THE EFFECT ON THE BIOLOGICAL ACTIVITY OF VARIABLE LIPOPHILIC CHARACTER OF SUBSTITUENTS ON FIVE NOVEL THIAZOLES

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CHAPTER I

INTRODUCTION

Studies of structure-activity relationships can provide a fundamental understanding of drug action at the molecular level and provide the foundation for rational drug design. A quantitative approach to drug action depends upon being able to express molecular structure in terms of numerical values, and then relating these values to corresponding changes in biological activity. This approach could facilitate the prediction of biological activity of novel compounds based on the physiochemical characteristics of existing related analogues.

Early attempts were made to quantitate a molecules interaction between a lipid phase and water and correlate this to its biological activity. The first parameters studied involved the physical measurement of the whole molecule and correlated activity that was largely independent of very small changes in structure. Meyer in 1899 and Overton in 1901 working independently were the first to draw attention to the significance of lipid solubility as a determinant of biological activity (Tute, 1971). They correlated the narcotic effect on amphibians of a wide variety of non-ionized compounds with the compounds oil-water partitioning; it was found that the compounds with a higher partition coefficient had greater narcotic activity. When lipid solubility became very high, and the substance was virtually insoluble in water, activity fell off. Ferguson (1939) later explained these results in

terms of a state of equilibrium where simple thermodynamic principles could be applied to drug activities; the important parameter to consider for the correlation of narcotic or depressant activities was the relative saturation of the substance in the applied phase (normally water). This has become known as Ferguson's principle. Expressing the results of earlier workers, Ferguson showed that for a large number of test objects, equi-toxic concentrations of both structurally related and unrelated compounds, possessed similar relative saturations in a particular biological system. Compounds active by virtue of highly specific chemical reactions or compounds interacting with different biological sites would not follow this principle.

The general anesthetics appear to be the only group of pharmacological compounds, for which structurally unrelated molecules have produced good correlations by Ferguson's principle (James, 1974). With homologous and analogous compounds, Ferguson's principle has found a more varied application. Burtt (1945) and Ferguson and Pirie (1948) demonstrated its validity with some volatile insecticide fumigants. Allawala and Riegelman (1954) investigating the bactericidal concentrations of some phenol analogues and Gilby and Few (1959) with the lysis of Micrococcus lysodeikticus protoplasts by alcohols, confirmed the applicability of Ferguson's principle to the activity of certain antimicrobial compounds.

In 1940 Hammett began looking at the contribution substituents made to the biological activity of the whole molecule. He showed that the chemical reactivity of meta and para substituted benzene derivatives could be correlated by

$$\log(K_x/K_H) = \rho\sigma x \qquad (1)$$

where K_H is the rate or equilibrium constant for the parent (unsubstituted) molecule and K_x is the rate for the substituted derivative. The substituent constant σ_x refers to the electronic effect of the substituent and is a parameter applied to many different reactions (characterized by different values of ρ) whose rate depends on the degree of electron release or withdrawal by the substituent. The equation is an example of a linear free-energy relationship (Ritchie and Sager, 1964) and is predictive in that knowledge of σ constants will enable an estimation to be made of the reactivity of an unknown compound. It is also diagnostic of a reaction type in that, if the equation is satisfied, one may say that an electronic effect of the substituent is important to the reaction mechanism.

Hansen (1962) attempted to set up a "biological Hammett equation" using data from the literature on the inhibition of growth of bacteria. The growth-inhibitory reaction was postulated to be a biomolecular one between the compound and an enzyme which either directly or by some feedback mechanism controlled growth. The approach gave rather disappointing results. Hansen admitted that some oversimplifications had been made in order to derive his equation. He had assumed that the same enzyme was involved for all members of the series, and that the substituents exerted their actions only by an electronic effect. Questions relating to varying ability to penetrate cell walls and variations in entropy due to different orientations on an enzyme had to be ignored. Hansen's approach still marked an important advance in the development of a theory for interdependence of biological activity and chemical structure.

