THE MICROBIAL INACTIVATION OF ERYTHROMYCIN A

BY

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I. Introduction

The ability of microorganisms to transform or inactivate macrolide antibiotics has recently begun to be studied. Nakahama (1, 2, 3, 4) in a series of investigations using the macrolide antibiotics maridomycin and josamycin has presented evidence for deacylation of maridomycin by both bacteria and actinomycetes, as well as both the microbial hydroxylation and reduction of these antibiotics.

The inactivation or transformation of erythromycin, a macrolide antibacterial antibiotic, has not previously been well established. Past investigations by Feldman (5) on microbial transformations of macrolide antibiotics only note the "disappearance" of erythromycin from the culture media of a small number of steroid-transforming strains of Streptomyces hygroscopicus, S. griseospirulis, and Nocardia corolina. The only other work demonstrating a biological inactivation of this antibiotic has concerned mammalian systems. Mao (6), Lee (7, 8), and Welles (9) have investigated the demethylation of erythromycin by the liver tissues of rabbits (in vitro), rats, and dogs (in vivo).

The purpose of this study concerns the isolation and characterization of a microorganism capable of enzymatic inactivation of erythromycin A. In investigating this inactivation it is hoped to define the chemical basis of enzymatic inactivation. Such an enzyme system could be used as a potential tool for bio-transformation and chemical modification of this macrolide antibiotic as well as a clinically important analytical means of assaying for the presence of erythromycin in antibiotic mixtures.

II. Chemistry of Erythromycin and the Macrolide Antibiotics

The antibacterial macrolides derive their name from a common chemical feature--a macrocyclic lactone. In addition to the large lactone, various ketonic and hydroxyl functions and glycosidically bound 6-deoxy sugars are structurally characteristic of this group of antibiotics. These macrolides are of great interest due to their antibacterial activity primarily against Gram positive organisms and mycoplasma.

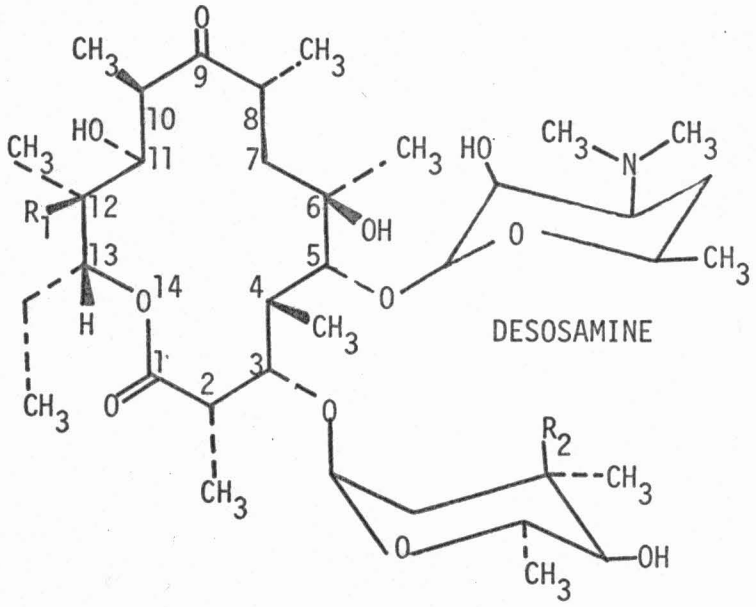
In Figure 1 and Table 1 are summarized the structural and chemical features of the erythromycins (10, 11, 12, 13). Information on other macrolide antibiotics can be obtained in several extensive reviews (14, 15, 16).

Erythromycin as elaborated in the fermentation broths of Streptomyces erythreus is a mixture of three closely related antibiotics; erythromycin A, B, and C. The major component produced by commercial high-yielding strains is erythromycin A; the second and third components are less prevalent with the latter present in only trace amounts in the fermentation.

The glycosides were the first portion of the erythromycin molecule to be identified. It was found that erythromycins A and B both contained the same sugar moieties, desosamine (a basic sugar) and cladinose (a neutral sugar) (11, 17, 18, 19). Both sugars were found to be 6-deoxypyranosides, with desosamine belonging to the D series and cladinose being an L sugar (20, 21, 22). The presence of a dimethylamino function on desosamine being responsible for its basic character. Later it was found that erythromycin C contained desosamine and has the same aglycone present in erythromycin A (13), the C form differing only by the presence of the unmethylated neutral sugar mycarose in place of cladinose (23).

Figure 1: STRUCTURE OF ERYTHROMYCIN A, B, AND C

Figure 1



ERYTHROMYCIN

CLADINOSE

A R_1 = OH
R_2 = OCH_3

B R_1 = H
R_2 = OCH_3

C R_1 = OH
R_2 = OH

TABLE I. CHEMICAL PROPERTIES OF ERYTHROMYCINS PRODUCED BY Streptomyces erythreus NRRL 2338

	Melting Point	Empirical Formula	D	U.V. Max
Erythromycin A	135 - 140°C	$C_{37}H_{67}O_{13}N$	-73.5° (CH_3OH)	289nm
Erythromycin B	198°C	$C_{37}H_{67}O_{12}N$	-78° (CH_3CH_2OH)	286nm
Erythromycin C	121 - 125°C	$C_{36}H_{65}O_{13}N$		

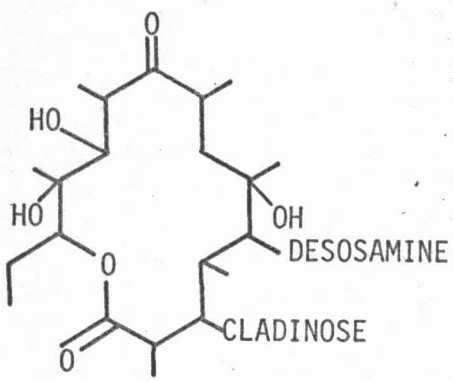
Although it was recognized that the aglycone portion of the erythromycins consisted of a large lactone ring containing a ketone function and numerous hydroxyl and methyl groups, the final structures were determined only after lengthy chemical degradations (10, 11, 24, 25, 26). The primary difficulty encountered is the extreme lability of the intact aglycone ring to acid or base. This acid lability was overcome by the use of the dihydro derivative obtained by borohydride reduction of the ketone function.

Some of the chemistry of erythromycin is summarized in Figures 2 and 3. Mild acid hydrolysis of erythromycin liberates cladinose and the basic compound erythralosamine. Desosamine can be obtained under more vigorous conditions with the concomitant destruction of the aglycone erythronolide producing propionaldehyde (15) (Figure 3).

Dihydro-erythronolide was isolated from the hydrolysis of dihydroerythromycin which was obtained by a $NaBH_4$ reduction of erythromycin. The structure of this moiety was elucidated by its cleavage with periodate, without loss of carbon, into the two fragments shown in Figure 2 (15). The structures of these were determined by further degradations. The possibility of fragments 1 and 2 being coupled in a reverse sense, which would lead to a nonalternating methyl group skeleton, was ruled out by the isolation of fragment 3 from the chromic acid oxidation of erythralosamine (15). The ketone group in erythromycin must be at C-9 since of the two possibilities C-9 or C-11 only the former explains the ready formation in acid of the ketal, anhydroerythromycin (15). Similar degradations have established the structures of erythromycin B and C (10, 13).

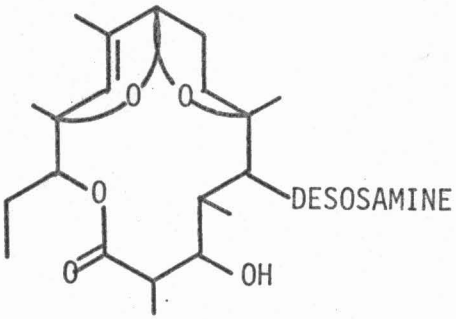
The study of the chemistry of the erythromycins has been completed by the x-ray analysis of erythromycin B providing the complete conformational assignments of the aglycone of this macrolide antibiotic (27, 28).

Figure 2: DEGRADATIVE REACTIONS LEADING TO THE STRUCTURE DETERMINATION OF ERYTHROMYCIN



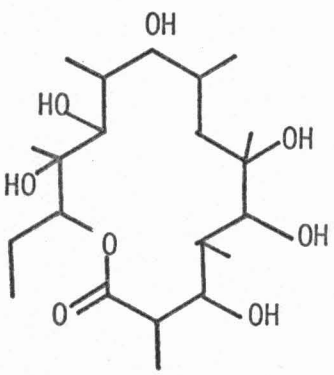
ERYTHROMYCIN A

[H+]



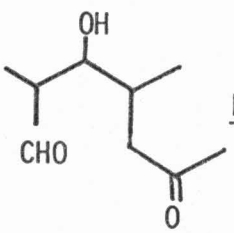
ERYTHRALOSAMINE

[$NaBH_4$]

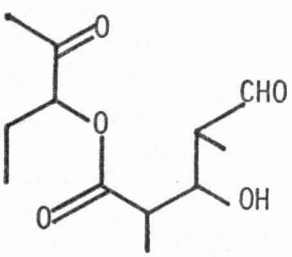


DIHYDROERYTHRONOLIDE

[IO_4^-]



FRAGMENT 1



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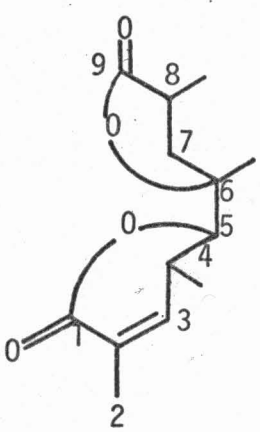

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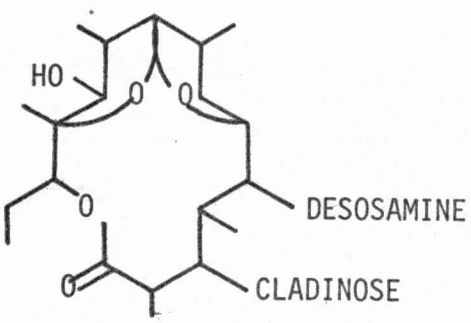
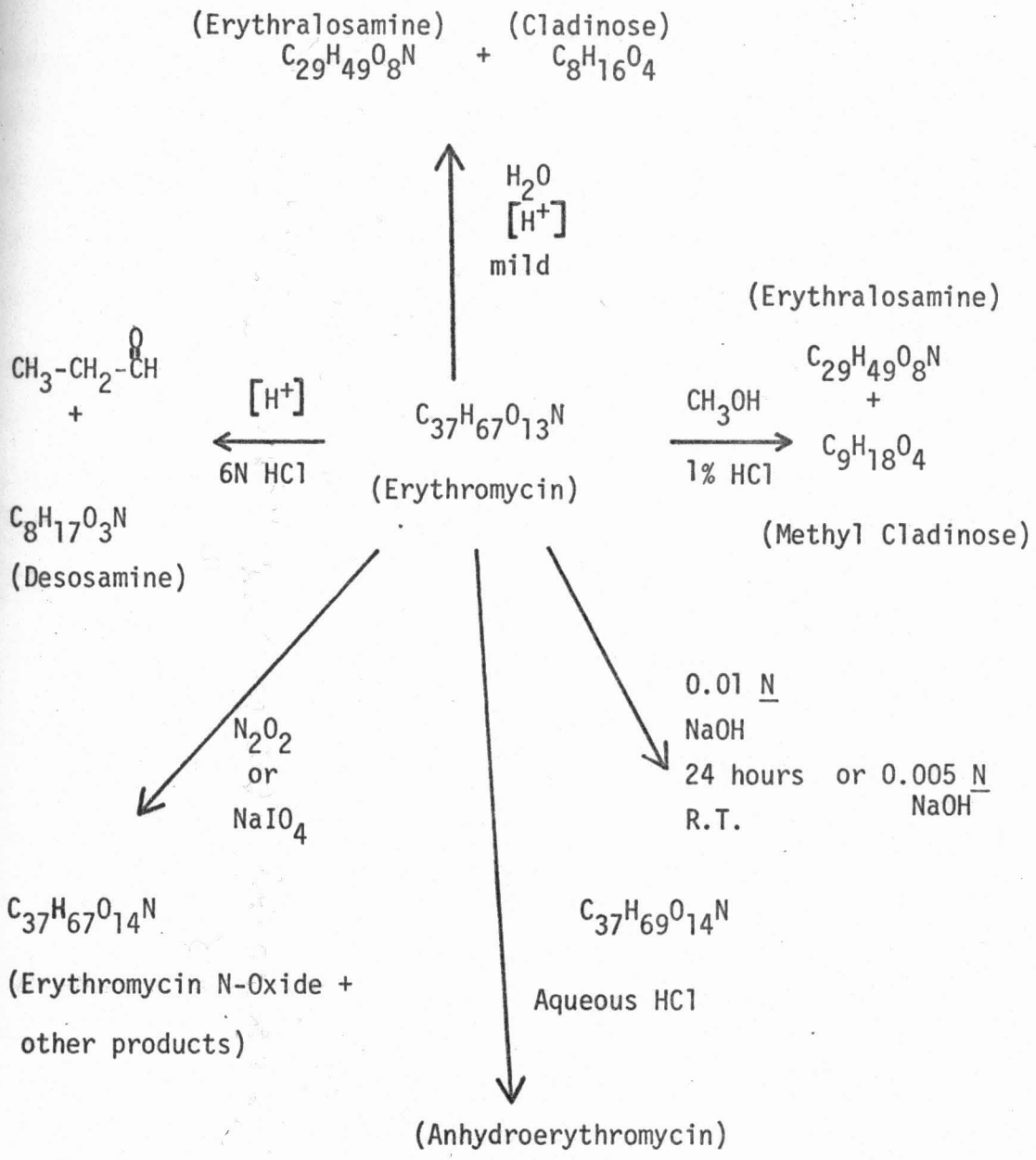


ANHYDROERYTHROMYCIN

Figure 3: SOME CHEMICAL REACTIONS OF ERYTHROMYCIN

Figure 3


(Erythralosamine) $C_{29}H_{49}O_8N$ + (Cladinose) $C_8H_{16}O_4$
H_2O
$[H^+]$
mild
(Erythralosamine)
$C_{29}H_{49}O_8N$
+
$C_9H_{18}O_4$
(Methyl Cladinose)
$CH_3-CH_2-\overset{O}{\overset{\|}{C}}H$
+
$C_8H_{17}O_3N$
(Desosamine)
$[H^+]$
6N HCl
$C_{37}H_{67}O_{13}N$
(Erythromycin)
CH_3OH
1% HCl
0.01 N NaOH
24 hours
R.T.
or 0.005 N NaOH
N_2O_2
or
$NaIO_4$
$C_{37}H_{67}O_{14}N$
(Erythromycin N-Oxide + other products)
$C_{37}H_{69}O_{14}N$
Aqueous HCl
(Anhydroerythromycin)

A. Stability of Erythromycin in Solution

It is imperative in an investigation of erythromycin to rigorously establish that the inactivation being observed is not chemical degradation but of biological origin. Erythromycin in aqueous solution loses microbiological activity as a function of pH, temperature, and time. This loss of activity has been discussed by Kavanagh (29) and by Korecka (30).

Erythromycin is unstable in highly acidic or alkaline solution and has its optimum stability in the range of pH 6.5 to 8.5. Aqueous or alcoholic solutions lose considerable antibacterial activity upon storage at 4°C for periods of several weeks. Solutions are usually made and diluted in buffers in pH 7 to pH 8 range. Acetone solutions of erythromycin base are favored as standard solutions as they are much more stable than alcoholic or aqueous solutions.

The chemical loss of biological activity under mildly acidic conditions probably follows the scheme suggested by Kurth (32) and Perun (33) shown in Figure 4. It has also been established (34) that loss of the neutral sugar decreases the biological activity of erythromycin and that only mild acidic conditions are required to chemically remove this sugar. This presents another possibility for the mechanism of chemical loss of biological activity in solution.

B. The Relationship Between Structure and Biological Activity: The Choice of an Assay

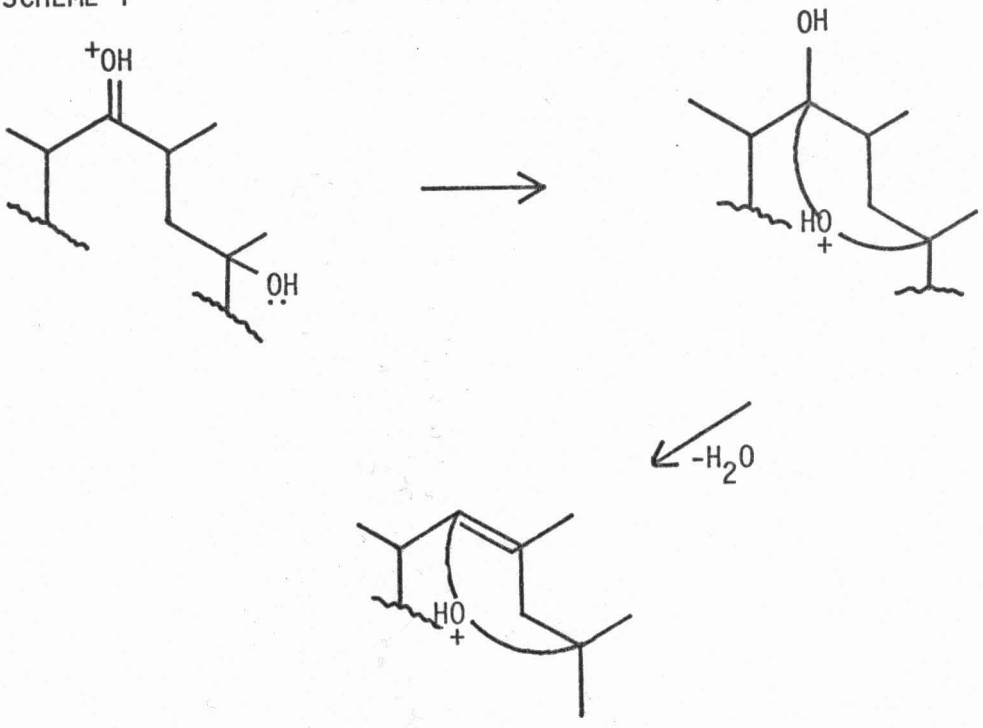
It has recently been demonstrated by Pestka and Le Mahieu (35) using a Staphlococcus aureus agar diffusion biological assay that

Figure 4: PROTONATION OF ERYTHROMYCIN IN ACIDIC SOLUTION

After Kurth (32) and Perun (33)

Figure 4

SCHEME 1



small chemical substitutions or deletions drastically alter the biological activity of erythromycin A. The microbial bioassay response of erythromycin B is approximately 50 percent that of erythromycin A. This demonstrates that the substitution of a hydroxyl group at a single position (C-12) in the A form doubles the biological activity over that of the B form where no C-12 hydroxyl exists. This response of the microbiological activity for structural changes makes the bioassay of erythromycin a most sensitive means of assaying for possible biological alterations of molecular structure.

III. Materials and Methods

A. Sources of Macrolide Antibiotics and Derivatives

The following is a listing of the macrolide antibiotics used in this study and their respective sources:

Macrolide or Derivative	Source
Erythromycin A	Abbott Laboratories
Erythromycin B	
Erythralosamine	
Desosamine	
Erythromycin A N-oxide	
Erythromycin B N-oxide	
Erythronolide B	
Oleandomycin base	Pfizer Corporation
Oleandomycin phosphate	
Methymycin	Squibb Institute for Medical Research
Tylosin tartrate	Eli Lilly & Company
Leucomycin base mixture	Ayerst Laboratories
Josamycin (Leucomycin A_3)	Yamanouchi Pharmaceutical Co. Ltd.
Maridomycin III	Takeda Chemical Co. Ltd.

B. Agar Diffusion Bioassay for Erythromycin and Other Macrolides

Bioassays were carried out using Staphylococcus aureus 209P or Sarcina lutea as the test organisms in an agar diffusion assay. The assays were in sterile 21 by 35 centimeter 3 quart Pyrex baking dishes with removable stainless steel covers. A two layer agar system was employed to enhance the sensitivity of the assay. Difco Antibiotic Medium 1 was used for the bioassays using S. aureus and Difco Antibiotic Medium 11 for those assays using Sarcina lutea. Two hundred ml of medium per baking dish was used for the base layer. The top agar layer consisted of 50 ml of the same agar with 1 ml of a 2 percent aqueous solution of 2, 3, 5 triphenyl tetrazolium chloride and 0.3 ml of a 24 hour suspension of the test organism as inoculum (10^7 cells per ml). The top agar was maintained at 52°C until inoculation. The pH of Antibiotic Medium 1 was 7 while that of Antibiotic Medium 11 was pH 8.

Antibiotic test solutions and standards were drawn up into 12.7 mm filter paper discs (Schleicher and Schuell) by capillary action and placed on the surface of the solidified agar. All samples were assayed with at least three filter paper discs. Each Pyrex dish could hold up to 56 discs without danger of overlapping inhibition zones. Of the 56 discs on each plate, at least nine were of known concentrations of erythromycin A. Plates of S. aureus were incubated at 37°C for 15 hours. (National Incubators) Sarcina lutea plates were incubated at 30°C under similar conditions for 15 hours. The antibiotic inhibition zones were read manually using a vernier calipers graduated in 0.1 mm divisions. The average value of three discs was used for each determination. Data was plotted as the logarithm of the antibiotic concentration in mcg per ml against the observed zone size in millimeters. A separate curve was

run for each plate and the slope of the line determined by the response of the standards on that plate. The accuracy of this assay was ± 15 percent for this data which is within expectations of a bioassay of this type.

When test solutions contained high bacterial populations efforts were made to 'sterilize' these discs by adding 40 microliters of methanol to the disc. In using this method, bacteria did not grow upon the discs being used for the bioassay. The same ± 15 percent precision was obtained from discs treated in this fashion in all cases.

C. Arsenomolybdate Assay

A colorimetric assay was developed from the Method of Perlman (36) to follow the disappearance of 'neutral macrolides' from test solutions:

A 1 ml sample of test solution at pH 8 containing about 200 mcg of erythromycin was transferred to a conical glass stoppered centrifuge tube at 0°C. 4.0ml of ethyl acetate was then added, the tube stoppered and shaken on a vortex mixer for 15 seconds. (Scientific Industries, Inc.) 1.0 ml of the ethyl acetate layer was transferred to a 18 x 150 mm Pyrex test tube. The contents of the tube were dried by a current of filtered air at room temperature. To this tube was then added 2.0 ml of 6N sulfuric acid and 1.0 ml of an arsenomolybdate color reagent devised by Nelson (37). The tube was then thoroughly mixed and incubated in a boiling water bath for 15 minutes to develop the color. 5.0 ml of water was added to the tube after it was removed from the bath and allowed to cool. The blue color was recorded as the absorbance at 660nm on a Bausch and Lomb Spectronic 20 spectrophotometer. This combination of reagents was run without added erythromycin as a control.

The response of this assay was linear between 0 and 80 mcg of erythromycin A per tube. A typical standard curve for the assay is shown in Figure 5.

A study of the efficiency of the extraction at pH 8 of erythromycin in the ethyl acetate layer revealed that the concentration of 'neutral macrolides' extracted into the non-aqueous layer did not exceed ± 5 percent of the concentration present originally in the aqueous phase.

Studies were also carried out to determine the correlation between the colorimetric assay and the agar diffusion bioassay. The correlation between the colorimetric assay and the concentration of erythromycin present in solutions of known concentration was within ± 5 percent. When these same standard solutions were assayed using the S. aureus bioassay only 80 to 90 percent of the original amount of macrolide antibiotic was found.

The 90 minute colorimetric assay had the advantage of elimination of the 15 hour incubation required by the bioassay.

Figure 6 shows the response observed with other macrolide antibiotics when tested using the arsenomolybdate colorimetric assay.

The arsenomolybdate reaction employed in this study measures the presence of reducing substances present in the samples after acid hydrolysis. The primary limitation of this assay is that it also reacts with carbohydrates and other reducing substances. When extracting enzyme reaction mixtures with ethyl acetate, care must be taken not draw up any cellular debris when removing aliquots of the non-aqueous phase for analysis. Small quantities of carbohydrate or protein adhering to the tip of the pipet or suspended in the ethyl acetate greatly interfere with the assay. It is this aspect of the assay method which limits

Figure 5: TYPICAL STANDARD CURVE FOR ARSENOMOLYBOATE COLORIMETRIC ASSAY

Figure 5

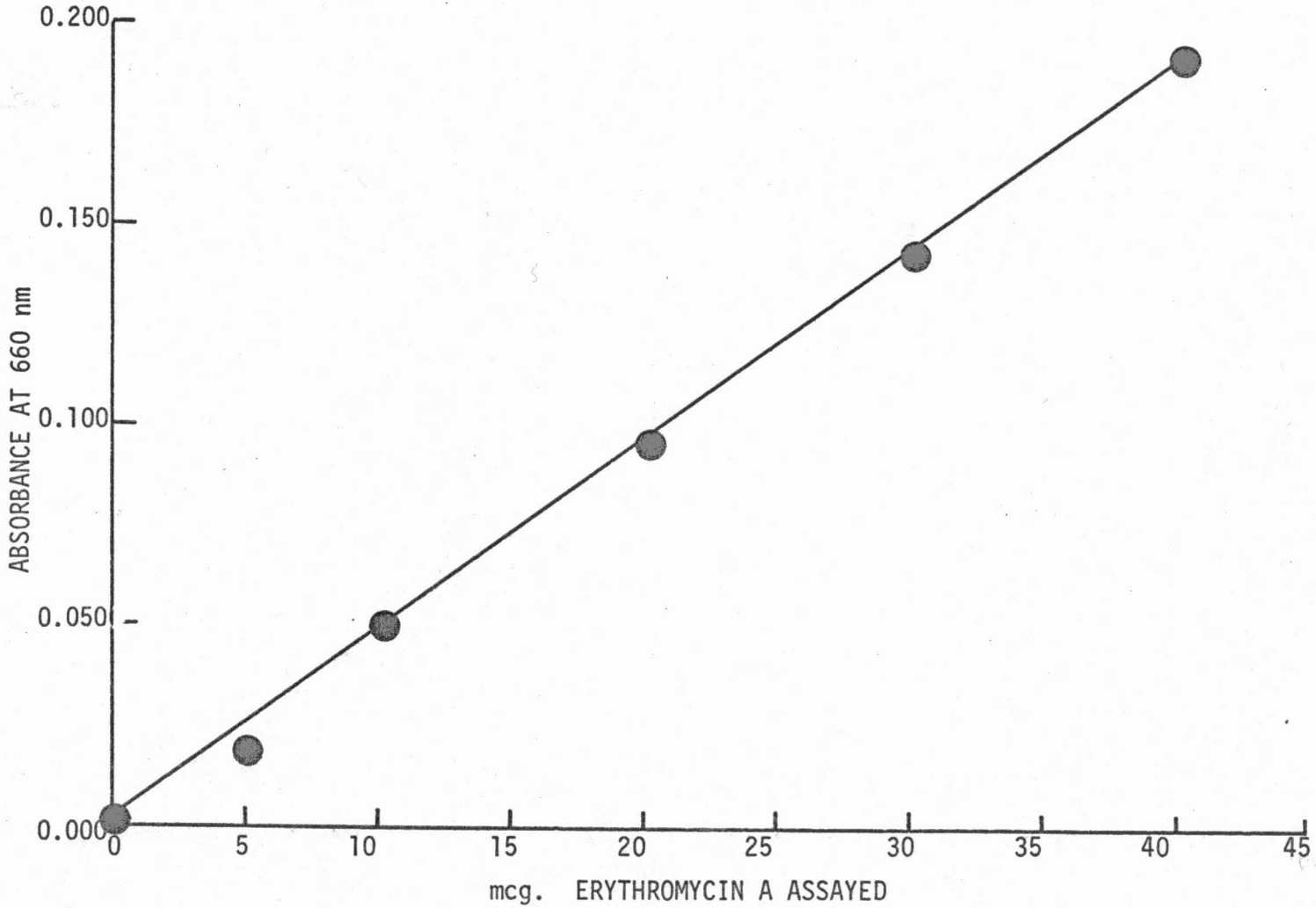

0.200
0.150
0.100
0.050
0.000
ABSORBANCE AT 660 nm
0
5
10
15
20
25
30
35
40
45
mcg. ERYTHROMYCIN A ASSAYED

Figure 6: ARSENOMOLYBDATE RESPONSE OF SOME MACROLIDE ANTIBIOTICS

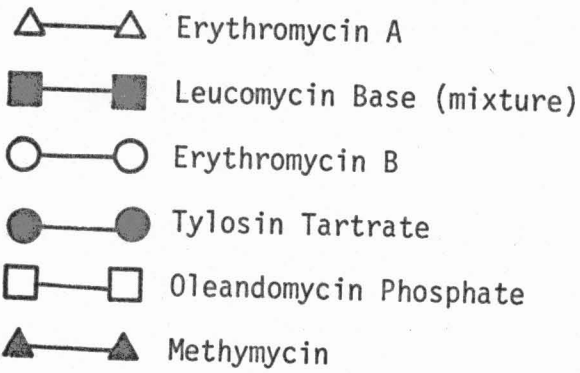
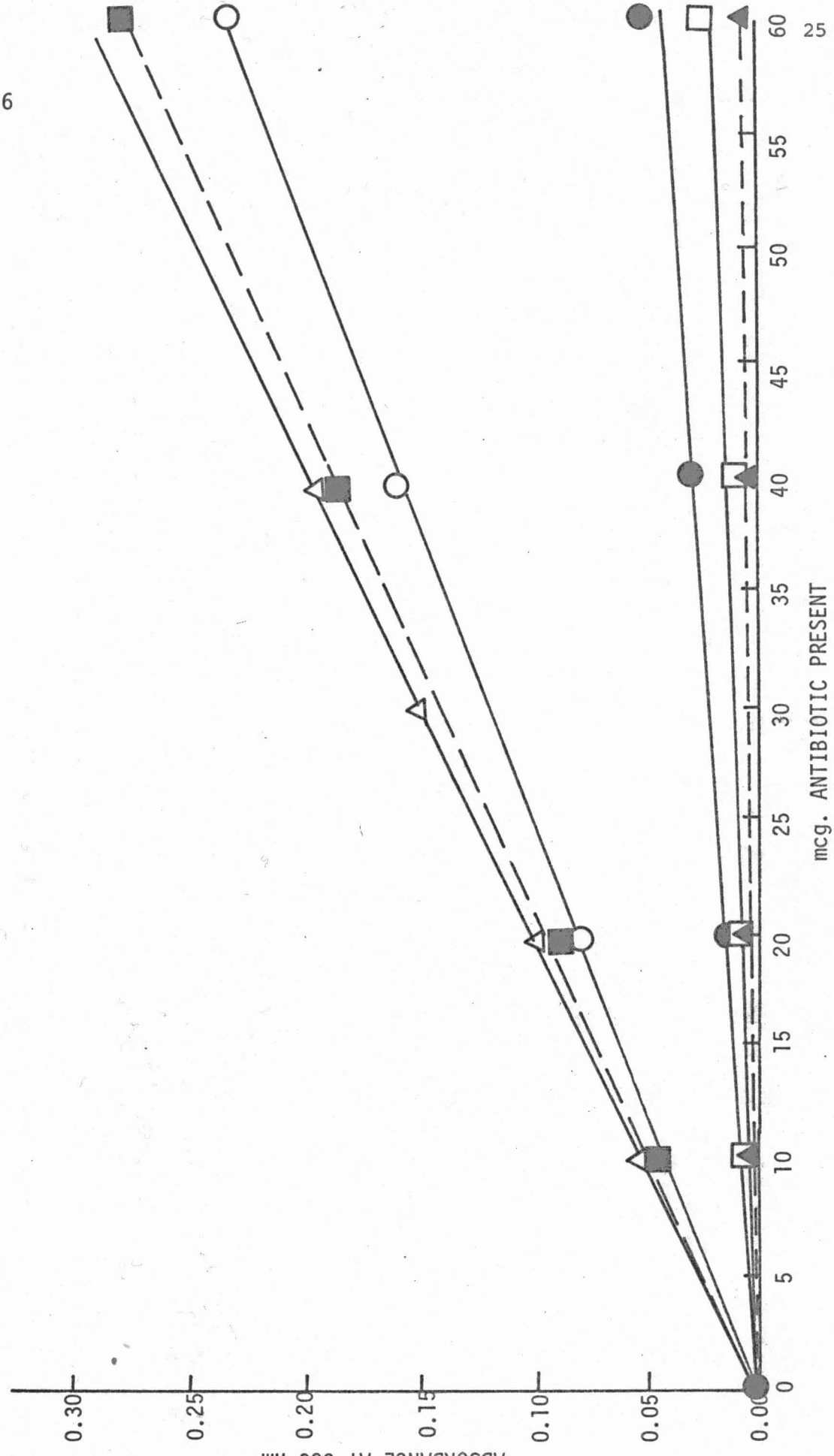


Figure 6


ABSORBANCE AT 660 nm
0.00
0.05
0.10
0.15
0.20
0.25
0.30
0
5
10
15
20
25
30
35
40
45
50
55
60
mcg. ANTIBIOTIC PRESENT

the study of any transforming enzyme to only very active preparations.

