THE BIOLOGICAL ACTIVITY OF TWO

NOVEL 15-AZASTEROIDS

By

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NOVEL 15-AZASTEROIDS

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ii

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I am dedicating this thesis to my wife, Donna, and my sons, Michael and David, whose understanding, encouragement and cooperation have made this goal possible.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

CHAPTER I

INTRODUCTION

The broad spectrum of biological activity associated with steroids and the multiplicity of actions expressed by some individual members has made these compounds one of the most widely studied groups in medicinal chemistry. Structural modification of the cyclopentaphenanthrene nucleus has permitted studies of the structure-action relationship and has led to the discovery of many very useful therapeutic agents.

Active steroid research began in the 1920's with the discovery of large amounts of estrogenic material in the urine of pregnant women (Aschheim, and Zondek, 1927) and a short time later with the isolation of this material (estrone) in a pure state (Doisy, Veler and Thayer, 1929). Numerous efforts were made to elucidate the structure of the new-found compounds but it was not until large quantities of equilenin were prepared in pure form that Cook and Girard (1934) were able to work out the complete steroid structure. The first total synthesis of a naturally occurring steroid was accomplished in 1940 by Bachmann, Cole, and Wilds (1940) using a twelve-stage process for the preparation of equilenin.

The earliest investigations dealing with nitrogen containing steroids involved the pharmacology of various plant alkaloids (Krayer, and Acheson, 1946). Since those early studies, steroids containing nitrogen atoms have been shown to possess a wide range of biological activities (Alauddin, and Martin-Smith, 1962; Martin-Smith, and Sungrue,

1

1964; Singh et al., 1970). Among this group of azasteroids a large

number have been tested for their antibacterial and antifungal activity.

Epimeric-7-amino-cholesterols and 4-aza-cholestane inhibited the growth

of several gram-positive bacteria but had no effect on the growth of

gram-negative organisms (Barnet, Ryman and Smith, 1946a; Smith, Shay and

Doorenbos, 1963). The potency of the synthetic cholesterol derivatives

was enhanced by the formation of the diamino-compound which, in addition,

showed activity against some gram-negative organisms (Barnet, Ryman and

Smith, 1946b).

Antibacterial activity was also found using various mono- and di-

aminocholestane derivatives and several hydroxylated 23-aminonorcholane

compounds (Barnet, Ryman, and Smith, 1946b; James et al., 1946).

Varricchio, Doorenbos and Stevens (1967) reported that 4-dimethyl-

aminoethyl-4-aza-5-cholestan-3-one methiodide nonreversibly inhibited

the growth of Bacillus subtilis and Sarcina lutea but had no effect on

the growth of Escherichia coli.

Irehdiamine, a plant alkaloid, first drew attention as an inhibitor

of bacteriophage growth in Escherichia coli (Harold, 1970). Silver and

Levine (1968) reported that at concentrations around 10^{-4} $\underline{M}$ this steroid

caused a rapid efflux of thiomethyl-galactoside and potassium ions as

well as inhibiting their uptake. These investigators also found that

while the action of irehdiamine on transport could be reversed by

removal of the drug or by addition of magnesium, the loss of cell

viability was apparently irreversible.

Smith and Shay (1965) reported the lysis of protoplasts prepared

from Sarcina lutea by the presence of 4-methyl-4-aza-5-cholestane thus

implicating the cell membrane rather than the cell wall as the probable

site of steroid action. Smith and Shay also showed that removal of or competition with magnesium ions at the membrane surface was not the primary factor in the action of the steroid and that the concentration of steroid necessary to cause lysis was several times that which was required to inhibit the growth of <u>Sarcina lutea</u>. Doorenbos and Bossle (1965), using this same cholestane derivative, substituted methyl groups at the 3 position or the 6 position and found that the antimicrobial activity was increased by a 3-methyl substitution but greatly reduced by the presence of the 6-β-methyl group.

In addition to antimicrobial properties, the nitrogen containing steroids produce an extremely wide range of other biological activities. Various members of this group possess anabolic capabilities, anti-inflammatory activity, and anti-hypercholesterolaemic properties while other azasteroids act as central nervous system depressants or vaso-cilatory agents (Martin-Smith, and Sugrue, 1964). In more recent years the antifertility, antihormonal and anticancer potential of the aza-steroids have been of great interest. A cytostatic effect has been found when 6-aza-3-5-cholestadiene was used to treat malignant tumors (Singh et al., 1970). 25-Azacholesterol has been found to be a cyto-static agent when tested in tissue cultures (Singh et al., 1970).

Only three azasteroids containing a nitrogen atom in the 15 position of the steroid nucleus have been reported in the chemical literature. These compounds, which are derivatives of 4,4-dimethyl-5 -androst-14-ene were first synthesized in the 1930's but apparently have not been studied for biological activity (Langenbeck, and Weissenborn, 1939; Fetizon, and Golfier, 1966).

The <u>de novo</u> synthesis of 1,10,11,11a-tetrahydro-7-methoxy-11a-

methyl-2H-naphth(1,2-g)indol (methoxyimine) and 1,10,11,11a-tetrahydro-11a-methyl-2H-naphth(1,2-g)indol-7-ol (hydroxyimine) was accomplished by Morgan et al. (1972) using the naturally occurring estrogenic hormone, equilenin, as a model for synthesis (Figure 1). Equilenin was chosen as a model compound because of its structural simplicity coupled with its potent biological activity. The steroid normally occurs in horses but has become medically important with its isolation from certain human adrenal carcinoma (Pincus, and Vollmer, 1960).

The methoxyimine and hydroxyimine both contain a nitrogen atom in the 15 position of the "D" ring and differ in having a methoxy or hydroxy group attached to the 3 position of the "A" ring. The methoxyimine has a molecular weight of 265 and a melting point of 181 C while the hydroxyimine has a molecular weight of 249 and a melting point of 288 C (Morgan et al., 1972).

This study was undertaken to characterize the biological activity of a new class of 15-azasteroid analogues. Bacterial and cell culture systems were utilized to investigate the molecular perturbations caused by the test compounds. The investigation is intended to elucidate both the mechanism of azasteroid action as well as the potential for utilizing these new compounds as therapeutic agents and as molecules to aid in the study of various biological phenomenon.

Figure 1. Structures of the Azasteroid Analogues (Methoxyimine and
          Hydroxyimine) and the Naturally Occurring Model Estrogen,
          Equilenin.

METHOXYIMINE

HYDROXYIMINE

EQUILENIN
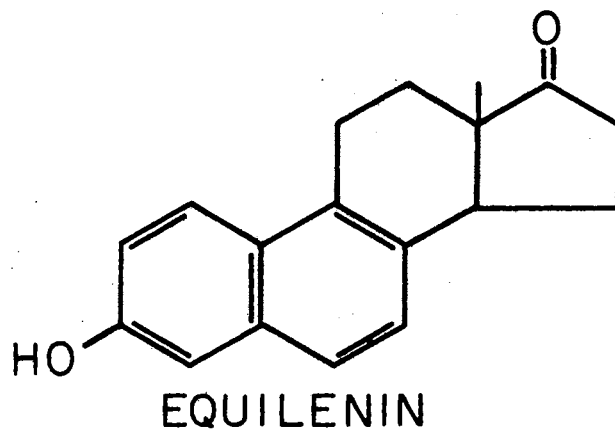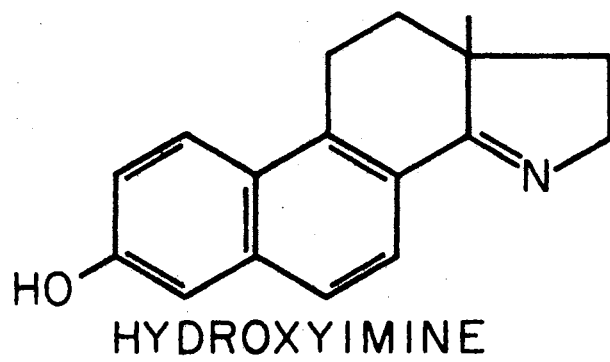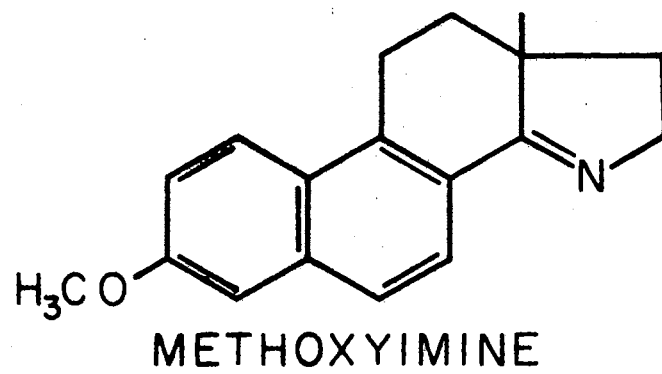
CHAPTER II

MATERIALS AND METHODS

Azasteroid Preparation for Bacterial Testing

The steroids tested during this study were synthesized in the laboratory of Dr. K. D. Berlin, Oklahoma State University (Morgan et al., 1971). The compounds 1,10,11,11a-tetrahydro-11a-methyl-2H-naphth (1,2-g)indol-7-ol (hydroxyimine) and 1,10,11,11a-tetrahydro-2-methoxy-2H-naphth(1,2-g)indol (methoxyimine) were supplied in crystalline form and stored at -5 C in screwtop vials flushed with nitrogen.

The steroids were prepared in stock solution by placing 1 to 3 mg of the crystals in a 10 ml volumetric flask, adding 2 ml of 0.1 N HCl and gently warming the contents until all crystals had dissolved. The solution was cooled to room temperature and filled to volume with glass distilled water. The acidic stock solutions were stored in the dark at 5 C. For experiments, the solution was diluted and neutralized with NaOH just prior to use. Ultraviolet absorption studies indicated the acidic steroid solution was stable for at least two months under these conditions.

Azasteroid Preparation for Cell
Culture Testing

A stock solution of the steroid was prepared as described for bacterial experiments except that the steroid was filtered through a

sterile 0.45 μ millipore HA filter prior to addition to the medium.

Radioisotope Precursors

Glucose-1-^{14}C (specific activity 3.0 mCi/mmole) was obtained from the California Corporation for Biochemical Research. Uracil-2-^{14}C (specific activity 40.6 mCi/mmole) and DL-alanine-1-^{14}C (specific activity 30.0 mCi/mmole) were purchased from Amersham/Searle. DL-Serine-3-^{14}C (specific activity 22.3 mCi/mmole) was obtained from Nuclear-Chicago. DL-Lysine-2-^{14}C (specific activity 10 mCi/mmole), DL-leucine-1-^{14}C (specific activity 20 mCi/mmole) and α-aminoisobutyric-1-^{14}C acid (specific activity 56 mCi/mmole) were purchased from ICN Chemical and Radioisotope Division.

An aqueous solution (5 μCi/ml) was prepared for each radioactive compound. This stock solution was divided into 2 ml aliquots and stored at -5 C until used.

Spectrophotometric Studies

Ultraviolet absorption experiments were performed using a Cary 14 recording spectrophotometer at room temperature (25 C) in 3 ml silicon cuvettes having a light path of 1 cm.

Spectrophotofluorometric Studies

Fluorescent experiments were performed using an Aminco-Bowman model A-8202 scanning spectrophotofluorometer. The circulating water temperature control was utilized in all experiments to maintain a cuvette temperature of 25 C.

Microscopy

Visible light and fluorescent photomicrography were performed on a
Wild M-20 research microscope equipped for dual-illumination of dark-
field, brightfield and phase contrast observations. Illumination was
provided by a 12 volt, 100 watt tungsten lamp and an HBO-200 watt high
pressure mercury burner. Filters used in fluorescent microscopy in-
cluded a GG-13 barrier filter, two UG-1 and one UG-2 exciter filters.

Photographs were taken with a Wild type MEL-13 photoautomat fitted
with a Wild 35 mm film back. Kodak daylight type High Speed Ektachrome
slide film was utilized for all fluorescent photography.

Microorganisms

Bacillus subtilis W23 and Escherichia coli were obtained from the
culture collection of Dr. F. R. Leach, Oklahoma State University.
Bacillus megaterium KM was provided by Dr. M. J. Wolin, University of
Illinois. A species of Flavobacterium and a strain of Pseudomonas,
tentatively identified (Ferguson, 1967) as Pseudomonas fluorescens NND,
were obtained from the culture collection of Dr. N. N. Durham, Oklahoma
State University. The pseudomonad was a gram-negative, motile rod which
forms smooth raised colonies on nutrient agar; negative reaction for
hydrogen sulfide production, indol production, and nitrate reduction;
acid but no gas in glucose; and produces fluorescin and pyocyanin in
Bacto-Pseudomonas agar F and Bacto-Pseudomonas agar P, respectively.

Bacterial stock cultures were maintained on slants of glucose or
succinate minimal agar medium and stored at 4 C.

Bacterial Culture Media

The glucose minimal medium utilized in this study was composed of 0.2 percent NaCl, 0.2 percent $(NH_4)_2SO_4$, 0.32 percent KH_2PO_4, 0.42 percent K_2HPO_4, and 0.2 percent or 0.5 percent glucose as indicated. The medium was prepared by dissolving the NaCl, NH_4SO_4, KH_2PO_4, and K_2HPO_4 in glass distilled water and the pH adjusted to 7.0 using KOH. A 10 percent aqueous stock solution of glucose was prepared in a separate container. All components were sterilized by autoclaving at 121 C with 15 pounds pressure per square inch for 15 minutes and cooled to room temperature (25 C) prior to use. The basal medium was supplemented with 0.1 ml of a minimal salts solution for each 100 ml of medium and glucose added to give the desired concentration. The minimal salts solution was composed of the following: 5.0 g $MgSO_4 \cdot 7H_2O$; 0.1 g $MnSO_4$; 1.0 g $FeCl_3$; and 0.5 g $CaCl_3$ in 100 ml of distilled water. This suspension was sterilized by autoclaving and cooled to room temperature prior to use. Agar (Difco) was added to a final concentration of 2.0 percent in preparing the solid medium.

Succinate minimal medium was composed of 0.2 percent NaCl, 0.2 percent NH_4Cl, 0.32 percent KH_2PO_4, 0.42 percent K_2HPO_4, and 0.2 percent succinate. The medium was adjusted to pH 7.0 using KOH and sterilized by autoclaving. The succinate medium was cooled to room temperature (25 C) and 0.1 ml of sterile mineral salts solution was added for each 100 ml of medium.

Growth Experiments

Glucose or succinate agar slants were inoculated with the appropriate culture and incubated 12-16 hours at 37 C. A portion of the

cells were transferred from the slant using a sterile transfer needle to a 250 ml flask containing 20 ml of glucose or succinate medium. The flasks were incubated 12-16 hours on a reciprocating shaker (100 cycles per minute) at 37 C. Growth studies were performed in tubes (15x150 mm) containing 5 ml of glucose or succinate medium and the test compound or water (control). The tubes were inoculated with cells from the growth flask to an absorbance of 0.05 at 540 nm and the contents adjusted to a final liquid volume of 6 ml. The tubes were incubated at 37 C with constant shaking in trays with a 45 degree incline. Growth determinations were made by measuring the increase in absorbance of the culture at 540 nm using a Coleman Junior II spectrophotometer (18 mm light path). When growth determinations were carried out using 250 ml side arm flasks, the flasks contained 20 ml final liquid volume and were incubated as described for tube studies.

Viable Cell Determinations

Glucose grown B. subtilis cells were inoculated into 20 ml of glucose minimal medium to an absorbance of 0.10. The flasks (250 ml) were incubated at 37 C in a shaking waterbath until the culture attained an absorbance of 0.13. Aliquots (1.0 ml) were removed, diluted in sterile 99 ml saline dilution blanks and plated in duplicate using nutrient agar pour plates. This sample served as an initial control to assure culture uniformity in relation to cell viability. The test compounds (1.0×10^{-4} M) or water (control) were added to the cultures, the absorbance recorded, samples (1.0 ml) removed and viable count made (0, 15, 30, 45, 60, and 120) minutes after steroid addition. The pour plates were allowed to solidify, inverted and incubated at 37 C for 15-18 hours.

Individual colonies were counted using a Quebec colony counter on those plates containing between 30 and 300 colonies per plate.

Protoplast Preparation

Protoplasts were prepared by a modification of the Patch and Landman method (1971). Bacillus subtilis cells were grown 14 hours in glucose minimal medium. The cells were washed and suspended to an absorbance of 0.1 in glucose minimal medium containing 0.6 $\underline{M}$ sucrose and 0.001 m$\underline{M}$ magnesium chloride (PM medium). Lysozyme (Sigma Chemical Co.) was added to a final concentration of 500 μg/ml and the cells were incubated at 25 C. Protoplast formation was judged complete by phase contrast microscopic observations (approximately 1 hour). The proto-plasts were centrifuged at 9,000 x g for 10 minutes, washed in PM medium and suspended in PM medium supplemented with the test compounds (8 x 10^{-5} $\underline{M}$ hydroxyimine and 1 percent v/v Triton X-100 final concentration or water (control).

PM medium containing the test compounds was prepared just prior to use by diluting double strength PM medium with an equal volume of water (control), or aqueous solutions of 1.6 x 10^{-4} $\underline{M}$ hydroxyimine or 2 percent Triton X-100.

Steroid-Antibiotic Potentiation

Studies were made to determine if the antibacterial potential of certain antibiotics could be enhanced by the presence of low concentrations of the steroid. Antibiotics used in this phase of the study were obtained from the following sources: penicillin G potassium (Mann Research Laboratories, Inc.), actinomycin D (Merck, Sharp and Dohme

Research Laboratories), chloramphenicol (Sigma Chemical Co.), vanco-
mycin (Eli Lilly and Co.), circulin (Upjohn Co.), mitomycin C (National
Biochemical Corp.).

A concentrated stock solution of each antibiotic was prepared by
dissolving the crystalline solid in sterile distilled water. The solu-
tions were stored at 5 C until used.

Potentiation experiments were conducted similar to the growth
studies described earlier using three steps. Step one consisted of
determining the maximum concentration of steroid which, when added to a
defined cell mass, produced little or no effect on growth as measured
by a change in absorbance at 540 nm. Step two was to determine a similar
non-inhibitory concentration for the antibiotic to be tested. Step
three was to test the combination of steroid and antibiotic at these
concentrations and determine the resulting growth inhibition when com-
pared to identical cultures containing water, steroid or antibiotic as
controls.

Bacterial Substrate Accumulation and Leakage

Experiments to measure accumulation of radioactive substrates were
carried out by inoculating 250 ml flasks containing 15 ml of minimal
medium, equilibrated to 37 C, with cells from a 12-16 hour liquid cul-
ture. The test compounds, or water, were added to the desired concen-
tration prior to, simultaneously or following addition of the labeled
substrate. The labeled compound (0.2 ml of 5 μCi/ml) along with the
appropriate carrier (0.5 ml of 5 mg/ml) was added to the flasks and
samples removed at the indicated intervals.

Samples (0.5 ml) were removed, placed on 13 mm Millipore filters

(0.45 μ pore size, HA) and washed immediately with 3 ml of minimal medium (37 C). The filters were placed in 25x45 mm counting vials and dried overnight at room temperature (25 C). Aquasol counting cocktail (New England Nuclear) was added to each vial (10 ml per vial) and incubated 4 hours at room temperature. Liquid scintillation counting was accomplished on a Nuclear Chicago Model 720 scintillation counter with a counting efficiency of 40 percent or a Packard Tricarb model 3320 scintillation counter with a counting efficiency of 70 percent for ^{14}C under these conditions.

Experiments to measure leakage of radioactive substrates were conducted similar to the procedures described above except that cells were first labeled 5 minutes with ^{14}C-α-aminoisobutyric acid. In some experiments, the cells were distributed into 5 ml aliquots and diluted with an equal volume of double strength hydroxyimine (3.4 x 10^{-4} $\underline{\underline{M}}$) or 2 percent Triton X-100 (Sigma Chemical Co.). Samples (0.5 ml) were removed and the radioactivity determined as described above.

In other experiments, the 5 ml aliquots were pelleted and suspended in fresh medium containing water, 1.7 x 10^{-4} $\underline{\underline{M}}$ hydroxyimine or 1 percent v/v Triton X-100. Samples were removed and counted as described above.

Cell Culture Lines

The human cell line KB, originally obtained from Dr. L. Vernon Scott, University of Oklahoma Medical School, was carried as a monolayer culture at 37 C in milk dilution bottles using medium 199 (Paul, 1972) supplemented with 10 percent calf serum. Mouse L-M cells were purchased from the American Type Culture Collection Cell Repository and were maintained in continuous culture at 37 C in milk dilution bottles using

McCoy's 5a (Paul, 1972) medium supplemented with 10 percent calf serum. WI-38 Human lung cells were obtained from the American Type Culture Collection Cell Repository. The lung cells were grown in 60x15 mm Falcon culture dishes on coverslips using medium 199 supplemented with 10 percent calf serum.

The rat mammary tumor, R3230AC, was obtained from rats supplied by Dr. K. E. Ebner, Oklahoma State University. The cells were isolated and grown in monolayer as described for WI-38 cells above (Paul, 1972).

Suspension Cultures

Mouse L-M cells were scraped from the stock culture bottles, suspended in fresh McCoy's 5a medium supplemented with 6 percent calf serum and centrifuged at 3000 x g for 5 minutes. The pelleted cells were washed, suspended in the medium and cell numbers were determined using a Coulter Cell Counter model B fitted with a 100 μ orifice.

The growth medium was prepared by diluting double strength McCoy's medium plus 12 percent calf serum with an equal volume of neutralized, filter sterilized steroid solution or sterile water (control). This procedure resulted in a final solution of single strength medium containing the desired steroid concentration (1 to 20 $\mu g/ml$).

Growth flasks (125-ml Erlenmeyer flasks fitted with silicone stoppers) containing 20 ml of the medium plus the steroid or water were inoculated to a final concentration of 1×10^5 cells per ml and incubated at 37 C on a New Brunswick G-10 gyrotory shaker at 50 oscillations per minute.

Growth was followed by removing samples (0.2 ml) from the growth flasks, diluting them in 0.9 percent sodium chloride and determining the

cell numbers on the Coulter counter.

Plating Efficiency

KB cells were scraped from the stock culture bottles, suspended in medium 199 plus 10 percent calf serum and centrifuged at 3000 x g for 5 minutes. The pelleted cells were washed with medium, suspended in fresh medium, and the cell number determined using the Coulter counter.

Double strength medium 199 plus 20 percent calf serum was diluted with the steroid solution or water as previously described to give the desired medium and steroid concentrations. The medium was inoculated with the KB cells to a concentration of 1×10^2 cells per ml, 5 ml was plated in 60x15 mm Falcon plastic culture dishes, and the dishes were incubated at 37 C in a CO_2 gas phase incubator. At the desired times, the medium was removed and replaced with fresh medium (5 ml) containing the test compounds or water as appropriate for the particular type of experiment (attached or nonattached cells) and the incubation continued for a total of 7 days. The medium was removed and the plates were washed with Hanks' salt solution (Paul, 1972). The cells were stained for 1 minute with a 0.5 percent aqueous crystal violet solution, rinsed with water and air dried. Colonies were counted microscopically and the relative plating efficiency determined using the colony count in the control (water) as 100 percent efficiency.

Growth of Cells on Coverslips

KB and L-M cells were grown on coverslips for microscopic examinations. The cells were removed from the stock culture bottles and counted

using a Coulter Counter. Medium 199 plus 10 percent calf serum was utilized for both cell lines to allow concurrent incubation in the same CO_2 gas phase incubator.

Coverslips were sterilized by washing in 50 percent nitric acid for 2 hours. The coverslips were removed from the acid, rinsed in sterile water, washed for 15 minutes in 95 percent ethanol and rinsed a final time in sterile water. One sterile coverslip was placed in each Falcon plastic tissue culture dish (60x15 mm).

The medium was inoculated with cells to a concentration of 8×10^4 L-M cells per ml or 4×10^4 KB cells per ml. The inoculated medium (5 ml) was added to the culture dishes taking care that the coverslips remained on the bottom of the dish. The dishes were incubated at 37 C in a 5 percent CO_2 gas phase incubator for 24 hours to allow the cells to firmly attach to the coverslips.

The steroid-containing medium was prepared as described with double strength medium being diluted with the steroid solution or water. When using coverslip grown cells, a final steroid concentration of 10 µg/ml was routinely used to allow some cell growth during incubation.

The steroid-containing medium (2 ml) was placed in Falcon plastic dishes (35x10 mm). The coverslips were removed from the 60x15 mm dishes and placed in the plates containing the steroid medium. The plates were incubated for 24 hours in the CO_2 incubator. The coverslips were removed from the medium, washed two times in 0.01 $\underline{M}$ Hepes buffered saline (0.8 percent NaCl; 0.04 percent KCl; 0.01 percent $NaHPO_4$; 0.1 percent glucose; and 0.238 percent 4-(2-hydroxyethyl)-1-piperazine-ethanesulfonic acid (Hepes), and either stained or mounted wet for use in different types of microscopic examination.

Cell Culture Substrate Accumulation

and Leakage

L-M cells grown in suspension were centrifuged at 2500 x g for 5
minutes and the pellet suspended in fresh McCoy's 5a medium. A sample
was removed and the cell number determined using the Coulter counter.
The cells were centrifuged and the pellet suspended in fresh medium to
a concentration of 8 x 10^5 cells per ml. The cells were divided into
30 ml aliquots per flask (125 ml). The flasks were stoppered and incu-
bated at 37 C on the gyrotory shaker at 50 oscillations per minute for
4 hours.

The medium from the growth flask was pooled and the cells were
pelleted by centrifugation. The pellet was suspended in an equal volume
of Hanks' salt solution (8 x 10^5 cells per ml) which was prepared Ca^{++}
and Mg^{++} free and the suspension divided placing 30 ml into 125 ml
flasks. The flasks were stoppered and incubated 15 minutes at 37 C to
allow temperature equilibration.

The flasks containing the cells were transferred to a 37 C shaking
waterbath (80 cycles per minute) and incubated for a second 15 minute
period. The radioactive substrate was added to each flask (0.3 ml of
5 μCi/ml) and the incubation continued. The test compounds, sodium
azide (Eastman Kodak Co.) or water, were added in the desired concentra-
tion prior to, simultaneously or following addition of the labeled
substrate.

Samples (2 ml) were removed from the test flasks at the indicated
times, placed on Millipore filters (47 mm, 0.45 μ, HA) and washed with
40 ml of Hanks' salt solution. The filters were placed in counting
vials, allowed to dry at room temperature, dissolved in 10 ml of Aquasol

counting cocktail and counted as previously described.

Leakage of metabolic precursors was measured similar to the experiments described above except that cells were labeled with ^{14}C-α-aminoisobutyric acid for 40 minutes. The cells were pooled, distributed into 30 ml aliquots and pelleted at 2500 x g for 10 minutes. The pellets were suspended in 15 ml of McCoy's medium containing water (control), 8 x 10^{-5} $\underline{M}$ hydroxyimine or 1 percent v/v Triton X-100. Samples were removed at various times, washed with Hanks' solution and counted as described above.