At the same time, Zahradnik was also looking at the possibility of a biological Hammett equation (Tute, 1971). He generated the biological equivalent of Hammett's σ constant, the β constant, by constructing an equation corresponding the magnitude of the biological effect of a homologous series of compounds RX to their structure. A second constant, α, characterized the sensitivity of the biological system. When he determined β values for 25 alkyl groups and α values for 39 different biological systems, a reasonably good fit was found for the equation, especially, as Hansch (1966) pointed out, where highly specific electronic effects were not critical. Kopecky and Bocek (1967) reported that the β constants of Zahradnik were linearly related to the π values of Hansch for alkyl substituents and that both were measures of a substituent "activity coefficient". Through the studies of Hansch it was evident that β values were measures of the relative free-energy change associated with transfer of molecules from an aqueous to a lipid phase; the α values characterized the relative importance of this effect to the particular system under study (Tute, 1971).

The major limitation of the approaches discussed so far was that they concentrated on one parameter to the exclusion of others. The parameter derived was not fundamental and so it could not be used for prediction or for the diagnosis of mechanism. Correlation as an end in itself is of no use in developing a rationale for drug design.

The method developed by Hansch began with his work on the growth-promoting activity of phenoxyacetic acids in 1962. The approach was multi-parameter, including any aspect of a group's contribution to overall activity, and generating parameters that were fundamental and able to be fitted to linear free-energy relationships. Further, the Hansch model

is not restricted to an equilibrium situation, but takes into account the probability of a molecule reaching its site of action within the time interval of the assay and is thus more likely to be applicable to an in vivo biological test.

The basic Hansch equation was derived by considering the general case of a drug applied to any biological system (Hansch and Fujita, 1964). There are two quantities that can be measured: the dose (the amount of compound given) and the response (the biological activity). The response is determined by the structure, that is, by the physiochemical properties of the compound. Within a closely related or so-called congeneric series of compounds, changes in structure can be related to changes in biological activity.

Collander (1954) had shown that the rate of transport of many organic compounds through the cellular material of Nitella cells was proportional to the logarithm of their partition coefficients between an organic solvent and water. Collander's work on log P and transport was re-examined by Milborrow and Williams (1968), who confirmed the original findings. This led to the assumption that absorption from the applied phase and ensuing transport to a sensitive site was highly influenced by the lipophilic-hydrophilic balance expressed as a partition coefficient.