A second limitation to this assay is that it will detect modifications of molecular structure which cause the molecule to favor association with the more polar aqueous layer when extracted. Biological transformations which retain the 'neutrality' of the macrolide will not be detected using this assay. A concomittant thin layer chromatogram of the reaction mixture is required to detect such transformations.

Biological or chemical transformations could also occur which produce a new compound having significantly high distribution coefficient between the ethyl acetate and aqueous phases at pH 8. In this case only a fraction of the compound's concentration will be detected by the arseonomolybdate color reagent. It must be noted by the molecular structure that even small alterations to erythromycin will favor its retention in the aqueous phase upon extraction with ethyl acetate at pH 8.

Even with the above limitations to this chemical assay it appears to be a relatively accurate, rapid means of monitoring the disappearance of erythromycin from culture broths and reaction mixtures with reasonable correlation to the biological activity present.

D. Medium Used for Growth of Cultures and Incubation Conditions

Isolates used in this study were grown in the following medium:

	grams per liter
Yeast Extract (Difco)	10
KH_2PO_4	2.3
K_2HPO_4	5.8
$(NH_4)_2SO_4$	0.5
Distilled Water	1000 ml
pH 7	

The medium was autoclaved for 20 minutes at 15 pounds pressure at 121°C.

The erythromycin A inactivating culture was maintained on slants of the above sterile medium with the addition of three percent Difco Bacto Agar. Slants were transferred every month with incubation for 24 hours at 30°C. The culture was also maintained as a frozen suspension stored in liquid nitrogen.

This culture was grown in 200 ml of the above medium in a 2000 ml Erlenmeyer flask on a New Brunswick Scientific Company incubator shaker operating at 270rpm with a one inch displacement.

Inoculum for the flasks was grown in 25 x150 mm Pyrex tubes with plastic closures (Bellco Glass Company). Each tube contained 10 ml of medium. The tubes were incubated over night at 30°C on a rotary shaker.

Measurement of cell growth was follows by the absorbance of the culture at 660 nm with a Bausch and Lomb Spectronic 20 Spectrometer. Growth was also correlated to cell dry weight. Two hundred ml of a 7 hour culture grown at 35°C on a 300 rpm shaker using a 10 percent inoculum was centrifuged and washed twice with 50mM pH 7 phosphate buffer and resuspended in the same. Aliquots of this dense suspension were diluted with buffer, their absorbance recorded at 660 nm and transferred quantitatively to pre-weighed and dried aluminum weighing pans. The pans were dried at 100°C for 15 hours, allowed to cool in a dessicator, and weighed to constant weight on an analytical balance. Aliquots of the cell suspension were adjusted so that at least 30 mgs dry weight of cells were weighed in each pan. Triplicate determinations were made at each dilution. The cell dry weight was corrected for the weight of the buffer salts.

E. Instrumentation

Infrared spectra were obtained on a Perkin Elmer 234 Infrared Spectrometer in liquid film or KBr pellet.

Mass spectra were obtained on a Finnigan 1015 GC-MS Mass Spectrometer at 60° or 200°c.

A New Brunswick Scientific Company water bath shaker was used in the enzyme studies. The stirred jar assembly and incubator shakers were also made by New Brunswick Scientific Company.

Disruption of whole cells was accomplished using a Aminco French Pressure cell or a Bronson Sonicator.

F. Thin Layer Chromatographic Methods

The separation of inactivation products was achieved on 0.5 mm thin layer chromatogram sheets (Eastman Chromagram Sheets, No. 13179-Silica Gel) using the following solvent systems:

System A - chloroform-methanol-acetone
(10:3:1)

System B - benzene-methanol-toluene
(85:10:5)

System C - n-butanol-water-acetic acid, (glacial)
(4:5:1)

System D - n-hexane-acetone-benzene-methanol-ethyl acetate
(30:10:25:10:20)

The tlc plates were used without heat activation. All solvent systems were freshly prepared. The chromatograms were developed under non-equilbrium conditions to obtain the best reproducibility of R_f values.

The products were visualized using a two spray method. The dried, developed, chromatogram was first sprayed with a fresh two percent alkaline solution of 2, 3, 5 triphenyl tetrazolium chloride in methanol. The color was developed at 100°C for two minutes. This reagent detected free sugars and other reducing compounds as a red color against a white background. The same chromatogram was then sprayed with a one percent solution of ceric sulfate in ten percent (v:v) sulfuric acid and charred at 100°C for three to five minutes. This spray detected all other compounds not reacting with the tetrazolium chloride as dark green to black spots against a light brown background.

IV. Results

A. Isolation and Characterization of Culture Number 56

Isolations were made from flower pot soil on the yeast extract medium described on page 26 in the presence of 100 mcg per ml of erythromycin A. One isolate demonstrated (agar diffusion assay) the ability to 'remove' the antibiotic from the growth medium. Ninety soil and water isolates able to grow in 100 mcg per ml of erythromycin A were screened for their ability to inactivate this antibiotic. Isolate 56 demonstrated the greatest ability to 'remove' the antibiotic from the medium. This isolate was a mixture of Gram negative rod shaped bacteria and fungal mycelium. Separation of these components demonstrated that only the bacterial component of the isolate was associated with all of the inactivating ability. This Gram negative rod shaped bacterium was designated as culture 56.

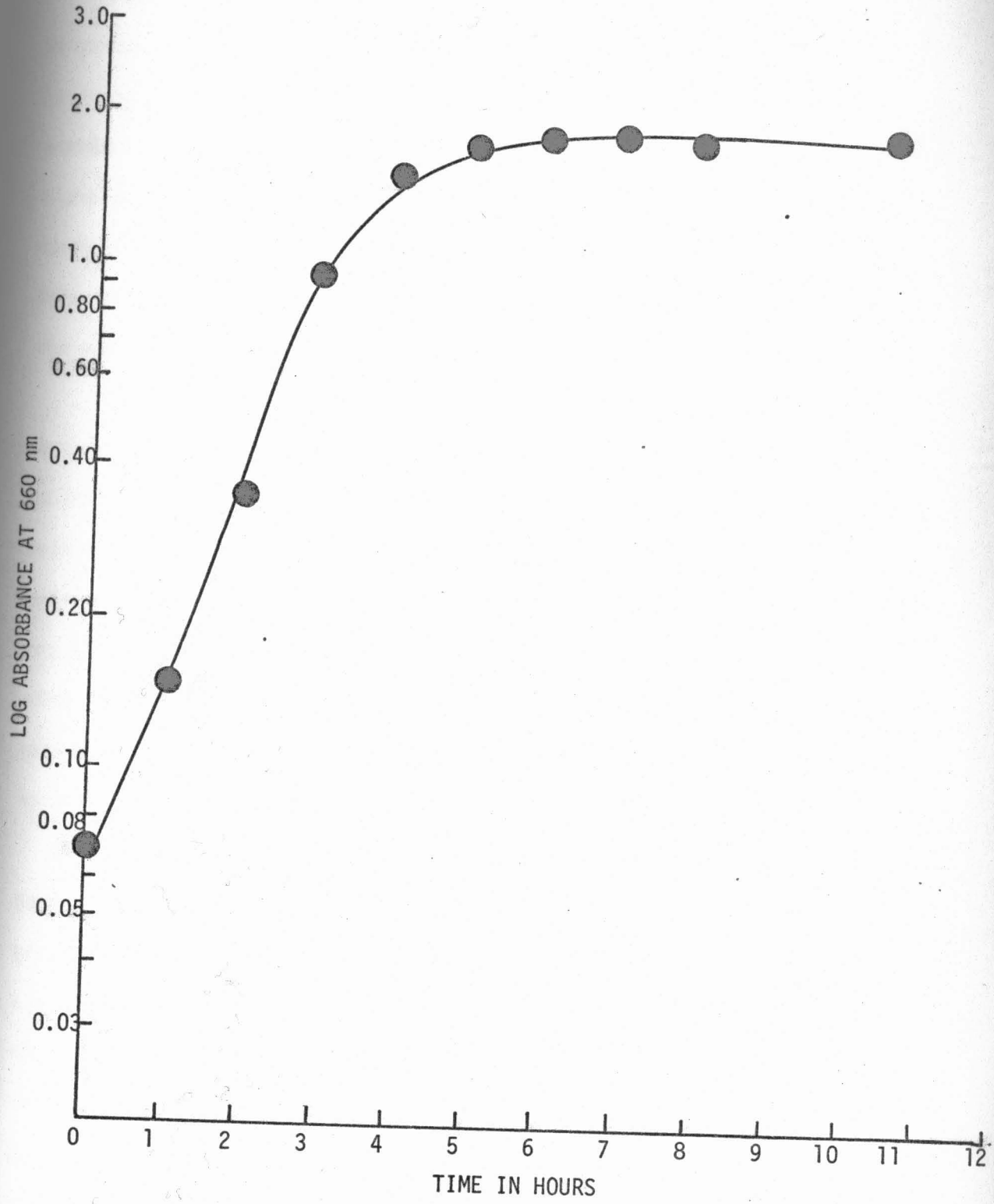
This culture was maintained on agar slants of the yeast extract medium without added erythromycin A.

Culture 56 grew at 27°, 30°, 35°, and 37°C; it would not grow at 41°C in liquid medium. Best growth rate in shake flasks was obtained at 35°C. Typical growth curve data is shown in Figure 6. Growth was measured by absorbance of the culture at 660 nm as was previously described. The exponential doubling time of the organism in shake flasks at 35°C was 42 minutes.

The cell dry weight of culture 56 was correlated with the absorbance of the culture at 660 nm by plotting this absorbance (in buffer) versus the cell dry weight as determined by the method described. An absorbance of 1.00 at 660 nm represented 1.03 grams cell dry weight per liter.

Figure 7: GROWTH OF CULTURE 56 ON A YEAST EXTRACT MEDIUM AT 35°C

Figure 7


3.0
2.0
1.0
0.80
0.60
0.40
0.20
0.10
0.08
0.05
0.03
LOG ABSORBANCE AT 660 nm
0
1
2
3
4
5
6
7
8
9
10
11
12
TIME IN HOURS

B. Taxonomic and Nutritional Studies on Culture Number 56

A taxonomic study of culture 56 was undertaken according to the programmed classification method of Skerman (59). The data obtained is summarized on Tables 2 and 3. The results of studies suggest the assignment of culture 56 to the genus Pseudomonas. However, in investigating the nutritional characteristics of members of this genus as elaborated by Stanier et. al. (38), the nutritional requirements of this organism were found not to correlate with any of the groups of the genus. This nutritional work is summarized in Tables 4 and 5.

It was discovered that culture 56 was unable to metabolize any carbohydrate, sugar, or keto acid as sole source of carbon and energy at 30°C, aerobically, in 24 hours. An auxotrophic nutritional requirement for one or a combination of amino acids was then suspected. Surprisingly, the culture failed to grow on a synthetic medium containing 10 mg of each of 20 amino acids in a medium supplemented with sufficient nitrogen source, phosphate buffer, and trace metals to support growth. Culture 56 failed to grow on each of these amino acids individually or all 20 in combination.

Investigation of a possible growth factor requirement followed. As the data in Table 5 suggests, the organism would not grow in response to the addition of any vitamin nor would it grow on a medium with a combination of all of the vitamins and growth factors. Interestingly, the organism did grow on a medium of Difco vitamin free Casamino Acids. This suggested that perhaps the growth of the organism could be stimulated by lipid. A small growth response was able to be demonstrated with additions of 0.01 and 0.1 percent (w:v) sodium oleate (Baker Chemical) to the synthetic combination of 20 amino acids which previously did not support growth.

Table II

SUMMARY OF PRELIMINARY TAXONOMIC INFORMATION

ON CULTURE NUMBER 56

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* For results on media commonly used for the study of pigmentation or fluorescence, see Table 4.

Table II

Gram stain: negative

Hydrolysis of agar in 14 days: none

Acid and gas from carbohydrates: no acid or gas

Morphology: small rod

Pigmentation on various media*: none

Fluorescence on various media*: none

Motility in hanging drop: motile

H_2S production: none

Growth at 27°, 30°, 35°, 37°C

No growth at 41°C

Slime or capsule formation: slime formation covering cells (47)

Table III

GROWTH OF CULTURE NO. 56 ON VARIOUS AGARS AND BROTHS COMMONLY USED FOR CLASSIFICATION OF PSEUDOMONADS

Table III

Broth	Description of growth at 30°C	
	48 hours	76 hours
Tryptone Yeast Extract	slight	diffuse-heavy
Proteose Peptone	slight	diffuse-heavy
Trypticase-Soy	very slight	diffuse-heavy

Agar	Growth and pigmentation at 30°C	
	48 hours	76 hours
Triple sugar iron	filiform, white no H_2S or pH change	same as 48 hour
Trypticase-Soy	very heavy, white to yellow	heavy, white
King's medium A	filiform and beaded, white	same as 48 hour
Citrate + phenol red	no growth	no growth
Hedberg's acetamide	no growth	no growth
King's Pseudomonas agar F	heavy filiform, white	heavy, white

Table IV

ABILITY OF CULTURE NO. 56 TO UTILIZE VARIOUS HYDROCARBONS AND CARBOHYDRATES AS A SOLE SOURCE OF CARBON AND ENERGY AT 30°C IN 24 HOURS

Table IV

Substrate	Culture 56	Pseudomonas aeruginosa A9843a
citrate	-	+
acetamide	-	+
D-glucose	-	+
maltose	-	+
D-galactose	-	-
D-mannose	-	-
D-xylose	-	-
D-ribose	-	-
L-rhamnose	-	+
sucrose	-	-
D-arabinose	-	-
D-fucose	-	-
D-mannitol	-	+
lactose	-	+
succinate	-	+
acetate	-	-
L-gluconic acid	-	+
pyruvate	-	+
naphthalene	-	-
inositol	-	-
phenol	-	-

Table V

GROWTH OF CULTURE 56 ON A MIXTURE OF AMINO ACIDS PLUS ADDED GROWTH FACTORS AT 30°C

*Added to 50 ml of medium containing a mixture of 100 mg/liter of each of 20 amino acids

Table V

Substrate	Growth in 24 hours
Mixture of 20 amino acids	-
Amino acids + inositol (100 mcg)*	-
" " + thiamine HCl (100 mcg)*	-
" " + nicotinamide (100 mcg)*	-
" " + folic acid (100 mcg)*	-
" " + riboflavin phosphate (100 mcg)*	-
" " + Ca pantothenate (100 mcg)*	-
" " + PABA (100 mcg)*	-
" " + nicotinic acid (100 mcg)*	-
" " + B_{12} (100 mcg)*	-
Mixture of amino acids, nucleotides all B complex vitamins and biotin, except B_{12}	-
Above + B_{12}	-
Mixture of 20 amino acids + 10 gm/liter Difco vitamin free Casamino Acids	+++
10 gm/liter vitamin free Casamino Acids	++
Mixture of 20 amino acids + 0.1 gm/liter sodium oleate (Baker Chemical)	+
Mixture of 20 amino acids + 1.0 gm/liter sodium oleate	++

The final absorbance of culture 56 on Difco vitamin free Casamino Acids appeared to be approximately 1/6 that on an equal weight of Difco yeast extract. Additions of sodium oleate (0.01, 0.1 percent) to the one percent yeast extract medium did not increase cell yields; however, additions of the same lipid source at the same levels to a one percent vitamin free Casamino Acids medium resulted in a three fold increase in cell yield. This demonstrated that culture 56 had a lipid requirement for growth. Growth of this organism on a one percent vitamin free Casamino Acids mixture appears to be growth factor (lipid) limited while growth on a similar concentration of yeast extract is not.

The fact that this organism requires lipid for growth proves to be of little value in classifying the organism as the lipid requirements of microorganisms in general and bacteria specifically are poorly defined (39). Even those Gram negative rod shaped organisms with 'fastidious' growth requirements do not fit the taxonomic characteristics of culture 56 as all of these organisms exhibit unusual morphology or pleomorphism absent with this culture.

Studies were undertaken to develop a defined medium for culture 56. Media based on tricarboxylic acid intermediates as carbon sources with additions of Tween 20, 40, 60, or 80 (Baker Chemical) did not promote growth of this organism.

A more definitive classification of this organism will have to await further electron microscopic or G-C ratio studies (47).

C. Demonstration of Inactivation of Erythromycin A in Fermentation Medium

The loss of biological activity due to chemical degradation of

erythromycin A was studied as a function of pH of the growth medium. The data presented in Figure 8 demonstrate that when screening for biological inactivation of this antibiotic at 35°C in shake flasks, the pH of the isolation medium must be greater than pH 7.0. When erythromycin A is added at an initial concentration of 100 mcg per ml to the sterile buffered yeast extract medium and placed on a rotary shaker at 35°C, chemical loss of biological activity was observed at pH values less than pH 7.0. After 45 hours only 30 percent of the original antibacterial activity was detected using the agar diffusion bioassay when the medium was buffered at pH 6.0. Utilizing a medium at pH 6.5 resulted in a 40 percent loss of antibacterial activity in the same time period and conditions. However, when the medium pH was greater than 7.0, no significant loss of bioactivity was observed over 45 hours at 35°C. It was decided that all screening and culture isolation in the presence of the antibiotic would be at pH 7.0 to minimize chemical loss of activity being confused with actual biological inactivation.

In this study and all following work, the medium was autoclaved separately, allowed to cool to room temperature, and the antibiotic was added aseptically after passage in phosphate buffer through a 0.2 micron membrane filter (Nalgene).

D. Demonstration of Inactivation of Erythromycin A with Growing Cells of Culture 56

Data showing the loss of bioactivity (by agar diffusion assay) by growing cells of Pseudomonad 56 is shown in Figure 9.

Addition of 80 mcg per ml of erythromycin A to growing cultures of number 56 just prior to the culture attaining its maximum absorbance

Figure 8: CHEMICAL INACTIVATION OF ERYTHROMYCIN A AT pH 6.0, 6.5, 7.0 and pH 7.5 AT 35°C USING A S. aureus BIOLOGICAL ASSAY

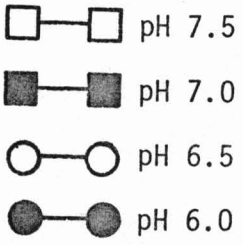


Figure 8

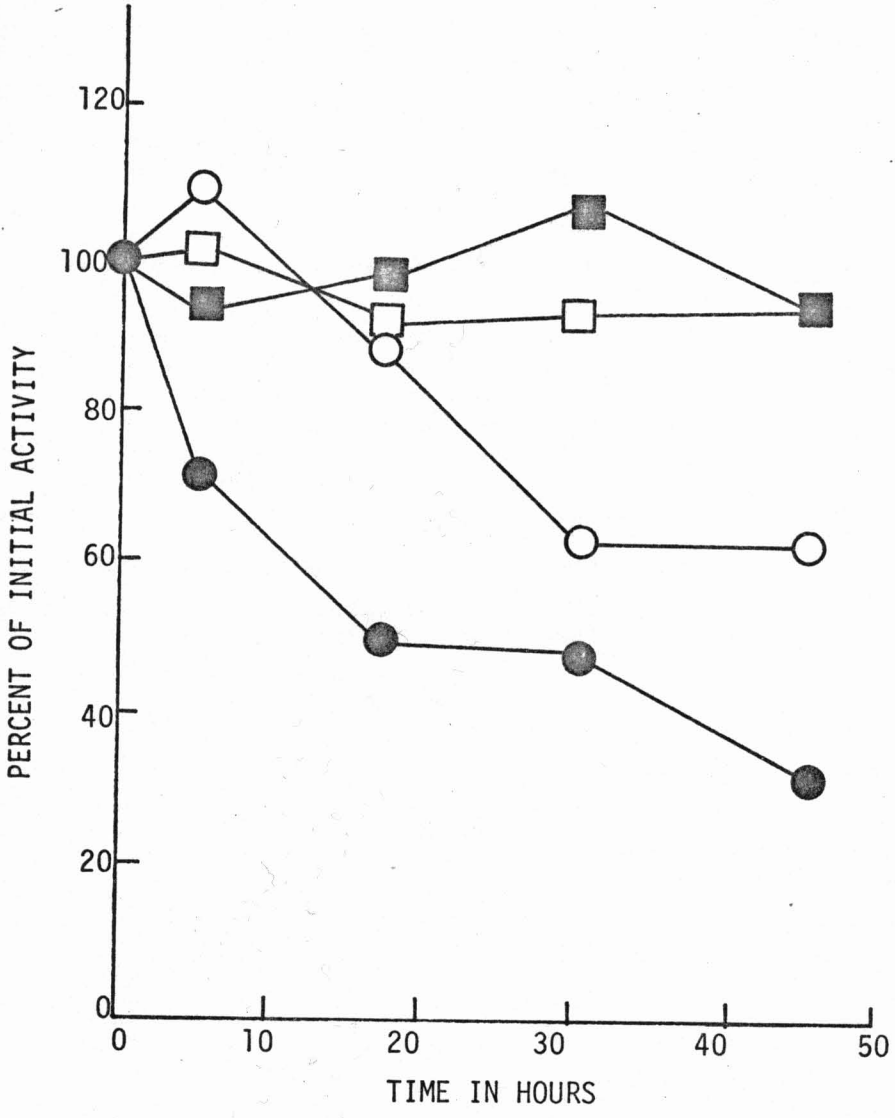
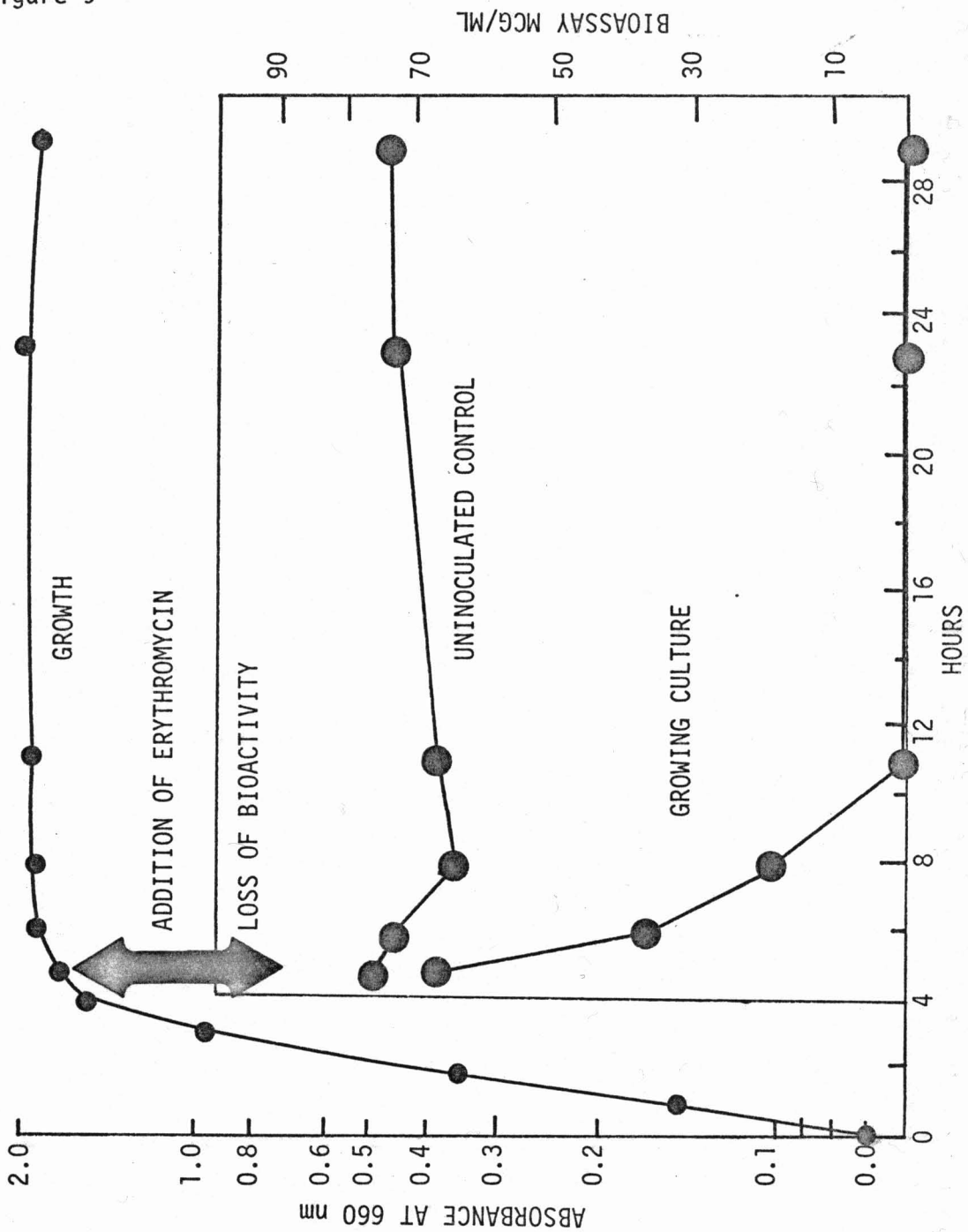

120
100
80
60
40
20
0
PERCENT OF INITIAL ACTIVITY
0
10
20
30
40
50
TIME IN HOURS

Figure 9: INACTIVATION OF ERYTHROMYCIN A BY PSEUDOMONAD 56 USING GROWING CELLS AT 35°C

Figure 9


ABSORBANCE AT 660 nm
2.0
1.0
0.8
0.6
0.5
0.4
0.3
0.2
0.1
0.0
BIOASSAY MCG/ML
90
70
50
30
10
0
4
8
12
16
20
24
28
HOURS
GROWTH
ADDITION OF ERYTHROMYCIN
LOSS OF BIOACTIVITY
UNINOCULATED CONTROL
GROWING CULTURE

resulted in complete inactivation of the antibiotic in 6 hours at 35°C. An uninoculated flask of the identical medium under the same conditions of pH, temperature, and agitation showed no significant loss of bioactivity (less than 10 percent) over the 24 hour incubation. Similar experiments were run at 27°C and 30°C showing similar loss of biological activity. The rate of inactivation at 30°C, however, was twice that at 35°C with all of the added antibiotic inactivated 3 hours after addition.