Preparation of L-M Cell Membranes

Mouse L-M cells were grown at 37 C in a spinner flask (Bellco Glass, Inc.) containing one liter of McCoy's medium plus 6 percent calf serum to a density of 7 x 10^5 cells per ml. Membranes were prepared by a modification of the "Tris Method" (Warren, Glick and Nass, 1966). The cell pellet (7 x 10^8 cells) was suspended in 20 ml of 0.05 $\underline{M}$ Tris-Cl buffer (pH 7.4) containing 2 ml of 0.05 $\underline{M}$ MgCl$_2$. The cells were pelleted by centrifugation at 1900 x g at 5 C for 10 minutes in a Sorvall RC-2B using an SS-34 rotor. The pellet was suspended in 10 ml of Tris-Cl and MgCl$_2$ buffer and allowed to stand for 5 minutes at 3 C. The suspension was placed in a 15 ml Dounce homogenizer tube (Kontes Glass Co.) and the membranes stripped from the cells with 10 strokes of type "B" pestle. The tube contents were suspended in an equal volume (10 ml) of 20 percent sucrose and layered (10 ml per tube) on 20 ml of 30 percent sucrose layered over 10 ml of 50 percent sucrose in a 50 ml tube. The tubes were centrifuged in an HB-4 rotor at 1600 x g for 20 minutes and the membranes which are located in the 30 percent fraction were harvested.

The harvested membrane fraction was pelleted in an SS-34 rotor at 5900 x g for 20 minutes. The pellet was suspended in 2 ml of 30 percent sucrose and layered on a 37 ml gradient consisting of 65, 55, 45, and 40 percent sucrose. The gradient was centrifuged in a Spinco SW-27 rotor at 90,000 x g for 2 hours and the membranes were harvested from the 40 and 45 percent bands. The sucrose was removed by dialysis at 5 C against 5 l of distilled water for 24 hours. The membranes were pelleted, washed with water and stored at 5 C until use.

Determination of Hydroxyimine
Estrogenic Activity

Mature random bred female mice (ICR-Swiss) were purchased from the Texas Inbred Mouse Co. The mice were used when they reached 70 ± 5 days of age and had an average body weight of 27 ± 2 g. The animals were housed in groups of 10 per cage in a light (14 hours light-10 hours darkness per 24 hours) and temperature (21 ± 1 C) controlled room and provided with food (Ralston Purina Laboratory Chow) and water ad libitum. All mice were ovariectomized 14 days prior to making the initial injections.

The hydroxyimine and 17β-estradiol (Sigma Chemical Co.) were dissolved in sesame oil (Fisher Chemical Co.) and stored at 5 C until just prior to use. Animals were weighed and then injected with 0.1 ml of the sesame oil solution subcutaneously once daily for 7 days. The complete injection series consisted of 5.1×10^{-9} $\underline{M}$ estradiol (estrogenic control), 1.6×10^{-9} $\underline{M}$ or 8.4×10^{-9} $\underline{M}$ hydroxyimine, sesame oil (carrier control) or no injection (ovariectomized control). The doses of hydroxyimine employed represent 3 or 15 times higher amounts than

required by estradiol to elicit a maximal response in terms of uterine weight by the assay procedure (Zarrow, Yochim and McCarthy, 1964).

All animals were weighed and then killed by cervical dislocation 24 hours after the last injection. The uteri were removed, trimmed of adhering tissue, blotted and weighed as soon as possible after removal from the body cavity. Uteri were then placed on tared planchets and dried at 110 C for 24 hours. The planchets were placed in a desiccator for 24 hours and weighed again. Other tissues (lungs, liver, spleen, intestines, kidney and mammary glands) were inspected for gross systemic lesions. Behavioral observations of the animals were made daily at the time of injection.

CHAPTER III

RESULTS AND DISCUSSION

Physical Data

Azasteroid Ultraviolet Absorption Spectra

and Standard Curves

Experiments were conducted to determine the physical properties of the new steroid molecules and to determine those properties which might aid in detection of the molecules during biological testing. The ultraviolet absorption spectrum was examined using the Cary 14 recording spectrophotometer at room temperature for both the methoxy- and hydroxyimines (Figures 2, 3) utilizing steroid suspended in minimal medium (pH 7.0).

Both compounds have a strong absorption peak at 260 nm and a shoulder at 280 nm. A second absorption peak was found at 334 nm and a prominent shoulder near 365 nm. Although there is a slight difference in relative peak intensities, the general shape and distribution of the spectra was indicative of the steroids close structural relationship.

To facilitate steroid quantitation, standard curves were constructed from the spectral data obtained above for the two major steroid adsorption peaks (260 nm and 334 nm) (Figure 4). A linear relationship was obtained for both steroid peaks within the concentration range used (7.6 x 10^{-6} $\underline{M}$ to 6.4 x 10^{-5} $\underline{M}$).

Figure 2. Ultraviolet Absorption Spectrum of the Hydroxyimine (3.2 x
          10^{-5} $\underline{M}$) Suspended in Minimal Medium at pH 7.0.
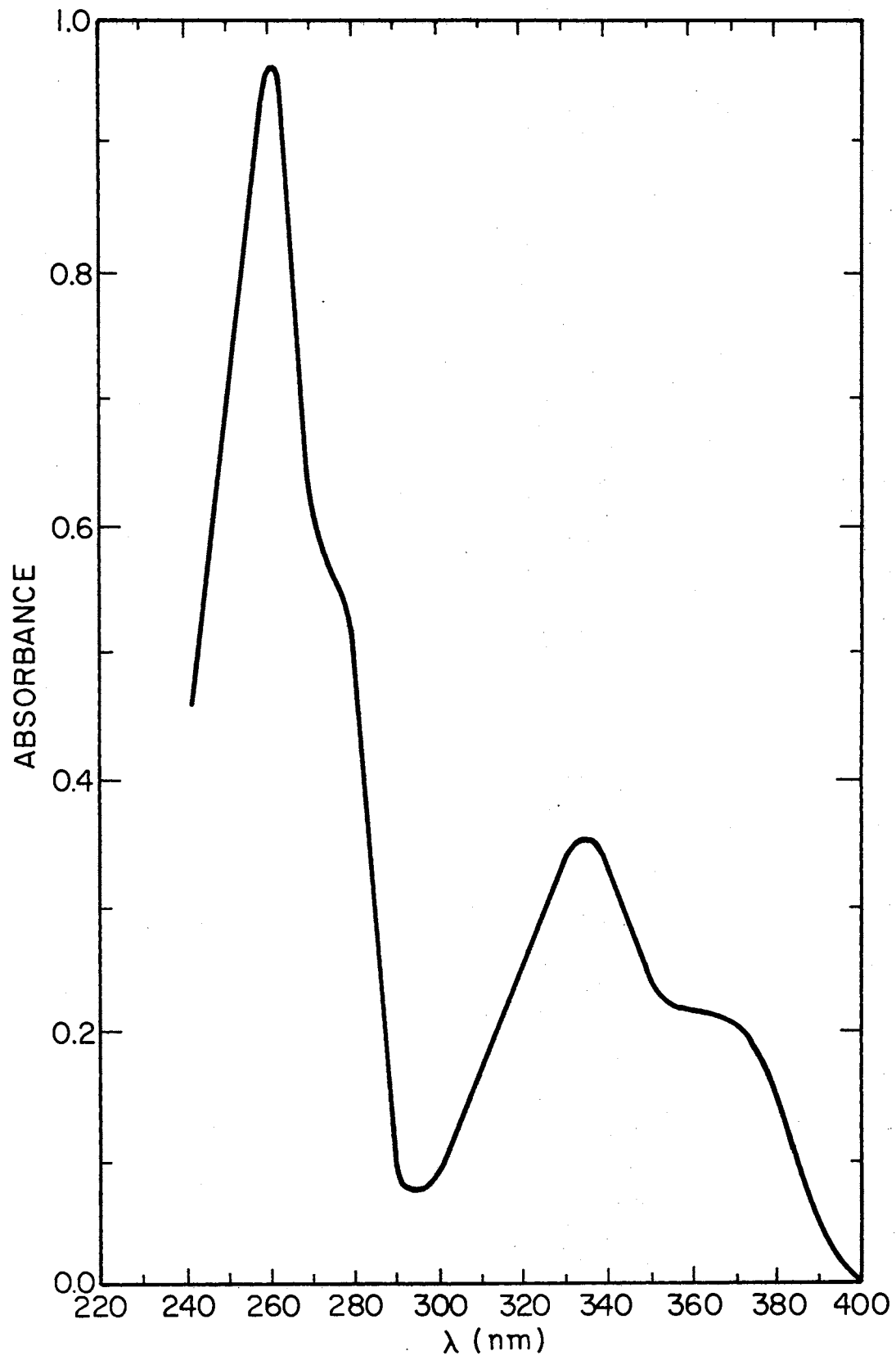
Figure 3. Ultraviolet Absorption Spectrum of the Methoxyimine (3.0 x
           10^{-5} $\underline{M}$) Suspended in Minimal Medium at pH 7.0.
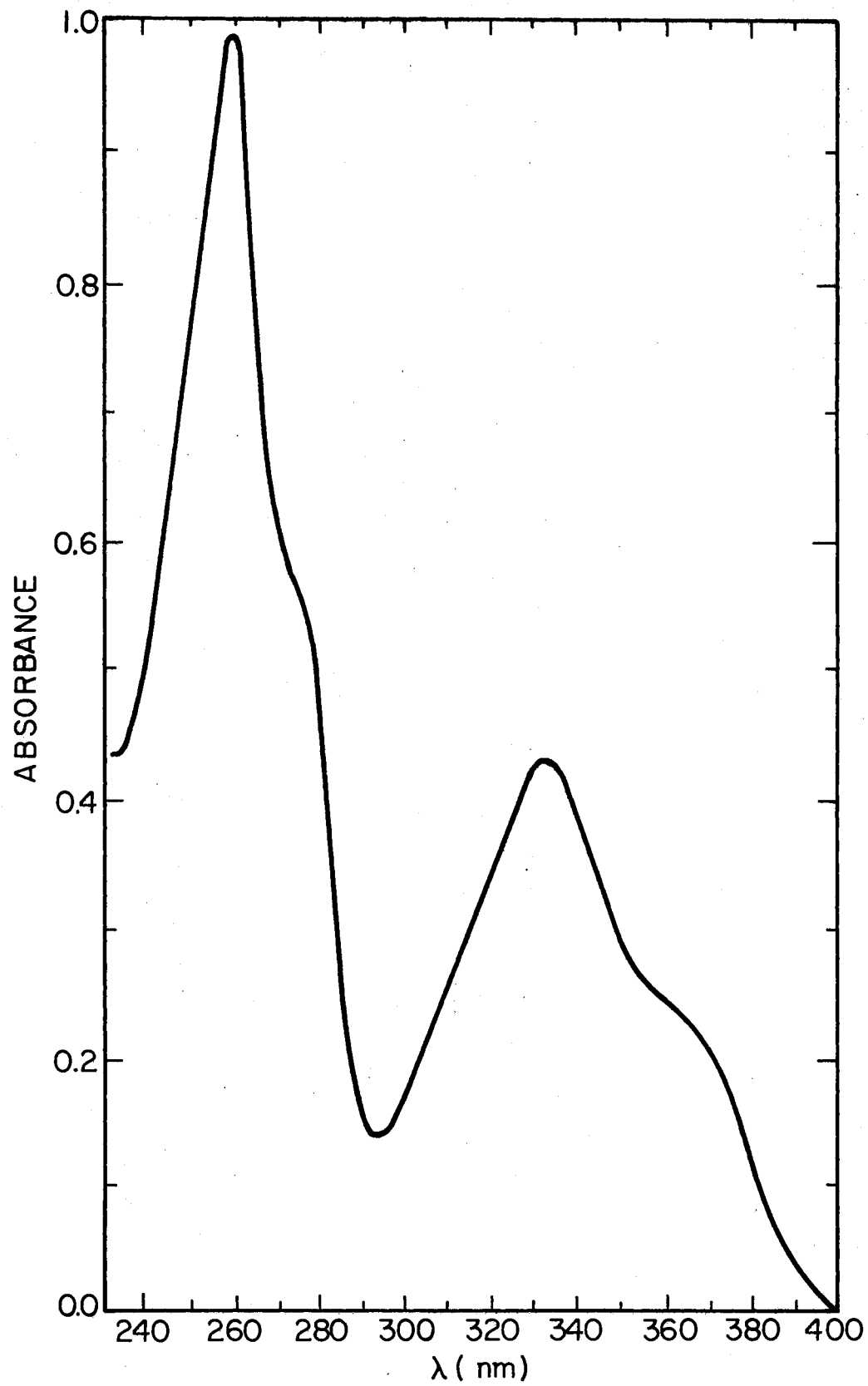
Figure 4. Standard Curves for Hydroxyimine and Methoxyimine Absorption
           at 260 nm and 334 nm.
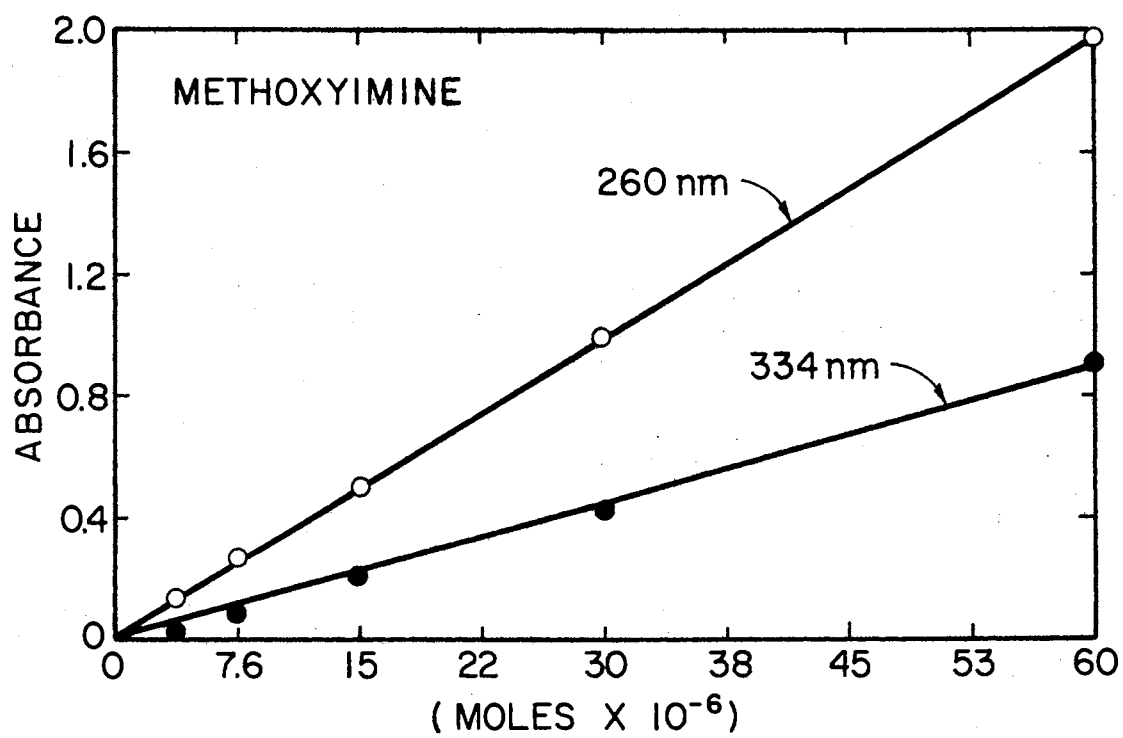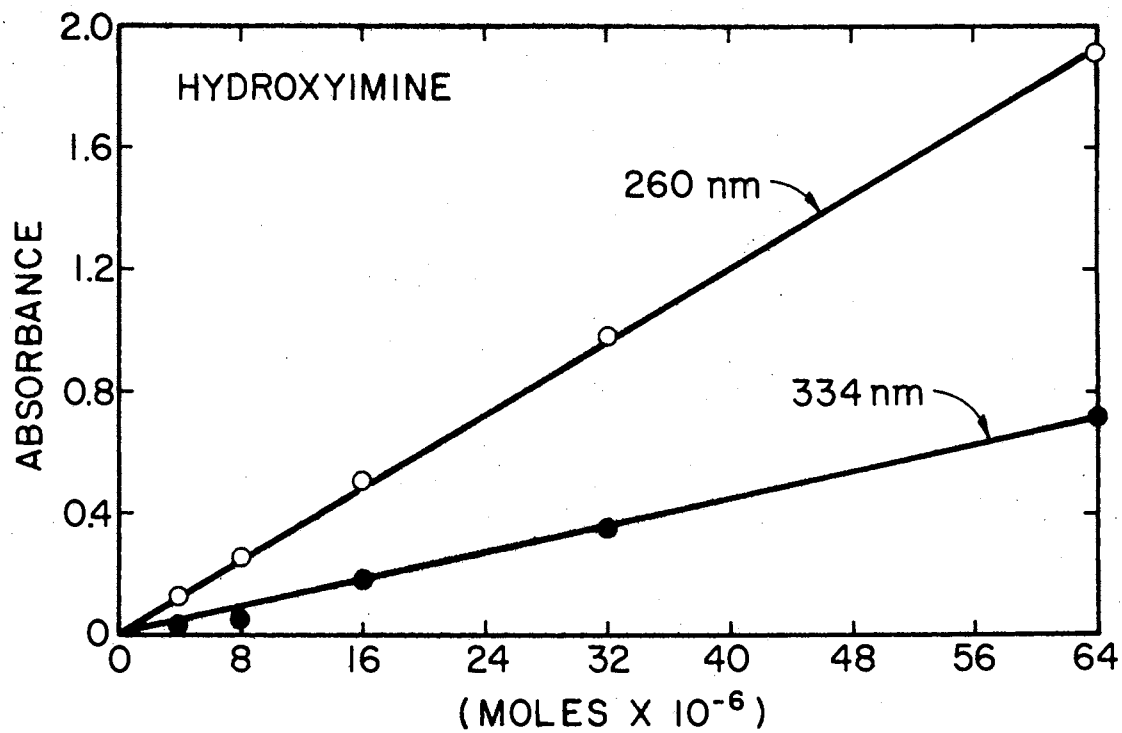
Hydroxyimine Fluorescent Properties

To determine the fluorescent properties of the hydroxyimine the steroid was suspended in various concentrations of dioxane and water. This condition allowed observations of spectral changes as the steroids environment was altered from a very polar solvent (water) to a nonpolar solvent (dioxane). Figure 5 shows the results plotted as the wavelength of maximum excitation and emission against the percent dioxane in water along with the relative quantum yield in percent.

From these results it appeared that both the wavelength of emitted light and the intensity of the emitted light were dependent upon the solvent polarity.

Because of these properties, a characterization of the fluorescence of the steroids when associated with biological samples may reveal evidence concerning polarity at the site of azasteroid/cell interaction.

Edelman and McClure (1968) utilized the dioxane and water technique to characterize the fluorescent properties of 2-p-toluidinylnaphthalene-6-sulfonate (2,6-TNS) and showed that this fluorescent compound was sensitive to changes in the solvent polarity. These experiments allowed an estimation of the microenvironmental polarity at the points of interaction between the fluorescent probe (2-6-TNS) and a test compound (cell protein).

Hydroxyimine Standard Fluorescence Curve

A determination of the fluorescence at various concentrations of the hydroxyimine was performed to facilitate steroid quantitation.

Solutions of the hydroxyimine were prepared in minimal medium minus the carbon source to final concentrations of 3.8×10^{-7} $\underline{M}$, 7.1×10^{-7} $\underline{M}$,

Figure 5. Fluorescent Properties of the Hydroxyimine (4×10^{-6} $\underline{M}$) in Water and/or Dioxane. ●, wavelength of maximum emission; ■, wavelength of maximum excitation; ▲, relative quantum yield at wavelength of maximum emission.
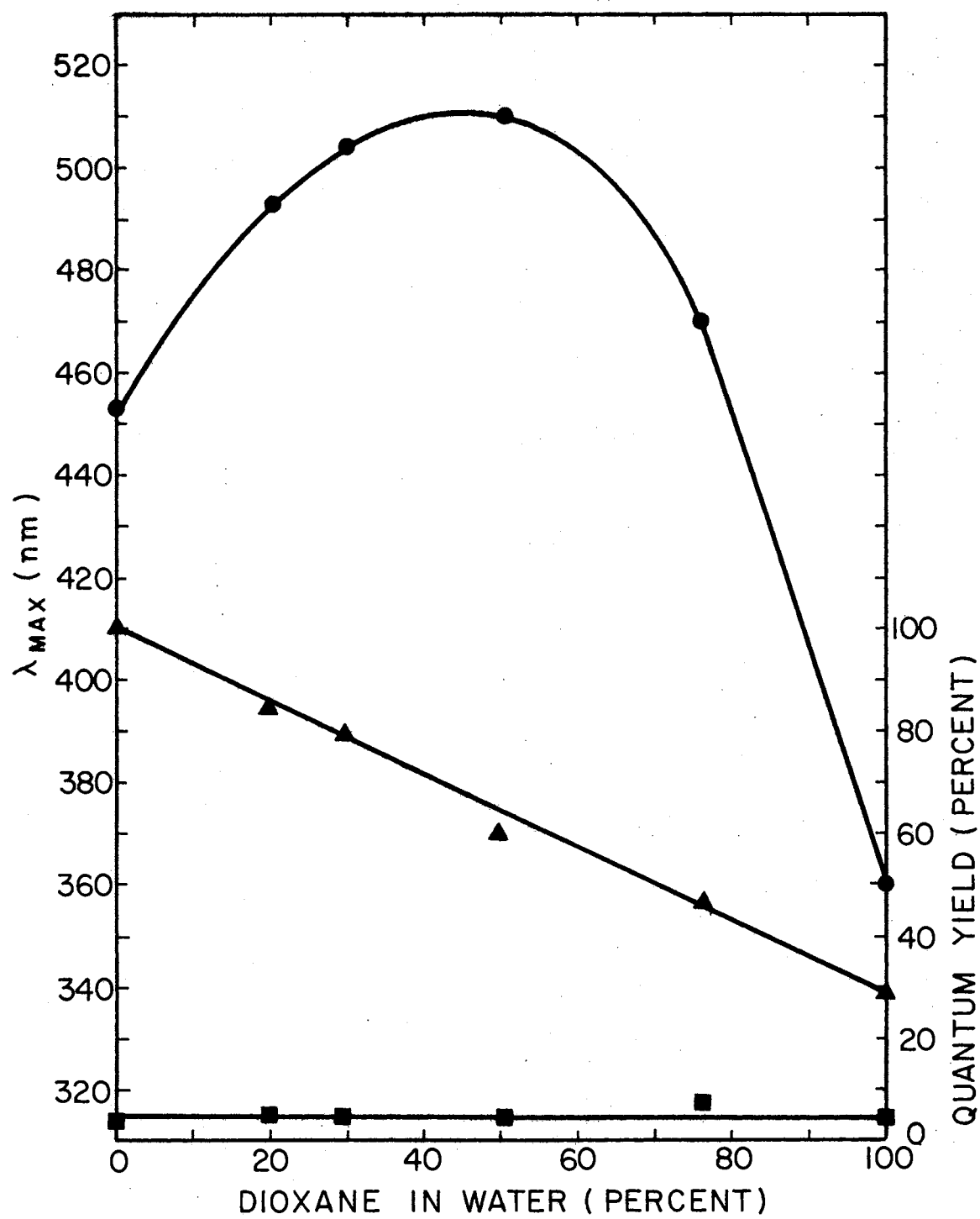
2.2×10^{-6} $\underline{M}$ and 5.7×10^{-6} $\underline{M}$. The percent emitted was determined on an Aminco-Bowman scanning spectrophotofluorometer at sensitivity 25, slit arrangement 3 and a photomultiplier setting of 1.0. The results (Figure 6) are shown as the percent transmittance of the emission peak at 456 nm when excited at 312 nm. A linear relationship was obtained within the concentration range tested.

<div align="center">Microbiological Studies</div>

Azasteroid Inhibition of Bacterial Growth

The possible antibacterial activity of the steroids was determined by adding the test compounds to cultures of $\underline{B}$. $\underline{subtilis}$ W23, $\underline{S}$. $\underline{aureus}$, $\underline{B}$. $\underline{megaterium}$ KM, $\underline{E}$. $\underline{coli}$, a species of $\underline{Flavorbacterium}$, and P. $\underline{fluorescens}$ NND. The growth response was followed by measuring the change in absorbance at 540 nm.

The growth of $\underline{B}$. $\underline{subtilis}$, $\underline{B}$. $\underline{megaterium}$ and $\underline{S}$. $\underline{aureus}$ was substantially reduced in the presence of the hydroxyimine and the methoxyimine. Figure 7 shows the results obtained using both control and steroid containing cultures of $\underline{B}$. $\underline{subtilis}$. Hydroxyimine (1.6×10^{-5} $\underline{M}$) caused only a slight decrease in growth rate while a 10 fold steroid concentration increase (1.7×10^{-4} $\underline{M}$) completely inhibited cell growth throughout the 11 hours of incubation. The methoxyimine, added to the $\underline{B}$. $\underline{subtilis}$ suspension at 1.9×10^{-5} $\underline{M}$ and at 1.9×10^{-4} $\underline{M}$ final concentration, caused virtually an identical inhibition in growth when compared to the response produced by the hydroxyimine. The growth of $\underline{B}$. $\underline{megaterium}$ and $\underline{S}$. $\underline{aureus}$ was inhibited by the hydroxyimine or the methoxyimine and results were similar to those obtained with $\underline{B}$. $\underline{subtilis}$.

The gram-negative microorganisms, $\underline{E}$. $\underline{coli}$, $\underline{Flavobacterium}$ sp. and

Figure 6. Standard Fluorescence Curve for the Hydroxyimine Determined
at 456 nm When Excited at 312 nm.

