammonia free medium, is able to support growth as well as that same amount of polymer distributed uniformly throughout the medium. argues that purely spontaneous hydrolysis is responsible for growth supporting ability. However, this argument is inconclusive, in that removal of NH_4^+ ions from polymer, as would occur in the dialysis system, would allow for continual spontaneous hydrolysis. This does not rule out the possibility of an enzyme catalyzing ammonia release.

It was thought initially that the enzyme involved in ammonia release might be the same as the low molecular weight amidase. Indirect and direct evidence, however, ultimately ruled out this possibility. The possibility remains that the postulated enzyme may be a protease.

Whether the polymer molecule is degraded (molecular weight greatly decreased) or undegraded does not seem to determine the extent to which bacteria are able to grow with the polymer as a nitroge source.

Release of ammonia may occur more readily with smaller molecules as substrate. This could be compatible with the growth observation if the additional amount of ammonia released from longer molecular weight polymer were not large enough to be critical for growth.

Several approaches to concentrating and conclusively demonstrating the existence of a polymer-hydrolyzing enzyme were unsuccessful. Our efforts were thwarted by the low level of activity of the enzyme, the instability of production of the enzyme along with high endogenous activity, and difficulties with the Nessler reaction. Use of the ammonia

electrode should be useful in future attempts to demonstrate such an enzyme.

We believe that we have shown conclusively that the postulated polymer-hydrolyzing enzyme is not the same as the well-characterized low molecular weight aliphatic amidase of Pseudomonas aeruginosa. Future work should concentrate exclusively on extracellular enzymes such as protease.

The concern of reservoir engineers regarding microbes is primarily the effect of microbial growth on the properties of EOR chemicals which are important in oil recovery.

In the case of polyacrylamides the viscosity (screen factor) of solutions u sed for flooding reservoir is the important property. We have done several experiments which show a reduction in screen factor (more than control) when growth of Pseudomonas has taken place in presence of polymer, but not enough data to reach definitive conclusions.

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