The above data suggests biological inactivation occurs with immediate addition of the antibiotic to the culture without a preliminary slow decrease in bioactivity or lag period. A lag would suggest a possible initial chemical modification in the medium before the molecule could be enzymatically inactivated.

E. Demonstration of Inactivation of Erythromycin A with Resting Cell Suspensions

Studies were continued on Pseudomonad 56 to determine whether the inactivation seen using the growing cells could be demonstrated with a resting cell suspension.

Cells obtained by centrifugation of 400 ml of an overnight culture grown at 30°C were washed with 50 mM pH 7 phosphate and resuspended in the same at a two fold concentration from the original culture broth. The cells used for this study were not grown in the presence of erythromycin A.

A tube containing 10 ml of the cell suspension was placed in boiling water for 15 minutes. The heat-treated suspension and an untreated cell suspension were cooled in an ice bath and 500 mcg per ml of erythromycin A was added. After thorough mixing, both samples along

with one control were incubated in a 45°C water bath without shaking. The temperature of 45°C was arbitrarily chosen in hopes of increasing the rate of inactivation. Samples were withdrawn every hour for bioassay using S. aureus 209 as the test organism for the agar diffusion assay. The results of this study are shown in Figure 10.

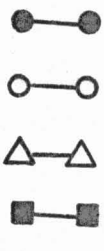
This is evidence for the biological nature of the inactivation. A total of 10 mg of erythromycin A was inactivated by the resting cell suspension in 8 hours at 45°C. However, when the cells were boiled, only chemical loss of activity was seen due to the high temperature: 30 percent of the bioactivity being lost in 8 hours. The results support the biological nature of the inactivation and that the enzyme(s) for the inactivation process are synthesized without the presence of erythromycin A during growth of the organism.

It is also noted that the rate of inactivation at 45°C was greatly increased over that seen using growing cells at 35°C. In addition, it is observed from Figure 8 that over short periods of time, less than one hour, less than 10 percent of the original activity was lost, presumably due to chemical inactivation. It appeared that the use of 45°C can be continued to study the optimization of reaction conditions and the inactivation by broken cell preparations over periods of 60 minutes or less without significant chemical inactivation occuring.

F. Determination of Reaction Conditions for Microbial Inactivation Using Broken Cell Preparations

Disrupted cell preparations were tested for their relative rates of inactivation to investigate the permeability of erythromycin in reaching the inactivation enzyme(s).

Figure 10: INACTIVATION OF ERYTHROMYCIN A BY WASHED CELLS



Washed Cells + Erythromycin

Boiled Cells + Erythromycin

Erythromycin Control

Sonicated Cells + Erythromycin

Conditions:

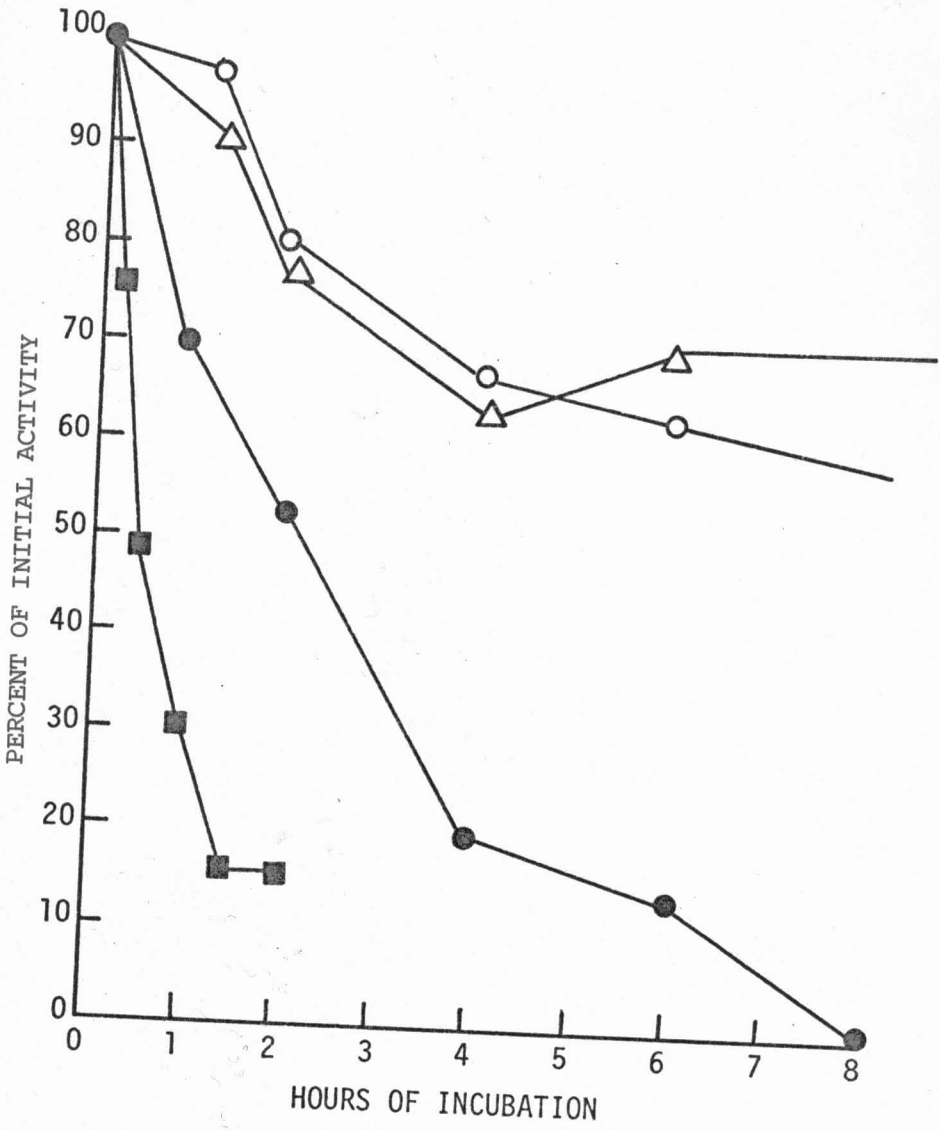
325 mg cells (dry wt.)

45°C Incubation

500 mcg/ml Antibitoic initially

pH 7.5

Figure 10


100
90
80
70
60
50
40
30
20
10
0
PERCENT OF INITIAL ACTIVITY
0
1
2
3
4
5
6
7
8
HOURS OF INCUBATION

The procedure of cell disruption that produced the most active broken cell preparation in 50 mM phosphate was sonication. A suspension of washed cells was sonicated 10 x 3 seconds using a Bronson Somicator at 0°C. This procedure produced a broken cell preparation that had greater inactivation ability at 45°C than resting cells. Data concerning the efficiency of disruption and inactivation activity of various methods of cell disruption is summarized in Table 6. While an Aminco French press provided the greatest disruption, the preparation was only one half as active as that from somicated cells. Hand grinding with sand at 0°C did not sufficiently disrupt the cells; the activity of this preparation being similar to that of the whole cells. Each procedure was corrected for chemical loss and possible absorption of antibiotic to the broken cell fragments by testing a boiled sample of each preparation. The activity of each preparation is expressed in change in mcg of erythromycin A per one hour per mg of protein used. The change in micrograms of erythromycin A was determined by the agar diffusion bioassay. Protein was determined by the method of Lowry et. al. (40) modified for whole cells.

The addition of dithiothreitol (DTT) (Sigma Chemical) to the disrupted cell preparations in a concentration of 0.1 mM was observed to produce slightly higher inactivation activities than without the addition of DTT.

Since the agar diffusion assay using S. aureus required a 15 hour incubation at 37°C as well as considerable time spent in preparing the two-layer plates and inoculum, a more rapid assay was necessary to follow the loss of erythromycin A from the disrupted cell preparations. It was also hoped to devise an assay that was more precise than the ± 15 percent of the bioassay.

With these objectives in mind, the arsenomolybdate assay was developed and tested for correlation with the agar diffusion assay as described

TABLE VI

LOSS OF BIOACTIVITY OF ERYTHROMYCIN IN THE PRESENCE OF DISRUPTED CELLS AND CELL FRAGMENTS OF CULTURE NO. 56

Procedure For Disruption of Cells	Change in Abs. of Disruption	mg protein per ml.	Change in mcg Eryth/ml. per 2 hr., 45 C	Change in mcg Eryth/ml. per 1 hr/mg protein
Washed Cells	---	105.	91	0.43
Boiled Washed Cells	---	105.	38	0.18
Phosphate buffer only pH 7.5	---	None	0	0
French Pressure Cell	26.0	31.2	18	0.29
Boiled-French Pressure Cell	26.0	31.2	12	0.19
Sonic Treatment	22.0	38.4	43	0.56
Boiled-sonic treated cells	22.0	38.4	0	0
Hand Grinding	10.0	48.0	43	0.45
Boiled Hand Grinding	10.0	48.0	0	0

in the section on "Materials and Methods". The new assay permitted the determination of residual erythromycin A in cell preparations in 90 minutes with good precision. This method was used in all of the following studies to assay for the loss of erythromycin A from reaction mixtures.

Using the active sonically disrupted cell preparation, the effect of temperature of incubation and pH on the initial reaction velocity were studied. Data collected in the study are summarized in Table 7. One ml aliquots of a sonically disrupted cell preparation with 500 mcg of antibiotic per tube at pH 8 were incubated at 0°C, 24°C, 29°C, 35°C, 45°C, and 53°C in water baths with shaking for one hour and then assayed for the loss of erythromycin using the colorimetric arsenomolybdate assay. Parallel tubes were incubated at the same temperature with the cell preparation to assay for chemical loss. The values shown in Table 7 represent the change in absorbance at 660 nm in the arsenomolybdate assay between a 0 time sample and after one hour incubation. Each tube contained 10 mg of a sonicated cell preparation. The data suggest that the rate of biological inactivation after 60 minutes of incubation increased with temperature. The initial rate of inactivation at 45°C being twice that at 35°C and four times as great as at 29°C. Insignificant inactivation was observed at 0°C. The greatest change in erythromycin A concentration observed was at 53°C, however, significant chemical loss was also evident at this temperature. This date suggests a temperature of 45°C for the most rapid inactivation of erythromycin A using broken cell preparation.

The effect of pH on initial reaction velocity was investigated using 50 mM phosphate and 50 mM tris-Cl buffer systems with broken cell preparations. The initial velocity of reaction mixtures at pH values of 7.5, 8.0, 8.5, 9.0, 9.5, and 10.0 were studied by sampling the reaction mixtures at 0, 15, and 30 minutes after the start of incubation at 45°C. The

TABLE VII. The Effect of Temperature of Incubation on the Biological Inactivation of Erythromycin A

TEMPERATURE	WITH ENZYME*	WITHOUT ENZYME
0 C	0.002	NONE
24	0.030	NONE
29	0.020	NONE
35	0.048	NONE
45	0.082	NONE
53	0.105	0.011

Values represent the change in absorbance at 660 nm in the arsenomolybdate colorimetric assay after 60 minutes of incubation. Initial concentration of erythromycin A: 500 mcg per tube. (*) denotes the addition of 10 mg of sonicated cell preparation + 0.01 mM DTT to the reaction mixture. The assay was carried out at pH 8.3.

initial velocity data, expressed as the change in the absorbance per minute (arsenomolybdate assay) suggest a pH optimum of 8.0 to 8.5. Similar results were obtained with both buffer systems. No attempt was made to study the reaction velocity below pH 7.0 as erythromycin is not chemically stable below this pH as shown from earlier studies. No results were obtained at pH values of 9.5 and 10.0 because the ethyl acetate extraction produced very stable emulsions of broken cell preparation when the extraction of residual erythromycin A was attempted at these high pH values.

This pH optimum data is summarized in Table 8. The reaction conditions apparently favored by this biological inactivation system at a pH of 8.3, temperature of 45°C and a reducing environment attained by the addition of DTT.

G. Localization of the Inactivation Activity Within the Cell: Attempts to Prepare a Cell Free System

Efforts were made to isolate the cellular fraction which contained the inactivation activity. These initial attempts at a purification of the enzyme(s) were made not with the intention of obtaining a homogenious protein for rigorous kinetic studies but to determine where the enzyme(s) is(are) located in the cell.

Studies using washed resting cell suspensions demonstrated that the enzyme was not strictly extracellular in nature. Washing of the cells and resuspension of the cells in buffer did not eliminate or diminish the activity of the inactivation process.

A differential centrifugation study utilizing the active sonicated cell preparations suggests that the inactivation enzyme(s) is(are) not

TABLE VIII

DETERMINATION OF pH OPTIMUM FOR
ENZYMATIC INACTIVATION OF ERYTHROMYCIN A

pH	Initial Velocity (0-30 minutes)
7.5	0.0017 Absorbance change/min.
8.0	0.0022
8.5	0.0021
9.0	0.0014
9.5	failure of ethyl acetate extraction
10.0	failure of ethyl acetate extraction

*cells grown not in the presence of erythromycin
Temperature: 45°C
Cell preparation: 26 mg sonicated cells*+ 0.1 mM DTT
Antibiotic concentration: 500 mcg in 1.5 ml.

cytoplasmic in location. Sonicated cell preparations with added DTT were centrifuged at 6000 relative centrifugal force for 20 minutes in a Sorvall refrigerated RC2-B centrifuge (DuPont). In these studies, all of the inactivation ability was recovered in the pellet; no activity was ever found in a soluble form.

Attempts were made to solubilize the active protein from the particulate cell fraction by chaotropic ions. Solutions of NaSCN (2 molar) were used to treat the sonicated cell preparations after the procedure of Hatefi (41). While 40 percent of particulate protein was solubilized by SCN treatment, the agent proved to be too strong a denaturant and all inactivation ability toward the antibiotic was lost. Cl (NaCl) ion did not denature the enzyme activity, however, it solubilized only 10 percent of the particulate protein leaving the inactivation ability still with the insoluble material. [Protein determination by Lowry assay (40)].

Treatment of the sonicated cell preparation with sodium deoxycholate failed as the deoxycholate interfered with the arsenomolybdate assay.

Several procedures from various literature sources were employed to isolate spheroplasts of Pseudomonad 56 (42, 43, 44, 45, 46). Stationary phase cells proved to be completely refractive to swelling with sucrose, lysozyme, and freeze-thaw treatments. Treatment of exponentially growing cells with sucrose, lysozyme, EDTA, and freeze-thaw did produce osmotically sensitive spheroplasts. The procedure which achieved some success was that summarized in Figure 11 modified from Kohn (43). When employing this technique, it was possible to produce spheroplasts whose lysis could be observed by the loss of absorbance over time in the spectrophotometer when they were diluted 10 fold with distilled water. An example of this data is shown in Figure 12. This procedure of a 10 fold dilution and monitoring of the absorbance change was used to check for the presence of spheroplasts.

Figure 11: PROCEDURE FOR SPHEROPLAST FORMATION FROM PSEUDOMONAD 56

Figure 11

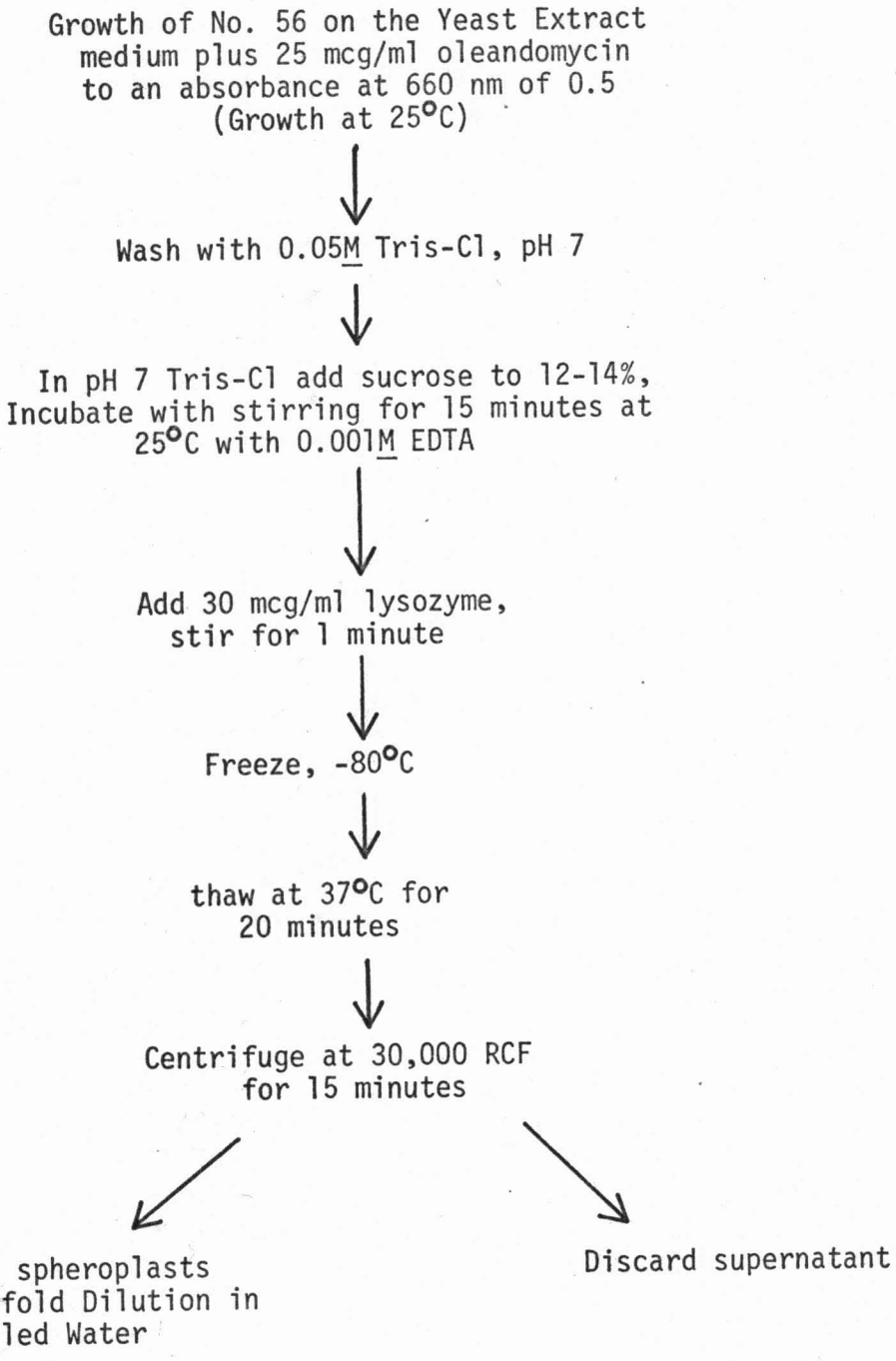
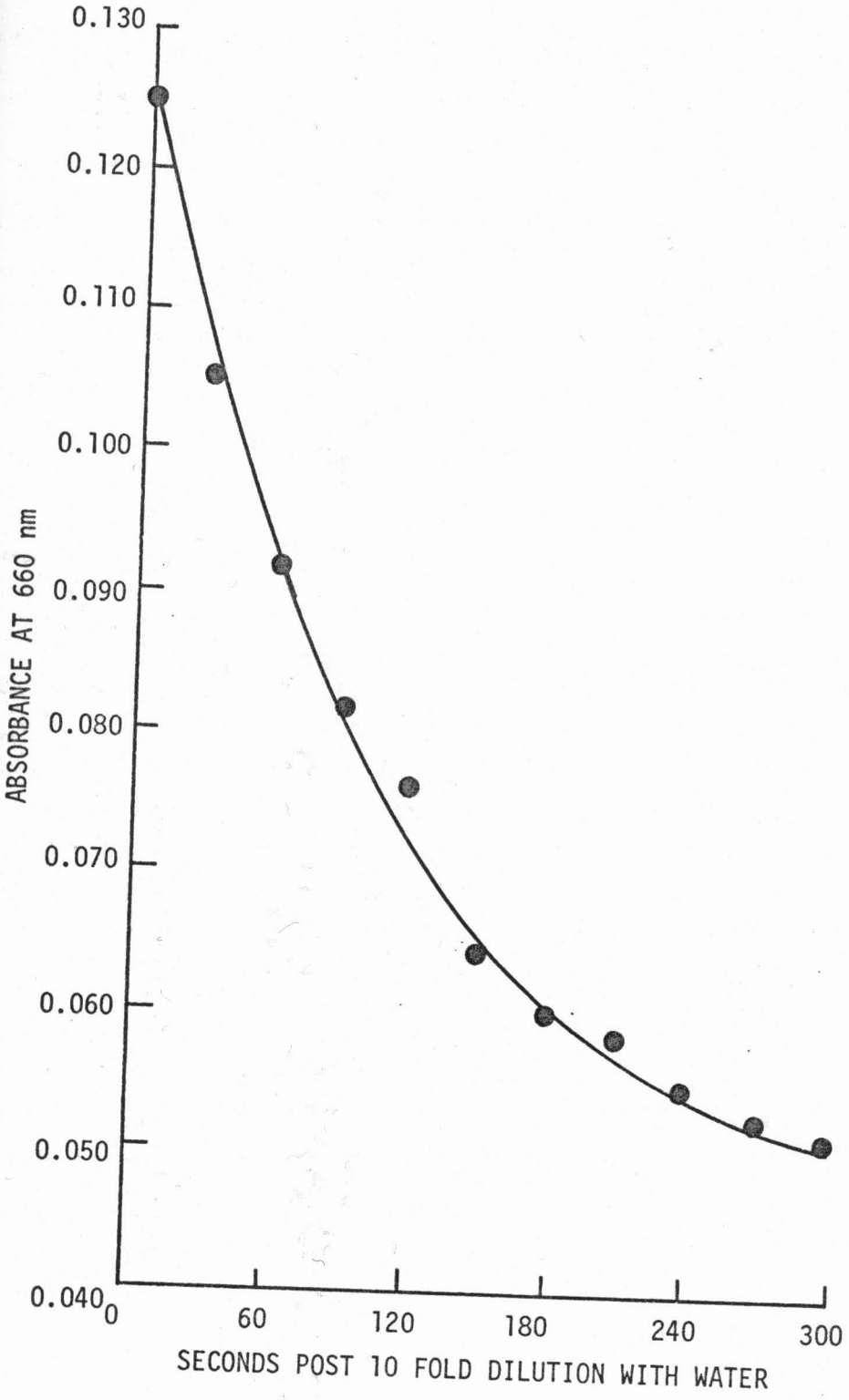

Growth of No. 56 on the Yeast Extract medium plus 25 mcg/ml oleandomycin to an absorbance at 660 nm of 0.5 (Growth at 25°C)
Wash with 0.05M Tris-Cl, pH 7
In pH 7 Tris-Cl add sucrose to 12-14%, Incubate with stirring for 15 minutes at 25°C with 0.001M EDTA
Add 30 mcg/ml lysozyme, stir for 1 minute
Freeze, -80°C
thaw at 37°C for 20 minutes
Centrifuge at 30,000 RCF for 15 minutes
Lyse spheroplasts By 10 fold Dilution in Distilled Water
Discard supernatant

Figure 12: CHANGE IN ABSORBANCE WHEN SPHEROPLASTS OF PSEUDOMONAD 56 ARE DILUTED WITH WATER

Exponential phase cells treated with sucrose swelling, EDTA, lysozyme, and freeze-thaw treatments

Figure 12


0.130
0.120
0.110
0.100
0.090
0.080
0.070
0.060
0.050
0.040
ABSORBANCE AT 660 nm
0
60
120
180
240
300
SECONDS POST 10 FOLD DILUTION WITH WATER

Microscopic observations of the spheroplasts prior to dilution and lysis showed cells in clumps. Intact exponentially growing cells not receiving any treatment did not demonstrate any change in absorbance over time when diluted 10 fold with distilled water.

As shown in Figure 11, the cellular debris remaining after lysis of the spheroplasts was centrifuged under refrigeration at a relative centrifugal force of 30,000 x g for 15 minutes. The pellet remaining after this treatment believed to be a 'membranes only' preparation from Pseudomonad 56 did show erythromycin A inactivation ability when assayed using the arsonomolybdate assay. The unit of enzyme activity used in this study was:

$$\text{one enzyme unit} = \frac{\text{change in absorbance at 660 nm in 30 minutes}}{\text{mg protein present}}$$

The initial enzyme activity of the exponential phase cells when harvested was 0.012 enzyme units with 0.48 total units in the sample (40 ml of suspended cells). The lysed spheroplast fraction showed 0.175 units in 10 ml of spheroplast membrane suspension. The spheroplast preparation demonstrated a 3 fold increase (in total activity) in ability to inactivate erythromycin A over exponentially growing cells.

This evidence does suggest that the enzyme or enzymes associated with the inactivation of erythromycin A are associated in some way with the cell membrane and are not periplasmic. If these enzymes were periplasmic, lysozyme and freeze-thaw techniques would have produced soluble inactivating activity. Pseudomonad 56 proved to be unusually resistant to the techniques commonly employed for spheroplast formation and membrane isolations from Gram negative cells. One possible explanation for this could be

that this organism produces a slime layer (47).

H. Fermentation Studies

1. Determination of the Time course of Enzyme Production

Fermentation studies on the production of the inactivation enzyme by Pseudomonad 56 were undertaken in 2000 ml Erlenmeyer flasks containing 200 ml of medium on a 300 rpm rotary shaker.

The time course of enzyme production by the organism was studied by following the cell mass (5 percent inoculum of a 24 hour culture) and enzyme levels. The growth of the culture was followed as the dry weight of cells per liter by the correlation method previously described between the absorbance of washed cells resuspended in buffer and the actual cell dry weight. Sufficient flasks were used in each study so that one flask could be removed for enzyme assay at each time point. The progress of the fermentation was monitored by removal of one flask every 2 hours for the first 12 hours and then once every following 12 hours. The one percent yeast extract medium (page 26) was employed in all studies with inducers being added aseptically, sterilized by filtration.

Enzyme levels in the fermentation were expressed on a per cell dry weight basis as defined below:

$$\text{enzyme units} = \frac{\text{change in absorbance at 660 nm in 30 minutes}}{\text{mg cell dry weight}}$$

This unit gives an indication of the enzyme level per cell during the fermentation. The procedure followed to determine enzyme units at each time point is as follows: the flask of cells harvested by refrigerated centrifugation, resuspended in 10 ml of 50 mM pH 8 phosphate and the

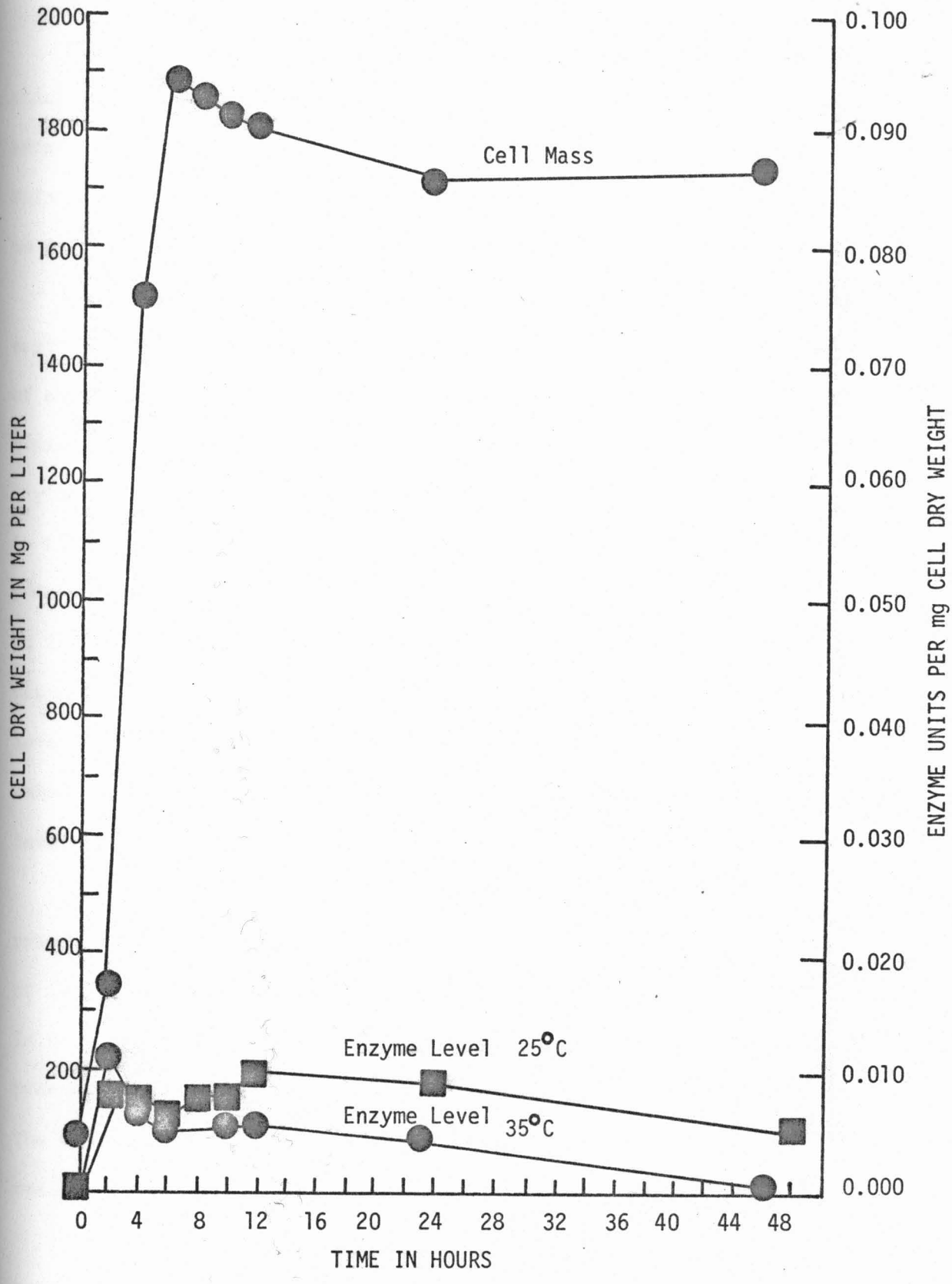
absorbance at 660 nm recorded for the conversion to cell dry weight. Two one ml samples of this cell suspension plus a one ml zero time sample were assayed using the arsenomolybdate technique. The zero time sample remained in ice while the two test samples were incubated for 30 minutes at 45°C in the presence of 500 mcg of erythromycin A. The samples were then extracted with ethyl acetate and the non-aqueous layer assayed for neutral macrolides using the colorimetric method.