PERCENT TRANSMITTANCE vs. HYDROXYIMINE (MOLES X 10^{-6})
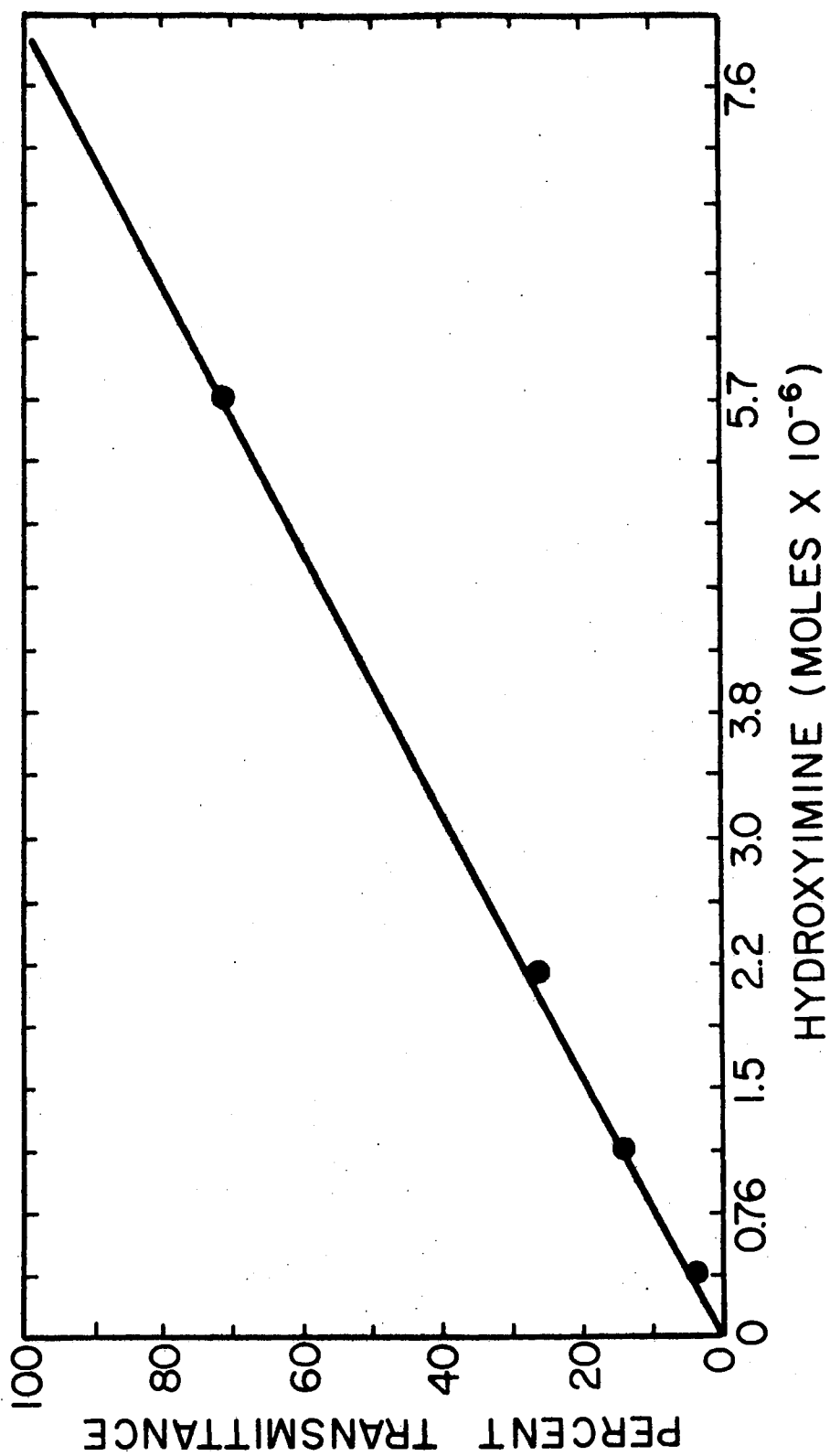
Figure 7. Effect of the Hydroxyimine on the Growth of _Bacillus_ _subtilis_.
▲, control; ■, 1.6 x 10^{-5} $\underline{\underline{M}}$ hydroxyimine; ●, 1.7 x 10^{-4} $\underline{\underline{M}}$
hydroxyimine.
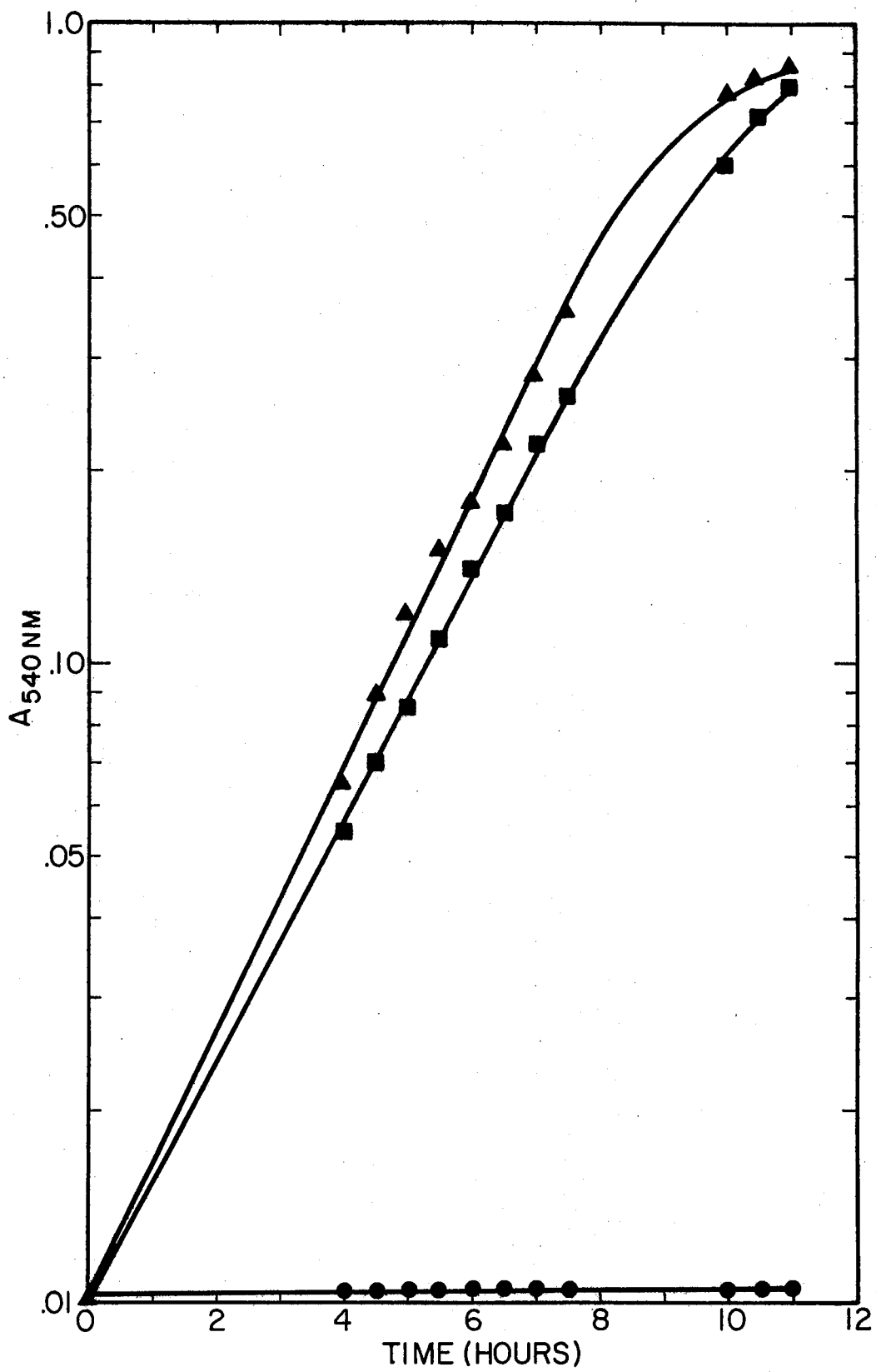
P. fluorescens, were less sensitive to the azasteroid.

E. coli incubated in 1.9 x 10^{-5} M methoxyimine (Figure 8) showed no growth inhibition while a 10 fold increase in methoxyimine concentration (1.9 x 10^{-4} M) produced a marked reduction in growth response.

Similar experiments containing 1.7 x 10^{-5} M and 1.7 x 10^{-4} M hydroxyimine produced very similar growth inhibition responses as did experiments utilizing Flavobacterium sp. when tested against both aza-steroids.

P. fluorescens was the least sensitive bacterium tested. Hydroxy-imine (1.7 x 10^{-4} M) caused only a small decrease in the growth rate (Figure 9) as did 1.9 x 10^{-4} M methoxyimine. The low sensitivity of the pseudomonad might be expected because this group of organisms is typically resistant to many inhibitor molecules perhaps in part a result of the complex composition of the outer wall.

Similar observations were made by Smith, Shay and Doorenbos (1963) who first noted that 4-aza-cholestanes inhibited the growth of various gram-positive bacteria although the growth of gram-negative organisms was unaffected. Varricchio, Doorenbos and Stevens (1966) showed that several nitrogen containing cholesterol derivatives completely inhibited the growth of B. subtilis and Sarcina lutea for up to 8 hours while the growth of E. coli was unaffected.

Reversal of Hydroxyimine Induced Growth

Inhibition

The hydroxyimine induced B. subtilis growth inhibition was tested for reversibility following removal of the test compound from the growth medium. B. subtilis cells were suspended in glucose minimal medium

Figure 8. Effect of the Methoxyimine on the Growth of <u>Escherichia</u> <u>coli</u>.
▲, control; ■, 1.9 x 10^{-5} $\underline{M}$ methoxyimine; ●, 1.9 x
10^{-4} $\underline{M}$ methoxyimine.
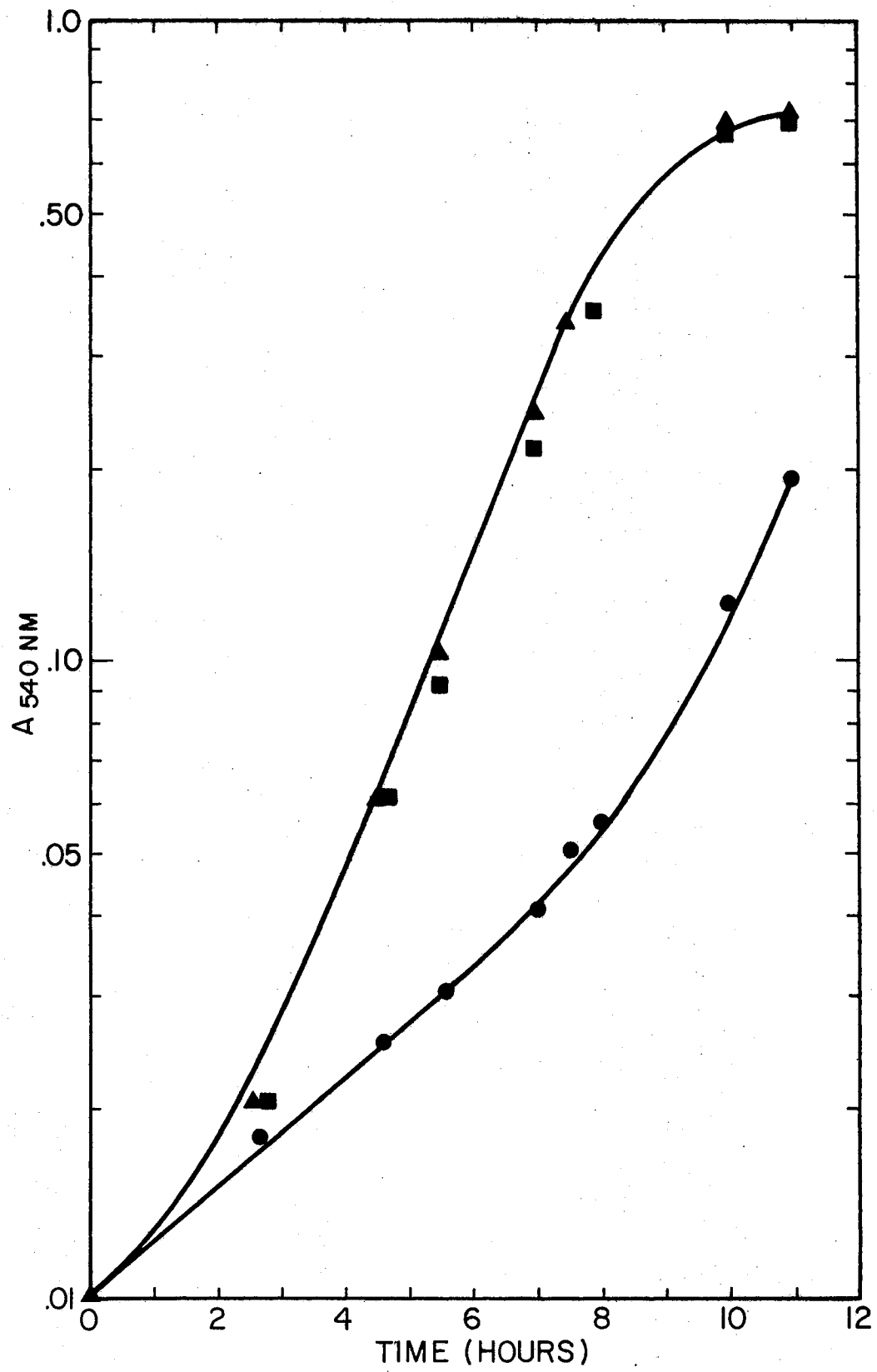
Figure 9. Effect of the Hydroxyimine on the Growth of Pseudomonas fluorescens. ▲, control; ■, 1.6 x 10^{-5} $\underline{M}$ hydroxyimine; ●, 1.7 x 10^{-4} $\underline{M}$ hydroxyimine.
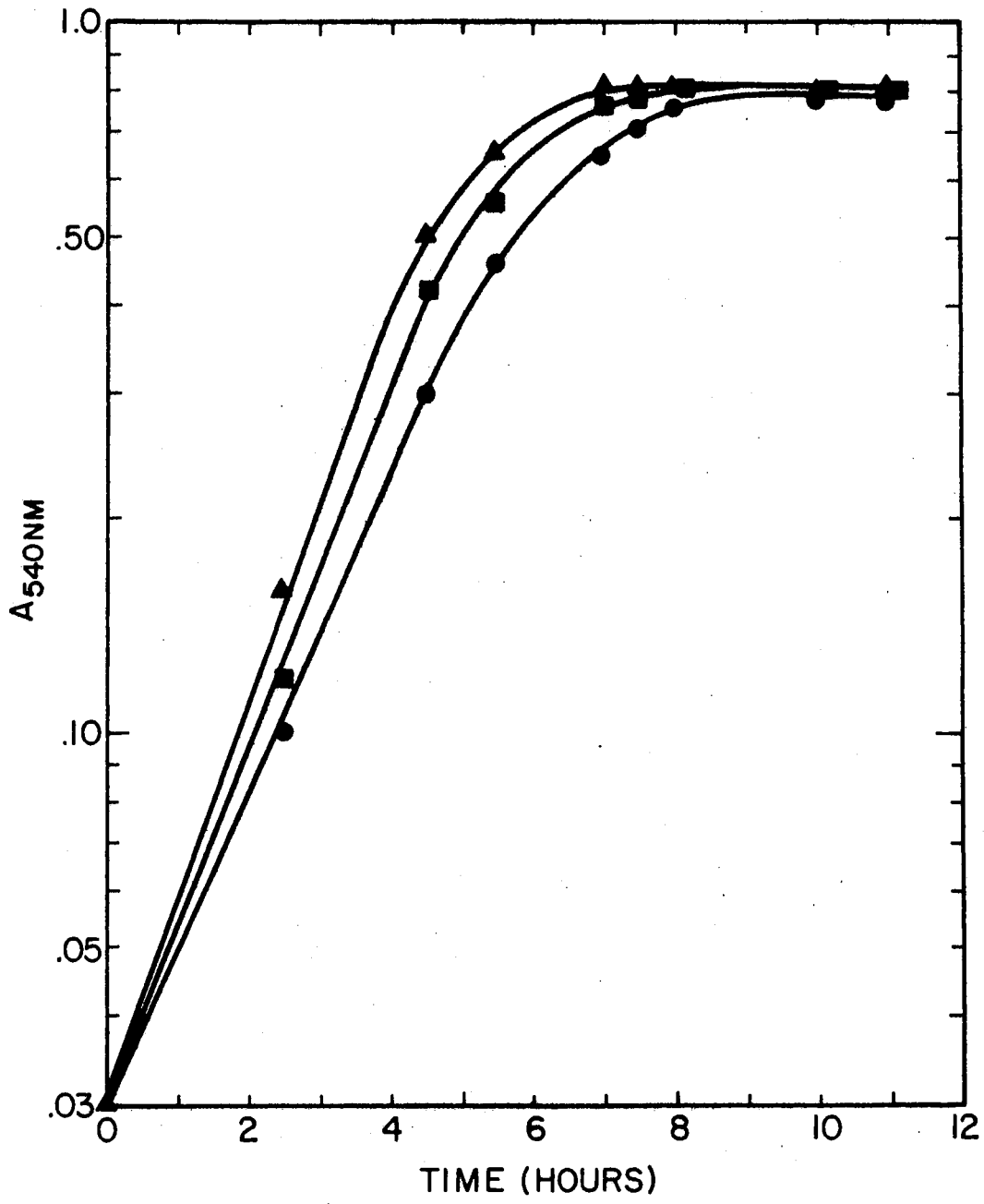
containing water (control) or 1.7×10^{-4} $\underline{M}$ hydroxyimine and incubated

under growth conditions for 2.5 hours. The cells were pelleted, washed

with medium and suspended to the same absorbance in fresh medium sup-

plemented with water or hydroxyimine, incubated and growth measured.

The results (Figure 10) show that control cells suspended in medium

lacking the hydroxyimine quickly resumed growth and reached stationary

phase at about 10 hours. In the reversal system, where steroid treated

cells were suspended in medium lacking the test compound, growth was

initiated only after approximately an 8 hour lag period. Once initiated,

growth of cells in the reversal system proceeded at a slightly reduced

growth rate although the culture did attain the maximum cell growth as

noted in the control. In the control and azasteroid treated cells

suspended in medium containing 1.7×10^{-4} $\underline{\underline{M}}$ hydroxyimine, the growth

inhibition was complete for 20 to 25 hours. Following this long lag

period, both cultures initiated growth and the hydroxyimine inhibited

culture showed a more rapid growth rate than the treated control.

These results indicate that the hydroxyimine induced $\underline{B}$. $\underline{subtilis}$

growth inhibition is reversible following removal of the steroid from

the growth medium although the growth rate is slightly reduced.

Effect of the Azasteroids on Cell Viability

The effect of methoxy- and hydroxyimine on the growth and viability

of $\underline{B}$. $\underline{subtilis}$ W23 was determined. The test compounds (1×10^{-4} $\underline{\underline{M}}$) were

added to actively growing cultures, samples were removed, diluted,

plated in nutrient agar and the colonies counted (Figure 11).

While the control cells steadily increased in numbers, the viable

Figure 10. Reversal of Hydroxyimine Induced <u>Bacillus subtilis</u> Growth Inhibition. △, control; ○, 1.7 x 10^{-4} $\underline{M}$ hydroxyimine; ▲, control suspended in medium lacking steroid; ●, steroid treated suspended in medium lacking steroid; ■, steroid treated suspended in medium plus 1.7 x 10^{-4} $\underline{M}$ hydroxyimine; ●, control suspended in medium plus 1.7 x 10^{-4} $\underline{M}$ hydroxyimine.
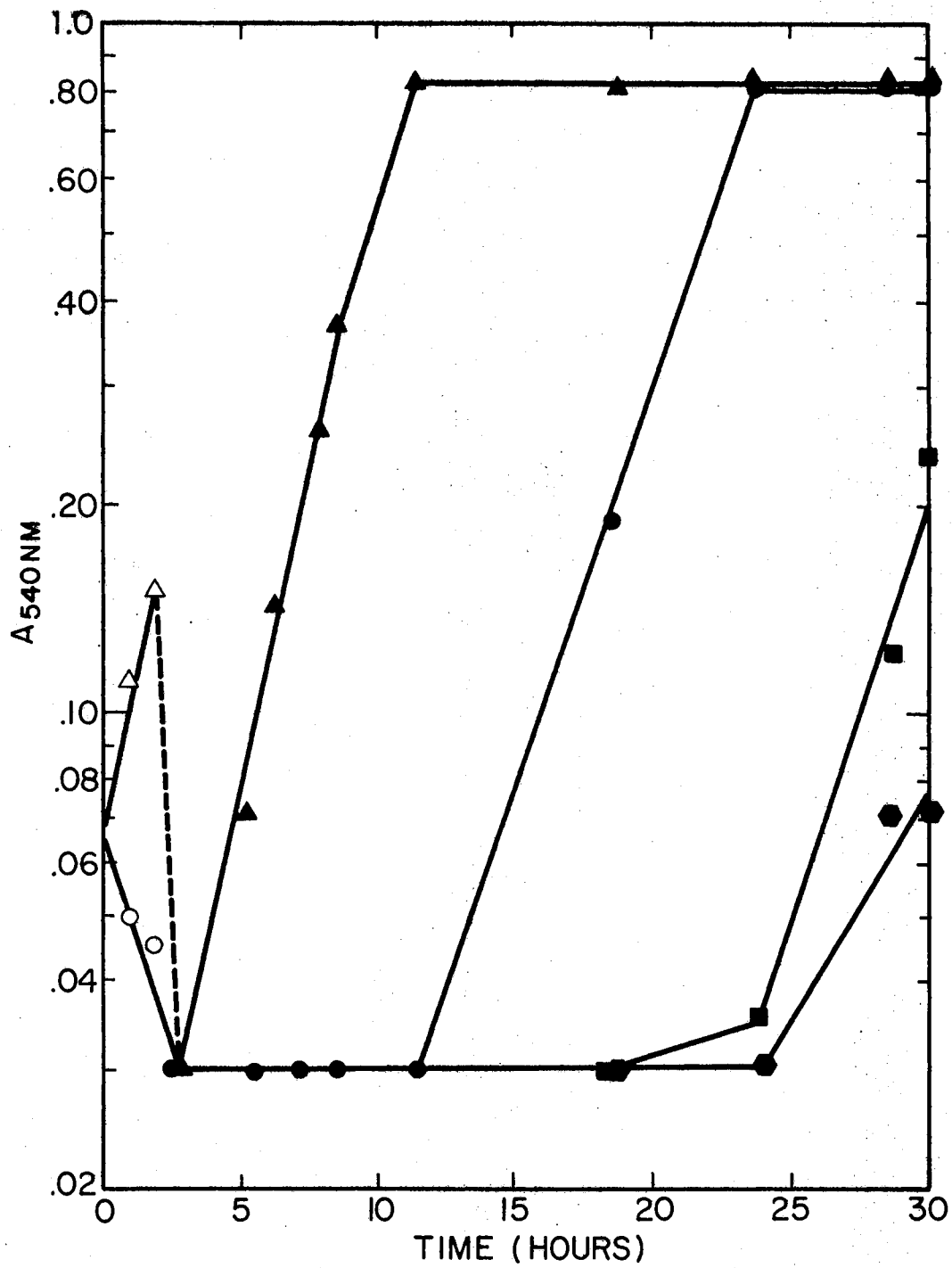
Figure 11. Effect of the Methoxyimine on <u>Bacillus</u> <u>subtilis</u> Cell
Viability. Viable cell numbers: ▲, control; ●,
1.0×10^{-4} $\underline{M}$ methoxyimine. Culture absorbance at
540 nm: △, control; ○, 1.0×10^{-4} $\underline{M}$ methoxyimine.
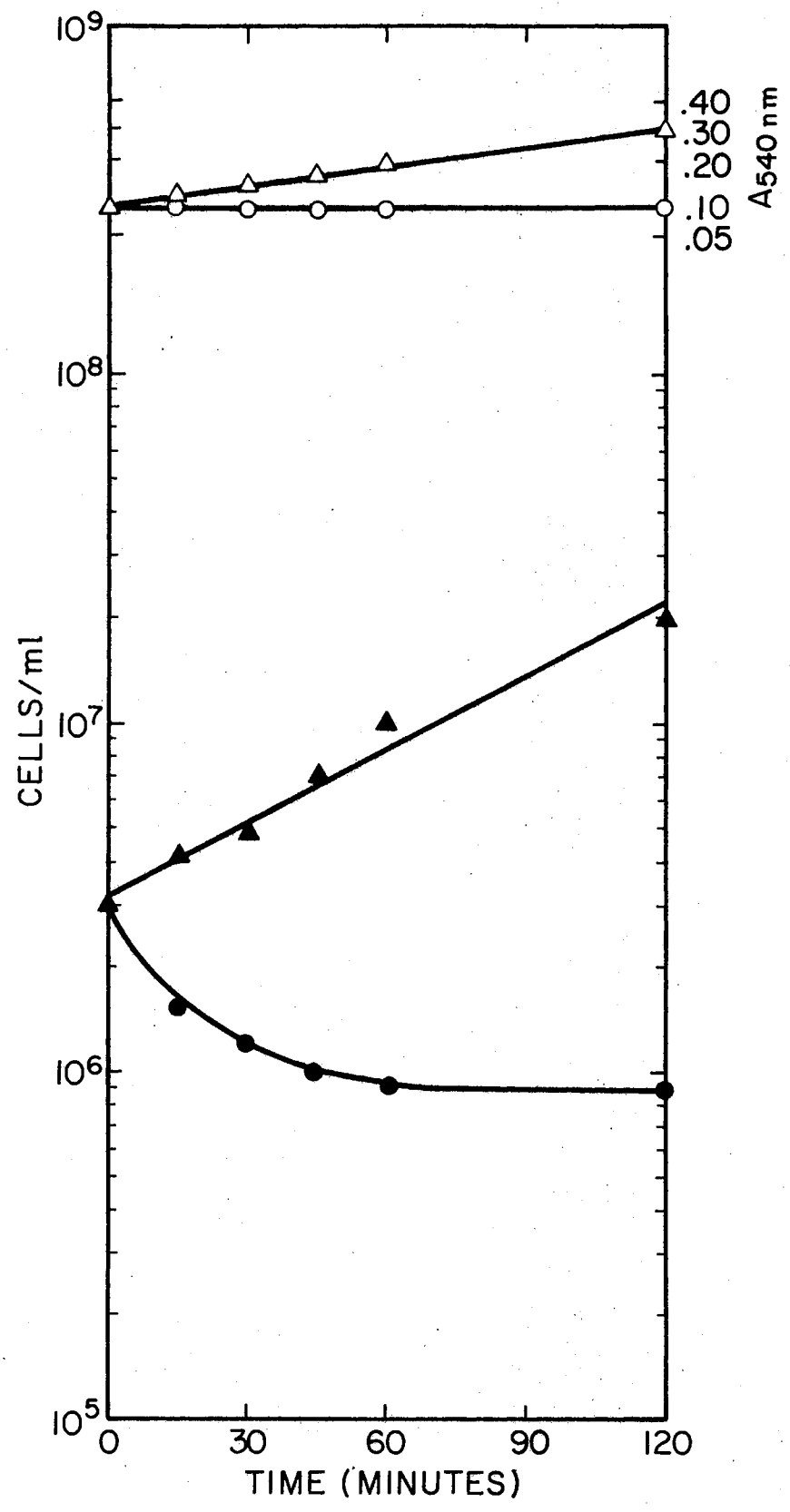
count in the methoxyimine treated culture showed an immediate decline.
The viable count in the steroid-containing cultures continued to drop
for about 45 minutes then reached a plateau level and remained rela-
tively constant throughout the additional 75 minutes of incubation.
Similar results were obtained with the hydroxyimine.