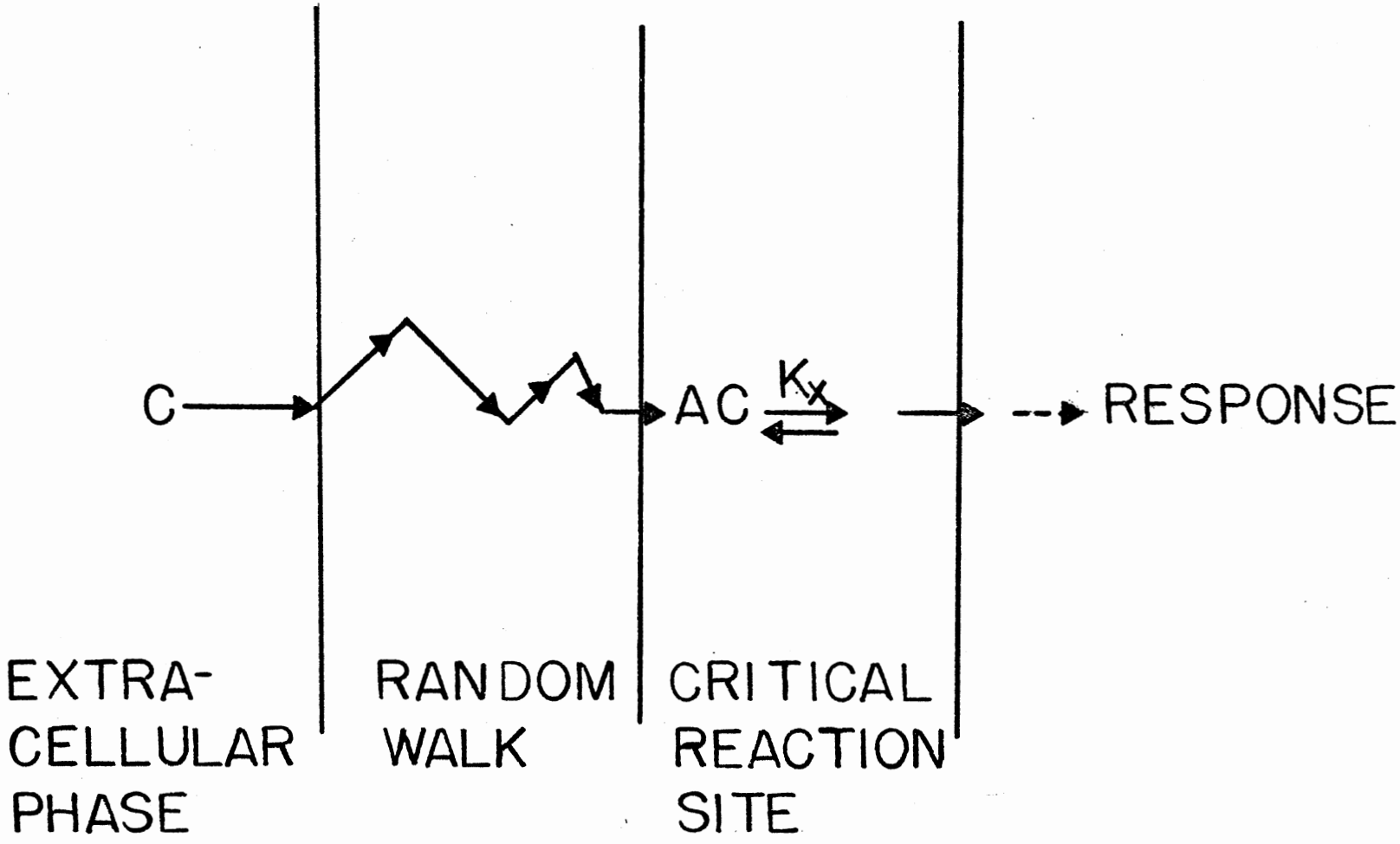
Although many factors may be involved in a biological system, Hansch assumed that for any congeneric series one particular "reaction" would be rate controlling. The development of his equation is then analogous to the derivation of equations expressing reaction rate from simple kinetic theory. Their initial equation, the basis for their model of drug-induced biological response, is an expression for the rate of biological response.

$$\frac{d(\text{response})}{dt} = ACK_x \qquad (2)$$

K_x is an equilibrium or rate constant for the rate-determining reaction which is not necessarily the site of action of the drug. C is the applied concentration and A represents the probability of a molecule reaching this critical site in a given time interval. Figure 1 expresses this idea: the molecules, after being given as a dose C, make their way through cellular material by what Hansch calls a "random walk" process, an effective concentration AC accumulating at the critical site (Hansch and Fujita, 1964). The passive permeation of a molecule would depend more on the general physical properties of the molecule and much less on the small differences, such as structure, stereochemistry, size and charge which are crucial in active transport (Penniston et al., 1969).

Inherent in this model is that during passive transport across the membrane, molecules are extracted by the lipid portion of the cell membrane on one side, and re re-extracted by the aqueous phase on the opposite side. The drug encounters the membrane during its random walk motion and its ability to be taken into the lipid matrix is proportional to its hydrophobic binding capacity. Its ability to leave the lipid phase is inversely proportional to its hydrophobicity. Hansch actually envisioned a series of aqueous and lipid layers through which a drug must pass in order to gain access to its site of action within. Substances of low water solubility would be unable to penetrate the aqueous layers and would accumulate in the lipid region (Hansch, 1966). Similarly, those with low oil solubilities would be unable to cross the lipophilic barriers. Hansch proposed that compounds between these two extremes must exist which possess the optimum balance between hydropholicity and lipophilicity for traversing cell barriers.