The results of the initial time course of enzyme production study are shown in Figure 13. This time course was made at 35°C, the temperature for optimum growth of the organism. The data suggests that under these conditions the enzyme level increases to a fixed level after 2 to 4 hours and then remains constant until sometime after 12 hours. The enzyme activity decreases after this time until by 46 hours into the fermentation the enzyme activity falls below that detectable by the assay. The cell dry weight of the fermentation reaches a maximum at 6 to 8 hours and decreases gradually probably due to cell lysis. The maximum cell dry weight attained in this study was 1.92 grams per liter with the enzyme level at 12 hours being 0.0058 units.

The decrease of enzyme level after 12 hours of fermentation suggested that perhaps a lower temperature for the fermentation would increase enzyme stability. The results of the same time course study at 25°C are shown in Figure 13. At this lower temperature the enzyme levels reached a maximum after 2 hours and this level remained constant throughout the 48 hour period assayed. The relative enzyme level after 12 hours was 1.8 that of the 12 hour enzyme level at 35°C, with the enzyme exhibiting much greater stability at the lower temperature. All further studies to enhance enzyme level production were run at 25°C for only a 24 hour period.

Figure 13: TIME COURSE OF ENZYME PRODUCTION BY PSEUDOMONAD 56

Figure 13


2000
1800
1600
1400
1200
1000
800
600
400
200
CELL DRY WEIGHT IN Mg PER LITER
0.100
0.090
0.080
0.070
0.060
0.050
0.040
0.030
0.020
0.010
0.000
ENZYME UNITS PER mg CELL DRY WEIGHT
Cell Mass
Enzyme Level 25°C
Enzyme Level 35°C
0 4 8 12 16 20 24 28 32 36 40 44 48
TIME IN HOURS

2. Induction of Higher Enzyme Levels

Efforts were made to increase enzyme production further by examination of possible inducers of enzyme synthesis. The inducers were added aseptically by filtration at a concentration of 25 mcg per ml. This concentration was chosen to insure that inducers if found would be displaying their maximum level of induction.

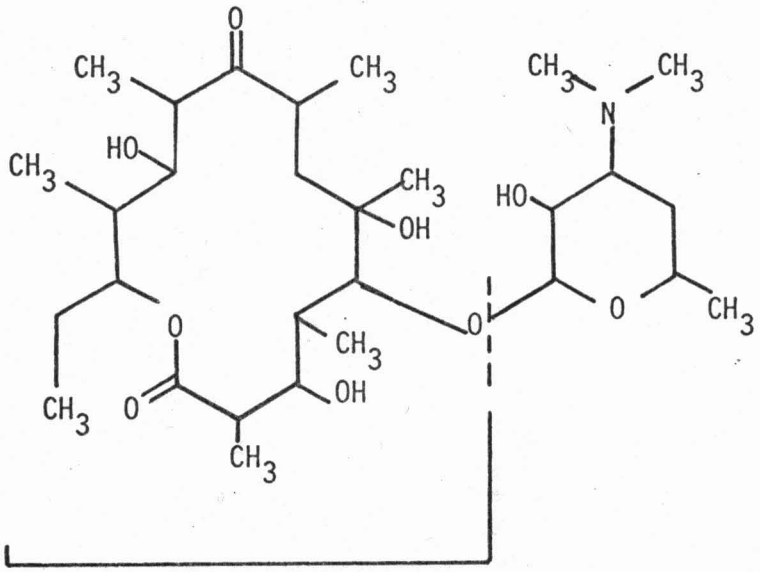
It was first suggested that if the inactivation enzyme degraded erythromycin then perhaps the products of this degradation--fragments of erythromycin might act as inducers of higher enzyme levels. Two available fragments of erythromycin were tried; erythronolide B and erythronolide B-desosamine. The structures of these inducers are shown in Figure 14. The results of induction experiments using these two compounds are shown in Figure 15. The twelve hour level of enzyme found at 25°C when only the lactone ring erythronolide B was added to the fermentation was 2.2 times greater than the uninduced level at 35°C. The level of induced response to the presence of the lactone ring plus the amino sugar desosamine attached was 2.3 times greater than the base level at 35°C.

Methymycin a 12 membered lactone antibacterial macrolide was tried. The structure of this inducer is shown in Figure 16. The level of induction due to the addition of this macrolide to the fermentation is shown in Figure 17. The 12 hour enzyme level with this compound present was 3.6 times that seen in the uninduced fermentation at 35°C. The enzyme then appears to be able to be induced by lactone rings of smaller size than the 14 membered ring of erythromycin A.

Even though a 3 fold increase in enzyme levels is significant, better inducers were sought. The substrate of the enzyme, erythromycin A, was considered for trial as an inducer. However, to insure that any

Figure 14: STRUCTURE OF ERYTHRONOLIDE B AND ERYTHRONOLIDE-B-DESOSAMINE

Figure 14



ERYTHRONOLIDE B

ERYTHRONOLIDE-B-DESOSAMINE

Figure 15: INDUCTION OF HIGHER ENZYME LEVELS BY ERYTHRONOLIDE B AND ERYTHRONOLIDE-B-DESOSAMINE

Temperature 25°C

Inducers added at the level of 25 mcg/ml.

● cell mass

■ Erythronolide-B-Desosamine

○ Erythronolide B

Figure 15

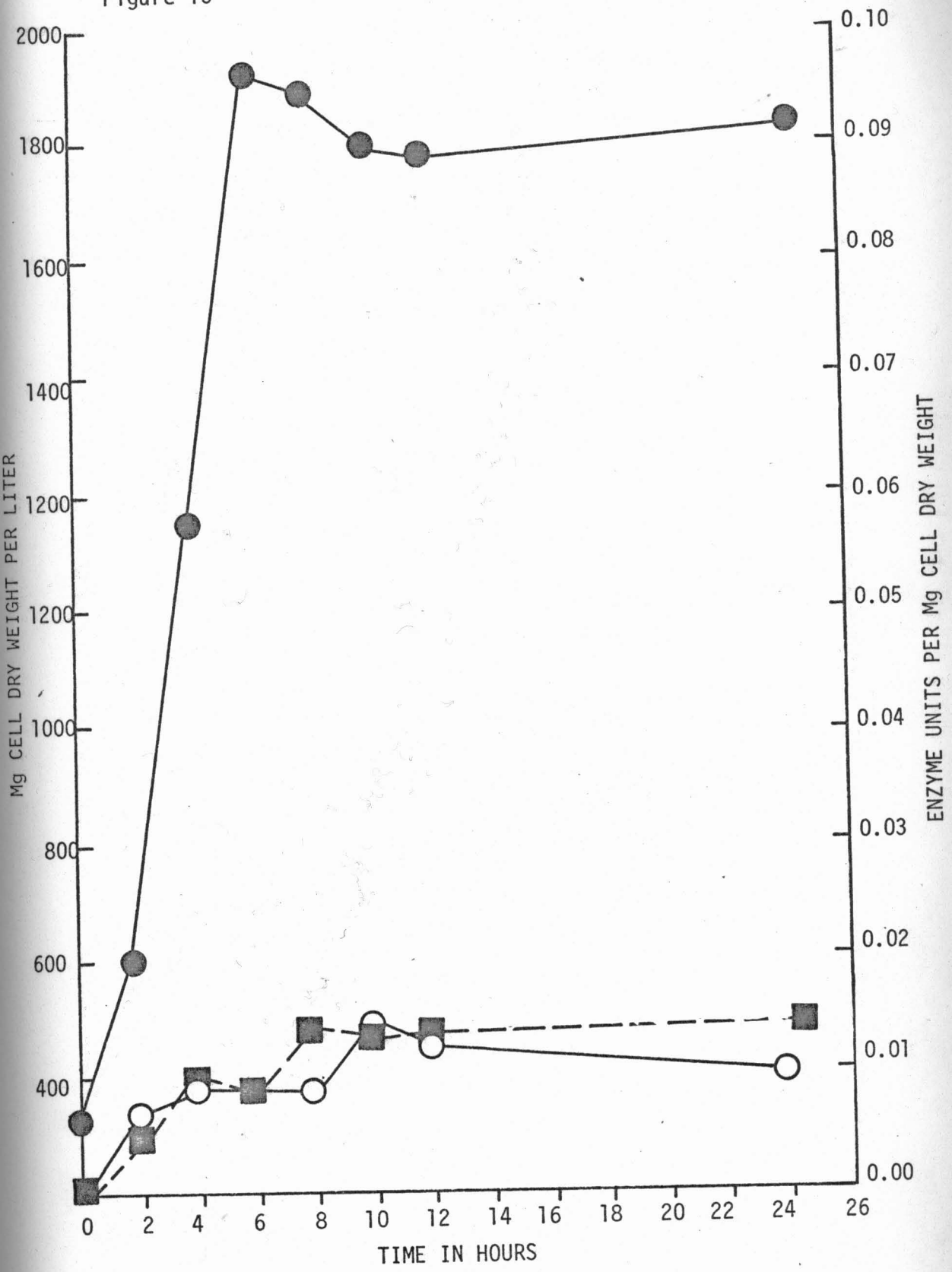
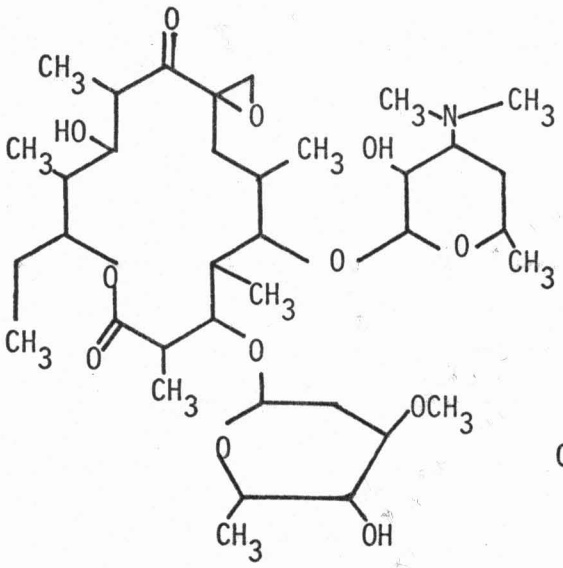
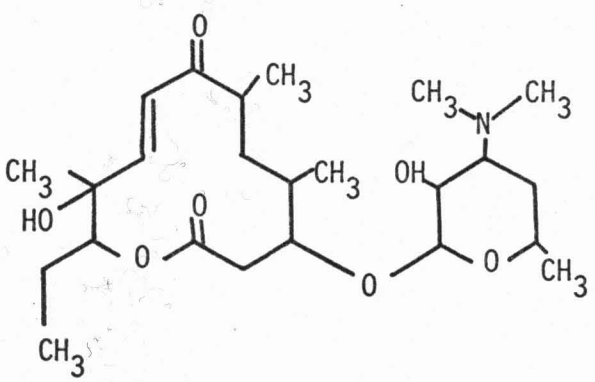

2000
1800
1600
1400
1200
1000
800
600
400
Mg CELL DRY WEIGHT PER LITER
0.10
0.09
0.08
0.07
0.06
0.05
0.04
0.03
0.02
0.01
0.00
ENZYME UNITS PER Mg CELL DRY WEIGHT
0
2
4
6
8
10
12
14
16
18
20
22
24
26
TIME IN HOURS

Figure 16: STRUCTURES OF OLEANDOMYCIN AND METHYMYCIN

Figure 16

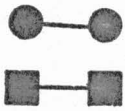


OLEANDOMYCIN



METHYMYCIN

Figure 17: INDUCTION OF HIGHER ENZYME LEVELS BY METHYMYCIN



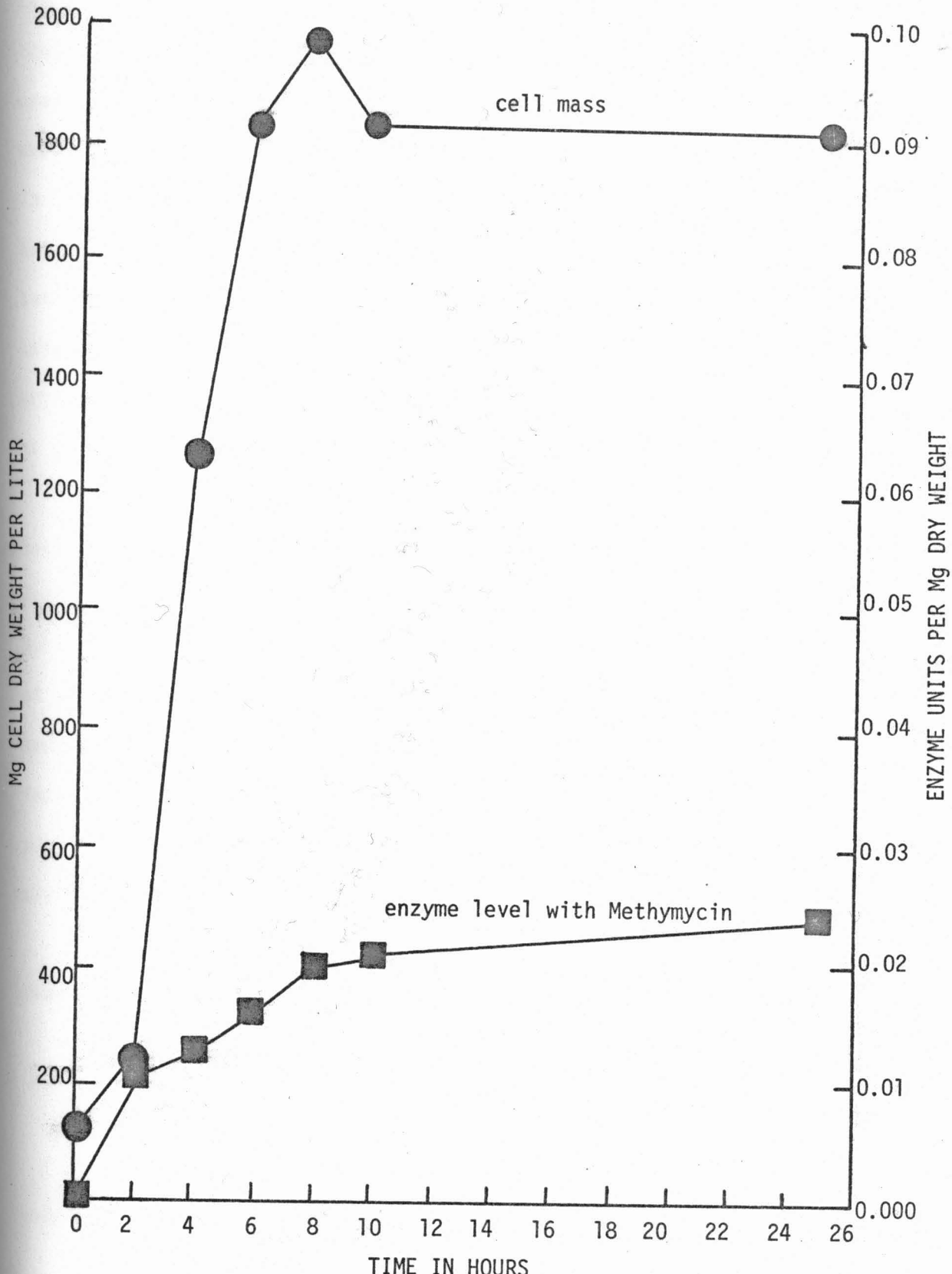
CELL MASS

ENZYME LEVEL WITH METHYMYCIN

Temperature 25°C

Inducer added at a level of 25 mcg/ml.

Figure 17


2000
1800
1600
1400
1200
1000
800
600
400
200
Mg CELL DRY WEIGHT PER LITER
0.10
0.09
0.08
0.07
0.06
0.05
0.04
0.03
0.02
0.01
0.000
ENZYME UNITS PER Mg DRY WEIGHT
cell mass
enzyme level with Methymycin
0
2
4
6
8
10
12
14
16
18
20
22
24
26
TIME IN HOURS

induction seen was due to the intact erythromycin molecule and not to any chemical degradation product which might occur over the 24 hour duration of the fermentation, the more chemically stable erythromycin B was used. (See Figure 1 for structure.) The results of this fermentation are shown in Figure 18. Erythromycin B proved to be a good inducer with the 12 hour enzyme level 13.5 times greater than the uninduced 35°C fermentation. This inducer also appeared to act somewhat as a growth stimulator as the maximum cell yield under its influence was 2.1 grams per liter of cell dry weight compared to the 1.9 grams per liter of cell dry weight seen with the poorer inducers in this system. This difference of 0.2 grams per liter of cell dry weight was reproducible.

The results of enzyme induction by oleandomycin, a 14 membered lactone macrolide, are shown in Figure 19. Oleandomycin contains desosamine but instead of cladinose the sugar oleandrose is attached to the macrolide ring. (See Figure 16) The lactone ring differs from that of erythromycin A by the presence of an epoxide at position 8. This macrolide proved to be an inducer. The 12 hour enzyme level of this fermentation was 14 times the uninduced level at 35°C, compared to the 13.5 times seen with erythromycin B as the inducer. Again, as seen for erythromycin B, oleandomycin also stimulated growth of the organism.

A composite of all of the induction level studies is shown in Figure 20.

3. Growth Factor Limited Fermentations

The fact that both of the inducers of high enzyme levels found also resulted in slightly increased cell yields suggested that perhaps the

Figure 18: INDUCTION OF HIGHER ENZYME LEVELS BY ERYTHROMYCIN B

● Cell Mass

■ Enzyme Level with Erythromycin B

Temperature 25°C

Inducer added at the Level of 25 mcg/ml.

Figure 18

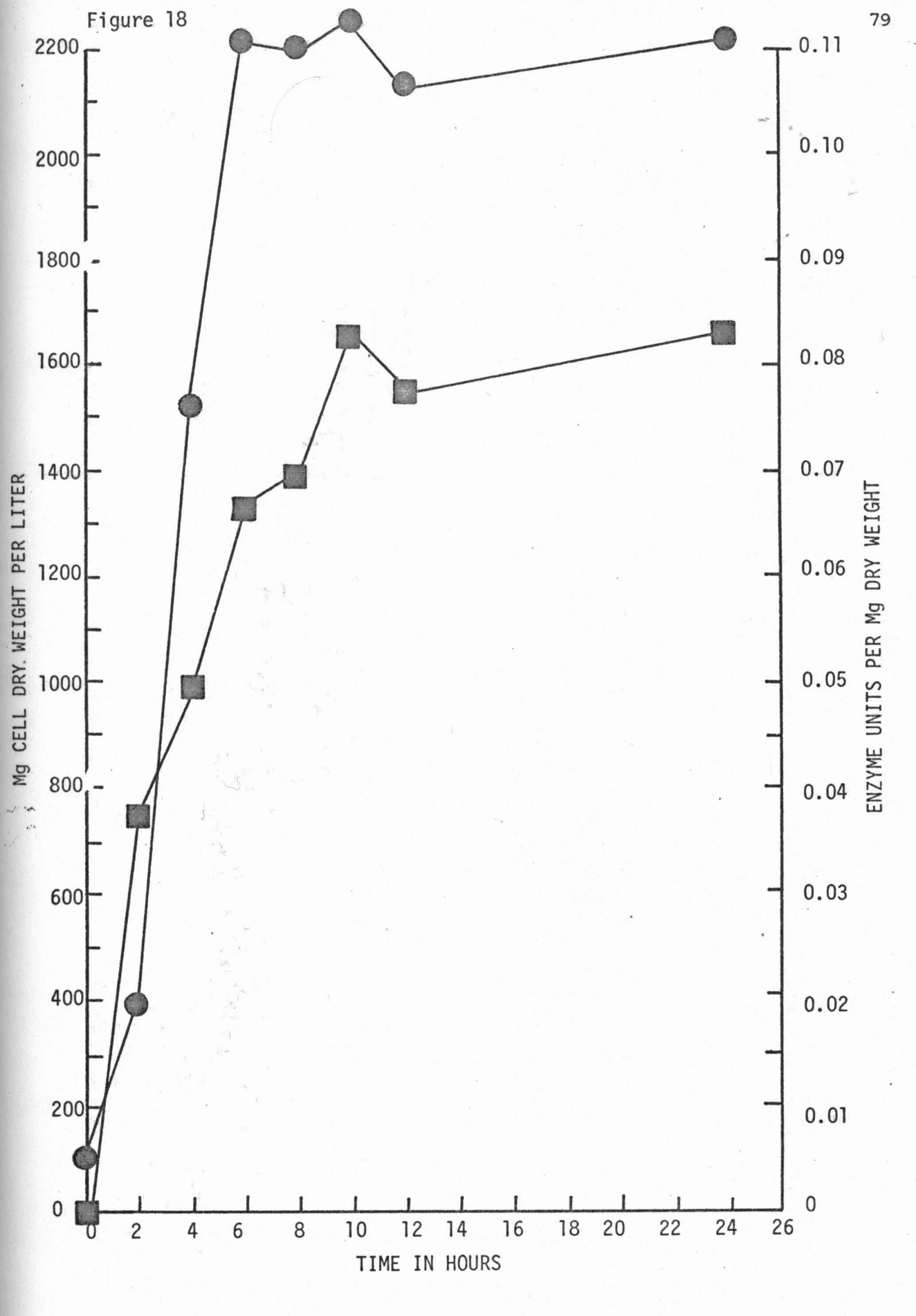

2200
2000
1800
1600
1400
1200
1000
800
600
400
200
0
Mg CELL DRY. WEIGHT PER LITER
0.11
0.10
0.09
0.08
0.07
0.06
0.05
0.04
0.03
0.02
0.01
0
ENZYME UNITS PER Mg DRY WEIGHT
0 2 4 6 8 10 12 14 16 18 20 22 24 26
TIME IN HOURS

Figure 19: INDUCTION OF HIGHER ENZYME LEVELS BY OLEANDOMYCIN

● Cell Mass

■ Enzyme Level with Oleandomycin

Temperature 25°C

Inducer added at the Level of 25 mcg/ml.

Figure 19

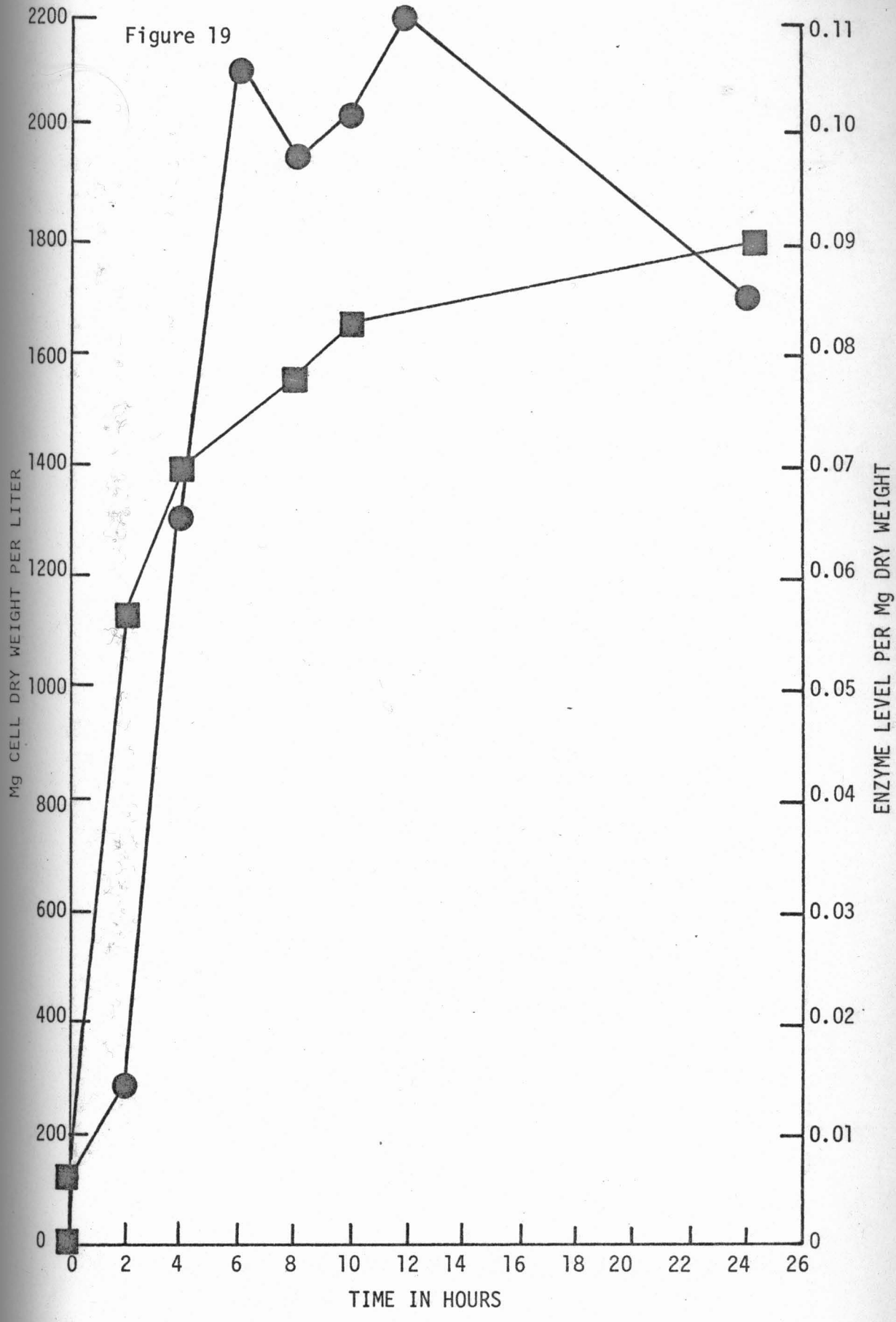

2200
2000
1800
1600
1400
1200
1000
800
600
400
200
0
Mg CELL DRY WEIGHT PER LITER
0.11
0.10
0.09
0.08
0.07
0.06
0.05
0.04
0.03
0.02
0.01
0
ENZYME LEVEL PER Mg DRY WEIGHT
0 2 4 6 8 10 12 14 16 18 20 22 24 26
TIME IN HOURS

Figure 20: SUMMARY OF ENZYME INDUCTION EXPERIMENTS

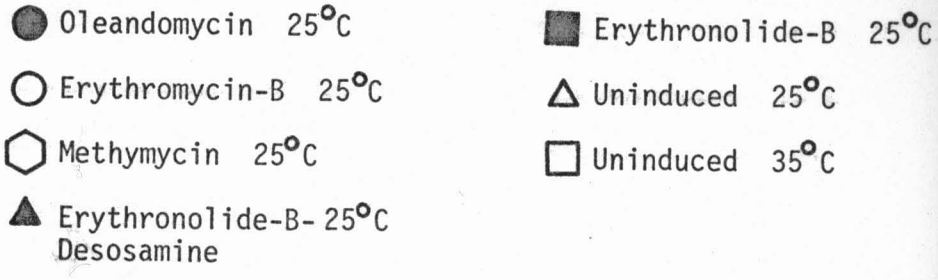
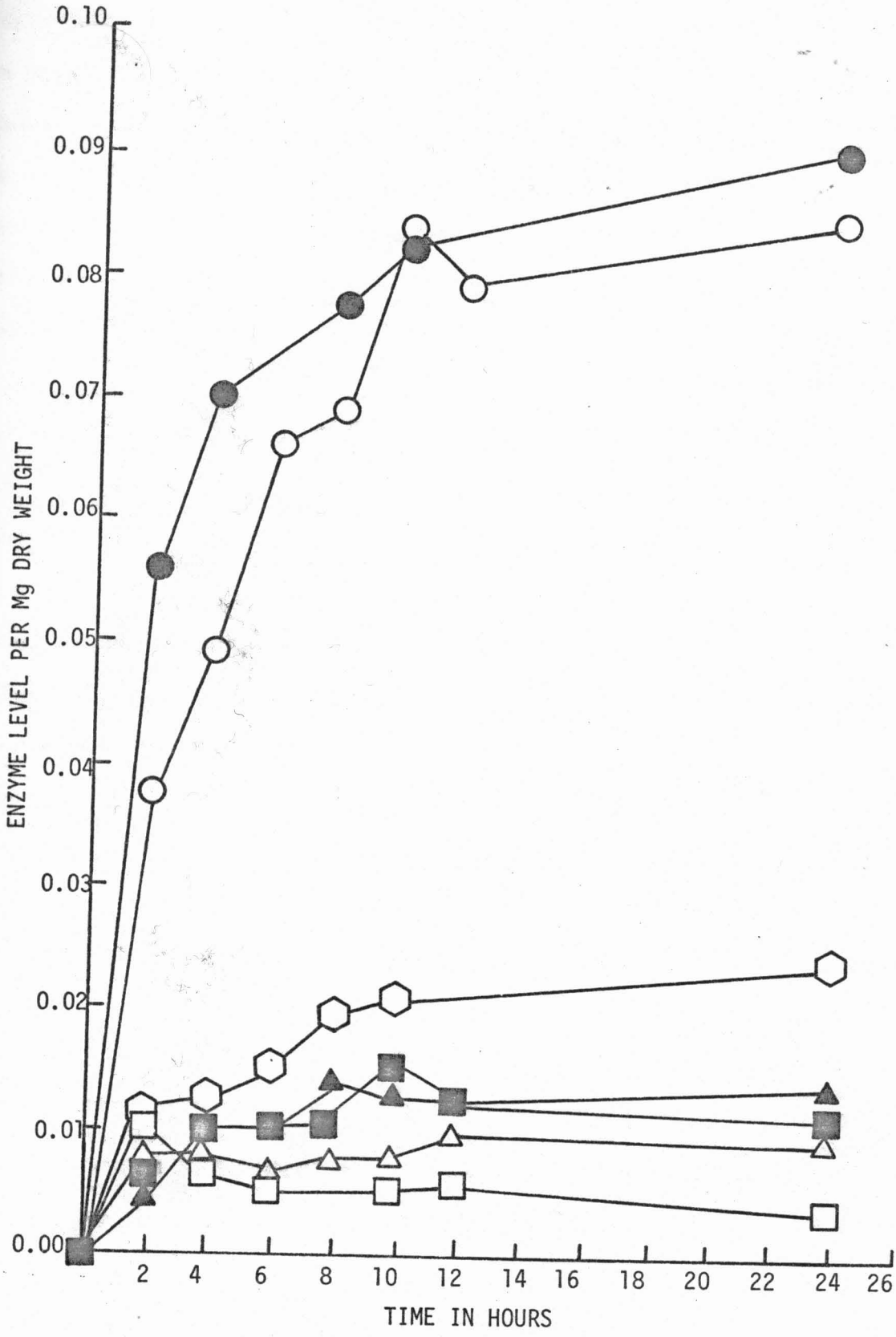


Figure 20


0.10
0.09
0.08
0.07
0.06
0.05
0.04
0.03
0.02
0.01
0.00
ENZYME LEVEL PER Mg DRY WEIGHT
2
4
6
8
10
12
14
16
18
20
22
24
26
TIME IN HOURS

opposite might be true. Because higher enzyme levels appear to correlate with higher cell yields and that the inactivation enzyme is produced even without the presence of any macrolide, then perhaps the production of this enzyme is necessary for the growth of the organism. It can be seen by the time course of enzyme induction studies (Figures 13, 15, 16, 17, 18) that enzyme production even under induced conditions is strictly growth associated.