Although addition of the methoxy- or hydroxyimine did cause an
initial decrease in the number of viable organisms, these results indi-
cate that at the concentrations tested the compounds are not bacterio-
cidal since a large number of microorganisms did remain viable.

Those cells remaining viable after 2 hours of incubation in the
presence of the steroid could represent a naturally occurring portion
of the original cell population (before steroid treatment) which was
less sensitive to steroid action. To test this hypothesis, the cells
incubated for 2 hours in water (control) or azasteroid as described
above were pelleted, washed (medium) and incubated in fresh medium for
12 hours. The organisms were then suspended in medium containing water
(control) or the test compounds and the viable cell numbers determined
as described above. The results were virtually the same as those shown
in Figure 11 indicating that no steroid-resistant cell population pre-
dominated in the surviving culture.

Azasteroid Stability Under Growth Conditions

An important aspect in determining the biological influence of a
compound is to measure its stability within the experimental environ-
ment. To learn more about the effects of medium and incubation condi-
tions on the test steroids, the stability of the hydroxyimine and
methoxyimine was determined by both ultraviolet absorption spectroscopy

and biological growth assay.

When the hydroxyimine was incubated in glucose minimal medium
under growth conditions and the steroid concentration measured spectro-
photometrically, the half life of the molecule was slightly greater than
1 hour. The apparent breakdown of the molecule continued and by 13
hours of incubation only 12 percent of the original steroid concentra-
tion remained in the medium (Table I). The apparent non-linear decom-
position following 4 hours of incubation is thought to be the result of
limiting spectrophotometer sensitivity. Absorption data for the methoxy-
imine obtained under identical conditions gave similar results.

To compliment the spectrophotometric studies, a biological assay
was utilized in which B. subtilis, the most steroid sensitive micro-
organism tested, was inoculated into: (1) medium containing the steroid
which had been incubated for 13 hours, (2) fresh steroid containing
medium and (3) incubated medium plus "fresh" unincubated steroid.
Figure 12 shows the results of tests using the hydroxyimine. While the
steroid medium incubated for 13 hours does cause some growth inhibition,
its effect is very moderate. In contrast, a complete inhibition of
growth was produced by the fresh steroid and confirms the decomposition
of the hydroxyimine during incubation under defined experimental con-
ditions.

Bio-assays utilizing the methoxyimine revealed a similar inhibition
pattern indicating that both test compounds have a rather short life
span under the defined growth conditions of this study.

Under these conditions, the action of the unassociated steroid
molecule must occur within the first few minutes of incubation. The
tests, as performed, reveal only the stability of the azasteroids in

TABLE I

HYDROXYIMINE STABILITY TESTED UNDER BACTERIAL GROWTH CONDITION

Incubation Time (Hours)	Hydroxyimine Concentration Present Following Incubation at 37 C Shaking in Glucose Minimal Medium*	
	Hydroxyimine Concentration Moles	Hydroxyimine Concentration Percent
0	2.3×10^{-4}	100
1	1.3×10^{-4}	57.9
2	5.2×10^{-5}	22.8
4	3.3×10^{-5}	14.5
13	2.8×10^{-5}	12.4

*Steroid concentration determined by ultraviolet absorption spectroscopy

Figure 12. The Inhibition of _Bacillus subtilis_ Growth by the Hydroxy-
imine Following 13 Hours of Steroid Incubation in Cell
Free Glucose Minimal Medium. ▲, incubated medium con-
trol; ■, incubated hydroxyimine (2.3×10^{-4} $\underline{M}$) medium;
●, incubated medium supplemented with unincubated
(2.3×10^{-4} $\underline{M}$) hydroxyimine.
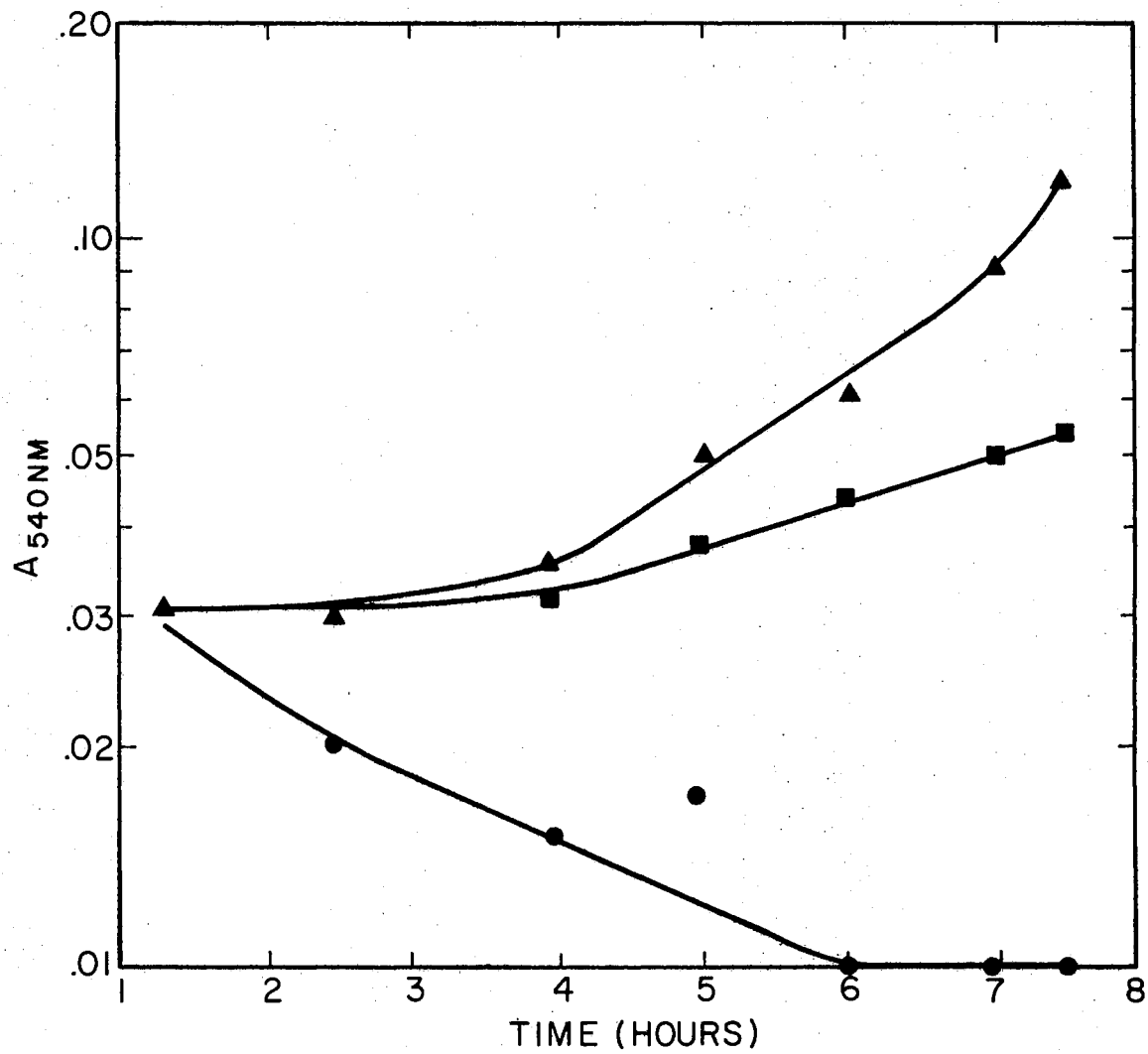
solution. Bound or intracytoplasmic steroid molecules may remain struc-
turally stable and therefore biologically potent for much longer periods
of time.

Biological assessment of azasteroid stability in the cell culture
system revealed that incubation of the test compound in cell free
medium (McCoy's 5a) for up to 7 days under growth conditions did not
alter the biological activity of the test compounds. The major dif-
ference between the growth environments of microorganisms and cultured
cell lines is the enhanced aeration required for aerobic bacterial
growth. This increased oxygenation may account for the decomposition
of the test compounds under bacterial growth conditions.

Hydroxyimine Effect on Protoplast Stability

Protoplasts of $\underline{B}$. subtilis prepared by lysozyme treatment of cells
suspended in 0.6 $\underline{M}$ sucrose were washed and suspended in glucose minimal
medium containing 0.6 $\underline{M}$ sucrose and supplemented with water, 8.0×10^{-5}
$\underline{M}$ hydroxyimine (maximum concentration soluble in sucrose medium) or 1
percent Triton X-100. The cells were incubated at 37 C and the proto-
plast integrity evaluated by measuring the absorbance at 540 nm (Figure
13). Triton X-100 caused a substantial decline in culture absorbance
but the hydroxyimine produced no change when compared to the control
suspension.

These data indicate that the hydroxyimine, at the concentration
tested, does not lyse $\underline{B}$. subtilis protoplasts.

Binding of the Hydroxyimine to Protoplasts

Hydroxyimine binding to the protoplasts was quantiated during

Figure 13. Hydroxyimine Effect on Protoplast Stability. $\blacktriangle$, control; $\bullet$, 8.0 x 10^{-5} $\underline{M}$ hydroxyimine; $\blacksquare$, 1 percent Triton X-100.
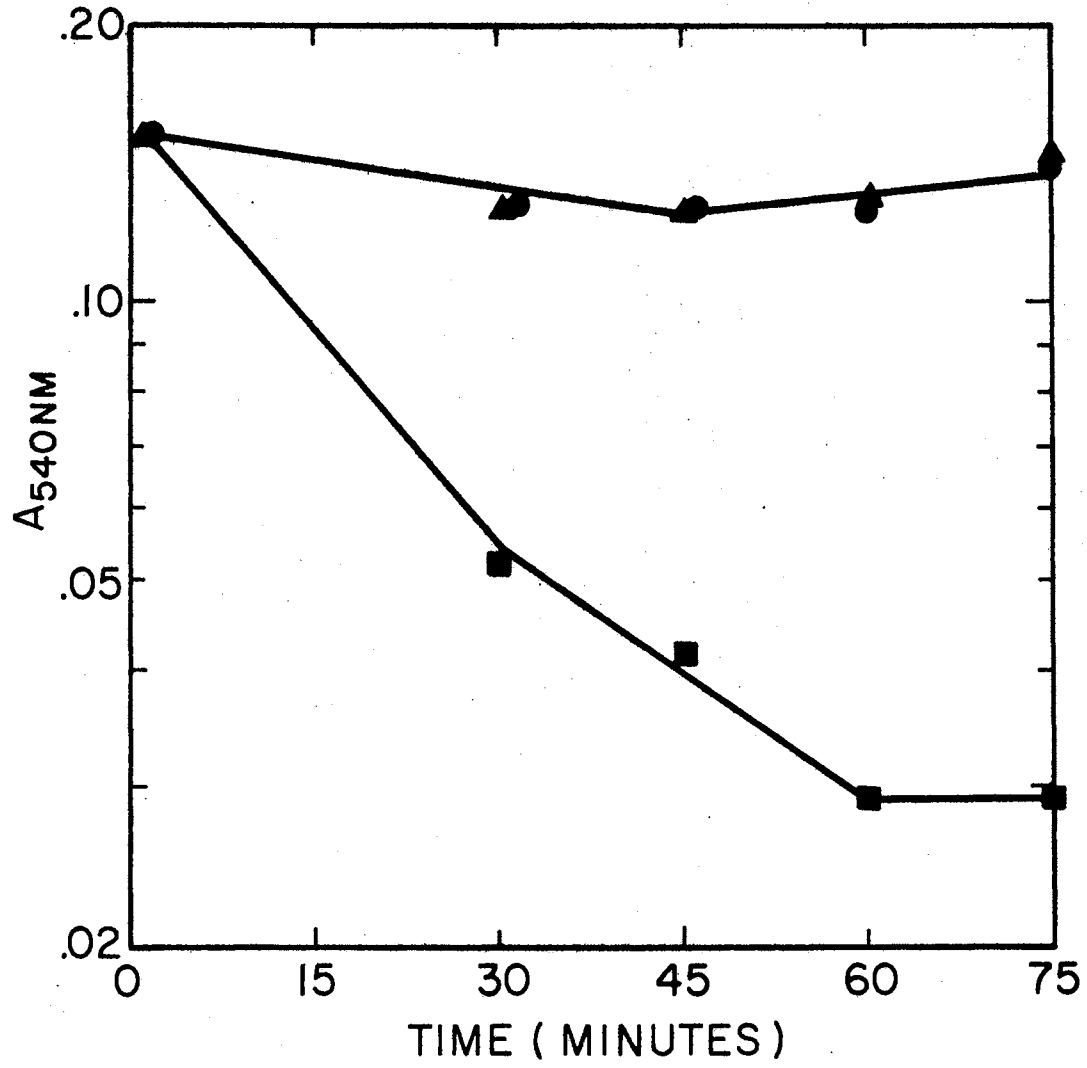
75 minutes incubation in 8×10^{-4} $\underline{M}$ hydroxyimine. The steroid-treated

suspension was pelleted, the supernatant solution diluted, and the con-

centration of hydroxyimine was determined by UV absorption spectroscopy

utilizing the 334 nm absorption peak. Results showed that 7.6×10^{-5} $\underline{M}$

steroid was recovered from the protoplast supernatant solution which

was close to the 8.0×10^{-5} M initial concentration. This indicates

that under the test conditions little hydroxyimine is bound to the

protoplasts.

Hydroxyimine Inhibition of Substrate
Accumulation

In an effort to determine the cause of steroid induced growth in-

hibition, studies were undertaken to evaluate the influence of the hy-

droxyimine on cell permeability. The accumulation of labeled substrates

was measured by adding the radioactive material to growing $\underline{B}$. subtilis

or $\underline{B}$. megaterium cultures following by addition of the steroid or

water (control). Samples were removed at the indicated times, washed,

counted and the accumulation of radioactivity was determined.

In the absence of the test compound, $\underline{B}$. subtilis cells rapidly con-

centrated ^{14}C-glucose (Figure 14). The addition of hydroxyimine $(1.7 \times$

10^{-4} $\underline{M})$ immediately inhibited accumulation, an effect which continued

throughout the 5 minutes of testing. In addition to glucose, an inhibi-

tion of substrate accumulation was observed using serine (Figure 15)

alanine, α-aminoisobutyric acid, lysine, leucine and uracil in the

presence of the hydroxyimine.

Figure 14. Inhibition of ^{14}C-Glucose Accumulation by <u>Bacillus subtilis</u> in the Presence of the Hydroxyimine. ▲, control; ●, 1.7 x 10^{-4} M̲ hydroxyimine.
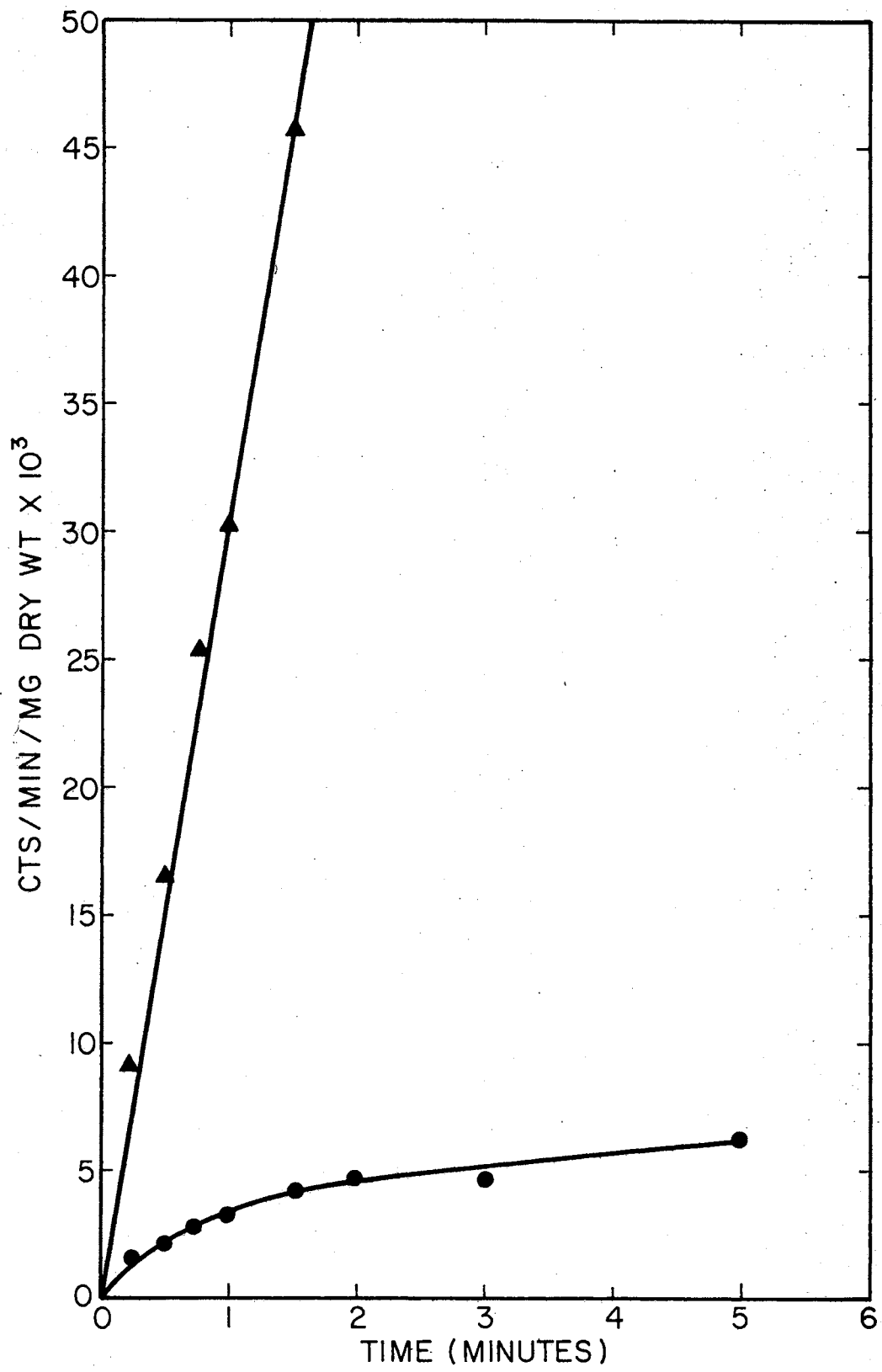
Figure 15. Inhibition of ^{14}C–Serine Accumulation by <u>Bacillus</u> <u>subtilis</u>
in the Presence of the Hydroxyimine. ▲, control; ●,
1.7 x 10^{-4} <u>M</u> hydroxyimine.
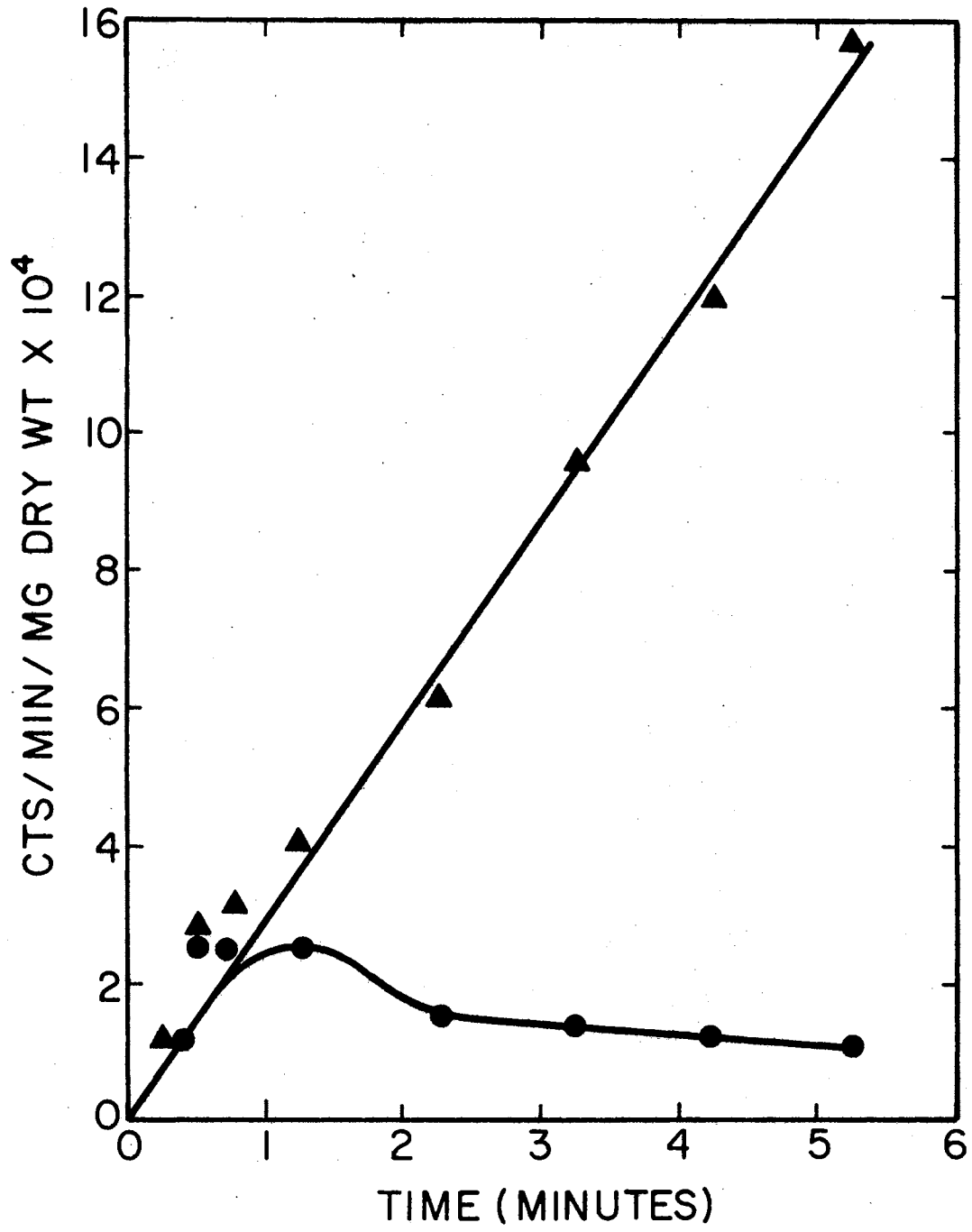
Effect of Various Hydroxyimine Concentrations

on Substrate Accumulation

The inhibition with <u>B</u>. <u>subtilis</u> of ^{14}C-serine accumulation was found to be dependent on the hydroxyimine concentration. Results obtained at steroid concentrations of 3.6×10^{-5} $\underline{M}$, 7.2×10^{-5} $\underline{M}$, and 1.4×10^{-4} $\underline{M}$ using ^{14}C-serine are presented in Table II. The hydroxyimine $(3.6 \times 10^{-5}$ $\underline{M})$ caused approximately a 44 percent reduction in accumulation within the first 0.5 minute but the cells recovered from this inhibition, and after 2 minutes of incubation, the cells contained 76 percent of the control radioactivity. The hydroxyimine at 7.2×10^{-5} $\underline{M}$ caused a 60 percent reduction in labeled serine accumulation after 0.5 minutes of incubation and again some recovery was observed in the 2 and 3 minute samples. At 1.4×10^{-4} $\underline{M}$ steroid concentration only 20 percent of the control radioactivity was accumulated in the first 0.5 minutes and this increased only 12 percent by the 3 minute sample.

These results (Figures 14, 15 and Table II) demonstrate the immediate inhibition of labeled substrate accumulation caused by the hydroxyimine and suggest an interaction of the steroid with the cell membrane. At the hydroxyimine concentrations $(3.6 \times 10^{-5}$ $\underline{M})$ which caused a slight delay in the initiation of growth (Figure 7), accumulation was initially inhibited (44 percent) but showed some increase after about 1 minute of incubation (Table II). Higher hydroxyimine concentrations $(1.4 \times 10^{-4}$ $\underline{M})$ which completely inhibit the growth of <u>B</u>. <u>subtilis</u> drastically inhibited the radioactive substrate accumulation (80-85 percent) and these levels increased only slightly upon continued incubation (Figures 14, 15 and Table II).

TABLE II

INHIBITION OF ^{14}C-SERINE ACCUMULATION IN BACILLUS SUBTILIS
BY DIFFERENT CONCENTRATIONS OF HYDROXYIMINE[+]

Time of Sampling After Azasteroid Addition (Minutes)	Percent of Control Radioactivity			
	Control	Hydroxyimine		
		3.6×10^{-5} $\underline{M}$	7.2×10^{-5} $\underline{M}$	1.4×10^{-4} $\underline{M}$
0.5	100 (15,000)*	56	40	20
1	100 (16,000)*	59	45	22
2	100 (17,000)*	76	58	29
3	100 (19,200)*	77	65	32

[+]cell mass determined by absorbance readings at 540 nm

*actual control count (CTS/min/mg dry wt.)

Reversal of Accumulation Inhibition

The reversal of inhibition of accumulation was determined using B. megaterium KM (Figure 16). The steroid (1.7×10^{-4} M) or water (control) was added to cultures, samples were removed from each and the rate of ^{14}C-serine accumulation was determined. The remaining cultures were pelleted, washed with glucose minimal medium and the pellet suspended in fresh medium without steroid. These cultures were incubated at 37 C and samples were removed at various time intervals to allow the determination of radioactive label accumulation. The results, plotted as the rate of accumulation (percent) of the steroid inhibited system compared to the control (as 100 percent), revealed that an initial 30 second exposure to the hydroxyimine reduced the cells ability to accumulate serine by approximately 60 percent. Subsequent removal of the steroid from the growth medium allowed reversal of the accumulation inhibition. The linearity of the ability of the cells to regain activity resembles a first order reaction in which approximately 70 minutes of incubation are required to attain the control rate.