Figure 1. Hansch's Proposed "Random Walk"
Model of Drug Activity


C
AC
K_x
RESPONSE
EXTRA-
CELLULAR
PHASE
RANDOM
WALK
CRITICAL
REACTION
SITE

Considering Collander's work and using his model as a working hypothesis, Hansch related A to log P and changes in A, in a congeneric series, to changes in log P. These changes were expressed in the form of substituent constants which Hansch et al. (1963) have termed π constants, defining π by the Hammett-like equation of

$$\pi = \log(P_x/P_H)$$

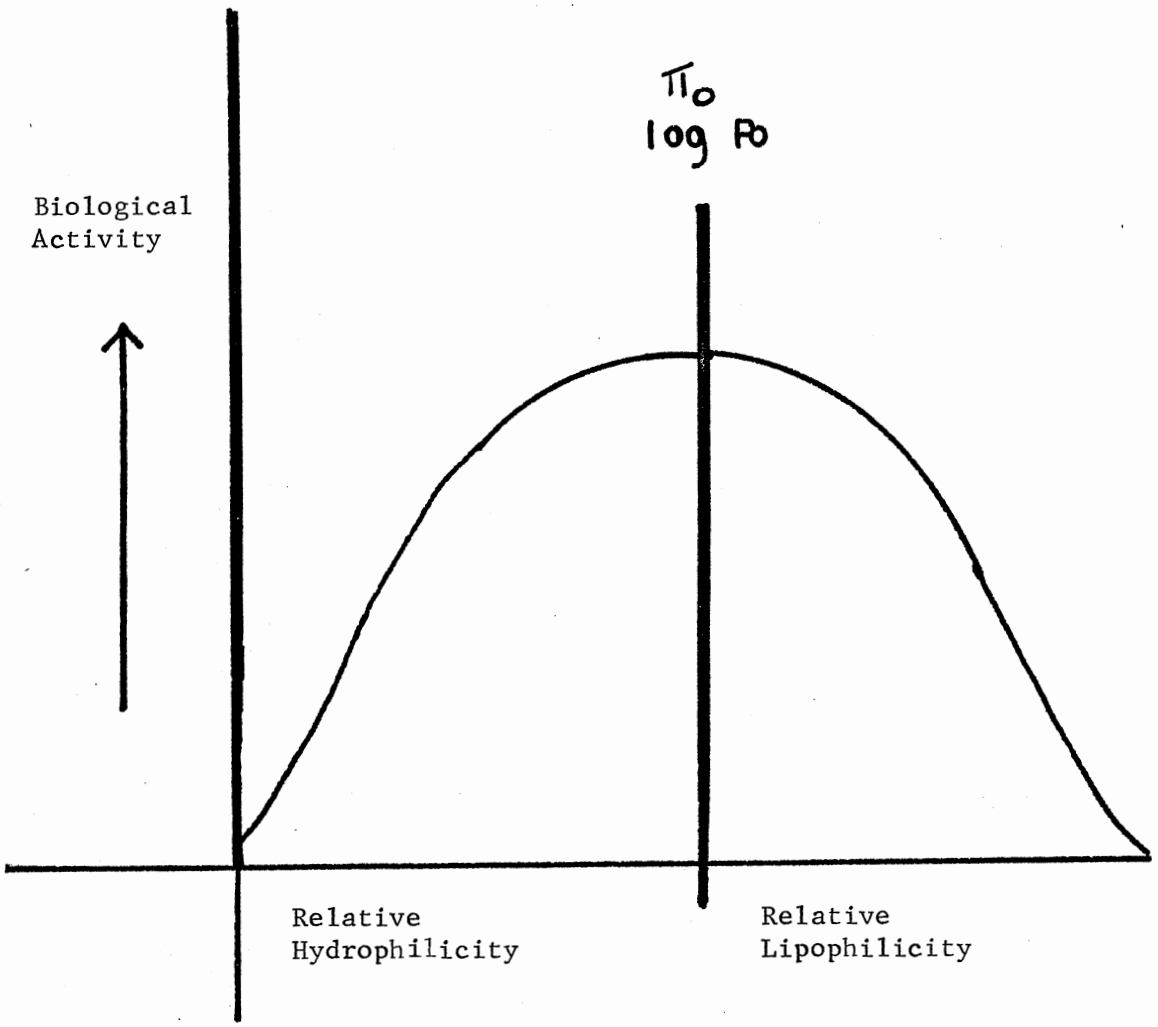
or

$$\pi = \log P_x - \log P_H \qquad (3)$$

where P_x and P_H are the partition coefficients of substituted and parent molecules. The π is a constant for the substituent X. Hansch also used the work of Collander, who correlated the partitioning of a compound in different oil-water systems, to show that log P derived from an octanol-water system was comparable to the partitioning of drugs into biological systems. This relationship has, however, been shown to deteriorate under certain circumstances and with particular drugs (Leo, 1972). A negative π value thus indicates a change towards greater affinity for the aqueous phase, and a positive value indicates greater affinity for the lipid phase. Just as σ is a free-energy related parameter, so too is π which expresses the relative free-energy change on moving a derivative from one phase to another.