The above considerations imply that growth factors could be inducers of higher enzyme levels. It has previously been suggested (48) that growth factor limited fermentations often exhibit greatly increased levels of enzyme synthesis.

At this point it is worthwhile to reconsider some of the results of earlier nutritional work (page 33). In attempting to determine the nutritional requirements of this organism (Pseudomonad 56) it was shown that its growth on a one percent Difco vitamin free Casamino Acids medium appeared to be growth factor limited. When this medium was used in the same system used for the enzyme level studies at 25°C, the 12 hour level of enzyme was the same as the value found at 25°C for the culture growing on the one percent yeast extract medium. The cell yield of the fermentation, however, was only half of that seen with the yeast extract medium. The growth factor limited growth did not in this case result in increased levels of the erythromycin A inactivating enzyme level only in decreased cell yield.

Since the only identified growth factor for Pseudomonad 56 was demonstrated to be sodium oleate, and that a small growth factor response has previously been demonstrated by adding sodium oleate to the Casamino Acids medium, the enzyme levels of growth factor limited

fermentations with added oleate were investigated. The addition of one percent sodium oleate to the Casamino Acids medium resulted in a 12 hour enzyme level per cell of twice that seen without added lipid. The level of erythromycin A inactivating enzyme then was responding to the presence of lipid in the medium. The addition of sodium oleate at the level of 10 gm/liter did not result in higher cell yields, only higher enzyme levels. (See Table 9)

Efforts to further increase the enzyme level on the Casamino Acids medium using a combination of both added sodium oleate and added oleandomycin resulted in increased enzyme levels of three times that seen without additions to the amino acids medium. These enzyme level studies are summarized in Table 9.

4. Study of the Minimum Level of Inducer Necessary for Full Enzyme Induction Using Oleandomycin as Inducer

A study was made to determine the minimum level of inducer necessary to effect maximum enzyme induction using oleandomycin as the inducer. The results of this study are shown in Figure 21. The conditions of cell growth and enzyme assay were identical to those for the induction experiments. The level of inducer needed for full enzyme induction suggested by this data appears to be 25 mcg per ml. Below this level, enzyme levels are directly related to the inducer levels.

5. Study of the Maintenance Level of Enzyme Induction

Enzyme induction in some systems has been described in the literature as an all or none process (49). In searching for ways of reducing

Table IX

SUMMARY OF 12 HOUR INDUCED ENZYME LEVELS

YE* = yeast extract medium described on page

CAA** = 10 gm/liter Difco Casamino Acids substituted for the yeast extract in the medium described on page

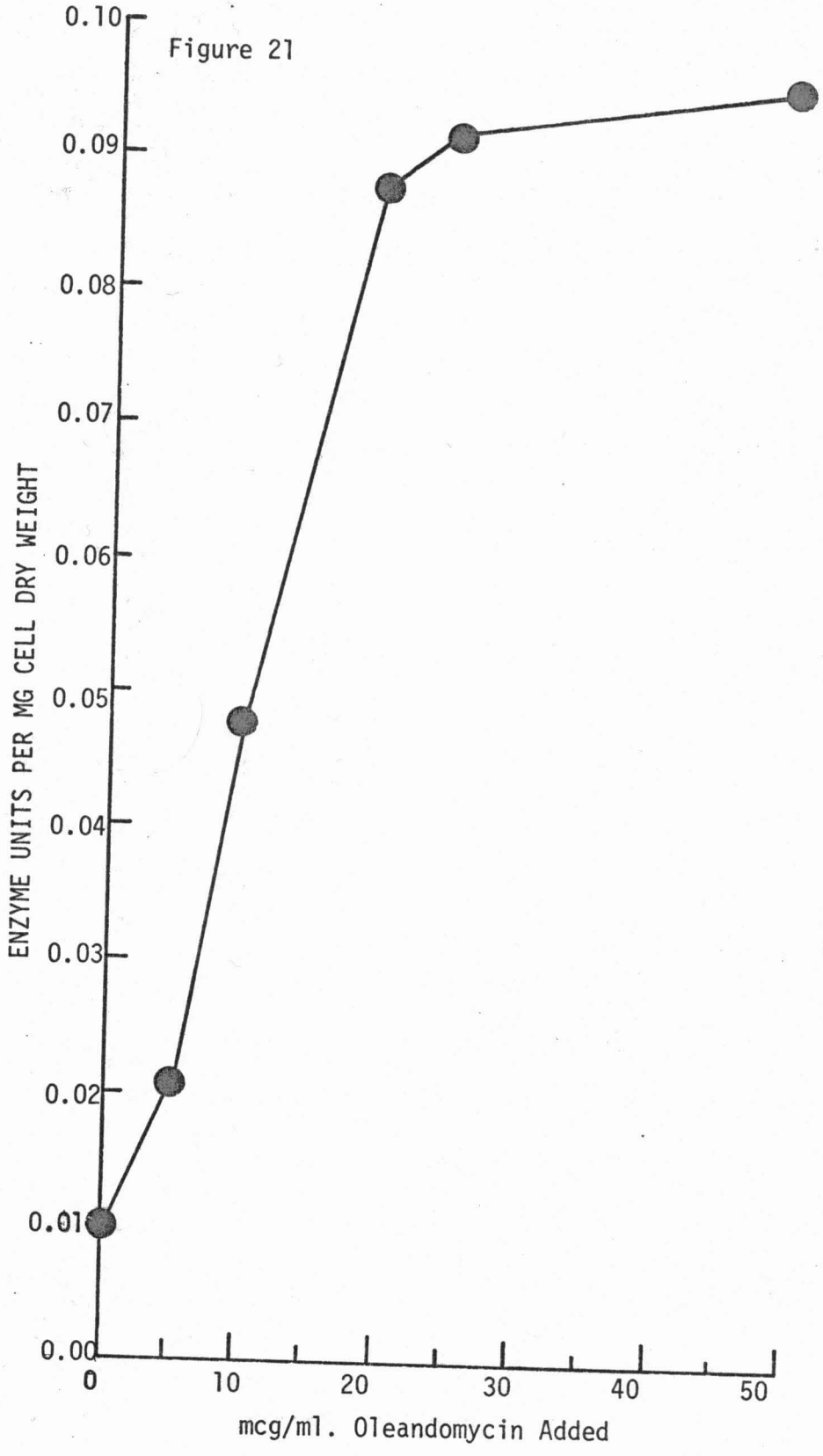
Table IX

Inducer	Medium	Relative Level	Enzyme Units (page 64)	Temp.	mg/liter Maximum Cell Yield
none	YE*	1.0	0.0058	35°C	1920
none	YE	1.8	0.010	25°C	1800
erythronolide B	YE	2.2	0.013	25°C	1920
erythronolide-B-desosamine	YE	2.3	0.014	25°C	1650
methymycin	YE	3.6	0.021	25°C	1850
erythromycin B	YE	13.5	0.078	25°C	2140
oleandomycin base	YE	14.4	0.083	25°C	2230
none	CAA**	1.9	0.011	25°C	970
sodium oleate	CAA	3.9	0.023	25°C	795
oleandomycin + sodium oleate	CAA	6.3	0.037	25°C	875

Figure 21: INDUCTION LEVEL STUDY USING OLEANDOMYCIN AS THE INDUCER OF HIGHER ENZYME LEVELS

Values Represent Enzyme Levels at 12 Hours of Incubation

Figure 21


0.10
0.09
0.08
0.07
0.06
0.05
0.04
0.03
0.02
0.01
0.00
ENZYME UNITS PER MG CELL DRY WEIGHT
0
10
20
30
40
50
mcg/ml. Oleandomycin Added

the quantity of the inducer oleandomycin necessary to fully induce enzyme production, the existence of the phenomenon of the maintenance level of induction was explored. A maintenance level of induction means that a fully induced culture can be inoculated into a medium containing less than the minimum level of inducer needed for full induction present and still produce the fully induced level of enzyme.

The results of the investigation of this phenomenon with Pseudomonad 56 are summarized in Figure 22. In this study, fully induced 12 hour inoculum was added to fermentation medium containing various levels of the inducer, oleandomycin, less than the 25 mcg per ml necessary for full enzyme induction. After 12 hours of incubation the level of enzyme induction at each inducer level was determined. As the results show, only when 25 mcg per ml of inducer is present in the fermentation flask is the full level of induction observed. This demonstrated, unfortunately, that no maintenance level effect is evident with this organism and oleandomycin as the inducer.

The fermentation studies on the erythromycin A inactivation enzyme of Pseudomonad 56 suggest that this is an inducible enzyme system and that significantly higher levels of enzyme can be obtained by the addition of oleandomycin at the level of 25 mcg per ml to the 10 gm/liter yeast extract medium. It is observed that the production of enzyme is strictly growth associated and that the maximum cell population is reached at 8 to 10 hours into the fermentation. Whether the inoculum is induced or uninduced does not appear to affect the final level of enzyme induction when 25 mcg of oleandomycin is present in the medium as the enzyme inducer.

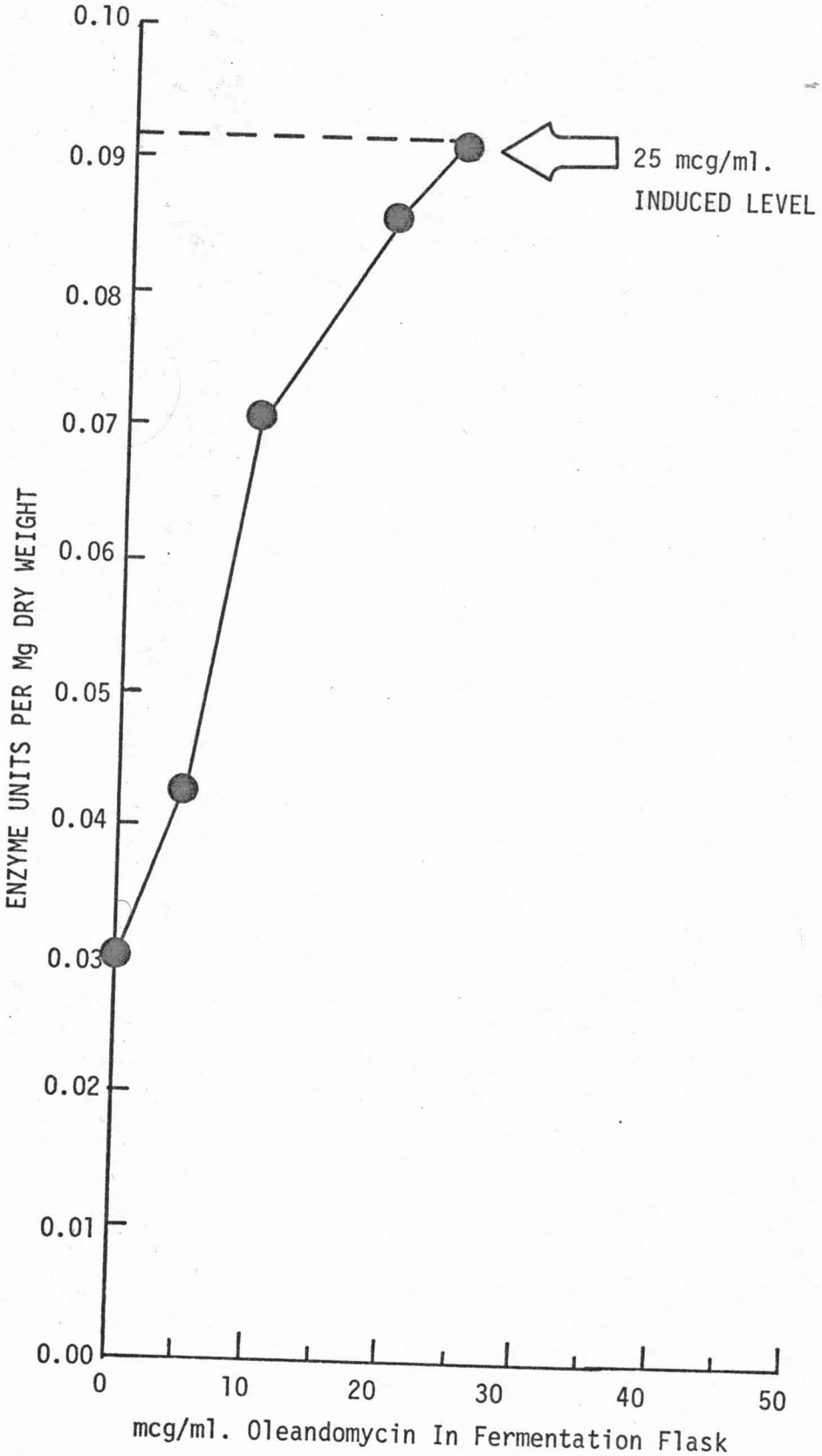
Figure 22: MAINTENCE LEVEL STUDY USING OLEANDOMYCIN AS INDUCER OF HIGHER ENZYME LEVELS

Inoculum Induced with 25 mcg/ml. of oleandomycin

Temperature 25°C

Values represent enzyme level at 12 hours of incubation

Figure 22


0.10
0.09
0.08
0.07
0.06
0.05
0.04
0.03
0.02
0.01
0.00
ENZYME UNITS PER Mg DRY WEIGHT
25 mcg/ml.
INDUCED LEVEL
0
10
20
30
40
50
mcg/ml. Oleandomycin In Fermentation Flask

6. Stirred Jar Fermentation of Pseudomonad 56

One fermentation of Pseudomonad 56 was run in a stirred jar assembly (New Brunswick Scientific) to determine what yield of enzyme could be obtained in a stirred jar using the fermentation conditions defined in the shake flask studies. The exact specifications of the 8 liter stirred jar run, the enzyme yield and its activity are given in Table 10. The 12 hour fermentation yielded a total of 38.6 grams of lyophilized cells (see page 96) with an activity of 0.113 units, higher than any previous preparation. The enzyme level in this material was 19.5 times that in the original 35°C uninduced shake flask experiments.

Table X

PARAMETERS AND ENZYME YIELD FROM A STIRRED JAR FERMENTATION OF PSEUDOMONAD 56

Table X

Volume	8 liters
Impeller Speed	400 rpm
Sparge Rate	16 liters/min.
Temperature	25°C
Medium	10 gm/liter yeast extract (Difco) 0.5 gm/liter $(NH_4)_2SO_4$ 4.56 gm/liter KH_2PO_4 11.6 gm/liter K_2HPO_4
Inducer	oleandomycin phosphate 25 mcg/ml
Antifoam	cotton seed oil (2 ml)
Inoculum	8% (12 hour culture, shake flask)
Duration	12.5 hours
Absorbance at Harvest (660 nm)	5.9
Yield	38.6 grams lyophilized cells (see page 96)
Activity	0.113 units per mg lyophilized weight

I. Storage of an Active Enzyme Preparation

Investigations of the erythromycin A inactivation ability of Pseudomonad 56 were impeded since active sonicated cell preparations when frozen at -20°C for 24 hours and thawed at 30°C lost all activity. This necessitated the production of fresh enzyme preparations for each separate experiment.

Attempts were made to achieve a 'stable' enzyme preparation which could be easily stored and handled and retain its full and original activity. The acetone dried powder technique after Morton (50) resulted in complete loss of erythromycin A inactivation activity when tried with both whole cell and disrupted cell preparations.

Techniques which proved to be successful for preparations of the active enzyme for storage were lyophilized whole cells and slow drying of cells. Twelve hour cultures with induced enzyme levels were washed and resuspended in 100 mM pH 8 potassium phosphate buffer and frozen in an ethanol-dry ice bath. The frozen cells were lyophilized on a New Brunswick Scientific Company cryolyzer for 15 hours. Upon removal from the lyophilizer, the cells were stored frozen at -20°C. When this procedure was followed, no loss of erythromycin A inactivation activity was noted in the thawed, reconstituted material. When cells were lyophilized in distilled water instead of buffer, a 12 percent loss of activity was observed in the reconstituted cell preparation.

The slow drying in vaccuum of whole cell preparations also produced active storable cell preparations. The cells in buffer were dried in an evacuated dessicator over $CaSO_4$ until dry. Disadvantages to this method are that it often required two to three days for the cell paste to thoroughly dry and that the cells tended to dry in a glass-like hard

substance which was difficult to resuspend in buffer.

The lyophilized cell preparation was observed to lose 50 percent of its inactivation ability upon storage at -20°C for four months.

J. Substrate Specificity of Pseudomonad 56 for Other Macrolides

1. Using Sonicated Cells Uninduced Preparations

The substrate specificity of a fresh, sonicated, uninduced cell preparation of Pseudomonad 56 was determined by incubation with six macrolide antibiotics at 45°C, pH 8, with 500 mcg per ml of each antibiotic present initially. The incubation was for 120 minutes with samples being removed every 15 minutes for assaying using the arsenomolybdate technique. The results of this study was shown in Figure 23. As can be expected, the sonicated cell preparation removed 85 percent of the erythromycin A initially present in the first 90 minutes of incubation. Oleandomycin and a mixture of leucomycins, however, were inactivated only slightly by Pseudomonad 56 with these two macrolides showing a decrease of 20 to 25 percent from their initial level in 120 minutes. Tylosin tartrate and methymycin were not inactivated at all by this sonicated cell preparation as no significant change in their level was observed during the incubation. The structures of tylosin and leucomycin A_3 are shown in Figure 24.

Of greatest interest is the observation that erythromycin B (differing from erythromycin A only by the absence of a hydroxyl group at C-12) was not significantly inactivated by this sonicated uninduced cell preparation of Pseudomonad 56. Ninety percent of the original erythromycin B present was recovered after 120 minutes. This data suggests that chemical instability contributes to the ability of Pseudomonad 56 to inactivate

Figure 23: MACROLIDE SUBSTRATE SPECIFICITY OF PSEUDOMONAD 56 USING UNINDUCED ENZYME LEVEL PREPARATION

Sonicated Cell Preparation - 23mg/ml
pH 8.0
45°C Incubation
500 Mcg/ml Antibitoic Initially

Figure 23

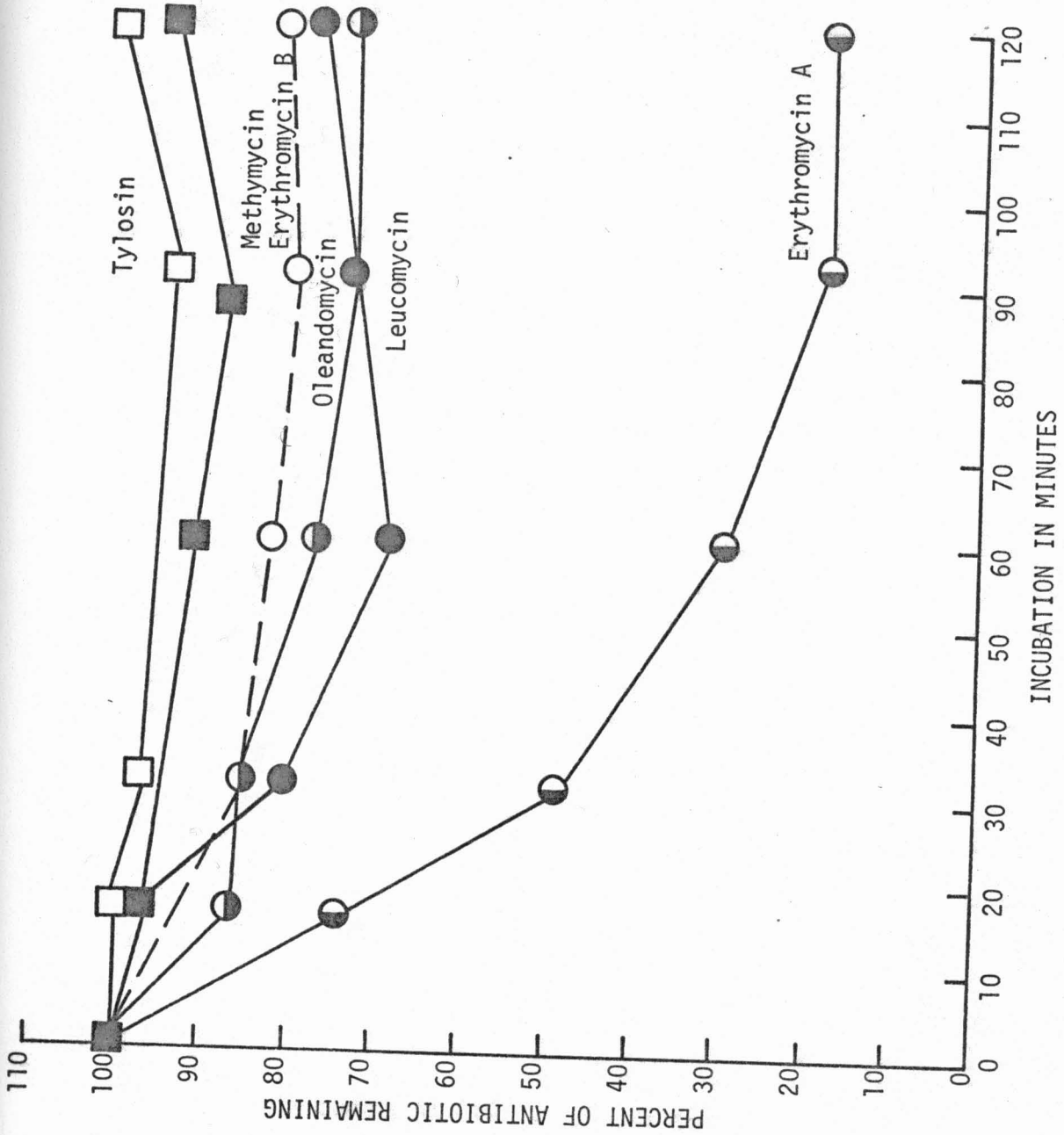
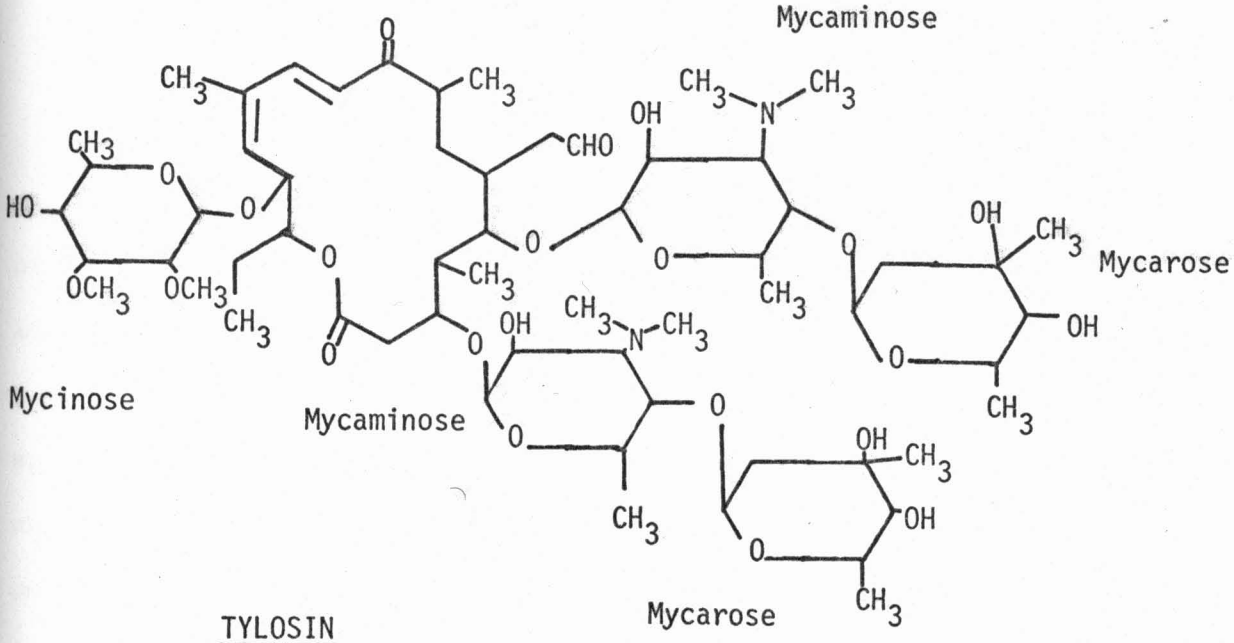
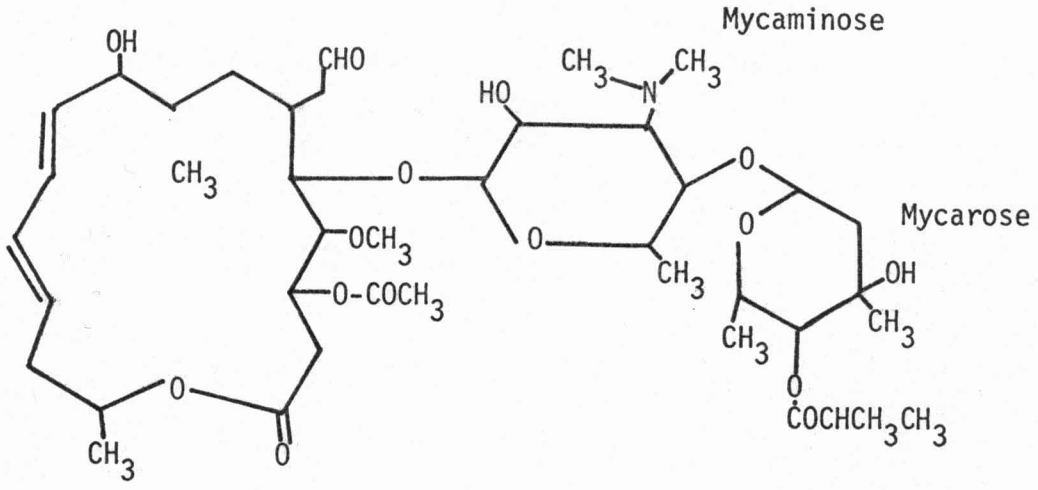

Tylosin
Methymycin
Erythromycin B
Oleandomycin
Leucomycin
Erythromycin A
PERCENT OF ANTIBIOTIC REMAINING
INCUBATION IN MINUTES
110
100
90
80
70
60
50
40
30
20
10
0
10
20
30
40
50
60
70
80
90
100
110
120

Figure 24: STRUCTURES OF TYLOSIN AND JOSAMYCIN

Figure 24



TYLOSIN



JOSAMYCIN (Leucomycin A_3)

erythromycin A. The more chemically stable erythromycin B and oleandomycin being successfully attacked only to a small degree compared to the more labile erythromycin A.