Effect of Magnesium on Azasteroid Induced
Growth Inhibition

Earlier results from this study suggested that the azasteroid exerts some action at the cell membrane. Since magnesium ions stabilize bacterial membranes (Weibull, 1956), experiments were conducted to determine if the observed growth inhibition and the inhibition of substrate accumulation could result from azasteroid chelation or competition with magensium ions. Growing cultures of B. subtilis were supplemented with 1.7×10^{-4} M magnesium sulfate 15 minutes prior to

Figure 16. Reversal of Inhibition of the Rate of ^{14}C—Serine Accumula-
tion by <u>Bacillus megaterium</u> Following Removal of the
Hydroxyimine. ▲, rate of serine accumulation of a
culture exposed 0.5 minutes to the hydroxyimine (1.7 x
10^{-4} <u>M</u>) then suspended and incubated in medium lacking
the azasteroid expressed as the percent of the control
rate.
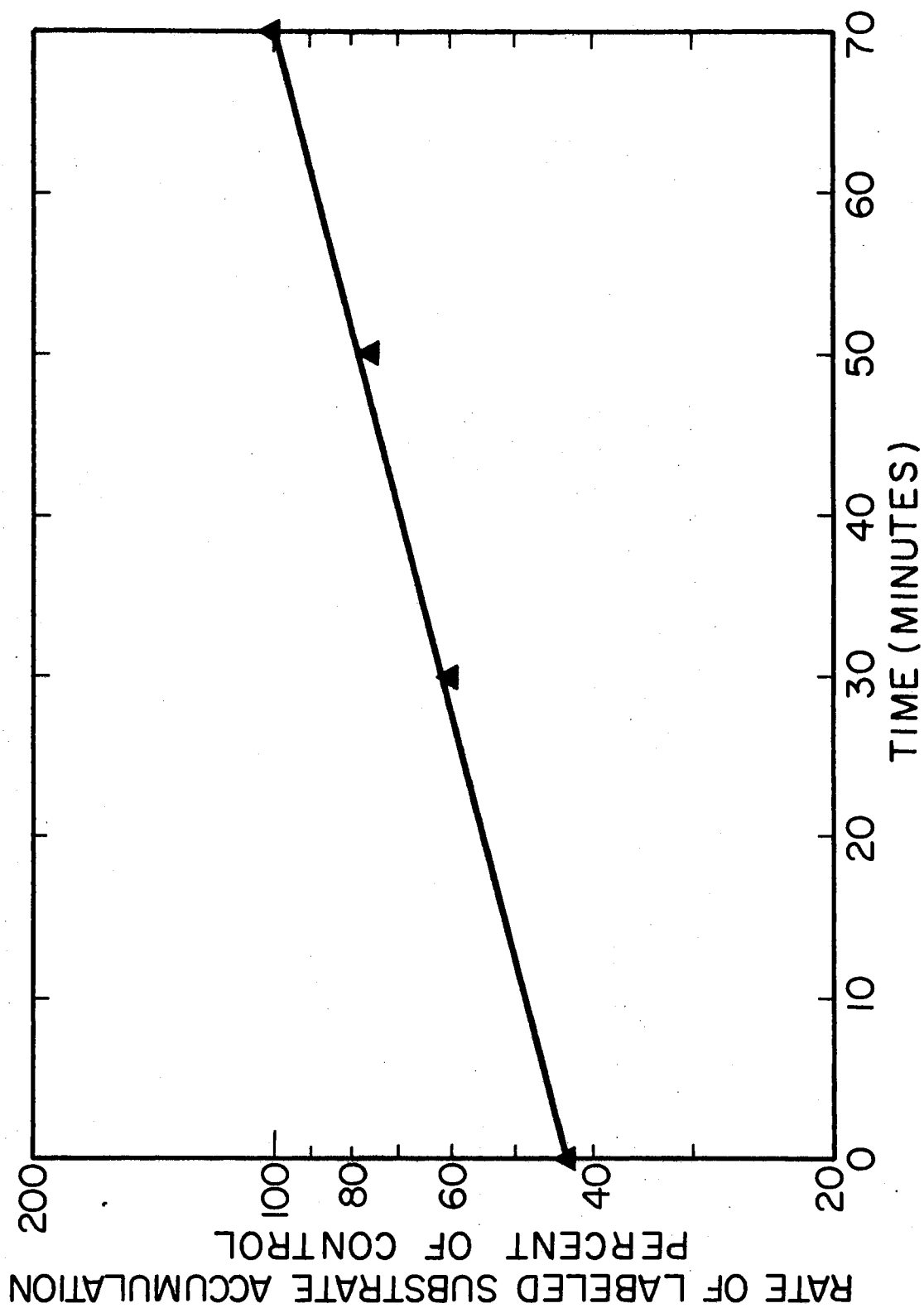
addition of the methoxy- or hydroxyimine (1.7×10^{-4} $\underline{M}$). The resulting

growth inhibition produced by the test compounds was not affected by

the magnesium treatment or the presence of the ion in the growth medium

(Figure 17). The results suggest that the molecular events taking place

between the cell and the steroid are not influenced by excess magnesium.

Smith and Shay (1965) reported that magnesium caused varying and

temporary degrees of protection for Sarcina lutea protoplasts when

treated with lytic concentrations of cholestane derivatives. Chelation

of the magnesium by ethylenediaminetetraacetate did not produce lysis

alone and caused little change in the lytic action of the steroids.

Results from this study differ from the report of Silver and Levine

(1968) who found that pretreatment or addition of magnesium for as long

as 10 minutes following treatment with the steroidal diamine, ireh-

diamine A, allowed not only protection of E. coli cells but actual

reversal of steroid inhibition. These observations suggest that the

test compounds and irehdiamine A probably differ in their mechanisms

of action.

Steroid Induced Leakage of Metabolite Pre-cursor Molecules from B. megaterium Cells

Azasteroids which have quaternary nitrogen can be loosely classi-

fied as cationic detergents (Harold, 1970). Although the test steroids

do not contain quaternary nitrogen, the inhibition of accumulation

caused by the test compounds could be produced from a detergent like

action resulting in an increased membrane permeability and leakage of

metabolic precursors. To test this hypothesis, B. megaterium cells were

labeled with ^{14}C-α-aminoisobutyric acid, a non-metabolizable substrate

Figure 17. Effect of Magnesium Ions on the Azasteroid Induced Bacillus
subtilis Growth Inhibition. All cultures were supple-
mented with 1.7 x 10^{-4} M magnesium sulfate 15 minutes
prior to addition of test compounds. ▲, magnesium
control; ■, methoxyimine 1.7 x 10^{-4} M; ●, hydroxyimine
1.7 x 10^{-4} M.
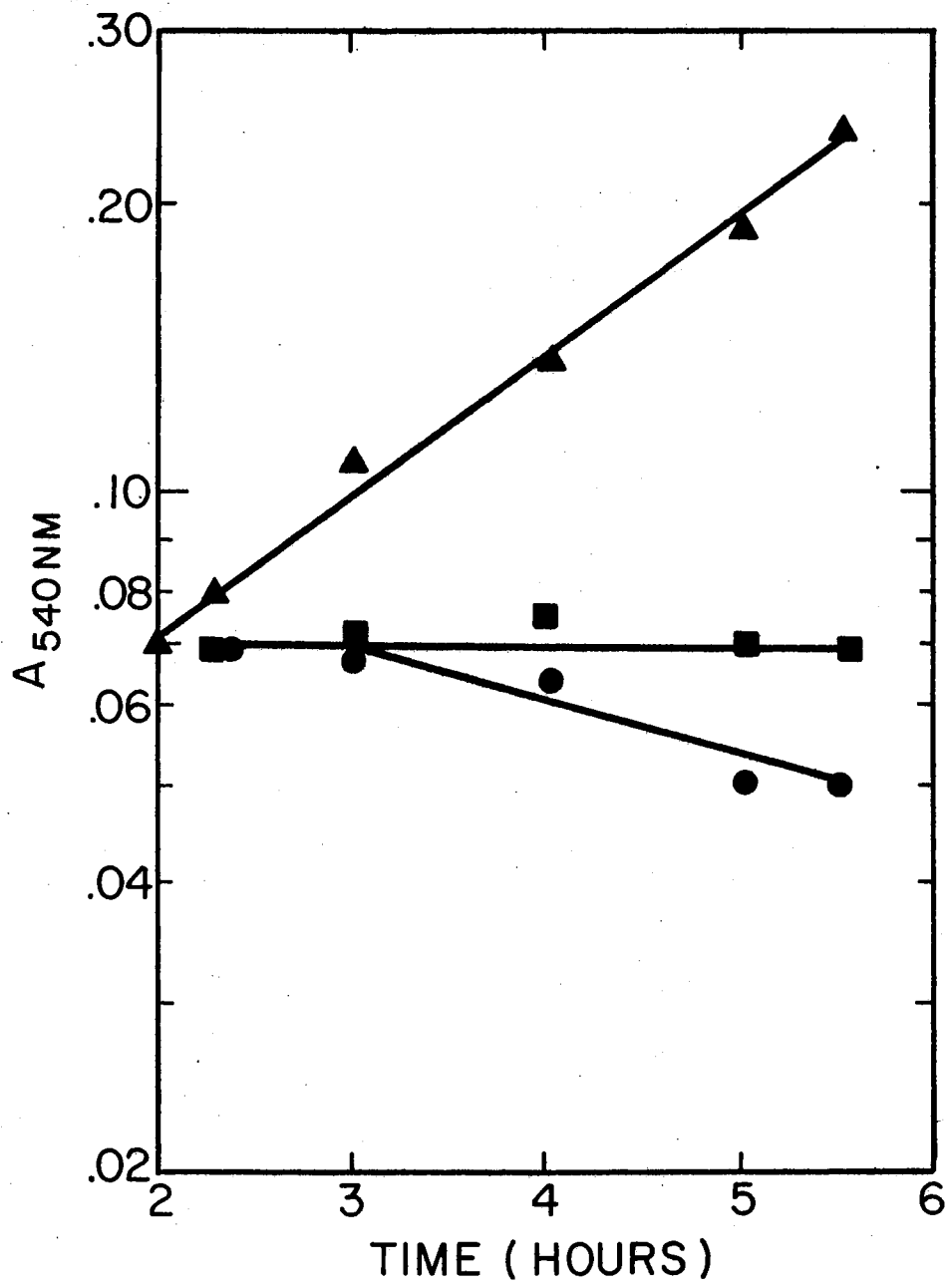
(Marquis and Gerhardt, 1964), and treated with water (control), 1.7 x 10^{-4} $\underline{M}$ hydroxyimine or 1 percent Triton X-100.

Initial studies were performed by labeling $\underline{B}$. megaterium cultures suspended in glucose minimal medium under growth conditions with 1 ml (5 μCi/ml) of α-aminoisobutyric acid for 7 minutes. The cells were pelleted, washed and suspended in 6 ml aliquots in medium supplemented with the test compounds. Samples were removed, filtered and the retention of radioactivity by the cells determined.

The results (Figure 18) indicated no leakage of labeled "pool" material in hydroxyimine treated cells when compared with the control culture. In contrast, the non-ionic detergent, Triton X-100, caused complete release of the labeled precursor.

To confirm these findings, a similar experiment was conducted in which $\underline{B}$. megaterium cells were suspended in medium in 6 ml aliquots and labeled with ^{14}C-α-aminoisobutyric acid for 5 minutes. In contrast to the previous leakage experiment, at time zero, 6 ml of medium containing water (control), 3.4 x 10^{-4} $\underline{M}$ hydroxyimine or 2 percent Triton X-100 was added directly to the appropriate culture, samples were removed and the retention of radioactivity determined.

These results (Figure 19) confirmed the results described in Figure 18 and revealed no labeled "pool" leakage of hydroxyimine treated or the water treated control cells. The detergent, Triton X-100, again caused a very rapid release of the labeled pool material.

These observation confirm that at the concentrations tested, there is no steroid induced membrane perturbations which allowed "pool" leakage. The action of the steroid on permeability does not appear to mimic the action of a detergent molecule.

Figure 18. Leakage of ^{14}C-α-Aminoisobutyric Acid From Hydroxyimine
Treated <u>Bacillus</u> <u>megaterium</u> Cells. Cells were incubated
with the radioisotope, pelleted, washed and pellet sus-
pended in the indicated solutions. ▲, control; ●,
1.7 x 10^{-4} M̲ hydroxyimine; ■, 1 percent Triton X-100.
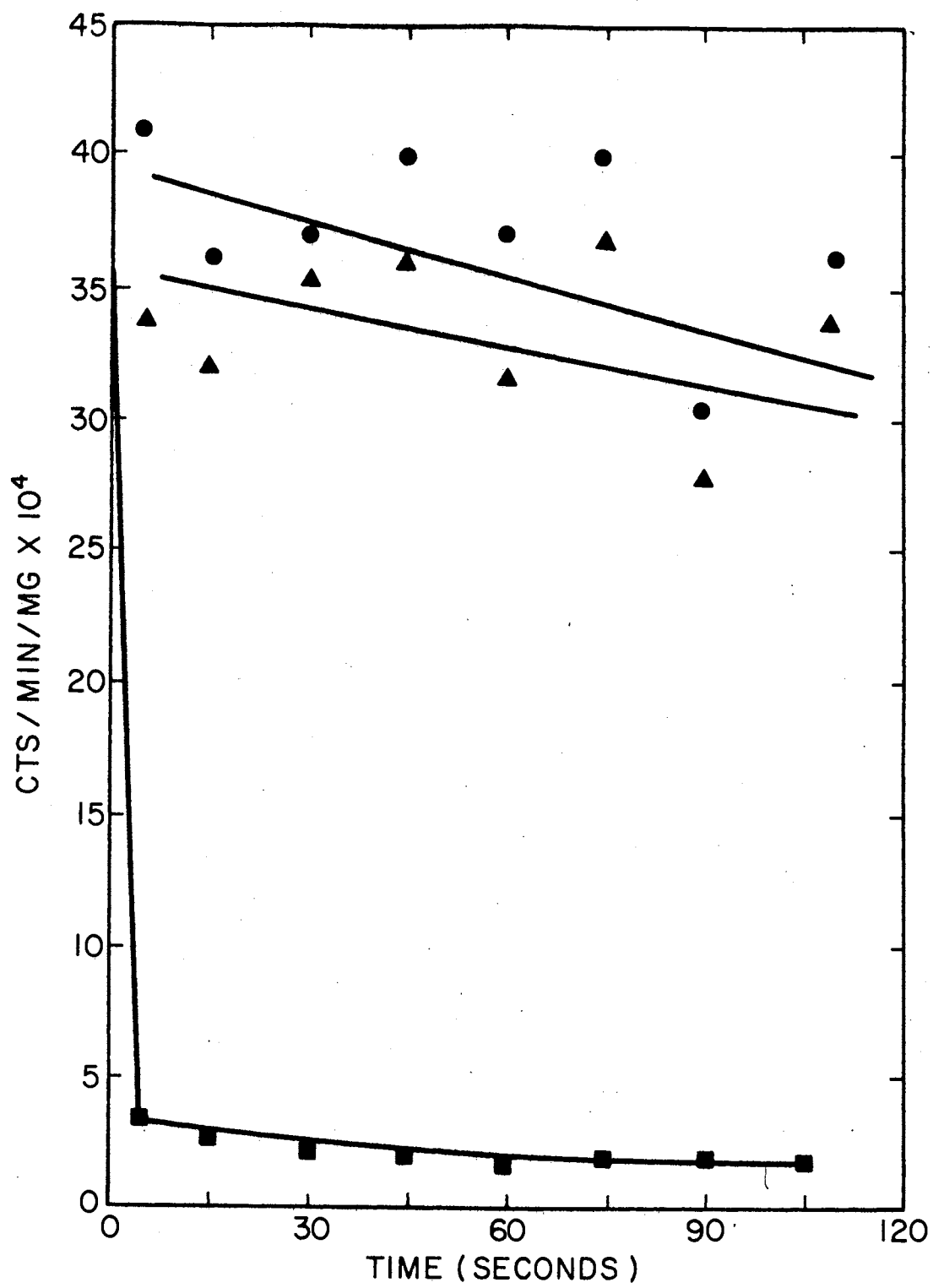
Figure 19. Leakage of $^{14}C-\alpha$-Aminoisobutyric Acid From Hydroxyimine
Treated <u>Bacillus</u> <u>megaterium</u> Cells. Cells were incubated
with the radioisotope and the indicated solutions added
directly to the cells to give the final concentrations.
▲, control; ●, 1.7 x 10^{-4} $\underline{M}$ hydroxyimine; ■, 1 per-
cent Triton X-100.
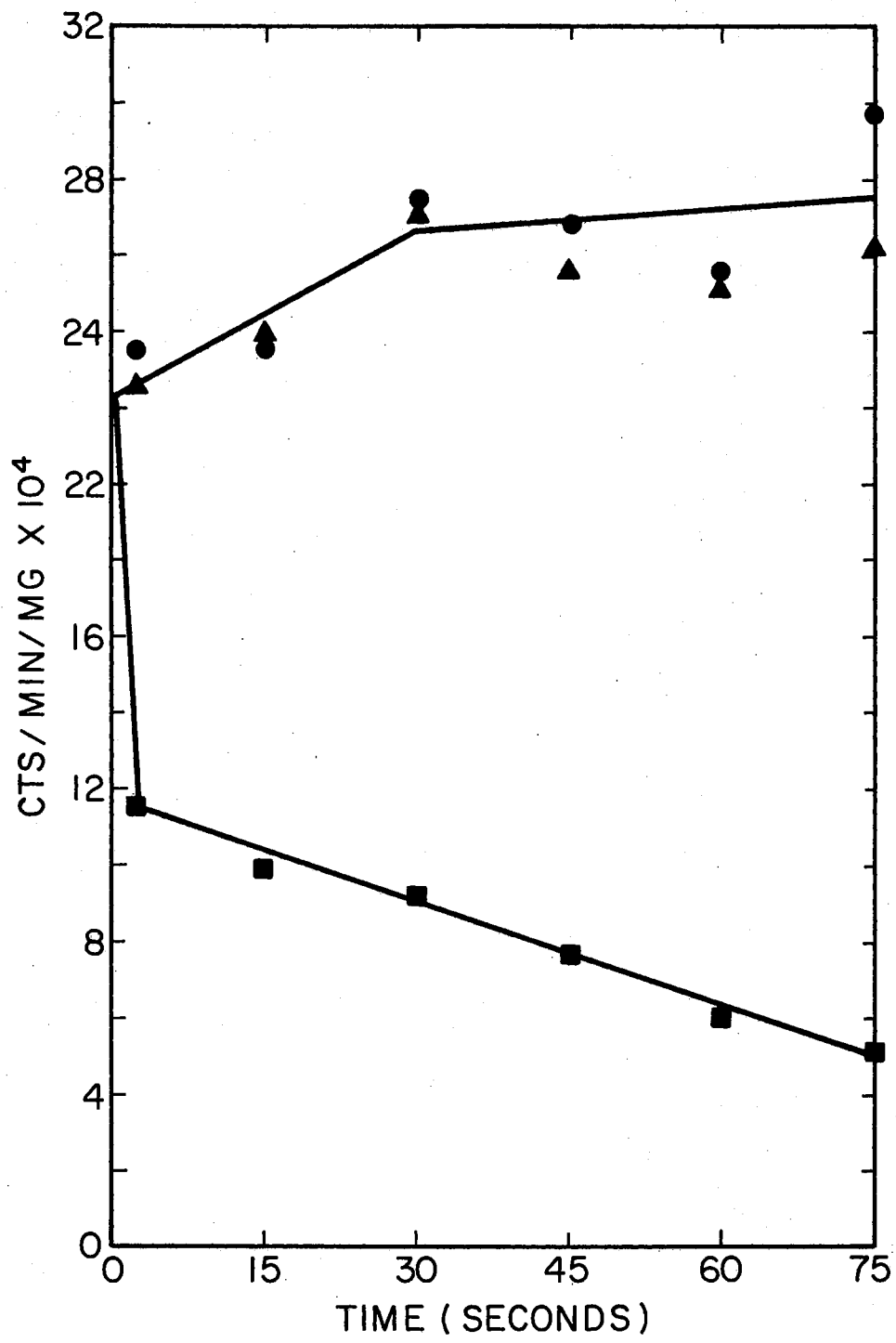
Methoxyimine Potentiation of Antibiotic

Activity

The methoxyimine has a lower toxicity for mammalian cells and was therefore used to determine if the azasteroid could potentiate the antibacterial activity of certain antibiotics. Growing cultures of B. subtilis or P. fluorescens were supplemented with the methoxyimine and/or polymyxin, circulin, vancomycin or chloramphenicol. The growth was followed by measuring the change in absorbance at 540 nm.

P. fluorescens cells were grown in succinate minimal medium supplemented with the methoxyimine (6.4 x 10^{-5} M) and/or polymyxin (5.6 x 10^{-7} M). The methoxyimine caused no detectable decrease in growth rate or total growth yield (Figure 20), as measured by this method, while growth of the organism in the presence of only polymyxin showed a slightly depressed rate and lower total growth. However, the culture supplemented with both the methoxyimine and polymyxin was completely inhibited throughout the 7 hours of incubation.

Action of the antibiotic, circulin, for P. fluorescens was also potentiated by the presence of the methoxyimine (Figure 21). The control and methoxyimine cultures grew at nearly the same rate and attained similar growth yields. The culture containing only circulin (1.2 x 10^{-5} M) showed a slightly decreased growth rate and a normal growth yield. The culture containing both the methoxyimine and circulin did not initiate growth and was inhibited throughout the 7 hours of incubation.

Similar experiments were conducted using B. subtilis with the methoxyimine and the antibiotics, vancomycin or chloramphenicol. Figure 23 shows the results obtained when the methoxyimine (9.5 x 10^{-7} M) was added in combination with chloramphenicol (5.2 x 10^{-7} M) to cultures of

Figure 20. Potentiation of the Antibacterial Activity of Polymyxin
Against Pseudomonas fluorescens by the Methoxyimine.
▲, control and 6.4 x 10^{-5} $\underline{M}$ methoxyimine; ■ 5.6 x
10^{-7} $\underline{M}$ polymyxin; ●, 5.6 x 10^{-7} $\underline{M}$ polymyxin plus
6.4 x 10^{-5} $\underline{M}$ methoxyimine.
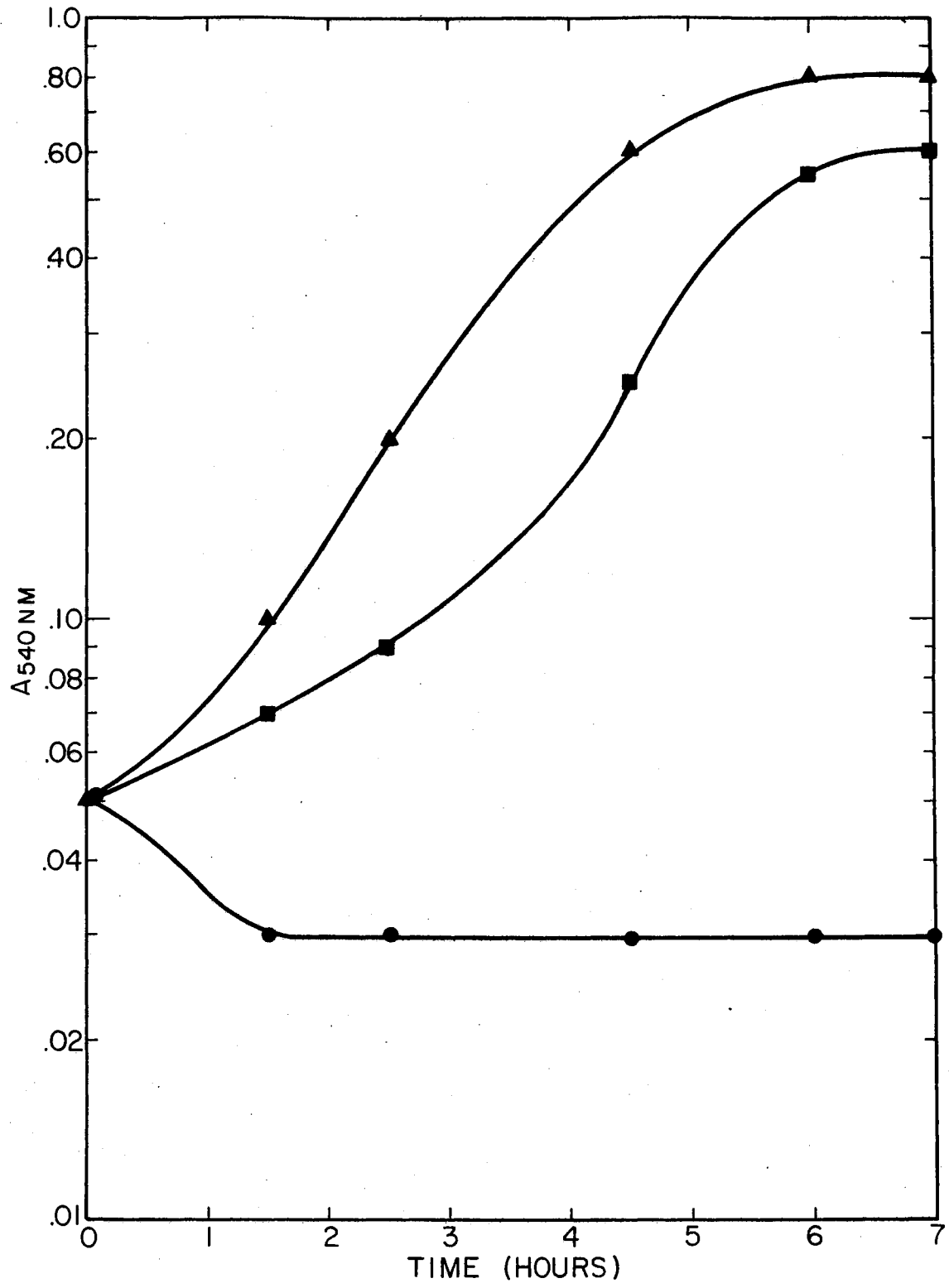
Figure 21. Potentiation of the Antibacterial Activity of Circulin
Against <u>Pseudomonas</u> <u>fluorescens</u> by the Methoxyimine.
▲, control and 6.4 x 10^{-5} $\underline{M}$ circulin; ■, 1.2 x 10^{-5}
$\underline{M}$ circulin; ●,1.2 x 10^{-5} $\underline{M}$ circulin plus 6.4 x 10^{-5} $\underline{M}$
methoxyimine.
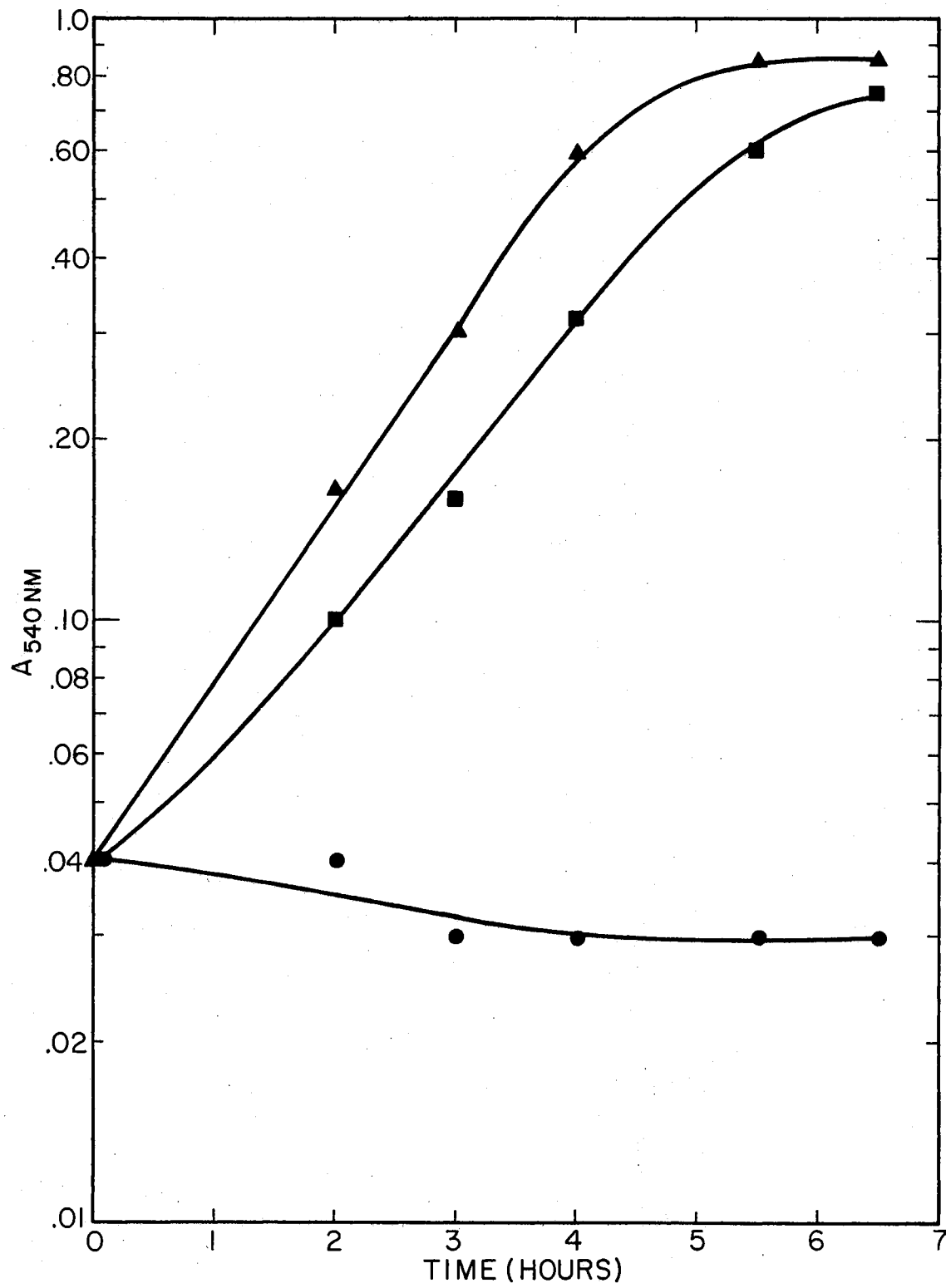
B. subtilis. The addition of methoxyimine alone did not influence growth while the addition of chloramphenicol produced an initial delay in growth but the rate was near normal after approximately 3 hours of incubation. Growth of the culture to which both the methoxyimine and chloramphenicol were added was completely inhibited throughout the 7 hours of incubation.