Hansch expanded his theory to include the concept that the relationship between the biological activity and structure was not linear but parabolic (Figure 2). Though largely empirical, the idea was based on the observation that in many series of compounds tested as the relative lipophilicity was increased activity rose to a maximum, fell off, and eventually reached zero. Hansch pointed out that approximately linear relationships of biological activity with log P could be

Figure 2. Proposed Parabolic Relationship Between Biological Activity and Structure


π_o
log Po
Biological
Activity
Relative
Hydrophilicity
Relative
Lipophilicity

expected when compounds within a limited range of log P values (either all higher or all lower than the optimum) were chosen for study, thus accounting for the results of Collander.

Hansch's original equation suggested that when a broad range of log P values are studied relative activity will depend parabolically on log P. This basic assumption could be considered another way; a fraction of the molecules from a fixed dose would reach the site of action in a given time interval, and this fraction would be at a maximum for some optimum value of log P. The number of molecules arriving at some critical site would affect the magnitude of the biological response. If one considered that the dose, C, was constant and that d(response)/dt varied, then a new equation could be derived representing the relative biological activity as a rate term for a constant dose and is normally written as log BR (Hansch et al., 1965b). The empirically predicted parabolic relationship between log BR and log (1/C) and π or log P has received both experimental and mathematical justification.

Hansch et al. (1965a) demonstrated the way log P determines concentration of a drug at the site of action using the work of Soloway et al. (1960) who had measured the actual concentration of boron in the brain and tumor of tumor-bearing mice that had been injected with a series of 25 substituted benzeneboronic acids. Hansch predicted which substituents would be worthy of further investigation based on π values. Those with values between -1.0 and -2.0 he said, would allow therapeutic concentration in tumor tissue while giving high selectivity.

By setting up a simple kinetic model, Penniston et al. (1969) demonstrated the validity of the concept of an optimum log P. The model

described the movement of molecules through a series of aqueous compartments separated by lipid barriers. The concentration of molecules in the different compartments after a chosen time was determined by differential equations using arbitrary values of time, number of barriers and initial drug concentrations. The results gave a parabolic relationship between the logarithm of the concentration in any chosen compartment and log P.

Using 19 different drug series in which log BR or log (1/C) had been used to express relative activity and log P_o had been derived, Hansch et al. (1965b) established the utility of this approach. From a summary of the results, it became clear that structurally different sets of compounds, acting at the same site, would all have the same log P_o value. Also this value could serve as a guide in the design of novel series of drugs to be used in the same system since it indicated the hydrophilic-balance to be preserved in the new series.

Bird and Marshall (1967) used π values to correlate the serum-protein binding of penicillins, enabling them to postulate a mechanism for this binding. In 1968, Hansch et al. studied the hypnotic activity of barbituates and non-barbituates with data taken from an extensive literature survey incorporating different methods of measuring a standard hypnotic response in a variety of animals. A log P_o value of 1.8 was determined for both types of hypnotics and Hansch made the assumption that almost any compound having a log P of about 2.0, provided it was not rapidly metabolized or eliminated, would have some hypnotic properties. This point seemed verified by the fact that well-known central nervous system depressants such as chloroform, chloretone and glutethimide have log P values of 1.97, 2.03 and 1.90 respectively. In a study of

thiobarbiturates the log P value was determined to be 3.1 (Hansch et al., 1968). The considerably higher value was thought to indicate that the thiobarbiturates site of action was a more lipophilic region, or on a more lipophilic type of receptor. In 1969, Hansch et al. correlated homolytic constants with the action of chloramphenicol against E. coli, and in 1973 Garret et al. extended this work, obtaining a trifluoro substituted chloramphenicol of greater activity than the parent compound, as a result of their predictions. Wedding et al. (1967) investigated the inhibition of beef-heart malate dehydrogenase by phenol analogues, correlating this with π and σ_x. They discussed possible mechanisms of inhibition on the basis of these relationships. Hansch and Steward (1964) related the antimicrobial action of some penicillin analogues with π and σ_x. They concluded that the electronic effect was not significant in the action of these penicillins.