2. Substrate Specificity Using Induced Enzyme Level Lyophilized Preparations

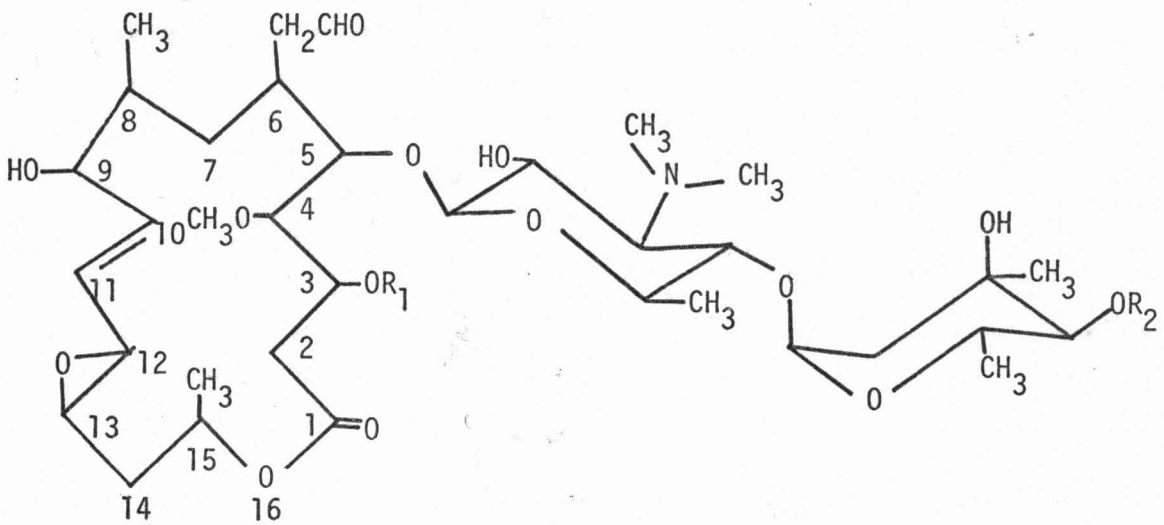
The substrate specificity of the lyophylized whole cell preparation of induced enzyme level was determined. The incubation conditions were identical to those used for the uninduced sonicated preparation except only 2 mg of cell protein was utilized in each assay tube. This substrate specificity study included josamycin (leucomycin A_3) and maridomycin III whose structures are shown in Figures 24 and 25. The results of the arsenomolybdate assay of this study was shown in Figure 26. These results using induced enzyme levels suggest that while maridomycin III, tylosin tartrate, methymycin, josamycin, and oleandomycin are not inactivated by Pseudomonad 56, erythromycin B is. Using the induced enzyme level preparation, 40 percent of the erythromycin B is inactivated in 120 minutes.

A confirmatory bioassay run on samples from the above substrate specificity study using a lyophylized whole cell preparation with induced enzyme levels was run. The best system found to bioassay all of the eight different macrolide antibacterial antibiotics simultaneously utilized the more sensitive Sarcina lutea instead of Staphyococcus aureus as the test organism. Oleandomycin could not be assayed on this S. lutea system as its response curve was nearly vertical.

The results of this bioassay are shown in Figure 27. The bioactivity data confirms the arsenomolybdate data showing that erythromycin A is rapidly inactivated and the other macrolide antibiotics tested were not inactivated. Only 15 percent of the biological activity of erythromycin B

Figure 25: STRUCTURE OF MARIDOMYCIN III

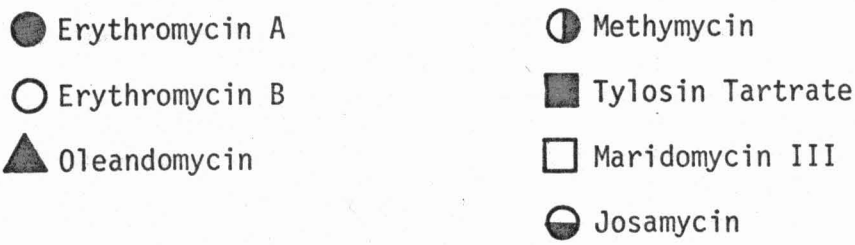
Figure 25



R_1 = propionyl

R_2 = propionyl

Figure 26: MACROLIDE SUBSTRATE SPECIFICITY OF PSEUDOMONAD 56 USING AN INDUCED ENZYME LEVEL PREPARATION



Lyophylized whole cell preparation - 2 mg/ml

pH 8.0

45°C Incubation

500 mcg/ml Antibiotic Initially

Figure 26

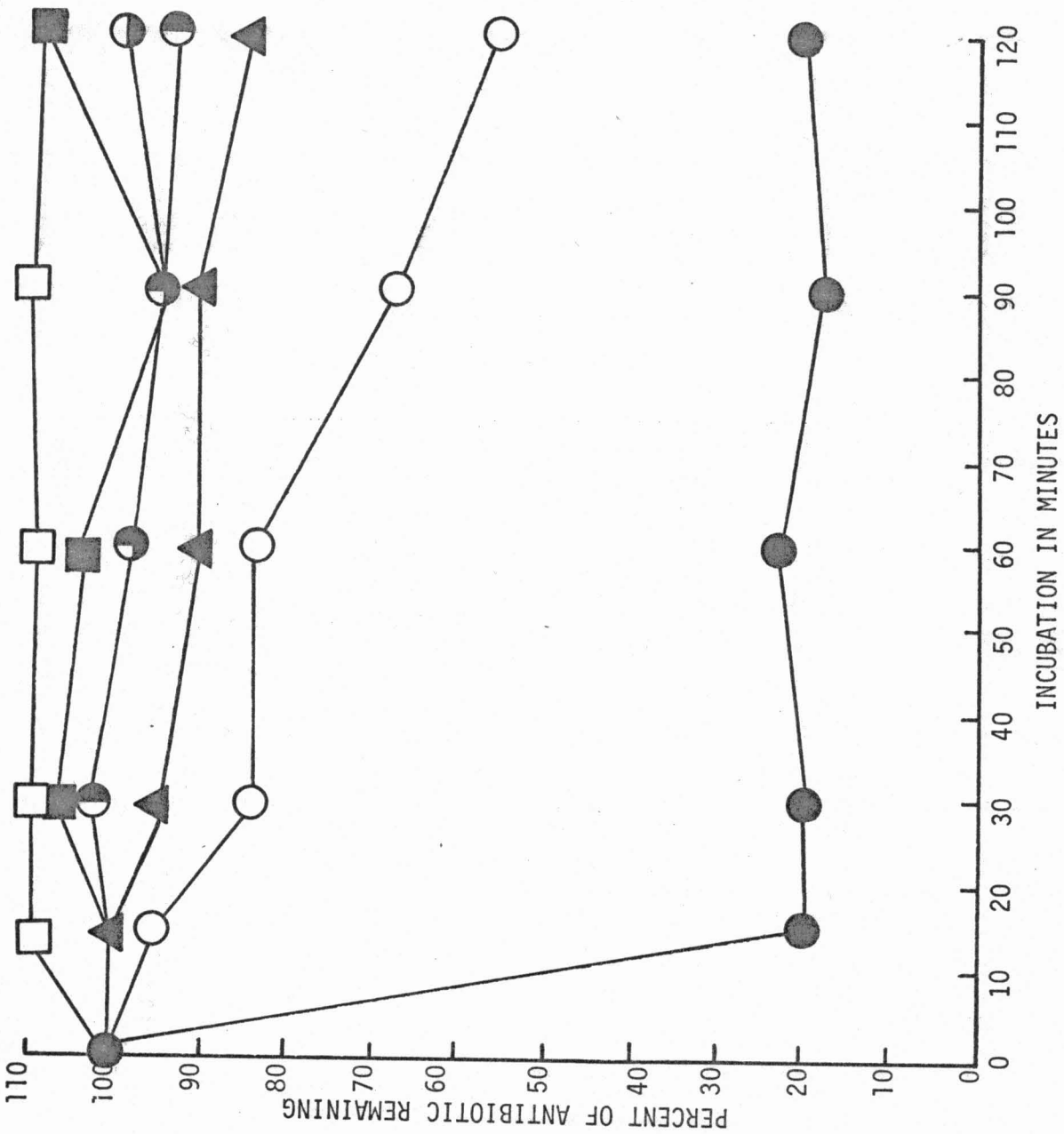

PERCENT OF ANTIBIOTIC REMAINING
110
100
90
80
70
60
50
40
30
20
10
0
0
10
20
30
40
50
60
70
80
90
100
110
120
INCUBATION IN MINUTES

Figure 27: Sarcina lutea BIOASSAY OF SUBSTRATE SPECIFICITY STUDY OF PSEUDOMONAD 56 WITH INDUCED ENZYME LEVELS

● Erythromycin A
○ Erythromycin B
□ Maridomycin III
◑ Methymycin
■ Tylosin Tartrate
◒ Josamycin

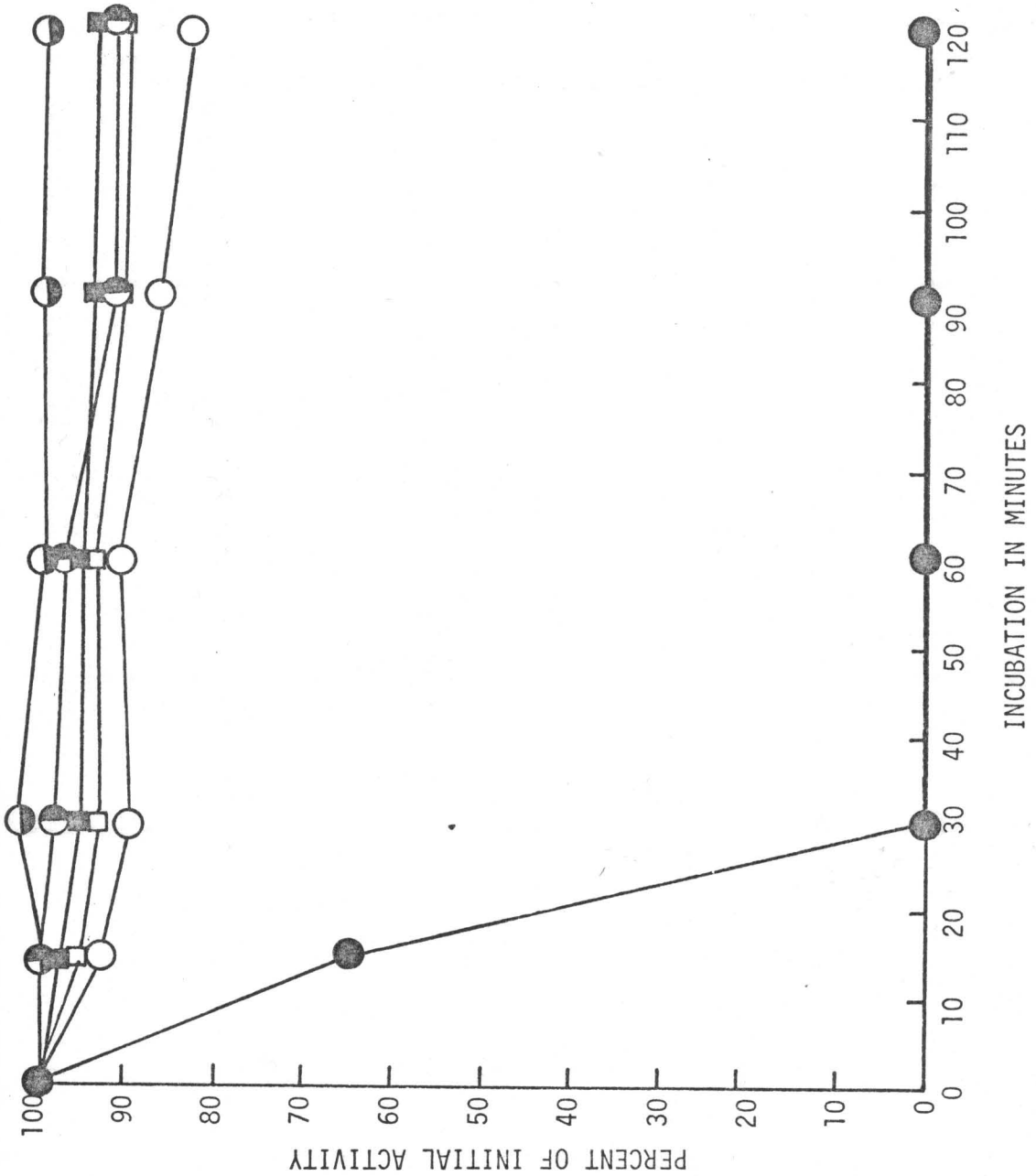
Lyophylized whole cells with Oleandomycin Induced Enzyme Levels

pH 8.0

45°C Incubation

500 mcg/ml antibiotic initially in 1.5 ml.

Figure 27


PERCENT OF INITIAL ACTIVITY
100
90
80
70
60
50
40
30
20
10
0
INCUBATION IN MINUTES
0
10
20
30
40
50
60
70
80
90
100
110
120

was lost in 120 minutes while the same sample assayed by the arsenomolybdate method suggested a 40 percent loss of activity. The differences between the two assays cannot be explained.

K. Chemical Study of the Mechanism of Inactivation of Erythromycin A by Pseudomonad 56

1. Isolation of Reaction Products

The inactivation of erythromycin A by Pseudomonad 56 was analyzed by the arsenomolybdate-ethyl acetate extraction method. 'Neutral macrolides' disappeared from the ethyl acetate extract when erythromycin A was incubated with cell preparations of Pseudomonad 56. The products of the microbial inactivation were then able to be easily separated from residual intact erythromycin A by this ethyl acetate extraction at pH 8. After extraction, the enzyme preparation was centrifuged and the protein pellet discarded leaving the inactivation products in the buffered aqueous extract.

Initial attempts using thin layer chromatography of the products of microbial inactivation were hampered by the necessity of desalting the aqueous reaction mixture. A simple spotting and development of the aqueous supernatant of the reaction mixture on 0.5 mm tlc plates (Eastman Chromagram Sheet) showed only salts which would not move from the origin. With inclusion of aqueous solvents, the spots simply moved with the solvent front without resolution.

The aqueous supernatant of the 10 mM pH 8 whole cell reaction mixture concentrated by lyophilization and resuspended in distilled water was passed through a Sephadex G-10 column (20 by 135 mm). This procedure produced only limited desalting as evidenced by thin layer chromatographic analysis; only salts were observed. Combinations of methanol precipitations

and G-10 desalting did not result in increased removal of phosphate from the reaction mixture.

Successful desalting of the mixture of inactivation products was obtained by the use of a strong anion exchange resin, Biorad Analytical Grade AG 1-X2, employing a volatile buffer system for elution. The lyophilized mixture of products was redissolved in a pH 10 volatile buffer system after Hirs (51) consisting of the following in four liters of solution: 60 ml N-ethyl morpholine, 80 ml 2-picoline, 40 ml pyridine, and acidic acid to adjust the pH. The concentrated product mixture was applied to a 21.5 by 0.7 cm pH 9.1 AG 1-X2 column (200-400 mesh) and eluted with the same buffer system at pH 7.0. This procedure resulted in the elution and desalting of all reaction products. No further products were removed from the column upon elution at pH 5 with 0.1 N HCl to remove the retained phosphate.

The volatile buffer was then removed under vaccuum and the aqueous mixture of inactivation products concentrated by lyophilization.

Further separation of the concentrated desalted products was achieved using an analytical grade silica gel column (500-200 micron) eluted with chloroform and increasing concentrations of methanol. The dimensions of the silica gel column were 24 by 1 cm with the sample applied in chloroform methanol (1:1). A flow sheet of the isolation procedure is given in Figure 28.

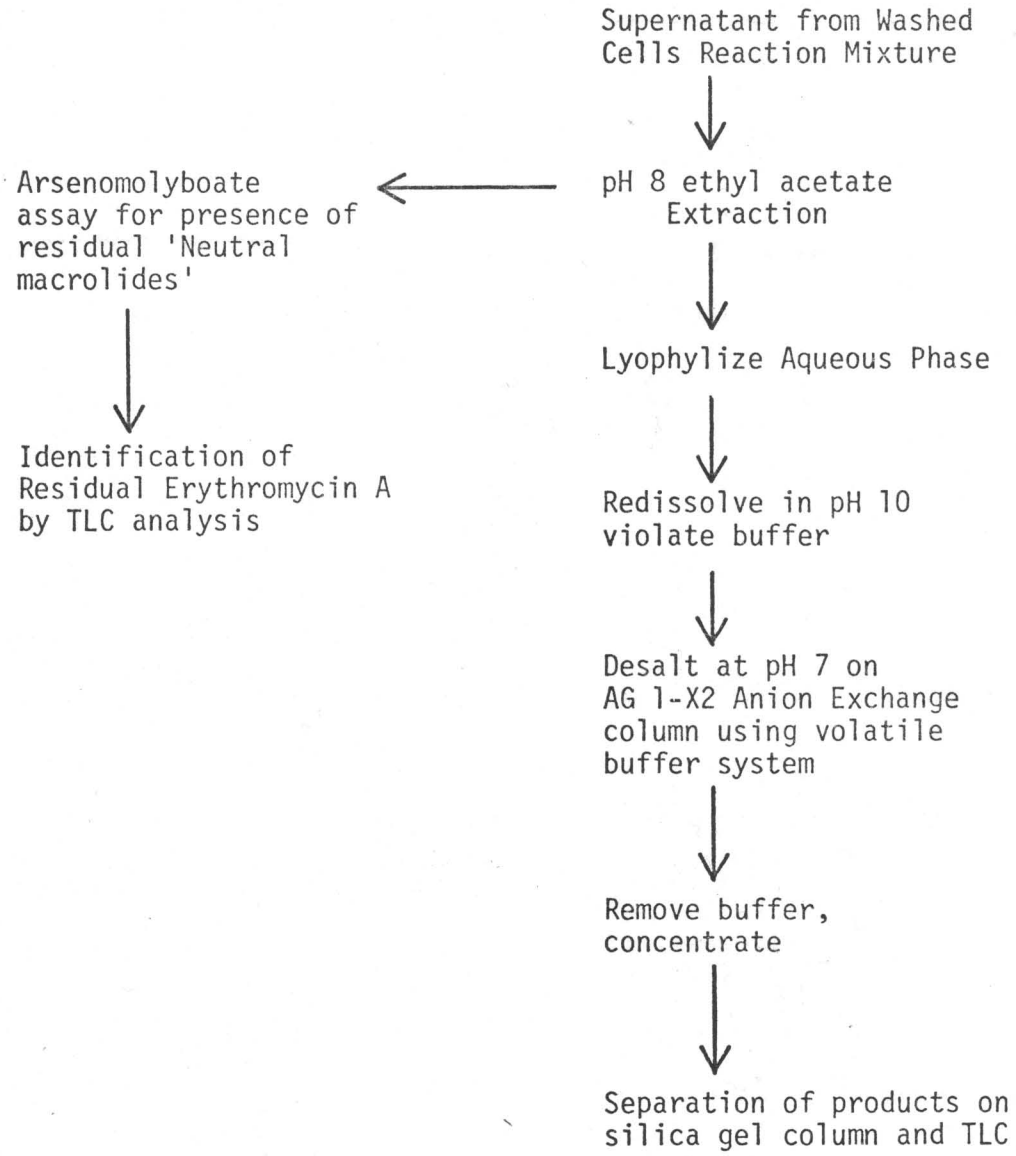
Concentration and tlc analysis of the pH 8 ethyl acetate extraction of the reaction mixtures showed only residual erythromycin A remaining in the non-aqueous phase.

2. Thin Layer Chromatographic Analysis of the Inactivation Products of Erythromycin A

Separation of the desalted reaction products on the silica gel column

Figure 28: ISOLATION OF INACTIVATION PRODUCTS FROM MICROBIAL INACTIVATION OF ERYTHROMYCIN A BY PSEUDOMONAD 56

Figure 28


Supernatant from Washed Cells Reaction Mixture
pH 8 ethyl acetate Extraction
Arsenomolyboate assay for presence of residual 'Neutral macrolides'
Identification of Residual Erythromycin A by TLC analysis
Lyophylize Aqueous Phase
Redissolve in pH 10 violate buffer
Desalt at pH 7 on AG 1-X2 Anion Exchange column using volatile buffer system
Remove buffer, concentrate
Separation of products on silica gel column and TLC

system were not always obtained. To determine the identity of the inactivation products relative to known compounds, four different solvent systems were developed. (See page 28) Identical R_f values of a specific product with a known compound on all four solvent systems was used as the criterion for identity.

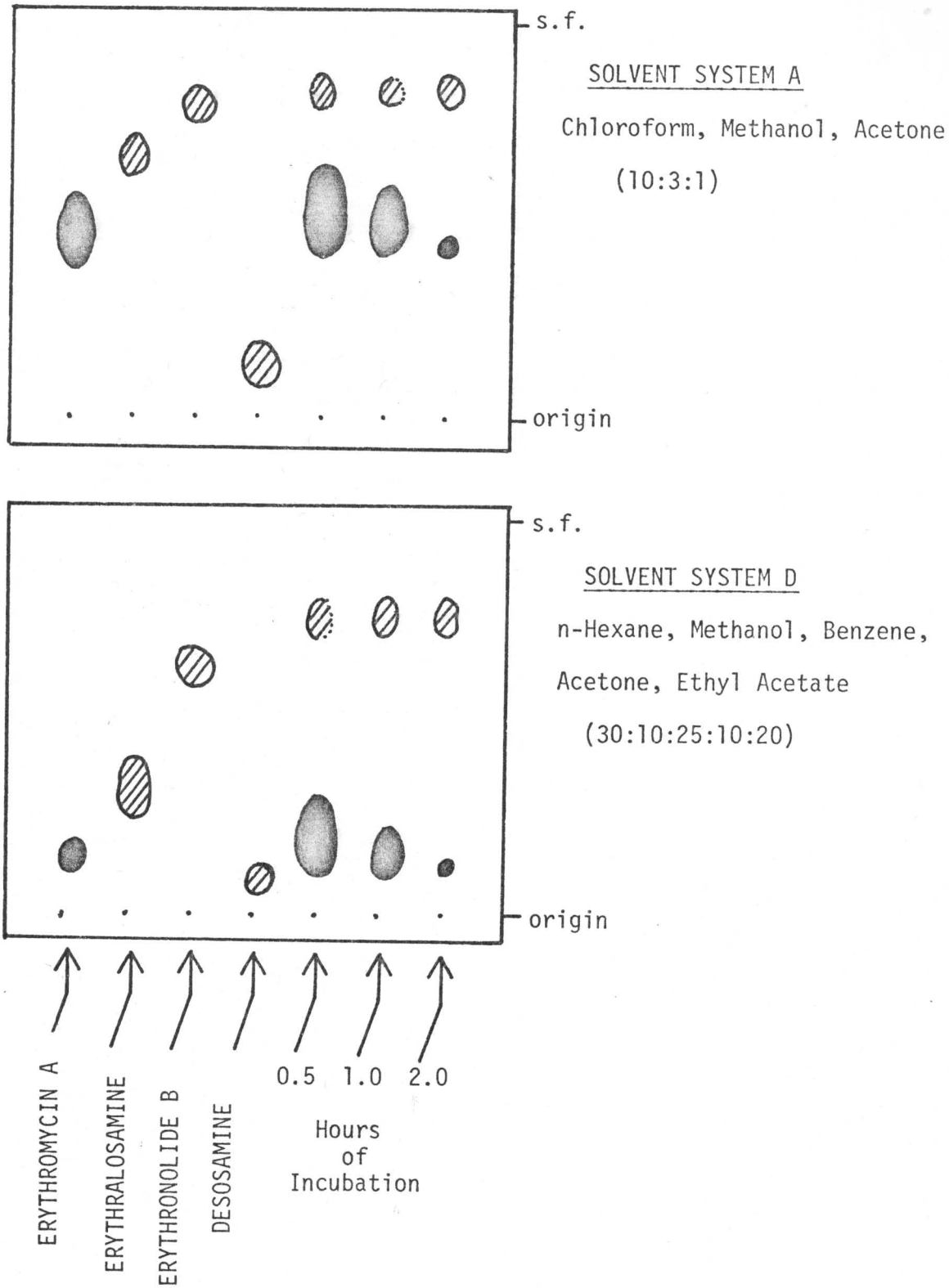
Initial thin layer plates of inactivation products from erythromycin A suggested a number of compounds in the reaction mixture. The identity and sequential formation of each of the products was followed by the appearance of each product during the time course of the reaction. A whole cell reaction mixture from 2.4 liters of culture 56 was incubated in the presence of 500 mg of erythromycin A for two hours at 45°C at pH 8.0. Forty milliliters were removed from this mixture at 0.5, 1.0, and 2.0 hours for tlc analysis. Each sample was extracted, desalted, and separated on the silica gel column as described in Figure 28. The eluted fractions from this column were spotted on the four tlc plates with four standard compounds on each plate. (Illustrated in Figure 29) One plate was developed in each of the four solvent systems. A record was then obtained of the appearance of inactivation products in the aqueous phase over the two hour period.

Erythromycin A, desosamine, erythronolide B, and erythralosamine were applied in concentrations of 20 micrograms per spot to each plate as known compounds for comparison with the inactivation products.

The ethyl acetate extractions of the reaction mixture were concentrated and submitted to tlc analysis on the same systems as the products in the aqueous phase. The results of following the disappearance of erythromycin A from the ethyl acetate extraction over time using solvent systems A and D are shown in Figure 29. The solid spots represent compounds visualized with the ceric sulfate-sulfuric acid spray while lined spots

Figure 29: THIN LAYER CHROMATOGRAPHIC ANALYSIS OF ETHYL ACETATE EXTRACTION OF AN ERYTHROMYCIN A - PSEUDOMONAD 56 REACTION MIXTURE DURING TWO HOURS OF INCUBATION

Figure 29


s.f.
SOLVENT SYSTEM A
Chloroform, Methanol, Acetone
(10:3:1)
origin
s.f.
SOLVENT SYSTEM D
n-Hexane, Methanol, Benzene,
Acetone, Ethyl Acetate
(30:10:25:10:20)
origin
ERYTHROMYCIN A
ERYTHRALOSAMINE
ERYTHRONOLIDE B
DESOSAMINE
0.5
1.0
2.0
Hours
of
Incubation

represent reducing compounds visualized with the tetrazolium spray.

The observation of the ethyl acetate extract over a period of two hours suggests that neutral erythromycin A does disappear from the ethyl acetate layer as incubation time increases. Secondly, a small amount of a new less polar reducing compound appears in the ethyl acetate extracts between the beginning of the incubation and 0.5 hours which does not appear to accumulate as the erythromycin is observed to disappear. This new compound was shown not to be erythronolide B, desosamine, or erythralosamine by comparing its R_f with these standards on all four solvent systems.

Composite representations of the chromatographic data from the aqueous phase after 0.5, 1.0, and 2.0 hours on two of the four solvent systems is presented in Figure 30. The products are numbered according to their mobility on system A.