The action of vancomycin (3.4×10^{-8} $\underline{M}$) was also potentiated by the addition of methoxyimine (9.5×10^{-7} $\underline{M}$). Although this low concentration of vancomycin delayed growth initiation, addition of both methoxyimine and vancomycin completely inhibited growth of B. subtilis throughout the 7 hours of incubation (Figure 22). The potentiation of antibiotic action was not noted when these same antibiotics were tested against other microorganisms. For example, the methoxyimine and chloramphenicol were not effective against P. fluorescens and the azasteroid with vancomycin had no influence on E. coli. Several other antibiotics were tested, (penicillin, erythromycin, neomycin, actinomycin D, and filipin) but showed no potentiation of antibacterial properties when combined with the methoxyimine. Because of its greater mammalian cell toxicity, the hydroxyimine was not tested for its ability to potentiate antibiotics. No nitrogen containing steroids have been previously reported to potentiate the action of antibiotics, although the steroid antibiotic, fusidic acid, potentiates the effects of amphotericin B against fungi (Medoff et al., 1972) and against transformed mammalian fibroblast cells (Kuwana et al., 1972).

gure 22. Potentiation of the Antibacterial Activity of Vancomycin
          Against Bacillus subtilis by the Methoxyimine. ▲,
          control and 7.9 x 10^{-7} $\underline{M}$ methoxyimine; ■, 3.4 x 10^{-8}
          $\underline{M}$ vancomycin; ●, 3.4 x 10^{-8} $\underline{M}$ vancomycin plus 7.9 x
          $\underline{10^{-7}}$ $\underline{M}$ methoxyimine.

Figure 23. Potentiation of the Antibacterial Activity of Chlor-
amphenicol Against Bacillus subtilis by the Methoxy-
imine. ▲, control and 7.9×10^{-7} $\underline{M}$ methoxyimine;
■, 5.2×10^{-7} $\underline{M}$ chloramphenicol; ●, 5.2×10^{-7} $\underline{M}$
chloramphenicol plus 7.9×10^{-7} $\underline{M}$ methoxyimine.
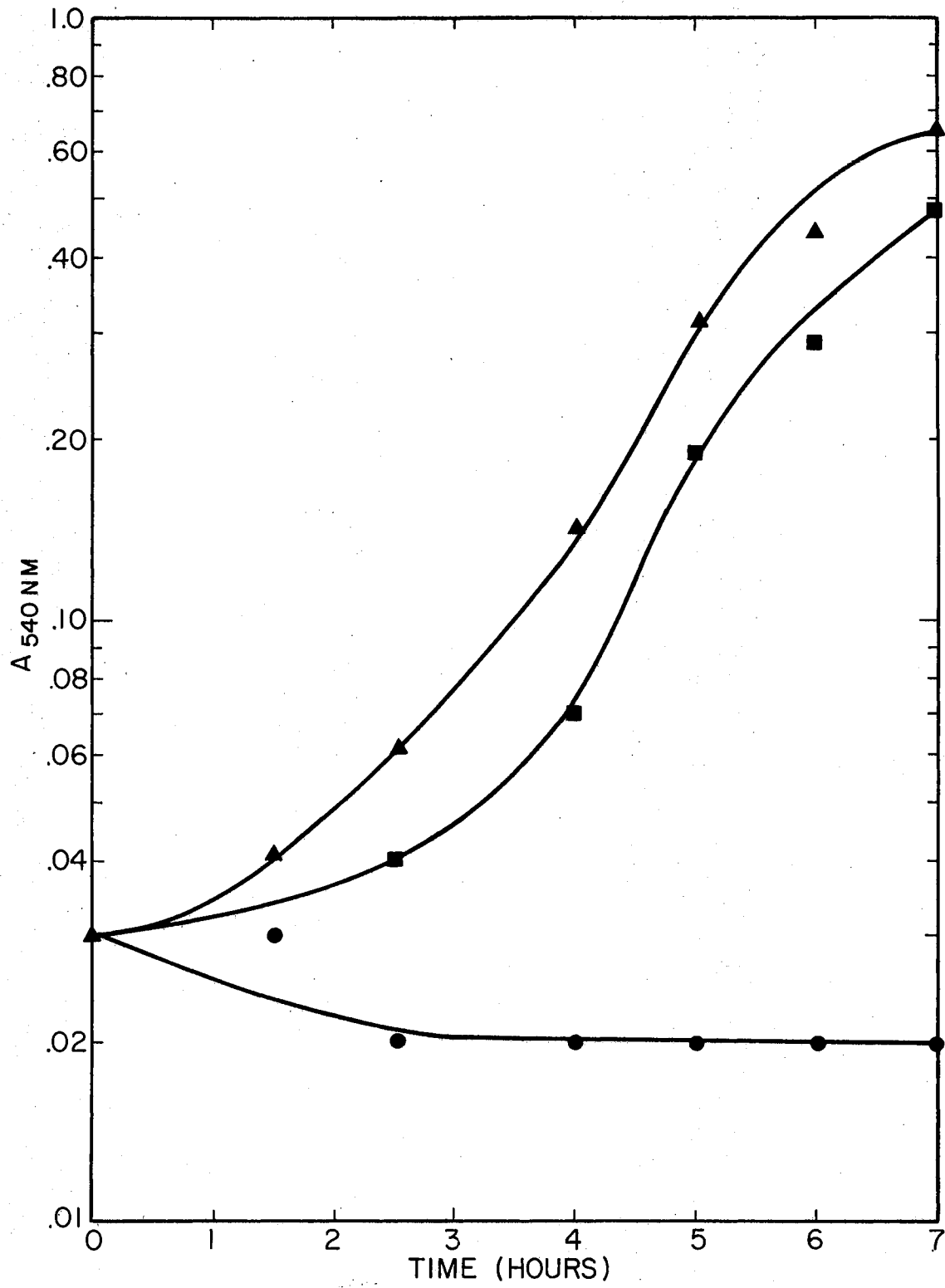
Cell Culture Studies

Azasteroid Inhibition of Mouse L-M Cell Growth

Suspension cultures of mouse L-M cells were supplemented with various concentrations of the methoxyimine, hydroxyimine or water (control) and the growth followed by determining the cell numbers for a period of 6 days.

In the presence of 4×10^{-6} $\underline{M}$ hydroxyimine the culture grew at a rate similar to the control while cells supplemented with 4×10^{-5} $\underline{M}$ hydroxyimine showed a depressed growth rate and lagged about 1.5 days behind the control (Figure 24). Hydroxyimine (8×10^{-5} $\underline{M}$) completely inhibited cell growth throughout the 6 days of incubation.

In contrast, the methoxyimine (3.8×10^{-6} $\underline{M}$, 3.8×10^{-5} $\underline{M}$, and 7.6×10^{-5} $\underline{M}$) caused only a slight depression of growth rate (Figure 24) with the highest concentration producing a lag of approximately 1 day when compared with the control.

These results indicate that the azasteroids do express biological activity against a mammalian cell line as well as exemplifying a high degree of molecular specificity in relation to their effects on cell growth.

Reversal of L-M Cell Growth Inhibition

Mouse L-M cells were grown in suspension culture in the presence and absence of the hydroxyimine (8×10^{-5} $\underline{M}$) for 36 hours. The cells were pelleted, washed and suspended in fresh medium in the presence and the absence of the steroid. The growth of each culture was followed and the results are shown in Figure 25.

Figure 24. Effect of Various Concentrations of Hydroxyimine and
Methoxyimine on L–M Cell Growth. Hydroxyimine: $\blacktriangle$,
control or 4.0×10^{-6} $\underline{M}$; $\bullet$, 4.0×10^{-5} $\underline{M}$; $\blacksquare$, 8.0×10^{-5} $\underline{M}$. Methoxyimine: $\blacktriangle$, control or 3.8×10^{-6} $\underline{M}$;
$\bullet$, 3.8×10^{-5} $\underline{M}$; $\blacksquare$, 7.6×10^{-5} $\underline{M}$.
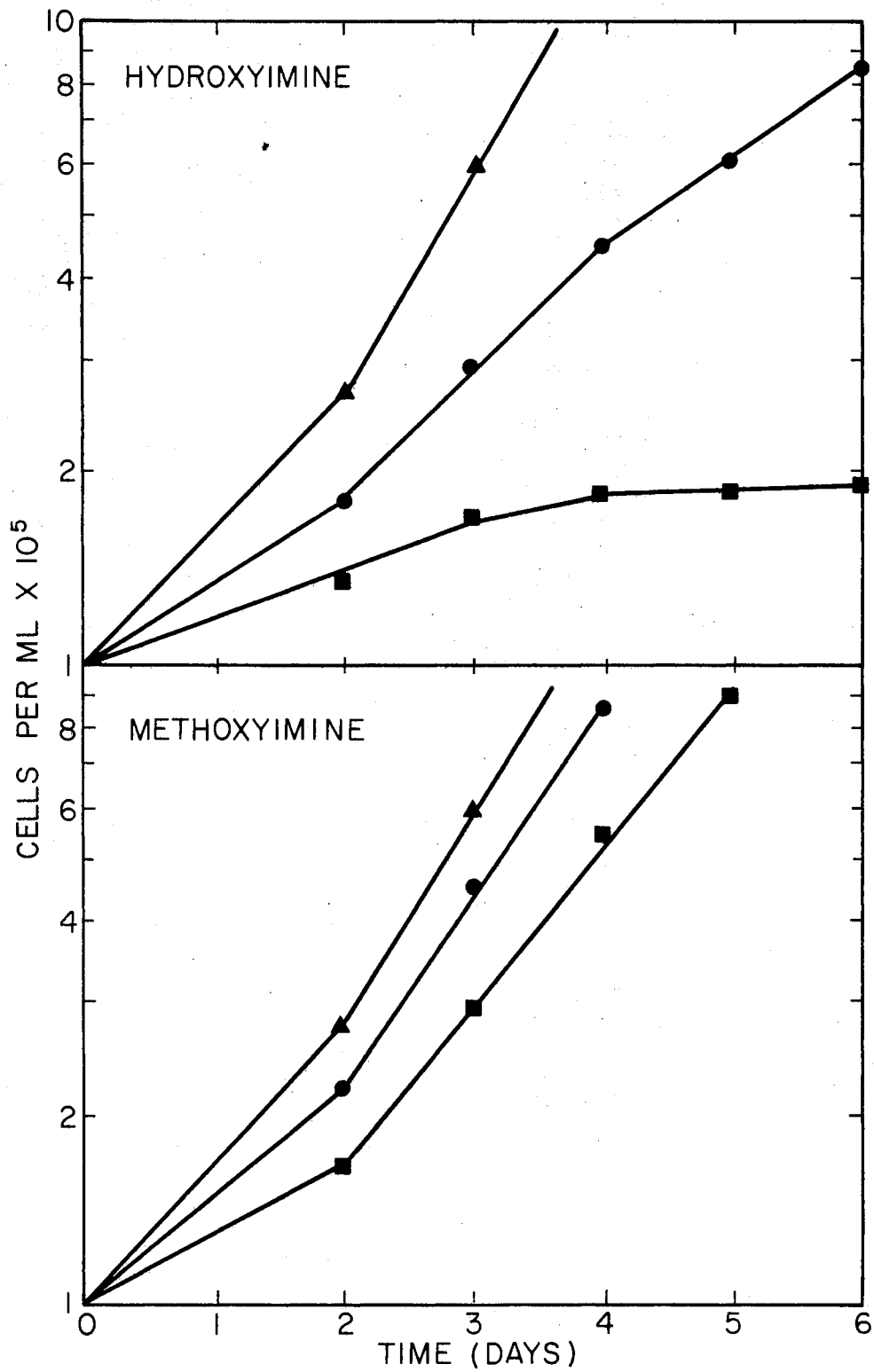
Figure 25. Reversal of Growth Inhibition Upon Removal of the Hydroxy-
          imine From the Culture Medium. ▲, control; ●, incubated
          36 hours in medium plus hydroxyimine 8 x 10^{-5} $\underline{M}$ and sus-
          pended in medium plus hydroxyimine 8 x 10^{-5} $\underline{M}$; ■, incu-
          bated 36 hours in medium plus hydroxyimine 8 x 10^{-5} $\underline{M}$,
          suspended in medium minus hydroxyimine; ↓, cells resus-
          pended to equal cell numbers.
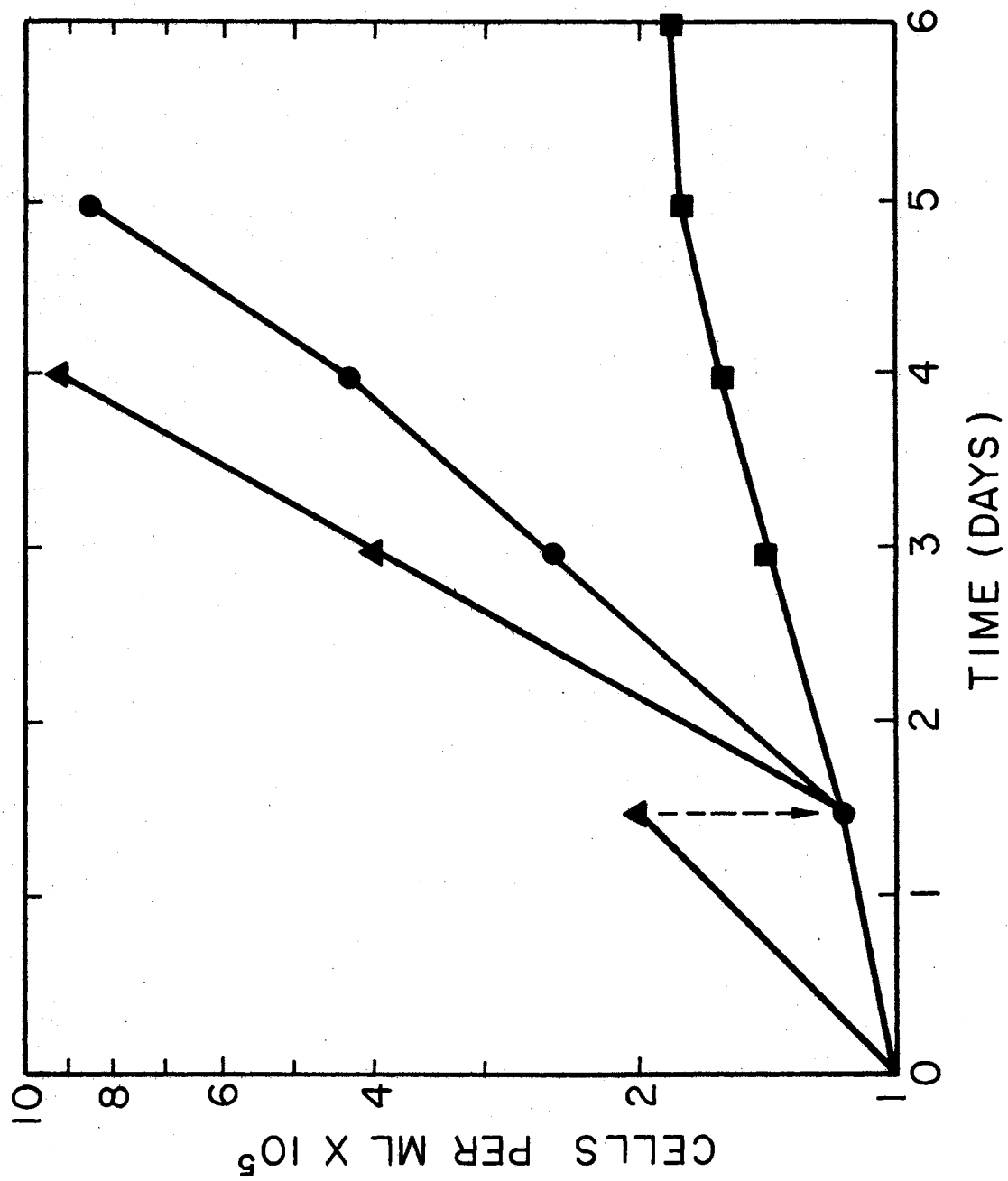
Cells initially growing in the absence of the hydroxyimine (control) grew at a normal rate when suspended in fresh medium lacking the azasteroid and following a slight lag period, the cells doubled approximately every 24 hours.

Those cells initially incubated in the presence of the hydroxyimine and placed into medium lacking the azasteroid began growing following a 1 day lag and showed a doubling rate near that of the control. When cells initially incubated in the presence of the hydroxyimine were placed into medium again supplemented with 8×10^{-5} $\underline{M}$ hydroxyimine, complete growth inhibition was noted and no cell doubling occurred throughout the 6 days of incubation.

The growth inhibition caused by the highest hydroxyimine concentration tested (8×10^{-5} $\underline{M}$) which caused complete cessation of cell growth was reversed following removal of the cells from the steroid-containing medium. The observations demonstrate the reversible nature of cellular damage caused by the steroid. In addition, because the reversal is not immediate, the results implicate the necessity for synthetic processes to occur prior to resumption of cell growth.

Effect of the Azasteroids on the Surface
Attachment of Fibroblast Cells

Since earlier results had implicated the plasma membrane as a possible site of azasteroid action, experiments were conducted to determine the effects of the methoxy- and hydroxyimines on the attachment of KB cells to the surface of Falcon plastic culture dishes and to determine the duration of steroid exposure required to cause cell detachment.

Cells were added to the culture dishes (approximately 500 cells per

dish) in the presence and absence of the methoxyimine (3.8×10^{-5} $\underline{M}$ or 7.6×10^{-5} $\underline{M}$) or the hydroxyimine (4×10^{-5} $\underline{M}$ or 7×10^{-5} $\underline{M}$). The cultures were incubated and the medium (containing non-attached cells) was removed at various times. The cells were washed, suspended in medium lacking the steroid and incubated for 7 days. The cells were stained and the cell numbers determined by direct microscopic count.

The results (Table III) show the percent of cells attached to the plates by the time of medium removal (0, 1, 3, 6 and 24 hours) compared to control plates. In the control cultures the majority of cells capable of attachment had attached after about 3 hours of incubation (95 percent) and that these cells remained attached throughout the additional 21 hours of incubation. The initial attachment of cells was not affected by either concentration of the methoxyimine. Most cells incubated in the hydroxyimine were also able to complete the initial attachment (73 percent). However, continued incubation for 6 or more hours in the presence of 7×10^{-5} $\underline{M}$ hydroxyimine caused cells which had initially become attached to be released from the plate surface and thus were removed with the medium. This effect was most evident in the culture incubated in the presence of the hydroxyimine (7×10^{-5} $\underline{M}$) for 24 hours prior to removal of the medium where only an 18 percent plating efficiency was observed.

Neither azasteroid at the concentrations tested significantly inhibited the initial surface attachment of the human fibroblast cells. However, cells incubated in the presence of the hydroxyimine which remained attached for approximately 3 hours, released by the 6 hours interval and had nearly all become detached by 24 hours. The evidence indicates that several hours of incubation are required to cause the

TABLE III

DETERMINATION OF KB CELL ATTACHMENT AND RELEASE OF CONTROL CELLS AND
CELLS INCUBATED IN THE METHOXY- OR HYDROXYIMINE*

| Incubation Time Prior to Medium Removal (Hours) | Percent Relative Plating Efficiency | | | | |
| | | Methoxyimine | | Hydroxyimine | |
	Control	3.8×10^{-5} $\underline{\underline{M}}$	7.6×10^{-5} $\underline{\underline{M}}$	4×10^{-5} $\underline{\underline{M}}$	7×10^{-5} $\underline{\underline{M}}$
0	8	4	3	4	0
1	85	87	85	80	70
3	95	96	96	97	73
6	94	94	98	98	55
24	95	98	95	88	18

*Values expressed as the percent of cells attached to the culture dish at the various time intervals compared to the total number of cells added to the plate.

cellular damage probably at the cell surface which is responsible for cell detachment.

Influence of the Azasteroids on KB Cell Plating Efficiency of Attached Compared to Suspended Cells

To test the differential effects of the test compounds on both suspended and attached cells, experiments were conducted in which the plating efficiency of KB cells was determined when the steroid was added prior to cell attachment (zero time) or following cell attachment (4 hours). Table IV shows the results obtained when the methoxyimine (3.8 x 10^{-5} $\underline{M}$ or 7.6 x 10^{-5} $\underline{M}$) or hydroxyimine (4 x 10^{-5} $\underline{M}$ or 8 x 10^{-5} $\underline{M}$) was added to the cultures at the indicated times and the effect on plating efficiency (cell viability) determined.

Addition of the hydroxyimine (7 x 10^{-5} $\underline{M}$) to suspended cells (zero time) reduced the plating efficiency to 3 percent while addition of the hydroxyimine (7 x 10^{-5} $\underline{M}$) to attached cells (4 hours) produced similar plating efficiency results (1 percent). In contrast, addition of the methoxyimine (7.6 x 10^{-5} $\underline{M}$) to suspended cells (zero time) only reduced plating efficiency to 88 percent while addition of the methoxyimine (7.6 x 10^{-5} $\underline{M}$) to attached cells (4 hours) lowered plating efficiency to 27 percent.

These data imply a distinct cellular difference between attached and suspended KB cells in response to the two closely related steroid analogues. This phenomenon might reflect a difference in the cell periphery between the attached and suspended KB cells which could result in an enhanced methoxyimine/cell interaction. In addition, the results

TABLE IV

RELATIVE PLATING EFFICIENCY OF KB CELLS TO WHICH STEROID WAS ADDED PRIOR TO CELL ATTACHMENT

| Time of Steroid Addition (Hours) | Relative Plating Efficiency in Percent | | | |
| | Methoxyimine | | Hydroxyimine | |
	3.8×10^{-5} M	7.6×10^{-5} M	3.5×10^{-5} M	7×10^{-5} M
0	100	88	76	3
4	88	27	85	1

validate the molecular specificity involved in steroid activity.

Azasteroid Inhibition of Radioactive Substrate
Accumulation

Experiments were conducted to determine the effect of the methoxy-
and hydroxyimine on tissue culture cell substrate accumulation. Mouse
L-M cells were suspended in Hepes balanced salts solution to a concentra-
tion of 10^6 cells per ml. At time zero, the radioactive substrate was
added to the culture followed by the addition of steroid or water (con-
trol). At the indicated times, samples were removed, washed, and radio-
activity of the cells was determined by liquid scintillation counting.

Addition of the hydroxyimine (8×10^{-5} $\underline{M}$) to the culture immediately
after addition of ^{14}C-alanine produced an immediate inhibition of sub-
strate accumulation (Figure 26). By the termination of the experiment
(60 minutes) the culture in the presence of hydroxyimine had amassed
only about 10 percent as much radioactivity as the control. Similar
results were noted with the mouse L-M cell system when measuring the
accumulation of ^{14}C-labeled lysine, leucine, serine, α-aminoisobutyric
acid or uracil.