Lien et al. (1968) generalized about the differential drug sensitivities of Gram-negative and Gram-positive bacteria. They calculated log P_o values for drugs against the two organism groups from the activities of 16 different compound series. They found log P_o for Gram-negative to be 4.0 and for Gram-positive cells 6.0. The value of these figures is dubious, however, since the biological data came from 25 separate sources; the average values were obtained using nine different Gram-negative organisms and 14 Gram-positive organisms, the taxonomic variation within the groups being vast. Hansch et al. (1972b) compared the sensitivity of groups of fungi, Gram-positive and Gram-negative bacteria to esters of p-hydroxybenzoic acid. The same overall criticism may be made of this work as that of Lien et al. Hansch, however, used the slope of the equations as the indicators of organism sensitivity.

He found mean slopes of 0.515, 0.863 and 0.540 for fungi, Gram-positive and Gram-negative bacteria, respectively.

Instead of measuring log P values by equilibration methods, partition chromatography may be used to estimate relative lipophilicities in a given series of compounds (Green and Marcinkiewicz, 1963). Boyce and Milborrow (1965) exploited the relationship between R_f values from thin-layer chromatography and partition coefficient proposed by Martin (1949) for liquid-liquid chromatography. Martin theorized that the partition coefficient P and R_f were related by the following equation:

$$P = K\,[(1/R_f) - 1], \tag{4}$$

where K is a constant for the system. Bate-Smith and Westall (1950) derived the term R_m defined by the equation

$$R_m = \log\,[1/R_f) - 1] \tag{5}$$

from which follows

$$\log P = \log K + R_m \tag{6}$$

so that R_m values are linearly related to log P. Using the molluscacidal activity of N-n-alkyltritylamines, Boyce and Millborrow (1965) demonstrated the linear relationship between log P and R_m. The theory was also confirmed by Iwasa et al. (1965) and Kopecky and Bocek (1967). Structure-activity correlations have been shown in a number of systems using R_m values: cardiac glycosides (Nover et al., 1968), bisdichloroacetamides (Biagi et al., 1969), vitamin K (Biagi et al., 1969), penicillins (Biagi et al., 1969 and Biagi et al., 1970), cephalosporins (Biagi et al., 1970), p-aminobenzoic acid (Seydel and Butte, 1977), and tetracyclines (Miller et al., 1977).

Since there is now much evidence that a correlation exists between lipophilicity and biological activity, the question becomes one of how to

modify chemical structure to increase activity. Price, in 1969, did an exhaustive study of structure-activity relationships with semisynthetic penicillins. With the isolation of the penicillin "nucleus", 6-amino-penicillanic acid (Batchelor *et al.*, 1959), structurally modified penicillins having greater activity and wider spectra could be produced. Three of the first such naturally produced penicillins had hydrocarbon sidechains attached to the amide-linked carboxylic acid of the β-lactam thiazolidene ring: a saturated five carbon chain, an unsaturated chain, and a saturated seven carbon chain. The potency of the sodium salt of these compounds against *Staphylococcus aureus* was found to decrease with saturation and the carbon length (Moyer and Coghill, 1947). The antistaphylococcal activity of the five carbon chain (*n*-amylpenicillin) is as active as penicillin G and is four times as active as the two-carbon ethyl penicillin (Price, 1969). Price also reported that penicillins with alkyl side chains tend to become somewhat less active as the alkyl chain length increases. In studies of trisubstituted derivatives, the presence of a long alkyl chain or an aryl group is required for maximal activity but maximal activity is only 10 percent of penicillin G.