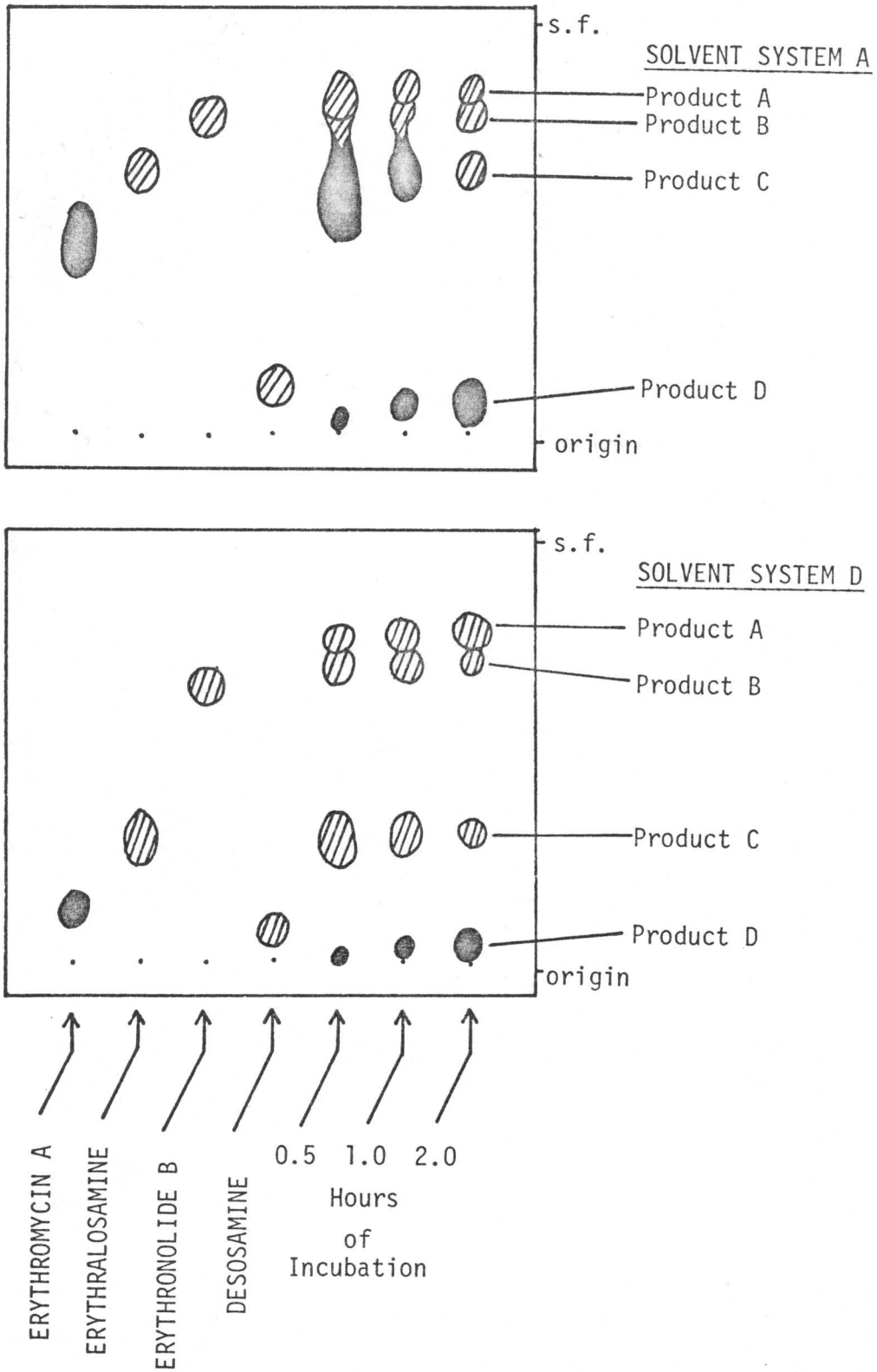
Analysis of these isolated reaction products using the four tlc systems suggests the following. Product A, as the most mobile product in solvent system A is designated, has the same mobility as the rapid moving reducing product seen in the ethyl acetate extract. This is true on all four solvent systems. In the aqueous reaction mixture this product accumulates over time. This product does not correspond to any known compound in the four solvent systems.

Product B, not present in the ethyl acetate extract and present only in relatively small quantities in the reaction mixture demonstrated the same R_f as erythronolide B in all four solvent systems. This suggests that it is possible to isolate the intact aglycone ring lactone from the enzymatic reaction mixture.

Product C has demonstrated the same R_f as erythralosamine in all four solvent systems. This would imply that the neutral sugar cladinose is perhaps removed enzymatically in the inactivation process. The removal

Figure 30: COMPOSITE THIN LAYER CHROMATOGRAPHIC ANALYSIS OF THE AQUEOUS PHASE OF THE ERYTHROMYCIN A - PSEUDOMONAD 56 REACTION MIXTURE DURING TWO HOURS OF INCUBATION

Figure 30


s.f.
SOLVENT SYSTEM A
Product A
Product B
Product C
Product D
origin
s.f.
SOLVENT SYSTEM D
Product A
Product B
Product C
Product D
origin
ERYTHROMYCIN A
ERYTHRALOSAMINE
ERYTHRONOLIDE B
DESOSAMINE
0.5
1.0
2.0
Hours
of
Incubation

of cladinose from the intact erythromycin would serve to abolish the biological activity of the molecule.

Product D appears to be a non-reducing very polar molecule which is observed to accumulate in the reaction mixture.

It remains unclear from this data whether the neutral sugar cladinose has actually been liberated from the erythromycin molecule. No standard cladinose was available to determine its R_f on these solvent systems. It is possible that since the reaction mixture for the inactivation products was run utilizing whole cells that this neutral sugar if cleaved from erythromycin was taken up by the cells.

The above observations suggest a possible mechanism of action for the inactivation of erythromycin A by Pseudomonad 56. Further evidence is needed to determine which products are liberated as the result of enzymatic activity and which are the result of chemical breakdown of the inactivation products due to the incubation conditions used for the reaction.

To test this erythromycin A, erythronolide B, erythralosamine, and desosamine were incubated in pH 8 phosphate at 45°C for three hours to test for evidence of chemical breakdown (100 mcg per ml). In all cases, no breakdown products suggesting chemical degradation of these standard compounds was identified by tlc analysis during this incubation. Desosamine was not detected as being removed from erythronolide-B-desosamine during the incubation. Desosamine itself did not show chemical breakdown during the two hour period. The erythronolide lactone ring did not appear to be opened at the incubation temperature of 45°C. Whether or not cladinose was removed chemically from the incubation sample of erythromycin A could not be detected rigorously from the tlc data alone due to the lack of a standard compound. No biological activity was lost during the incubation of erythromycin A implicating that cladinose was not liberated.

The mechanism of inactivation implied by the tlc observations above is that the intact erythromycin A molecule is attacked by Pseudomonad 56. It appears that the neutral sugar cladinose is probably cleaved to result in the immediate loss of biological activity. The degradation of the molecule after this step might proceed by the removal of the amino sugar desosamine which is rapidly converted into some degradation product. This product appears to accumulate in the aqueous reaction mixture and distributes itself between the ethyl acetate and aqueous phases upon extraction at pH 8. Free desosamine does not appear to accumulate in the reaction mixture. The cleavage of desosamine and formation of the degradation product might by concerted steps. The intact lactone ring which also does not appear to accumulate in the reaction mixture is opened enzymatically producing an anion (polar product D) which does accumulate in the mixture. A table showing the R_f values of erythromycin A and various degradation products in the four solvent systems is presented in Table 11.

3. Structure Determination of Inactivation Products of Erythromycin A

The structure of erythromycin was determined after a lengthy series of chemical degradations (10, 11, 24, 25, 26). It has not been until recently with the application of mass spectrometry and chemical ionization mass spectrometry that structural information on macrolide antibiotics could be obtained utilizing only small amounts of sample material.

The recent mass spectral work of both Foltz and Mitscher (52, 53, 54, 55, 56) has concerned the application of electron impact and chemical ionization techniques on the 14 membered lactone ring macrolide antibiotics and erythromycin in particular. These studies have demonstrated that much

TABLE XI. R_f Values for Erythromycin A, Erythromycin Derivatives, and Inactivation Products on Four Thin Layer Chromatographic Systems

Solvent Systems ⟶	A	B	C	D
Erythromycin A	0.35	0.07	0.67	0.11
Erythromycin B	0.34	0.07	0.66	---
Erythronolide B	0.67	0.31	0.83	0.61
Erythralosamine	0.58	0.17	0.55	0.24
Desosamine	0.22	0.06	0.21	0.08
Product A	0.78	0.63	0.79	0.79
Product B	0.69	0.31	0.83	0.61
Product C	0.60	0.18	0.55	0.24
Product D	0.07	Origin	0.33	0.0-0.06

0.5 mm Silica Gel Plates (Kodak)

SOLVENT SYSTEMS:

A - Chloroform, methanol, Acetone (10:3:1)

B - Benzene, Methanol, Toluene (85:10:5)

C - n-Butanol, Acetic Acid, Water (4:1:5)

D - n-Hexane, Benzene, Methanol, Ethyl Acetate, Acetone (30:25:10:20:10)

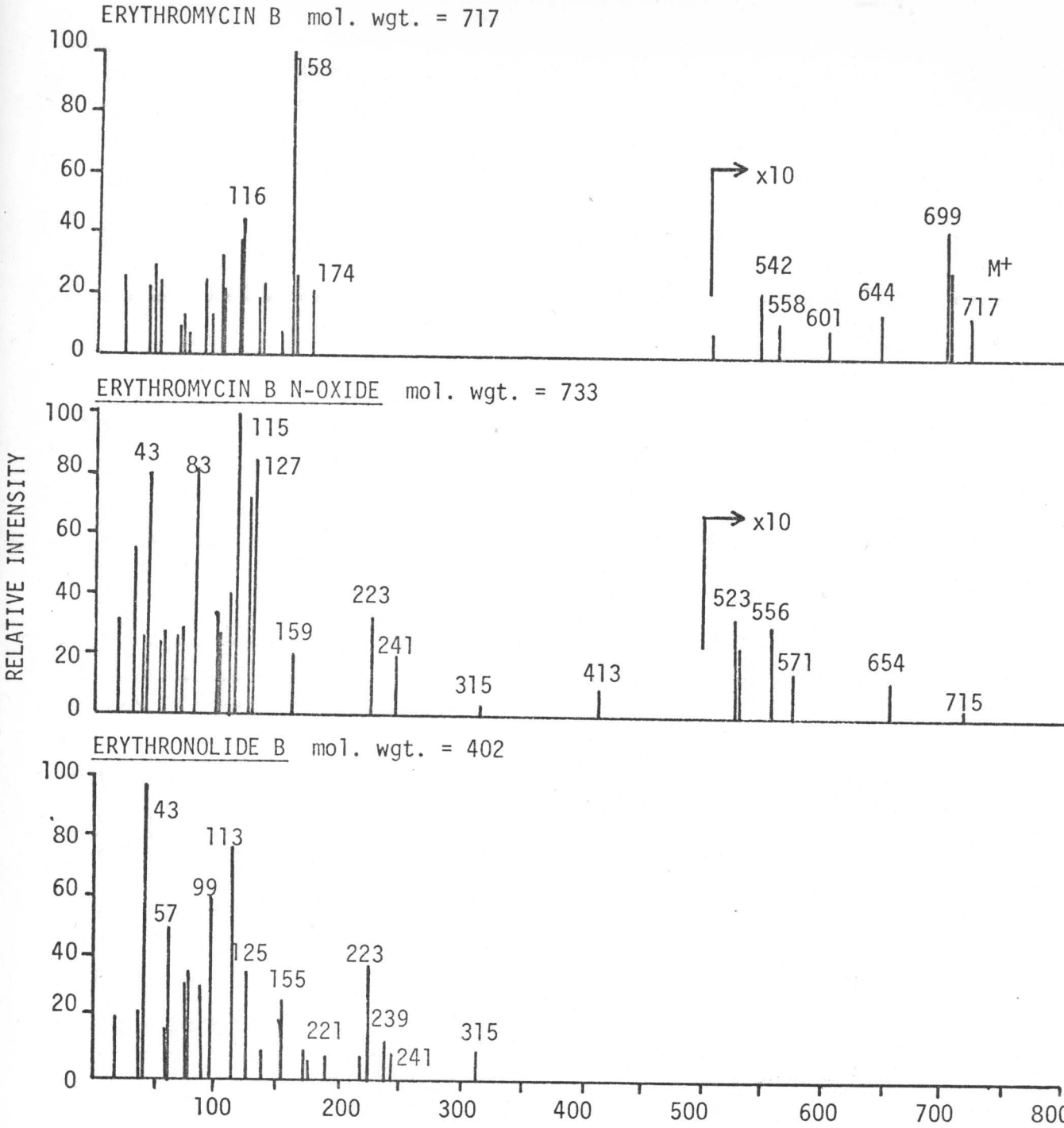
greater structural information could be obtained from the chemical ionization mass spectra than from the electron impact mass spectra. The chemical ionization mass spectra have a characteristically more intense molecular ion peak and a greatly 'simplified' spectrum from a more 'mild' fragmentation. Unfortunately, however, only electron impact mass spectrometry was available for use in this study.

The eludication of the structure of inactivation products of erythromycin A is further complicated by the fact that the only detailed mass spectral fragmentation work published on erythromycin has concerned the more chemically stable erythromycin B. In general the reason for this appears to be the combined difficulty of obtaining molecular ions for such large polyfunctional molecules and the occurance of an amino sugar. Fragmentation of the amino sugar usually dominates the spectrum and reduced the utility of mass spectrometry for structural analysis. This can be demonstrated by the spectrum of erythromycin B in Figure 31. The peaks above M/e 174 are all very weak and all of the peaks above M/e 80 with a relative intensity of greater than 20 percent either contain nitrogen and are then associated with the fragmentation of desosamine or are derived from cladinose the other sugar (56).

Because of this difficulty detailed fragmentation information concerning the lactone ring was unable to be obtained from electron impact mass spectrometry until Mitscher and Foltz (56) developed the utility of the N-oxide derivative in enhancing fragmentation of the non-sugar portion of the molecule. In this technique, the N-dominating effect of the desosamine moiety is suppressed by production of the N-oxide derivative which would tie-up the nonbonding electrons of the offending nitrogen atom and render it less basic. The results of their work has demonstrated that

Figure 31: MASS SPECTRUM OF ERYTHROMYCIN B, ERYTHROMYCIN B N-OXIDE, AND ERYTHRONOLIDE B

Figure 31


ERYTHROMYCIN B mol. wgt. = 717
158
116
174
x10
699
542
M+
644
558
601
717
ERYTHROMYCIN B N-OXIDE mol. wgt. = 733
115
43
83
127
x10
223
523
556
159
241
571
654
315
413
715
ERYTHRONOLIDE B mol. wgt. = 402
43
113
99
57
125
223
155
239
221
315
241
RELATIVE INTENSITY
100
80
60
40
20
0
100
200
300
400
500
600
700
800
m/e

the N-oxide of erythromycin B has the desired mass spectral properties. The N-oxide behaves more analogously to the neutral erythronolide B. This can clearly be seen in the mass spectra obtained on the Finnigan 1015 mass spectrometer of erythromycin B, erythromycin B N-oxide, and erythronolide B in Figure 31. Assignments agree with published spectra of these compounds. It should be noted that neither the N-oxide of erythromycin B nor erythronolide B show the molecular ion M+. In effect, the spectrum of erythromycin B N-oxide is essentially a composite of the spectrum of erythromycin B and that of erythronolide B.

Utilizing this approach the detailed mass spectral data of the fragmentation of the more chemically labile lactone ring of erythromycin A was sought. There are no published studies on this 14 membered lactone ring known as erythronolide A. Information on the fragmentation of this molecule is important for comparison with the spectra of the products of biological inactivation implicated by the thin layer chromatographic data to contain this lactone moiety.

The electron impact mass spectral study of Thompson (57) using an AEI MS-9 mass spectrometer suggested the fragmentation pattern shown in Figure 32 for the loss of desosamine and cladinose from erythromycin A and the appearance of desosamine and its fragments. The origin of the intense M/e 87 and M/e 71 fragments in the spectrum of erythromycin A can be observed to be from desosamine and the dimethyl amino group as was previously seen for erythromycin B. (56)

The detailed fragmentation of erythronolide B of Mitscher (56) is presented in Table 12. Careful study of this fragmentation has lead to proposal of an analogous set of major fragments for the fragmentation of erythronolide A shown in Table 13. Most of the proposed fragments are similar except for the addition of the hydroxyl group at position 12. The

Figure 32: FRAGMENTATION OF ERYTHROMYCIN A

Fragmentation Scheme from Thompson (57)

Figure 32

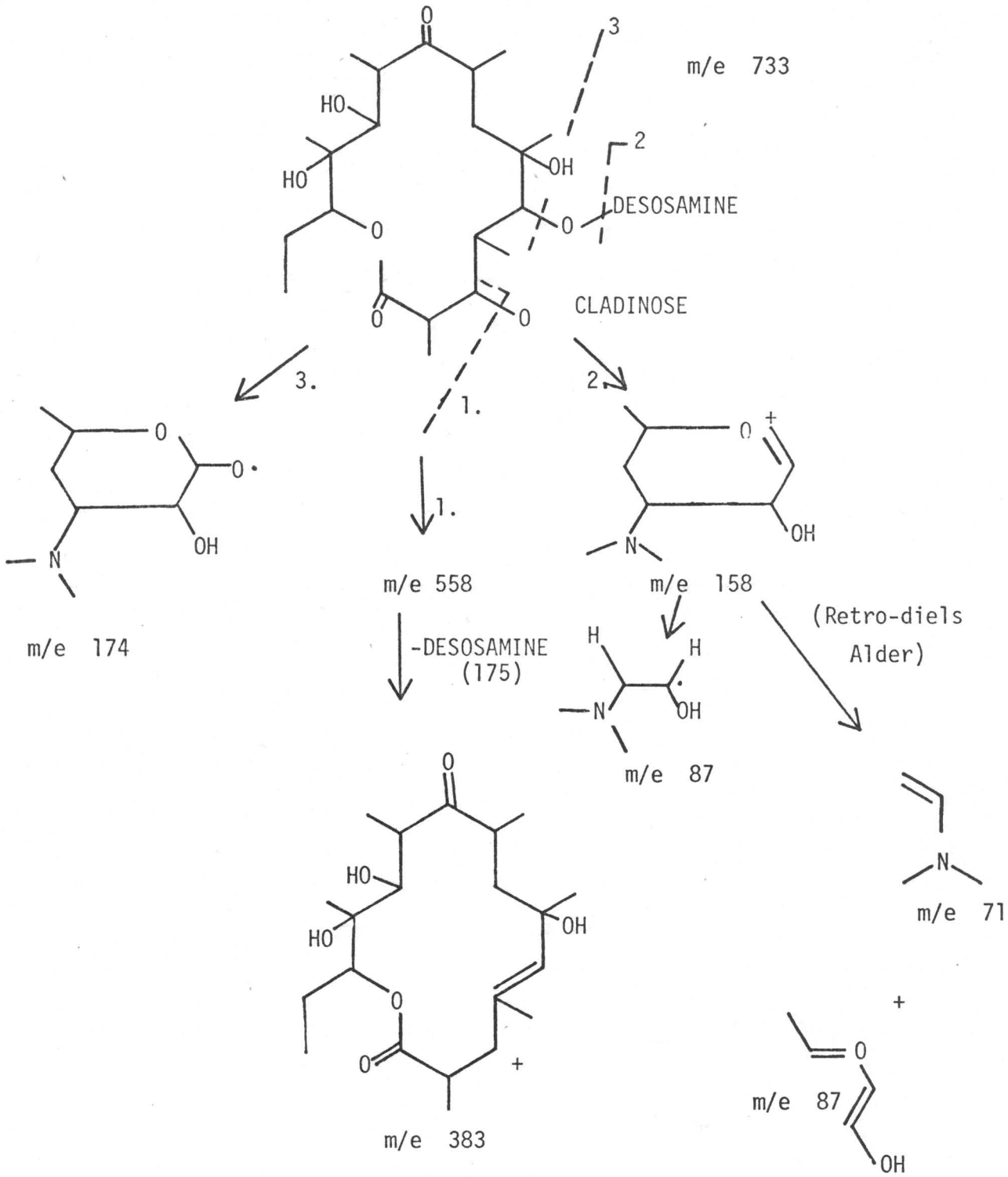
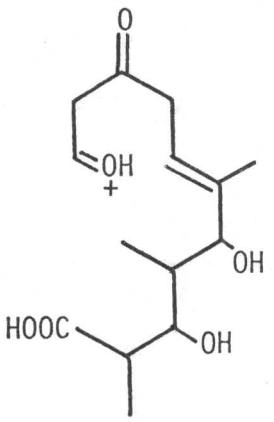
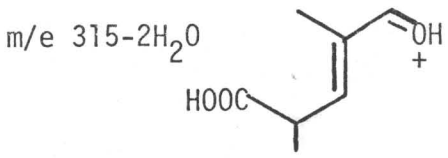

m/e 733
DESOSAMINE
CLADINOSE
3.
2.
1.
1.
m/e 174
m/e 558
m/e 158
-DESOSAMINE
(175)
(Retro-diels
Alder)
m/e 87
m/e 71
m/e 383
m/e 87

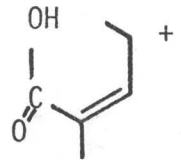
TABLE XII. FRAGMENTS OF ERYTHRONOLIDE B



m = 402

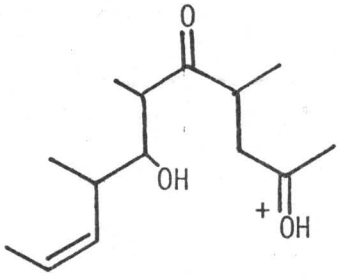


m/e 315-$2H_2O$

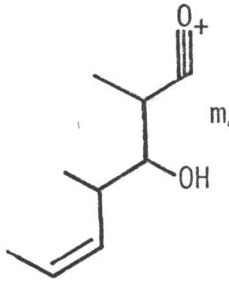


m/e 279 m/e 143 m/e 113

m - 87
m/e 315

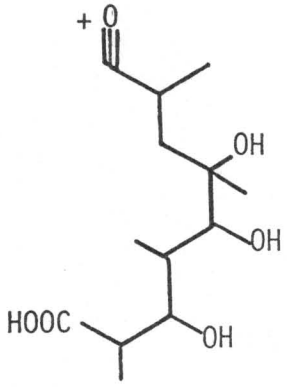


m/e 241-H_2O



m/e 155-H_2O

m/e 241 m/e 223 m/e 155 m/e 137



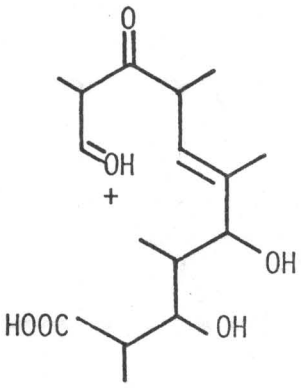
m/e 275-H_2O m/e 275-$2H_2O$ m/e 275-$3H_2O$

m/e 275 m/e 257 m/e 239 m/e 221

Fragments proposed by Mitscher (56)

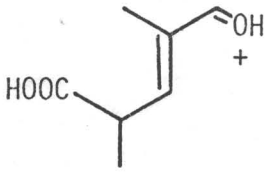
TABLE XIII. Proposed Fragments of Erythronolide A

m = 418

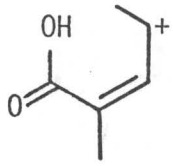


m-103
m/e 315

m/e 315-$2H_2O$

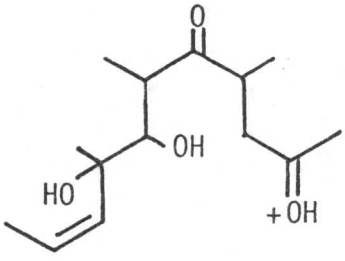


m/e 279



m/e 143

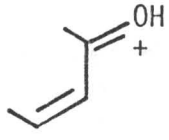
m/e 113



m/e 257

m/e 257-H_2O

m/e 239

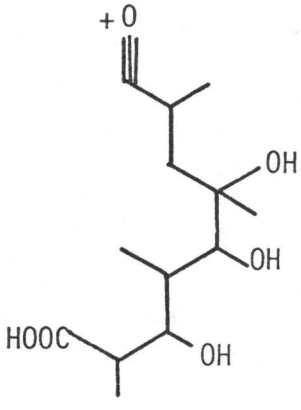


m/e 85

m/e 85-H_2O

m/e 67

m^+-H_2O



m/e 400

m/e 275

m/e 275-$2H_2O$

m/e 239

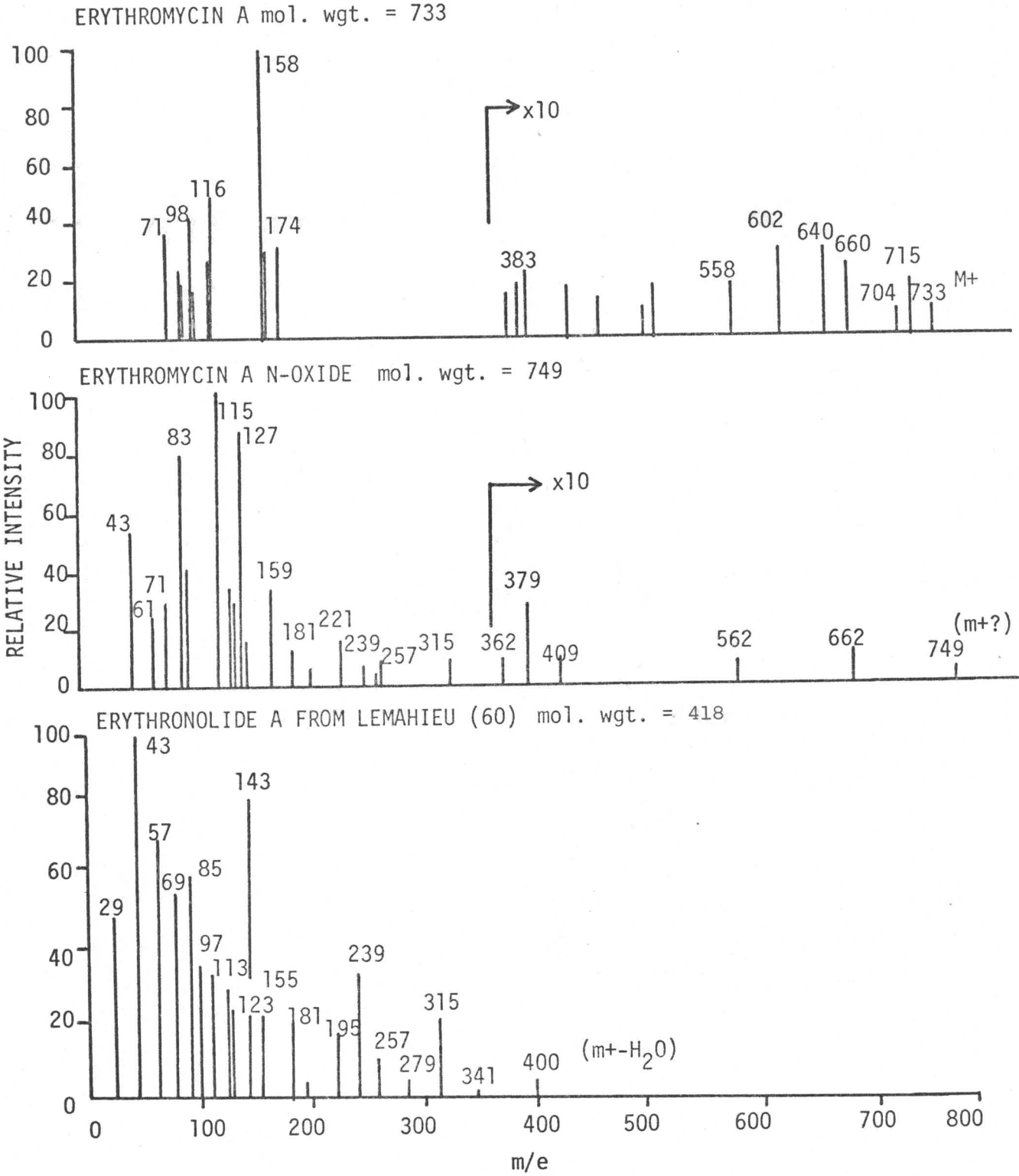
M/e 155 and M/e 137 fragments suggested from the erythronolide B fragmentation probably would not be seen in the fragmentation of the A form as the C-11, C-12, diol would not be stable. Instead cleavage of the C-11, C-12 bond would occur resulting in the M/e 85 and M/e 85 - H_2O fragments proposed.

Recently LeMahieu (37, 60,unpublished spectra) has made available electron impact mass spectra of the aglycone of erythromycin A. This spectrum shown in Figure 33 agrees with the proposed diagnostic fragments derived from the study of erythronolide B. This spectrum is also consistent with fragmentation studies of Mitscher, Foltz, and Maezawa (58) on similar 14 membered lactone ring aglycones.

The major diagnostic peaks on this electron impact mass spectrum of the aglycone of erythromycin A are : M/e 400 ($M^+ - H_2O$), 341, 315, 279, 239, 221, 181, 155, 143, 123, 113, 97, 85, 69, 57, 43 (parent), and 29. Using the above spectra and the proposed fragmentation as a guide, the spectra of inactivation products of erythromycin A obtained on the Finnigan 1015 mass spectrometer have been evaluated to give some suggestion of the structures of these products. It is only when larger quantities of purified inactivation products are available for both chemical ionization mass spectrometry and high resolution mass spectrometry that the true mechanism of inactivation of erythromycin A by this enzyme can be rigorously determined.