To confirm the immediate effect of the azasteroid on uptake, ^{14}C-
leucine was added to duplicate L-M cultures at time zero and the accumu-
lation of leucine followed for 25 minutes. At this time, the hydroxy-
imine (8×10^{-5} $\underline{M}$) or water (control) was added to the cultures and the
measurement of leucine accumulation continued (Figure 27). Accumulation
of ^{14}C-leucine ceased almost immediately following addition of the
steroid and did not change throughout the remaining 30 minutes of the
experiment. In contrast, the control culture continued to rapidly

Figure 26. Accumulation of ^{14}C-Alanine by L-M Cell Cultures in the Presence and Absence of Hydroxyimine. ▲, control; ●, 8 x 10^{-5} M hydroxyimine.
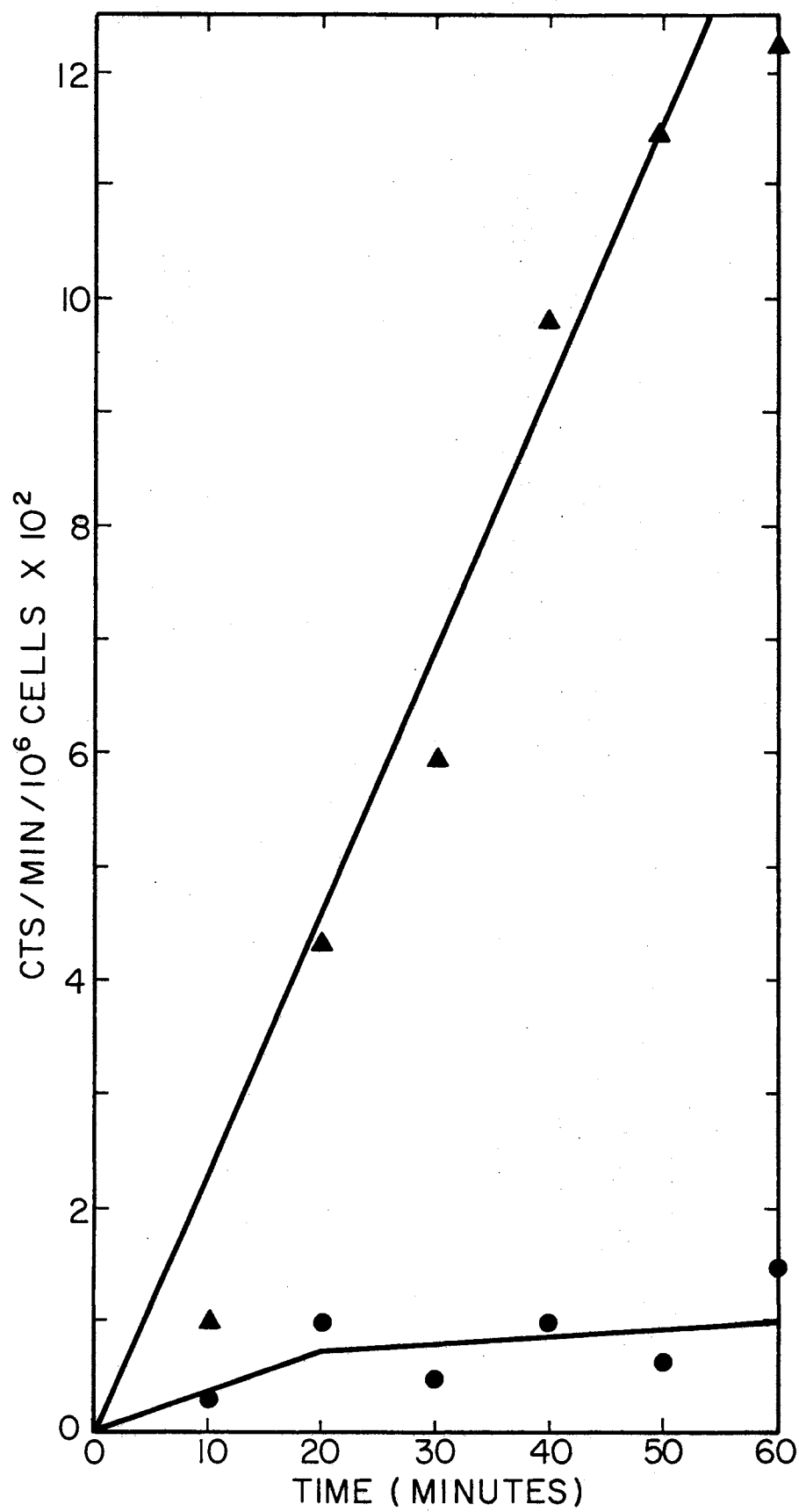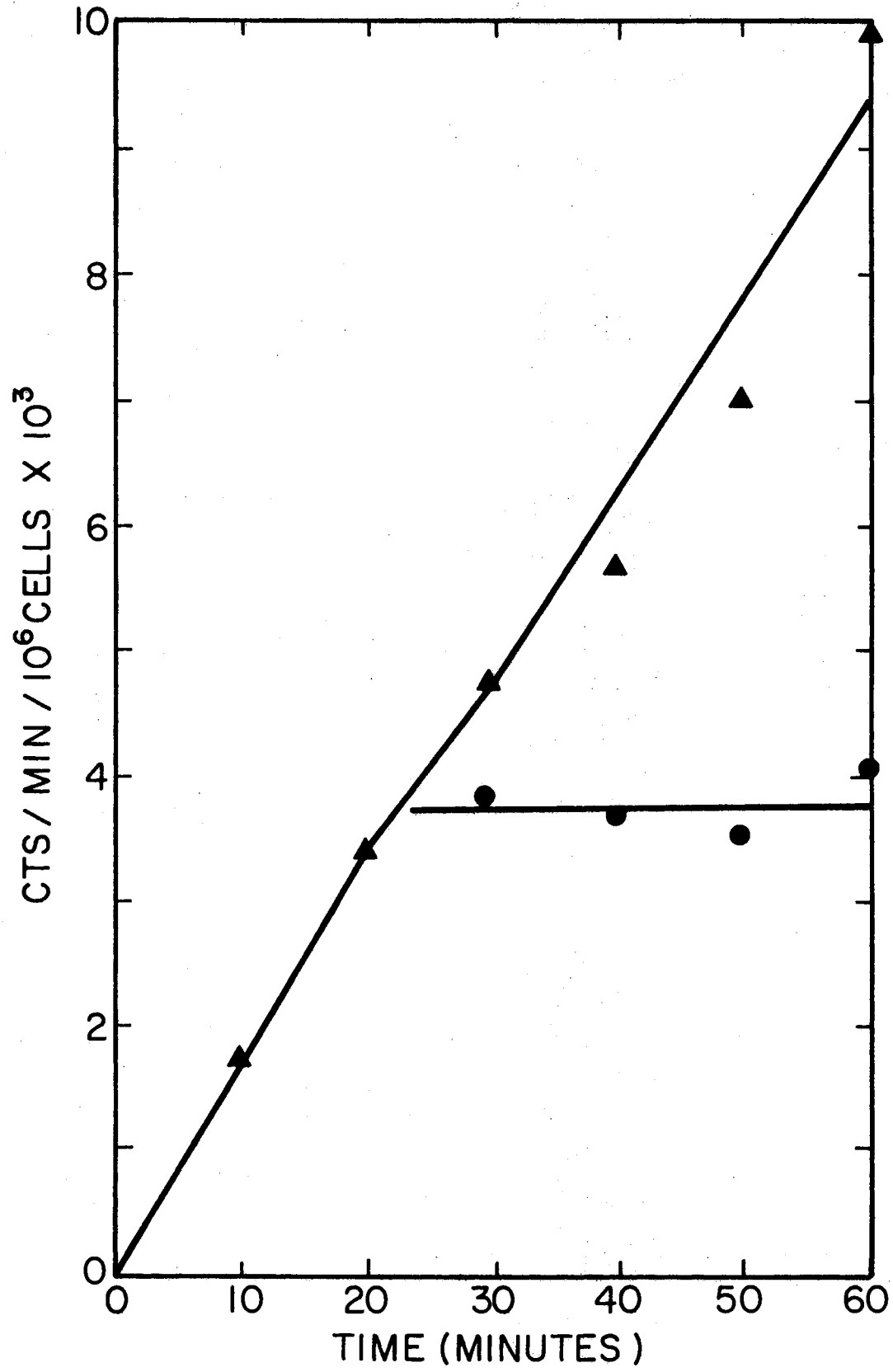
Figure 27. Accumulation of ^{14}C–Leucine by L–M Cells Prior to and Fol-
lowing Hydroxyimine Addition. ▲, control; ●, 8 x 10^{-5} $\underline{M}$
hydroxyimine added at 25 minutes.

concentrate the labeled leucine.

These results indicate that the action of the hydroxyimine on L-M cells is almost immediate which would imply a direct interaction with the plasma membrane resulting in blockage of permeation.

Experiments were conducted in which the methoxyimine (7.6×10^{-5} $\underline{M}$) was added to L-M cells immediately following addition of ^{14}C-leucine (Figure 28). As observed with the hydroxyimine, the methoxyimine greatly reduced the ability of the mouse cells to accumulate the labeled leucine. Only about 10 percent of the radioactivity was accumulated by the methoxyimine treated culture as compared to the control.

Comparison of Hydroxyimine Inhibition Kinetics
With Sodium Azide

Highly conjugated molecules possessing polarizable groups like the imine and hydroxy functions of the test compounds are capable of acting as electron donor-acceptor molecules and could interfere with electron transfer thereby blocking energy production. To test the influence of steroids on the energy available for accumulation and maintenance of precursor "pool" materials, the accumulation of ^{14}C-leucine was measured in mouse L-M cells treated with water, hydroxyimine or sodium azide, a known metabolic inhibitor (Packer et al., 1959).

Triplicate L-M cultures were supplemented with labeled ^{14}C-leucine and the accumulation of radioactivity followed for 20 minutes. The cultures were then supplemented with water (control), 8×10^{-5} $\underline{M}$ hydroxyimine or 8×10^{-5} $\underline{M}$ sodium azide and the determination of radioactivity continued. Hydroxyimine (8×10^{-5} $\underline{M}$) caused an immediate inhibition of substrate accumulation while the same concentration of sodium azide

Figure 28. Accumulation of ^{14}C–Leucine by L–M Cell Cultures in the Presence and Absence of Hydroxyimine. ▲, control; ●, 7.6 x 10^{-5} M methoxyimine.
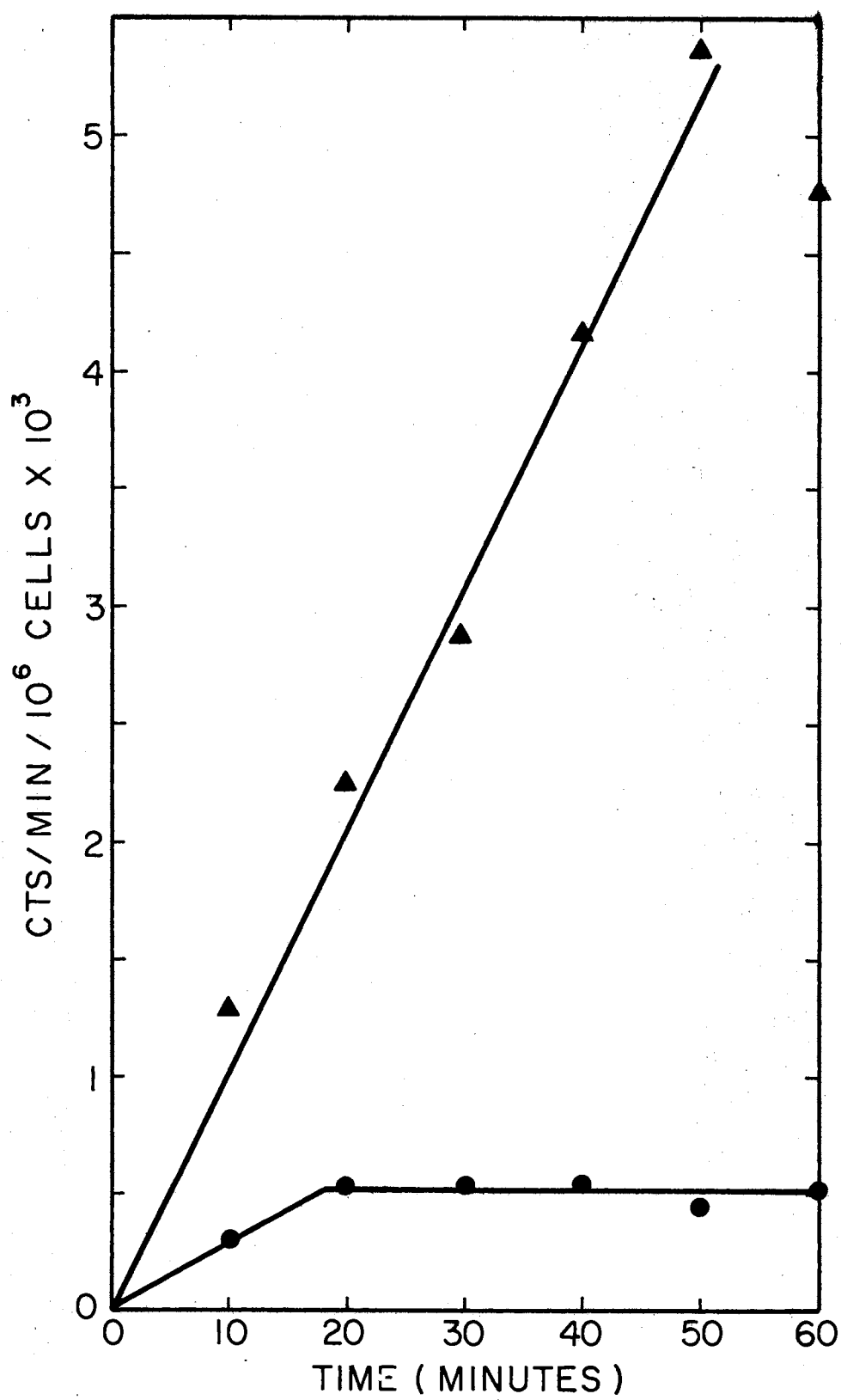
$(8 \times 10^{-5} \underline{\underline{M}})$ caused no inhibition of leucine accumulation (Figure 29). These data indicate that on a molar basis, the action of the hydroxy-imine is much more efficient in affecting the transport mechanism than the election transport inhibitor, sodium azide.

A comparison of the kinetics of accumulation of labeled leucine in L-M cells is shown in Figure 30. The cells accumulated ^{14}C-leucine for 25 minutes at which time $8 \times 10^{-5} \underline{\underline{M}}$ hydroxyimine or $1 \times 10^{-3} \underline{\underline{M}}$ sodium azide was added to the cultures and the determination of radio-active accumulation continued. At the increased azide concentration, the inhibition patterns caused by the hydroxyimine and the azide were initially similar but an increase in the release of labeled precursors from the azide treated culture was noted between 50 and 90 minutes of incubation.

Because the inhibition patterns between the two molecules are similar, no definitive evaluation of steroid action can be made. The inhibition pattern similarities support the hypothesis that the steroids interfere with energy coupled transport.

Hydroxyimine Induced Leakage of Metabolic Pre-cursor Molecules From Mouse L-M Cells

To augment the bacterial studies on steroid induced "pool" release, the leakage of the non-metabolizable substrate, α-aminoisobutyric acid (Christensen, Parker and Riggs, 1958) was tested in mouse L-M cells.

Cells were labeled for 40 minutes with ^{14}C-α-aminoisobutyric acid, divided into 3 equal volumes, centrifuged, washed and suspended in medium supplemented with water (control), $8 \times 10^{-5} \underline{\underline{M}}$ hydroxyimine or 1 percent Triton X-100. Samples were removed, filtered and the retention of

Figure 29. The Effects of Equal Molar Concentrations of Hydroxyimine
and Sodium Azide on the Accumulation of ^{14}C-Leucine by
Mouse LM Cells. ▲, control; ■, 8 x 10^{-5} $\underline{M}$ sodium azide;
●, 8 x 10^{-5} $\underline{M}$ hydroxyimine; test compounds added at 25
minutes.
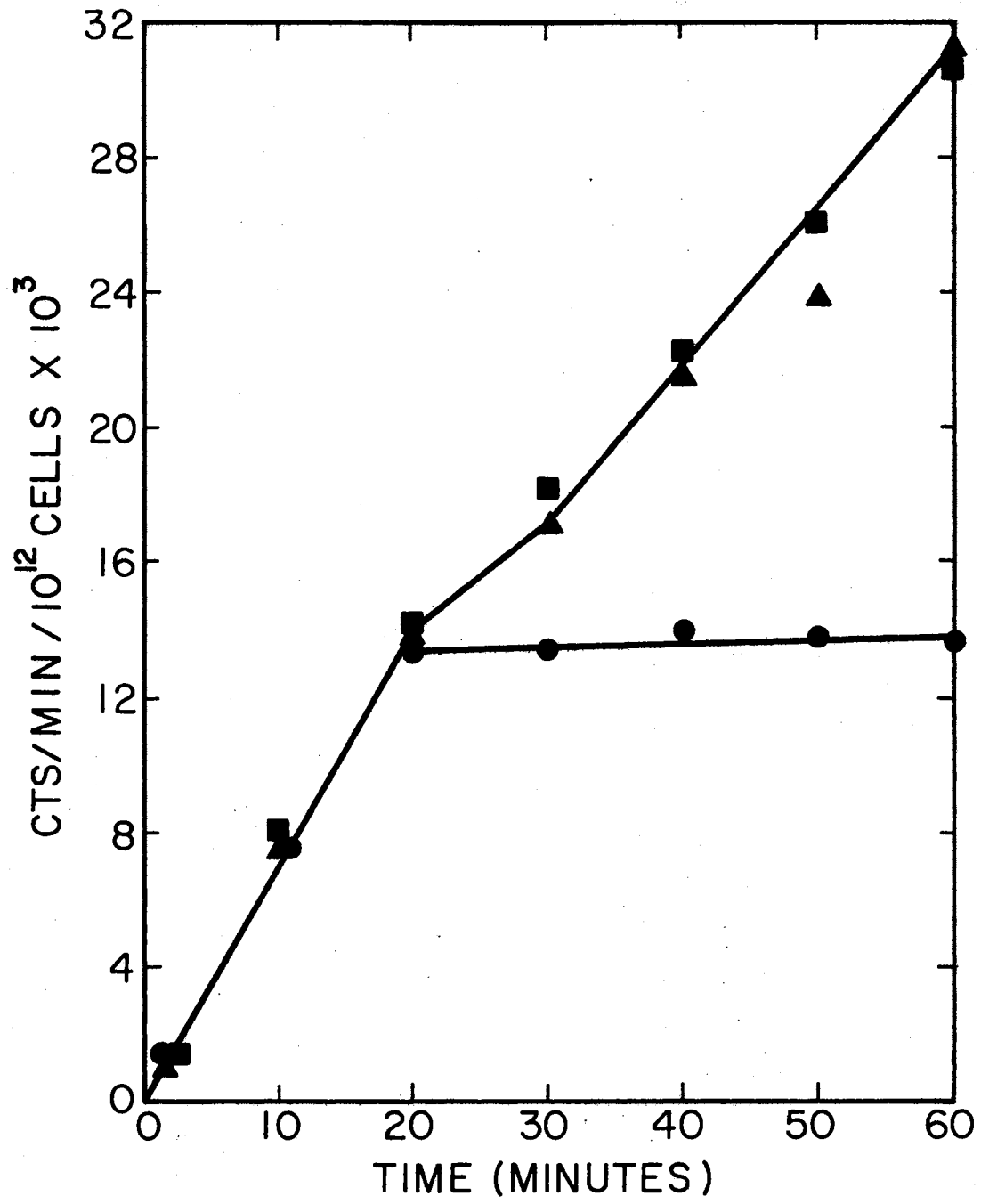
Figure 30. A Comparison of the Effect of 10^{-5} $\underline{M}$ Hydroxyimine and 10^{-3} $\underline{M}$
Sodium Azide on the Accumulation of ^{14}C-Leucine by Mouse
LM Cells. ▲, control; ●, 8 x 10^{-5} $\underline{M}$ hydroxyimine; ■,
1 x 10^{-3} $\underline{M}$ sodium azide; test compounds added at 25
minutes.
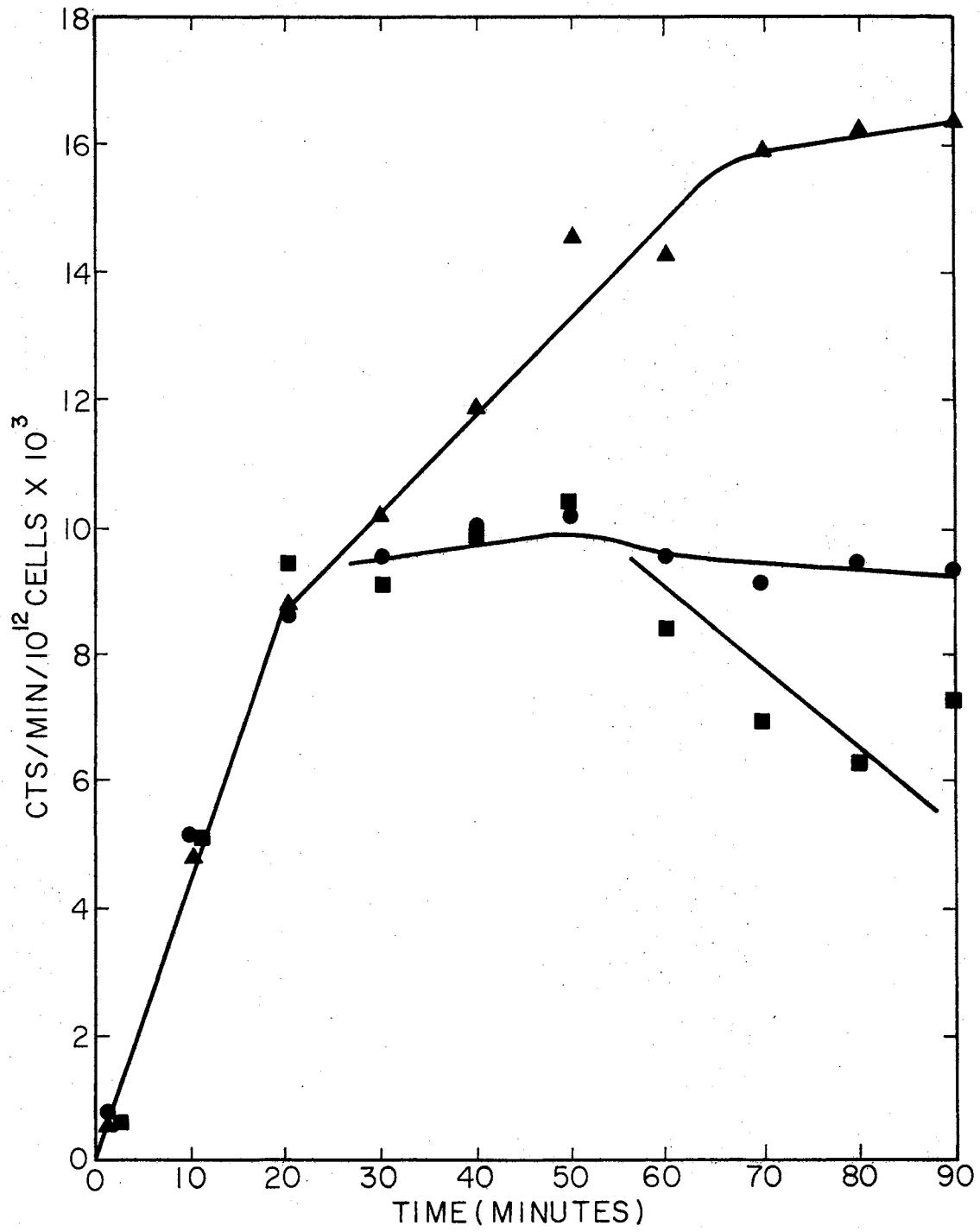
radioactivity determined.

The results (Figure 31) indicated no "pool" leakage from steroid treated cells when compared to the water treated control culture. The detergent, Triton X-100, caused a very rapid and complete release of the labeled substrate.

The observations shown in Figure 31 represent events occurring within 2 minutes following steroid addition. Similar experiments conducted for as long as 40 minutes indicated that the hydroxyimine does not affect the loss of labeled substrate from the cell.

Hydroxyimine Binding to Isolated L-M Cell
Membranes

To ascertain the extent of hydroxyimine association with L-M cell membranes, binding to isolated cell membranes was studied utilizing the azasteroids fluorescent properties and isolated cell membranes.

Samples of prepared membranes (5 ml containing approximately 20 mg dry wt of membrane material) were treated with medium containing water (control) or 1.8×10^{-4} $\underline{M}$ hydroxyimine. The suspension was incubated for 30 minutes at 5 C, pelleted and washed with fresh cold medium. The pellet was suspended in medium at 25 C and the percent emission determined photofluorometrically.

Approximately 1.2×10^{-6} moles of hydroxyimine (0.3 μg) was associated with each milligram dry weight of cell membrane. This would indicate that about 4 percent of the initial hydroxyimine concentration was associated with the cell periphery. Binding of steroids to the L-M cell membrane must be of a highly specific nature and indicates that the compound must act efficiently in establishing its effects.

Figure 31. Leakage of ^{14}C-α-Aminoisobutyric Acid From Hydroxyimine
            Treated Mouse LM Cells. ▲, control; ●, 8 x 10^{-5} $\underline{M}$
            hydroxyimine; ■, 1 percent Triton X-100.
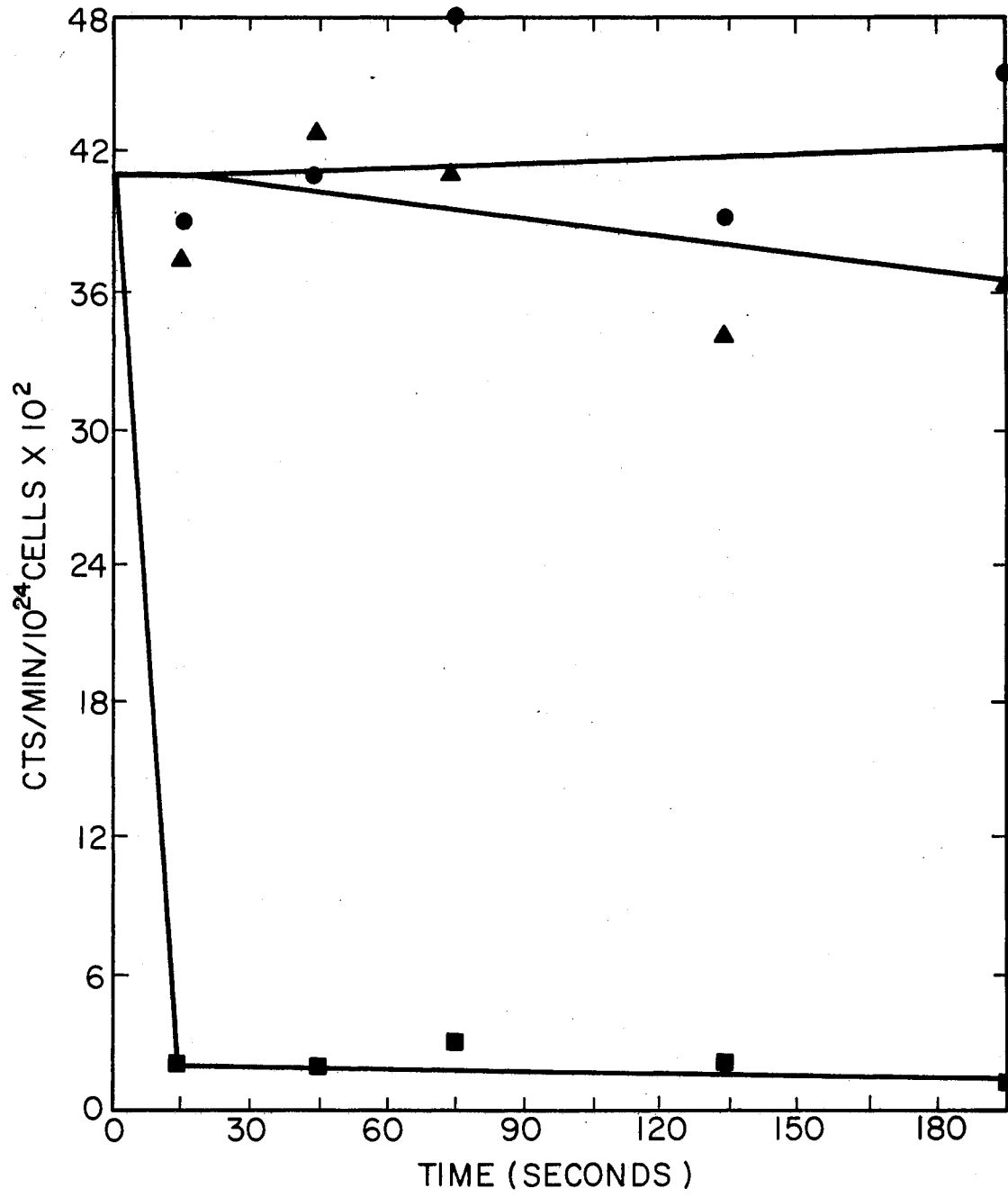
Fluorescent Microscopic Determination of the

Test Compounds in Cell Culture Systems

The fluorescent properties of the test compounds were utilized to determine the distribution of the steroids within the different cell lines.