Figure 33: MASS SPECTRUM OF ERYTHROMYCIN A, ERYTHROMYCIN A N-OXIDE, AND ERYTHRONOLIDE A

Figure 33


ERYTHROMYCIN A mol. wgt. = 733
100
80
60
40
20
0
158
x10
116
71
98
174
383
602
640
660
715
558
704
733
M+
ERYTHROMYCIN A N-OXIDE mol. wgt. = 749
RELATIVE INTENSITY
115
83
127
x10
43
71
61
159
379
181
221
239
257
315
362
409
562
662
749
(m+?)
ERYTHRONOLIDE A FROM LEMAHIEU (60) mol. wgt. = 418
43
143
57
69
85
29
97
113
155
123
181
195
239
315
257
279
341
400
(m+-H2O)
0
100
200
300
400
500
600
700
800
m/e

Proposed Structure of Product A

From the tlc study it was determined that product A was a relatively less polar molecule probably derived from one of the attached sugars either desosamine or cladinose. It was also learned that this product could be extracted into ethyl acetate at pH 8.

The infrared spectrum and mass spectrum of product A are shown in Figure 34.

The infrared spectrum of this compound (liquid film) isolated from the reaction mixture shows the presence of the amine nitrogen by an absorption band at 2460 cm^{-1}. This immediately would suggest that the origin of product A is desosamine. Other features of the infrared spectrum that support this are the absorption of an aldehyde carbonyl at 1710 cm^{-1} as well as a carbonyl showing a resonance effect at 1670 cm^{-1} probably due to internal hydrogen bonding. Intermolecular hydrogen bonded O-H stretching and methylene stretching absorption bands are also evident at 3420 cm^{-1} and 2800-2900 cm^{-1} respectively.

The mass spectrum of this product shows peaks consistent with those suggested by Thompson and Mitscher for the fragmentation of desosamine (56,57). Figure 35 proposes one possible structure for product A consistent with both the infrared and mass spectral data. Major fragmentations are also suggested.

Figure 34: IR AND MASS SPECTRUM OF PRODUCT A

Figure 34

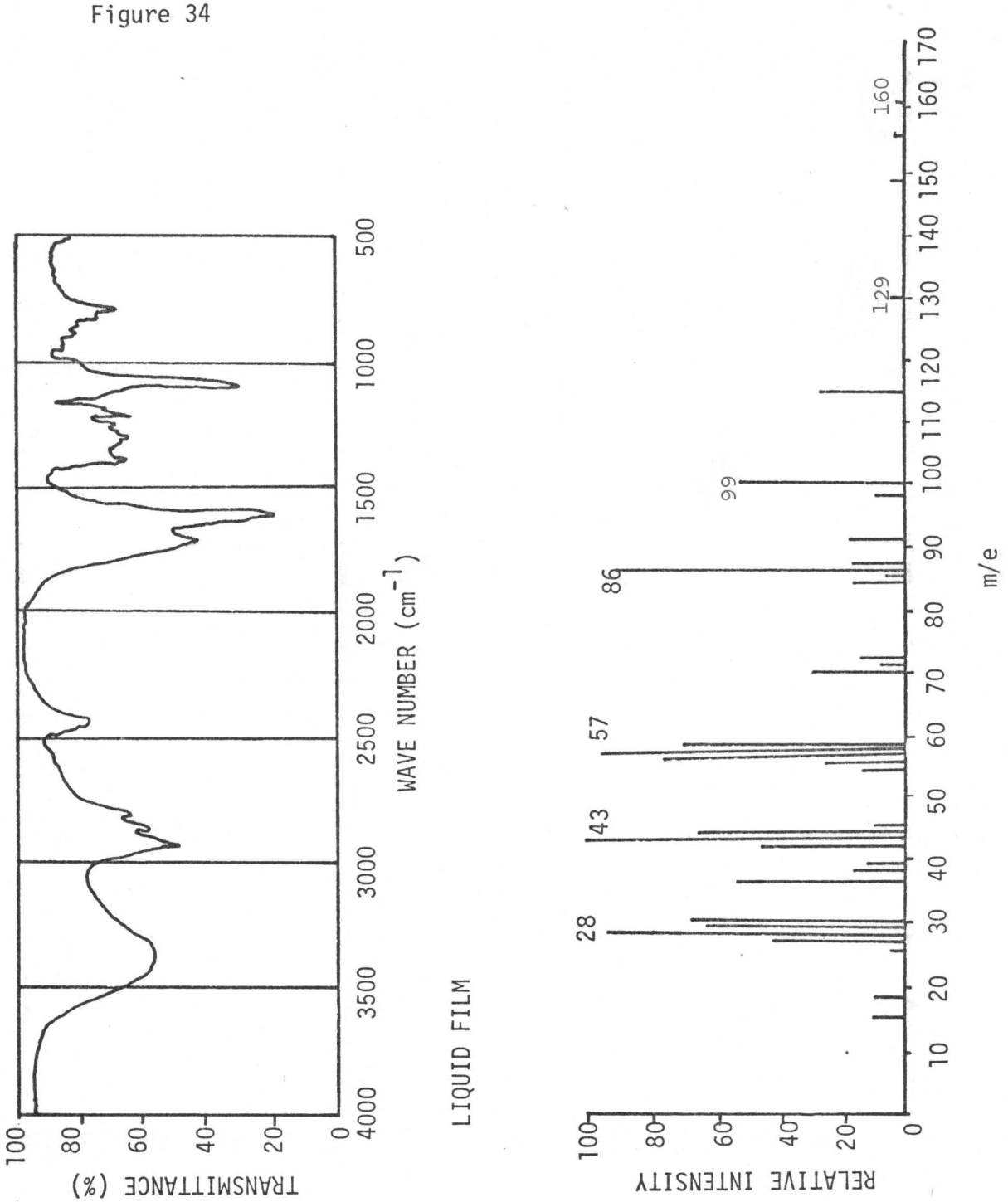
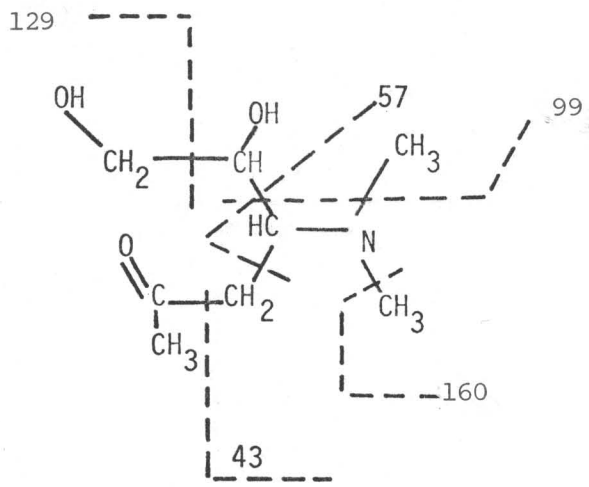

TRANSMITTANCE (%)
100
80
60
40
20
0
4000
3500
3000
2500
2000
1500
1000
500
WAVE NUMBER (cm^{-1})
LIQUID FILM
RELATIVE INTENSITY
100
80
60
40
20
0
10 20 30 40 50 60 70 80 90 100 110 120 130 140 150 160 170
m/e
28
43
57
86
99
129
160

Figure 35: PROPOSED STRUCTURE OF PRODUCT A

Figure 35

molecular weight = 175



Structure Analysis of Product B

Product B was implicated from tlc studies to be the intact lactone ring of erythromycin A without attached sugars. This product was not observed to accumulate in the reaction mixture with only small amounts being present. Its isolation in sufficient quantities to permit structural analysis was tedious. Only enough material was recovered to permit IR and mass spectral studies. These spectra are shown in Figure 36.

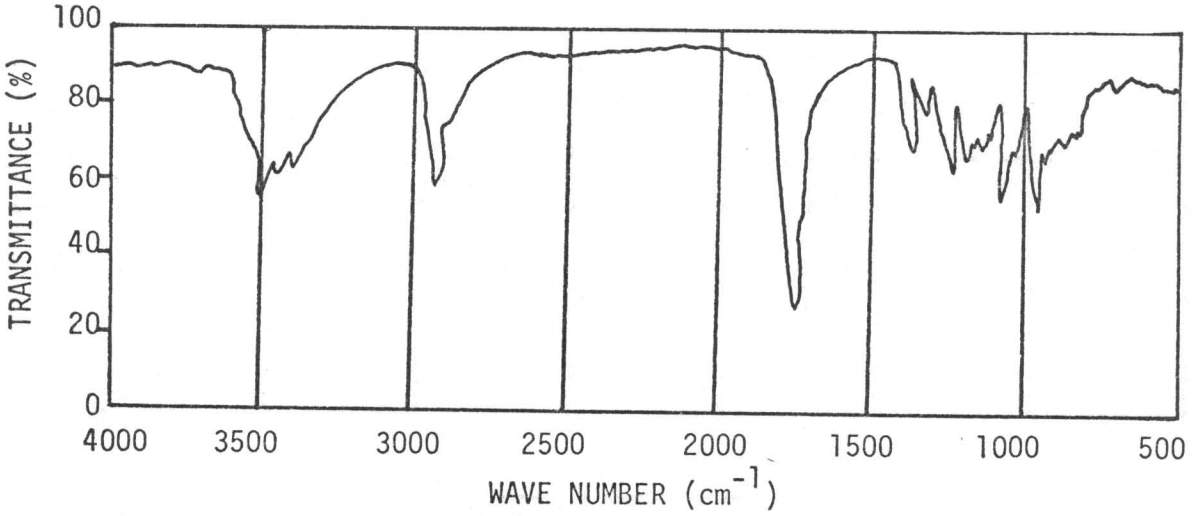
The IR spectrum indicates hydroxyls (3400-3500 cm^{-1}), methylene absorption in the region 2960 cm^{-1} and the absorption of a lactone carbonyl at 1720 cm^{-1}. The strong glycosidic C-O-C absorption that can be seen in the IR spectrum of erythromycin A is absent in this spectrum.

The mass spectrum of product B can in part be compared with that of erythronolide A. However, since the structure of product A (Figure 35) suggests that the cleavage of desosamine from the molecule occurs between C-5 and the glycosidic oxygen, two possible structures exist for the isolated lactone. The two possibilities are either erythronolide A lacking the C-5 hydroxyl (cleaved with desosamine) and including a hydroxyl at C-3 or the erythronolide lacking hydroxyl groups at both C-3 and C-5. The latter structure would imply that cladinose was cleaved from the aglycone between C-3 and the glycosidic oxygen.

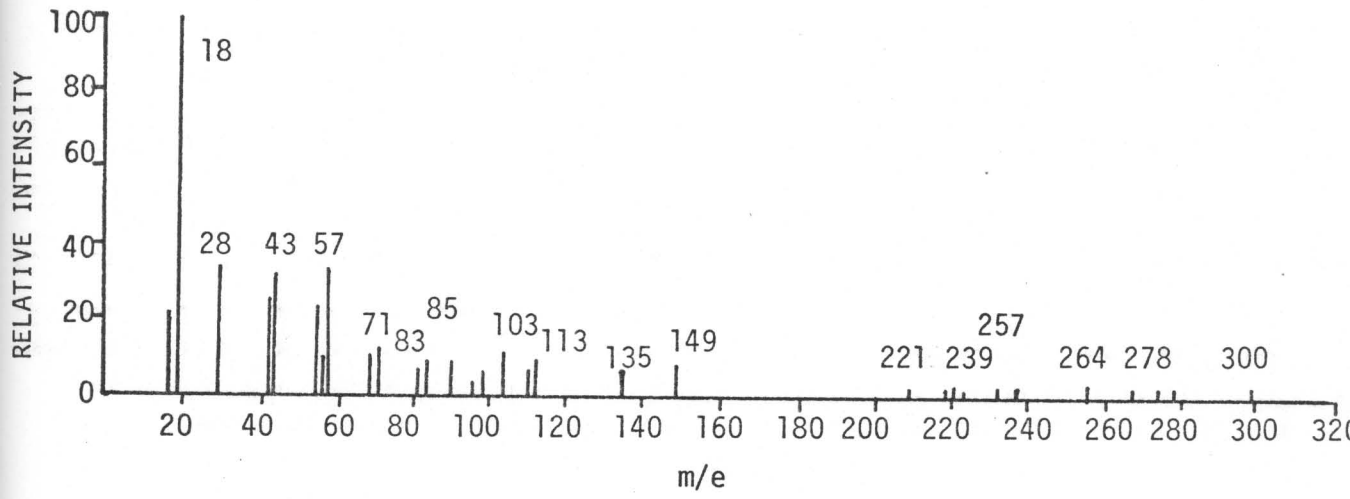
Of the two possible structures, only the former is consistent with the mass spectrum obtained for product B. The molecular weight of erythronolide A containing the C-3 hydroxyl and lacking the C-5 hydroxyl would be 403. The initial M-103 fragmentation seen in erythronolide A would result in a high mass peak of 403-103 or M/e 300 for product B. This is the high mass peak observed in the spectrum of this isolated product. The analogous M-103 fragmentation for the aglycone without hydroxyl groups at both C-5 and C-3 would show a high mass peak of 386-103 or M/e 283.

Figure 36: MASS SPECTRUM AND IR SPECTRUM OF PRODUCT B

Figure 36



KBr Pellet (1%)



Other major fragments and the suggested structure of product B are presented in Figure 37.

Structure Analysis of Product D

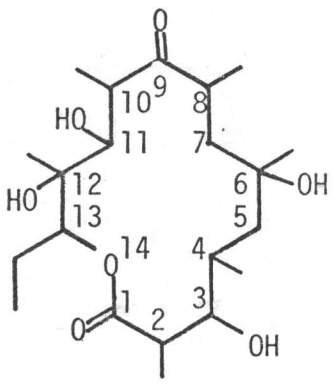
Product D was shown by tlc data to be a very polar product which accumulated in the reaction mixture over time. The major problem in the isolation of this product was to obtain a sample completely desalted. The IR spectrum and mass spectrum of a thoroughly desalted sample of product D are shown in Figure 38.

The IR spectrum shows strong carboxylate anion absorption at 1550 cm^{-1} and 1400 cm^{-1}, the absence of the lactone absorption (1720 cm^{-1}) and the presence of hydroxyl (3400 cm^{-1}) and methylene absorption (2490 cm^{-1}). This functional group analysis would suggest that product D is the enzymatically opened lactone aglycone.

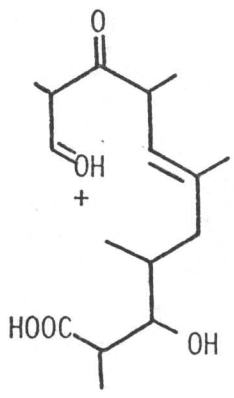
No evidence for the attachment or absence of cladinose to this carboxylate anion product is given in the IR data. The mass spectrum of this product, however, shows a high mass peak of 300 suggesting that this product is the opened ring form of product B. In addition to this high mass peak, product D exhibits the following fragments common to product B: M/e 278, 271, 210, 149, 135, 113, 103, 85, 57, 42, and 28. The mass spectrum of this product is suggestive of that of a long chain carboxylic acid which would show stepwise cleavage and accumulation of small fragments along the chain differing by mass units of 12-15. The proposed structure of this product and some suggested fragments are shown in Figure 39.

Figure 37: PROPOSED STRUCTURE OF PRODUCT B AND MAJOR DIAGNOSTIC FRAGMENTS

Figure 37



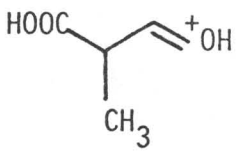
PRODUCT B mol. wgt. = 402



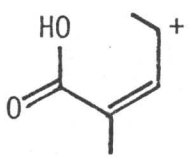
m/e 300

m/e 300-$2H_2O$

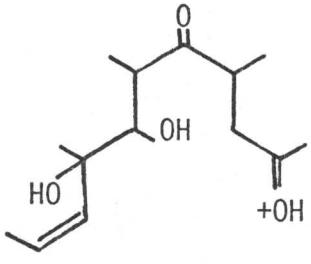
m/e 264



m/e 103



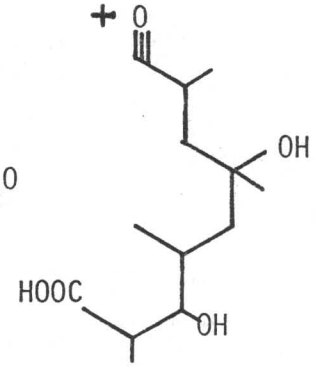
m/e 113



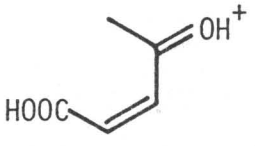
m/e 257

m/e 257-H_2O

m/e 239



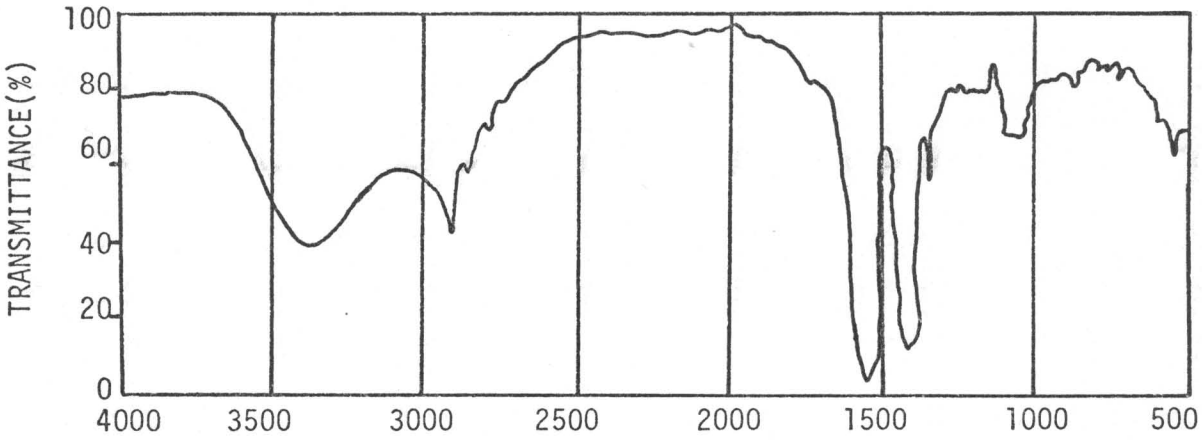
m/e 259



m/e 85

Figure 38: IR AND MASS SPECTRUM OF PRODUCT D

Figure 38



KBr Pellet (1%)

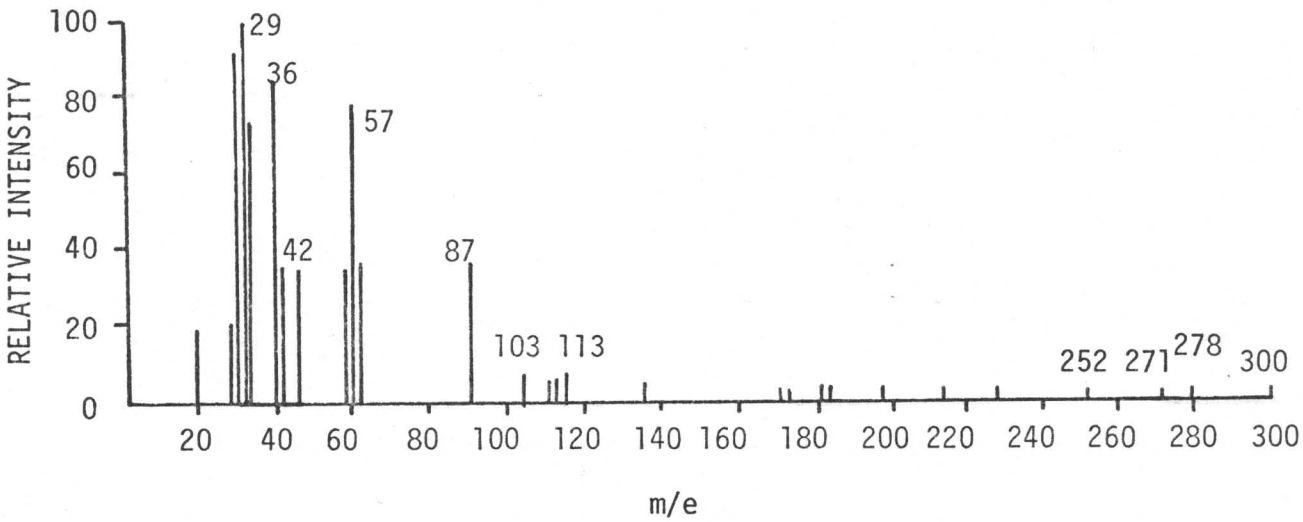
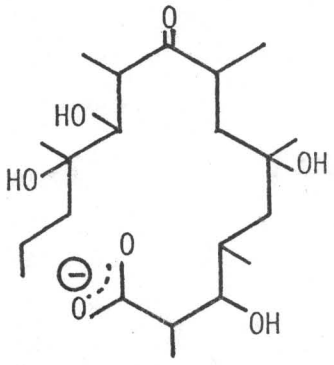


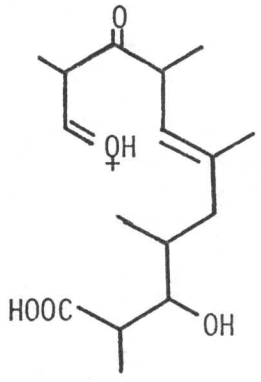
Figure 39: PROPOSED STRUCTURE OF PRODUCT D AND MAJOR DIAGNOSTIC FRAGMENTS

Figure 39

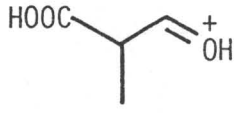


PRODUCT D

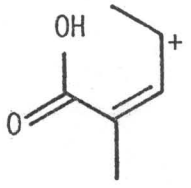
mol. wgt. = 402



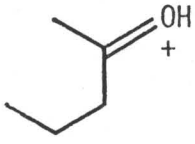
m/e 300



m/e 103



m/e 113



m/e 87

L. The Mechanism of Inactivation of Erythromycin A by Pseudomonad 56

The proposed mechanism of inactivation of erythromycin A by Pseudomonad 56 is presented in Figure 40. The analysis by thin layer chromatography and the limited mass spectral data suggest that cladinose is initially cleaved from the intact molecule leaving a C-3 hydroxyl group. Cladinose itself was never isolated from the reaction mixture.

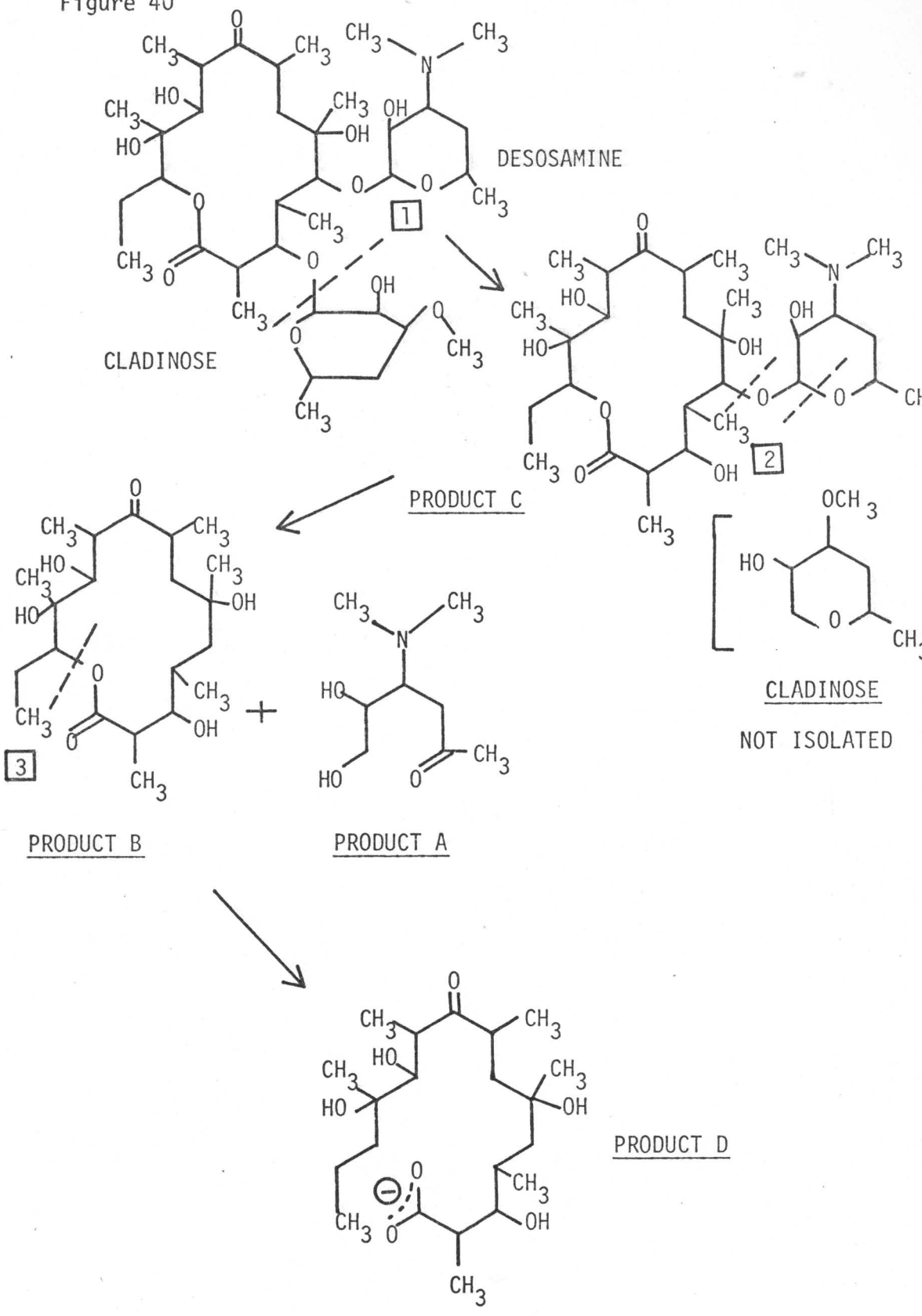
The second step in the inactivation appears to be the concerted cleavage of desosamine from the aglycone and its cleavage to yield product A. This product is not degraded further.

The remaining 14 membered lactone is enzymatically cleaved to produce the anion, product D, which accumulates in the reaction mixture.

The microbial inactivation of this molecule appears to follow a stepwise glycosidic cleavage. This would imply that the enzyme(s) present in Pseudomonad 56 functions as a glycosidase. However, due to the chemical lability of the 14 membered lactone of erythromycin A, this same glycosidase is probably able to function as the a lactonase cleaving the lactone and producing the carboxylic acid.

Figure 40: PROPOSED MECHANISM OF INACTIVATION OF ERYTHROMYCIN A BY PSEUDOMONAD 56

Figure 40


DESOSAMINE
CLADINOSE
1
2
3
PRODUCT C
CLADINOSE
NOT ISOLATED
PRODUCT B
PRODUCT A
PRODUCT D

CONCLUSIONS

The results of this study give strong evidence to suggest that erythromycin A can be inactivated by a bacterial enzyme.

The biological nature of this inactivation has been rigorously demonstrated by the following facts:

1. Pseudomonas 56 is capable of effecting the loss of biologically active antibiotic from the medium under conditions of pH and temperature at which no significant chemical loss of activity occurs.
2. The ability of whole cells or broken cell preparations of Pseudomonas 56 to inactivate erythromycin A is completely lost when these preparations are boiled. This shows that the inactivation agent is heat labile. Simple chemical inactivation would not be affected by treatment of the cell preparation if this type of inactivation was all that occurred.
3. The biological inactivation activity was able to be localized first in the particulate fraction in broken cell preparations and later associated directly with the cell membrane itself. Inactivation by the production of some inactivating chemical agent from the cell is thus eliminated by demonstration of inactivation ability in the absence of biosynthetic capacity.
4. Demonstration that the ability of the cells to inactivate erythromycin A could be increased by the addition of inducers to the growth medium is conclusive evidence of the biological nature of the inactivation. There is no chemical inactivation scheme which could show this induction effect.

The organism which demonstrates this capacity to inactivate erythromyc A has been shown to be unique. The enzyme that inactivates this molecule is perhaps not episomal as erythromycin A was not required in the medium fo maintenance of the inactivating ability of this culture.

The inactivation enzyme appears to be directly associated with the growth of Pseudomonas 56. The discovery of the unusual lipid requirements for growth and that higher levels of this enzyme are induced by the presence of lipid in the medium directly suggest that this enzyme is necessary for the growth of the organism. It is also noted that the production of this enzyme is strictly associated with the growth phase of this culture and that low levels of enzyme are present in the absence of any macrolides

The limited chemical study of the mechanism of inactivation of erythromycin A by Pseudomonas 56 suggests that this inactivating enzyme functions perhaps as some type of glycosidase. In the case of erythromycin A this glycosidase is also able to function as a lactonase. This is perhaps possible because erythromycin A has the most chemically labile lacton ring of all of the 16 membered lactone macrolides. It is this chemical lability that facilitates enzymatic cleavage of the lactone. The substrate specificity studies on various macrolides present evidence that this inactivating enzyme does not have true lactonase activity.

Because of the apparent substrate specificity of this enzyme for erythromycin A it may be of use in the biological assay of blood levels of erythromycin in the presence of other antibiotics. In assaying the blood levels of patients receiving multiple antibiotic therapy, the enzyme from Pseudomonad 56 could be used to selectively remove erythromycin from the sample so that the other antibiotic present could be accurately assayed without interference from erythromycin A.

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