Coverslip-grown cells were incubated 24 hours in medium supplemented with 4×10^{-5} $\underline{\underline{M}}$ hydroxyimine, methoxyimine or water (control). The coverslips were washed, sealed in wet mounts with warm petrolatum and observed immediately.

Figure 32a shows a WI-38 human lung cell photographed using dark-field microscopy and Figure 32b shows this same lung cell photographed by darkfield fluorescent microscopy. The extensive intracellular fluorescence exemplifies the wide distribution of the steroid within the cytoplasm. Control cells under these same conditions do not fluoresce.

The methoxyimine tested under these same conditions gave similar results although the fluorescent intensity was partially diminished. The decreased intensity could result from a lower intracytoplasmic concentration of methoxyimine although quantitation using this method has not been perfected.

Figure 33a shows a mouse L-M cell photographed by a combination darkfield and darkfield fluorescence technique in which the non-fluorescent areas of the cell are illuminated by the tungsten lamp and appear as a golden color. The fluorescence of the hydroxyimine is seen as a blue green color and has a rather wide distribution throughout the cytoplasm. Distinct bodies of more intense fluorescence can also be seen within the cytosol and probably represent vacuoles of the concentrated steroid.

Figure 32a. Darkfield Photomicrograph of a WI-38 Human Lung Cell
             (2800 X)

Figure 32b. Darkfield Fluorescence of the Same WI-38 Cell Shown in
             Figure 32a (2800 X).
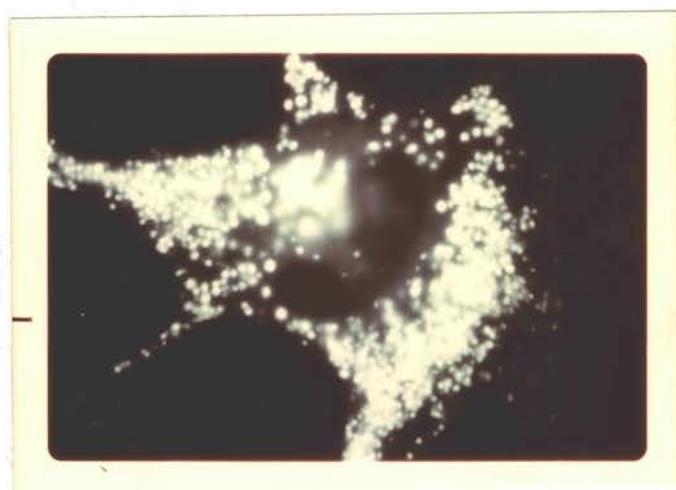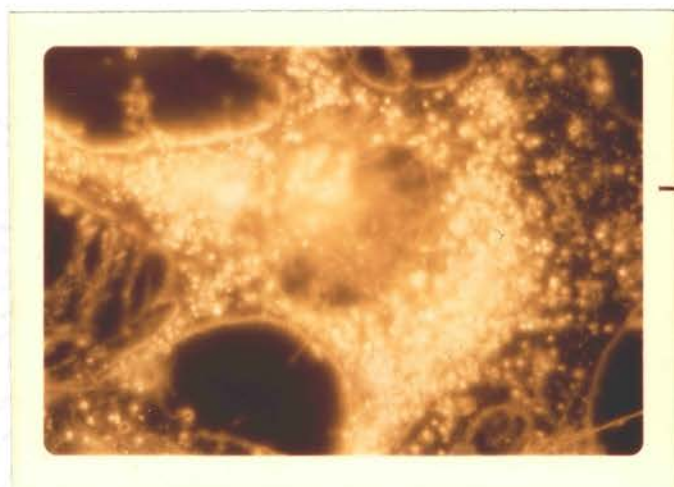
Figure 33b shows hydroxyimine treated cells growing in a monolayer which were isolated from the rat mammary tumor R3230 AC and photographed via brightfield fluorescence microscopy. As with the previous cell types, there appears to be a wide distribution of fluorescent steroid within the cytoplasm. The results of such microscopic observations indicate that the steroid molecule readily enters a variety of different human and rodent cell lines.

Determination of Hydroxyimine Estrogenic Activity, Effect on Animal Behavior and Assessment of Organ Toxicity

Studies, conducted under the direction of Dr. C. Desjardins, Department of Physiological Sciences, Oklahoma State University, were made to test the hydroxyimine for estrogenic activity and at the same time assess the effect of the compound on animal behavior and major body organs.

Ovariectomized female mice (ICR-Swiss) were weighed and then injected (0.1 ml) once daily for 7 days with sesame oil solutions of the various test compounds. Total injections consisted of 5.1×10^{-10} moles estradiol (estrogen control), 1.6×10^{-9} moles or 8.4×10^{-9} moles hydroxyimine, sesame oil (carrier control), or no injection (ovariectomy control). Twenty-four hours following the last injection the animals were weighed then killed by cervical dislocation. Uteri were removed, weighed (wet weight) then dried at 110 C for 24 hours and weighed (dry weight).

Table V shows the effect of estradiol and hydroxyimine on the body and uterine weight. The body weight under all test and control conditions

Figure 33a. Combination Darkfield and Darkfield Fluorescence Photo-
             micrograph of a Mouse L-M Cell (2800 X).

Figure 33b. Brightfield Fluorescence Photomicrograph of Cells Isolated
             From an R-3230 AC Rat Mammary Tumor (450 X).
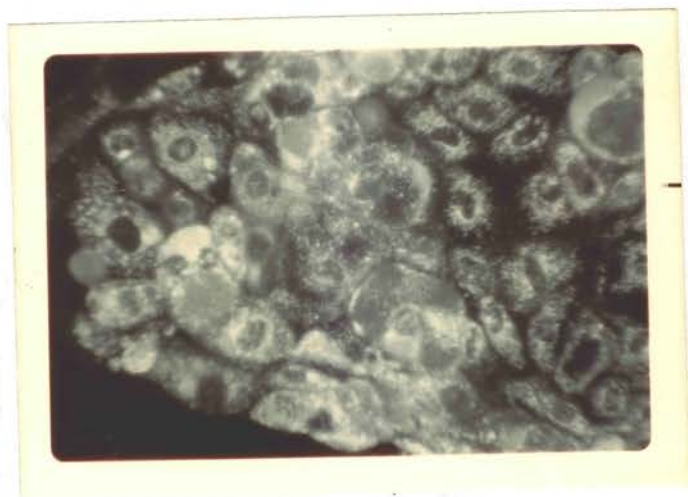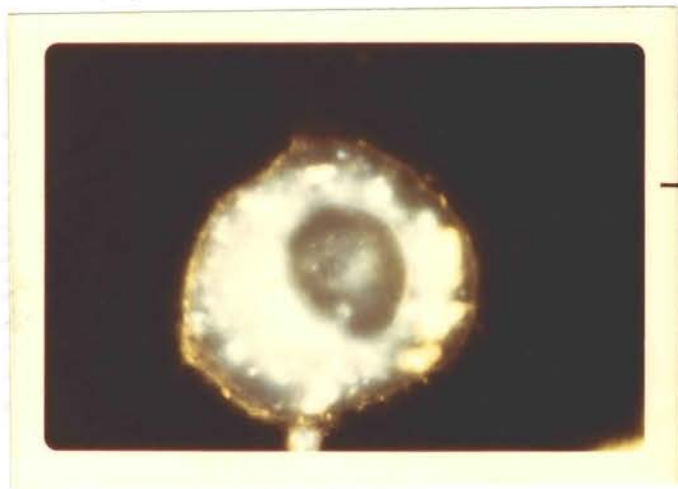
showed little change while the uterine weight increased nearly 5 fold

in response to estrogen stimulation. Mice injected with 1.6×10^{-9} moles

or 8.4×10^{-9} moles hydroxyimine showed no significant differences in

uterine weight when compared to the non-estrogenic stimulated animals

indicating that the hydroxyimine possesses no estrogenic activity.

Studies performed in conjunction with the estrogenic activity in-

cluded a subjective analysis of the behavior or treated and non-treated

animals and an inspection of major organs (lungs, liver, spleen, in-

testine, kidney and mammary gland) for gross lesions.

Observations, made daily at the time of injection, failed to reveal

any noticeable behavior differences between treatment groups. Inspection

of major organs following uterus removal failed to yield any detectable

or striking differences between treated and non-treated animals.

It should be noted that dissimilar results would probably be ob-

tained if a different vehicle (saline or water) had been used which

would have allowed a more rapid dissemination of the test compounds.

Nevertheless, the procedures employed in the present study are routinely

used to assess toxicity and/or efficacy on all types of steroid prepara-

tions (Zarrow, Yochim and McCarthy, 1964).

TABLE V

EFFECTS OF ESTRADIOL AND HYDROXYIMINE ON BODY AND UTERINE
WEIGHT OF MATURE OVARIECTOMIZED MICE*

Material Injected	Amount Injected Per Mouse Over 7 Days (moles x 10^{-9})	Body Weight (g)		Uterine Weight (mg)	
		Initial	Final	Wet	Dry
Estradiol	0.51	27.2 ± 1.1	28.3 ± 0.9	67.4 ± 4.3	16.6 ± 0.5
Hydroxyimine	1.6	26.9 ± 1.8	28.0 ± 1.5	17.3 ± 1.9	3.4 ± 0.5
Hydroxyimine	8.4	27.1 ± 1.5	27.5 ± 1.4	16.4 ± 2.4	3.6 ± 0.3
Sesame Oil	—	27.3 ± 1.6	28.4 ± 1.2	15.5 ± 2.1	3.5 ± 0.2
No Injection	—	26.4 ± 1.2	27.0 ± 2.0	12.8 ± 2.4	3.1 ± 0.3

*Mice were injected with the indicated compounds in 0.1 ml of sesame oil for 7 days. Body and uterine weight values represent the mean ± standard error of 10 mice.

CHAPTER IV

SUMMARY AND CONCLUSIONS

Physical data regarding the ultraviolet absorption properties of the azasteroids revealed prominent peaks at 260 and 334 nm. Quantitation of steroid concentrations by measuring absorption peak intensities allowed construction of standard curves which were used to moniter test compound stability under growth conditions as well as quantitating various steroid solutions.

The compounds are highly fluorescent molecules; both the emission wavelengths and the quantum yield of the hydroxyimine varied greatly in relation to the solvent polarity. These properties make possible the use of the hydroxyimine as a fluorescent probe or reporter molecule (at 10^{-5} to 10^{-9} $\underline{M}$ concentration) which could reveal the nature of the microenvironment of the compound when associated with a biological component.

Since the azasteroids were structurally related to the potent estrogen, equilenin, whole animal studies were conducted to evaluate the estrogenic potential of the new azasteroids. The azasteroids showed no estrogenic activity or toxicity for mice at concentrations up to 16 times that required for estradiol to produce maximum estrogenic response. While these data are not adequate to assess mammalian toxicity, they suggest the potential of the new compounds in chemotherapy without undesirable hormonal side effects.

The stability of the hydroxyimine was determined under growth conditions. Incubation of the hydroxyimine in a cell free preparation of minimal medium revealed that the half-life of the steroid was slightly greater than 1 hour. Continued incubation resulted in further alteration(s) of the steroid molecule until after 13 hours of incubation only about 12 percent of the original compound remained in the medium as measured. A biological assay was also performed. B. subtilis cells were inoculated into the 13 hour incubated hydroxyimine medium and showed only a 2 hour lag and slightly decreased growth rate while the incubation medium supplemented with fresh hydroxyimine caused complete inhibition of B. subtilis growth.

Biological assay of the hydroxyimine stability when incubated in cell culture medium under growth conditions for up to 7 days revealed no loss of L-M cell growth inhibition activity.

These data indicate the susceptibility (probably towards oxidation) of the hydroxyimine molecule when aerated by shaking. Figure 34 shows one such possible mechanism by which the hydroxyimine could undergo this type of oxidation. Steroid oxidation is probably less prominent in the presence of cells or the complex cell culture medium which, as a result of weak interactions, might afford some steroid protection.

The azasteroids inhibit the growth of several different bacteria as well as the human KB and mouse L-M cell lines. Growth of the gram-positive microorganisms tested (B. subtilis, B. megaterium and S. aureus) was most susceptible to the azasteroid induced inhibition. Test compound concentrations of approximately 2×10^{-4} $\underline{M}$ caused complete growth inhibition in these organisms. The gram-negative bacteria tested (E. coli, Flavobacterium sp. and P. fluorescens) were, as a group, less

Figure 34. A Possible Mechanism of Hydroxyimine Oxidation.

OXIDATION PRODUCTS
(QUINONES, ETC.)
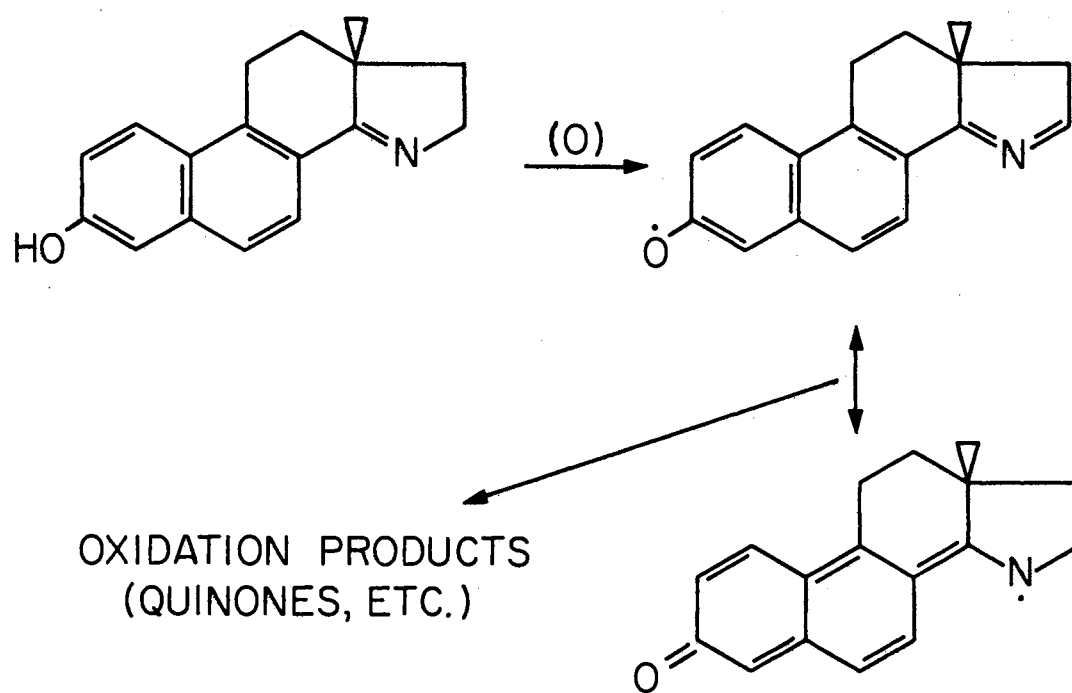
sensitive to the steroid induced inhibition. The highest azasteroid concentrations tested (approximately 2×10^{-4} $\underline{M}$) caused substantial reduction in the growth rate of $\underline{E}$. $\underline{coli}$ and $\underline{Flavobacterium}$ sp. cultures while the same steroid concentration in $\underline{P}$. $\underline{fluorescens}$ showed little effect. The decreased sensitivity of gram-negative microorganisms to azasteroid effects had been noted by earlier workers in this area (Smith, Shay and Doorenbos, 1963; Varricchio, Doorenbos and Stevens, 1967) and probably represents the inability of the steroid molecule to permeate the complex outer gram-negative cell wall.

Viable cell determinations of $\underline{B}$. $\underline{subtilis}$ in the presence of the test compounds showed that, at the maximum concentrations tested, neither steroid was bacteriocidal. Further studies revealed that incubation in the test compounds for up to 1.5 hours did not significantly alter the steroid sensitivity of the viable cell population.

The hydroxyimine inhibition of $\underline{B}$. $\underline{subtilis}$ growth was reversed on removal of the steroid from the growth medium although the growth rate of the reversal system was slightly less than the control. These data augment the viable cell count results and show that the action of the steroids on the bacterial cell is of a more subtile nature than the complete disorganization caused by many detergent molecules.

The growth of mouse L-M cells incubated in suspension culture was completely inhibited by 8×10^{-5} $\underline{M}$ hydroxyimine while 7.6×10^{-5} $\underline{M}$ methoxyimine caused only about a 1 day growth lag. In a different cell system, the human tumor cell line, KB, was utilized and neither azasteroid inhibited the initial attachment of KB cells to the culture plate surface. Once attached, cells incubated in the hydroxyimine (7×10^{-5} $\underline{M}$) began to detach from the plate surface and, therefore, were

unable to grow and form colonies. After 6 hours of incubation with hydroxyimine, approximately 50 percent of the cell population had released and by 24 hours only 18 percent of the cells remained attached to the plate surface. The methoxyimine at approximately the same concentration (7.6×10^{-5} $\underline{M}$) had little, if any, effect on the ability of the cells to remain attached to the culture plate surface and grow to form colonies.

Supplementary experiments in which the plating efficiency of cells in suspension at the time of steroid addition were compared to cells which had been allowed to attach prior to steroid addition revealed that the inhibition of plating efficiency established by the hydroxyimine was equally effective against suspended compared to attached KB cells. In contrast, the methoxyimine had little inhibitory effect on plating efficiency when added to suspended KB cells, but reduced their plating efficiency to 27 percent when the cells were allowed to attach prior to methoxyimine addition. These data could be interpreted as a reflection of the differences in the cell periphery between attached and suspended cells which might result in an altered steroid/cell interaction.

As with $\underline{B}$. $\underline{subtilis}$, the growth inhibition of L-M cells by the hydroxyimine (8×10^{-5} $\underline{M}$) was readily reversed upon removal of the test compound from the growth medium.

Tests concerning the effects of the hydroxyimine on $\underline{B}$. $\underline{subtilis}$ protoplast stability revealed no lytic action at the steroid concentrations tested. Subsequent binding studies of the hydroxyimine to the protoplasts showed little steroid/protoplast association.

The methoxyimine was less toxic to cell culture cells and was

therefore tested for its ability to potentiate the action of several selected antibacterial compounds. The methoxyimine greatly enhanced the antibacterial activity of vancomycin and chloramphenicol against B. subtilis and the action of polymyxin and circulin against the growth of P. fluorescens. Several other antibiotics tested showed only additive or less than additive effects.

The inhibition of substrate accumulation was reversible. B. megaterium cells, initially exposed to the hydroxyimine for 0.5 minute, required approximately 75 minutes of incubation in steroid-free medium before regaining the ability to accumulate substrates at the control rate.

The azasteroids, several of which can be classified as weak cationic detergents (Harold, 1970), were studied to determine if the compounds caused an increase in cell membrane permeability which could result in "pool" leakage and thus lower the accumulation levels. B. megaterium and mouse L-M cells were labeled with the non-metabolizable substrate, α-aminoisobutyric acid, and treated with the test compounds. No leakage occurred following hydroxyimine treatment in either the bacterial or the cell culture system indicating no general permeability alterations of the cell.

In the bacterial system, the membrane stabilizing effects of magnesium were tested using B. subtilis. Addition of equimolar concentrations of magnesium prior to or following hydroxyimine or methoxyimine treatment did not influence the growth inhibition.

The hydroxyimine rapidly inhibited the accumulation of lysine, leucine, serine, alanine, α-aminoisobutyric acid and uracil by mouse L-M cells. Further, addition of the steroid to L-M cultures for as long

as 25 minutes after initiation of radioactive substrate accumulation caused rapid termination of label accumulation.

The methoxyimine, while not tested in the bacterial system, was equally effective in inhibiting the accumulation of labeled precursors in the cell culture system.

Binding experiments, in which isolated mouse L-M cell membranes were treated with the hydroxyimine, revealed that only 1.2×10^{-6} moles (4 percent of the total added) hydroxyimine per mg dry wt of membrane remained associated after one washing. Thus large quantities of the azasteroid molecules do not associate with the cell membrane and that the action of the steroid is probably of a specific nature.

Since the inhibition of substrate accumulation could not be explained by membrane leakage, experiments were performed to compare the kinetics of uptake inhibition between the hydroxyimine and a known electron transport inhibitor, sodium azide. Hydroxyimine (8×10^{-5} $\underline{\underline{M}}$) completely inhibited the uptake of labeled leucine in mouse L-M cells, while an equimolar concentration of sodium azide had no effect on the uptake. These data suggested that the steroid was either effecting the uptake system in a manner different from sodium azide or that the azasteroid molecule was much more efficient in uncoupling the energy-related process. Increasing the sodium azide concentration to 1×10^{-3} $\underline{\underline{M}}$ produced an inhibition similar, although not identical, to the hydroxy-imine. Because of the similarity between the azasteroid and sodium azide inhibition kinetics, azasteroid disruption of electron transport remains a distinct possibility.

This investigation establishes that the new 15-azasteroid molecules possess a wide spectrum of biological activity. Both molecular

specificity and variations in organism susceptibility were observed within the bacterial and cell culture systems employed.

The inhibition of growth may result from the steroid induced inhibition of substrate accumulation. Since no evidence for membrane disruption or "pool" leakage was found, the movement of molecules into the cytoplasm must be inhibited by the test compounds. Bacterial and cell culture growth inhibition as well as the inhibition of substrate accumulation in the bacterial system were reversible.

Studies showed no lytic effect following addition of the hydroxy-imine. These data augment the results obtained with leakage studies showing that the perturbations caused by the steroid molecules, while sufficient to produce the observed effects, are not of a detergent-like or dissagregative nature.

The inhibition of substrate accumulation was rapidly established following addition of the test compounds suggesting the initial hypothesis that the steroid acts at the cell membrane. A wide variety of substrate molecules were affected. The uptake of various amino acids, glucose and uracil were all inhibited by the test compounds in both the bacterial and cell culture systems. These findings strongly implicate a general effect on overall cell permeability distinct from a competitive inhibition or specific interaction with binding proteins or translocation mechanisms.

Binding studies in which the association of the hydroxyimine with both protoplasts and isolated L-M cell membranes revealed that a relatively small quantity of the steroid is bound to the membrane. As a result, the action of the steroid on the membrane must be specific and efficient in nature.

There are several possible mechanisms by which the steroid molecules could bring about the general inhibition of uptake. One such mechanism could involve the inhibition of energy available to active transport systems. Results from the comparison of inhibition kinetic patterns between the hydroxyimine and sodium azide showed that the steroid was a much more efficient inhibitor since much higher concentrations of sodium azide (13 fold) were required to produce similar although not identical inhibition patterns.

Both the hydroxy- and methoxyimine molecules have the potential to act as an electron donor-acceptor molecule forming a relatively stable benzylic type radical (Figure 35). Once formed, this compound would not readily lose the electron to other molecules and could thereby block electron transfer. Alternatives to radical formation might involve the interaction of the azasteroids with other aromatic electron transfer molecules such as nicotinamide adenine dinucleotide or flavine adenine dinucleotide through charge transfer of pi orbital overlap. These interactions could alter or destroy the electron transfer capabilities of these vital electron transfer molecules.

Another mechanism by which the azasteroids could cause a non-specific uptake inhibition would involve the direct interaction of the steroid molecule with cell membrane structural components. Through charge transfer interactions or pi orbital overlap with aromatic amino acids, the steroid molecules could directly alter the local charge of protein or lipid molecules which could bring about sufficient conformational changes to completely disrupt the uptake process. In addition to direct interaction with the protein or lipid moieties of the surface membrane, disruption of the structured water (Drost-Hanson, 1971)

Figure 35. Models of Possible Benzylic Type Electron Acceptor Forms of
the Hydroxyimine.

ETC.
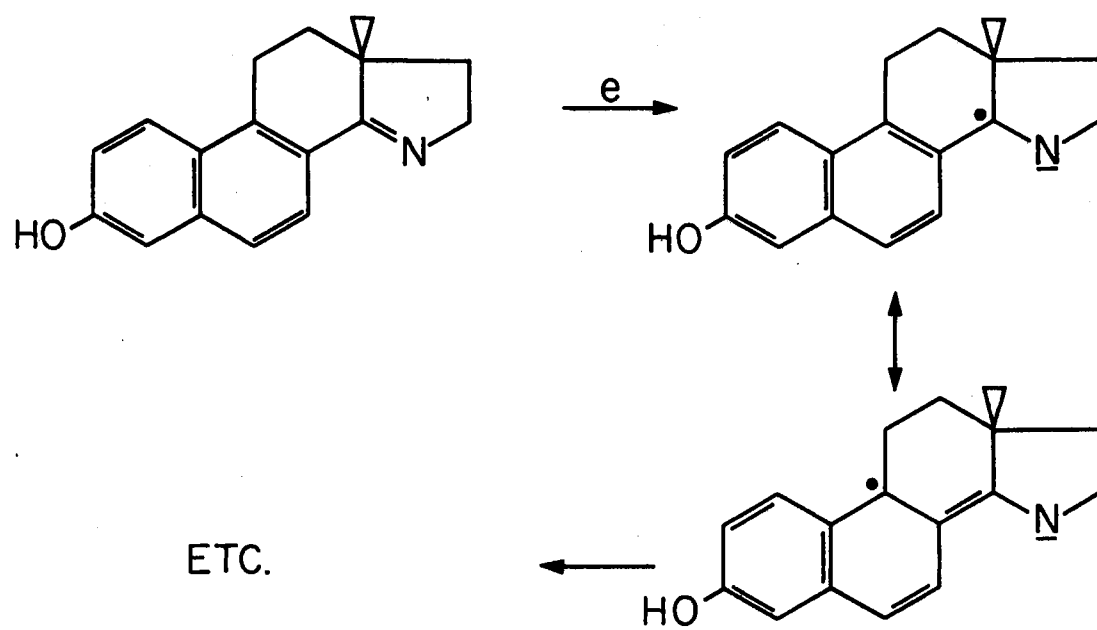
surrounding the membrane could cause sufficient loss in stabilization of proteins and/or lipids to produce conformational alterations.

Further studies concerning the effect of the steroid molecules on the energy production system within the cell as well as the influence of the test compounds on membrane and protein conformation will be necessary to explain fully the observed biological effects.

Fluorescent observations revealed that the steroid enters cell culture cells and is widely distributed throughout the cytoplasm. Based on this observation, it seems likely that the inhibition of uptake described above represents only one of many probable azasteroid biological activities. Manometric as well as specific enzyme studies will be necessary to fully elucidate the biological effects produced by these unique molecules.

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