Godfrey and Price (1972) also studied structure-activity relationships of coumermycins. *In vitro* and *in vivo* studies were done with derivatives having up to six carbons in a straight chain in the number three position of the coumarin moiety. Compounds having three, four, five, or six carbons were up to 20 times more effective against *S. aureus* than the 3-methyl homolog. The Gram-negative activity, based on *Klebsiella pneumoniae* studies, was also considerably affected by the size of the *n*-alkyl side chain. Much the same activity pattern was

observed with branch-chain derivatives. A number of derivatives were prepared by acylation of the coumarine amine with aliphatic chlorides. Chain length of this series varied from 2 to 22 carbons. Chain length had a profound affect on antistaphylococcal activity; potency varied over a 1000-fold range. An increase in chain length starting from the 2-carbon acetyl group resulted in an increase in potency of 10- to 20-fold that peaked with the 10-carbon acyl radical. Activity then fell off rather precipitously as the number of carbons increased still further. None of the series displayed significant activity against K. pneumoniae.

In 1974, Chihara et al. probed the effect of carbon number on the activity of α-N-fatty acyl colistin nonapeptide. They divided all organisms tested into three groups (i.e., type I, II, and III). Type I includes colistin-sensitive Gram-negative bacteria for which the most effective carbon numbers of n-fatty acyl moiety is limited to a very narrow range of 10 to 12. This includes Escherichia coli, Enterobacter aerogenes, Salmonella enteritidis, Salmonella infantis, Shigella flexneri, Pseudomonas aeruginosa and Pseudomonas desmolitica. Type II includes Gram-positive bacteria and one Gram-negative bacterium, Xanthomonas oryzae, for which carbon numbers more than 10 in n-fatty acyl moieties facilitate antimicrobial activity with their increase. They postulate that a carbon increase beyond 14 might be more effective. The Gram-positive organisms of group II are M. lysodeikticus, S. auerus, Sarcina lutea, Bacillus subtilis and Bacillus cereus.

The purpose of this study was three-fold: (1) to measure the effect of various hydrocarbon chain substituents of novel organic compounds on the lipophilicity of the compound as measured by R_m values;

(2) to determine the mode of action of the compounds against bacteria; and (3) to ascertain if there is a correlation between the lipophilicity of the compounds with their degree of activity or mode of action.

CHAPTER II

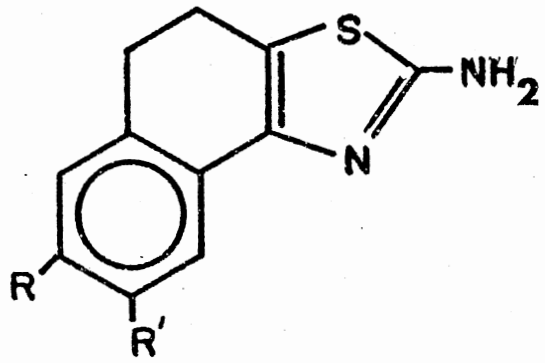
MATERIALS AND METHODS

Compound Preparation for Bacterial Testing

The compounds (Figure 3) tested during this study were synthesized in the laboratory of Dr. K. D. Berlin, Oklahoma State University (Ramalingam et al., 1977). The compounds 2-amino-4,5-dihydro-7-hexyloxynaptho(1,2-d)thiazole (hexyloxythiazole), 2-amino-4,5-dihydro-7-hexyloxynaptho(1,2-d)thiazole hydrochloride (hexyloxythiazole hydrochloride), 2-amino-4,5-dihydro-7-methoxynaptho(1,2-d)thiazole (methoxythiazole), 2-amino-4,5-dihydro-7,8-dimethoxynaptho(1,2-d)-thiazole (dimethoxythiazole) and 2-amino-4,5-dihydro-7-methoxynaptho (1,2-d)-thiazole hydrochloride (methoxythiazole hydrochloride) were supplied in cystalline form and stored at -15 C in screwtop vials.

The compounds were prepared in stock solution by placing 5 mg of the crystals in a test tube, adding 0.5 ml of DMSO, and allowing the compounds to go into solution. This solution was further diluted by adding 4.5 ml of sterile glass distilled water, and the solutions were stored in the dark at 5 C. For experiments, solutions were either added directly to the test system or further diluted. Ultraviolet absorption studies indicated the stock solution was stable for at least two months under these conditions.

Figure 3. Structure of Five Novel Thiazole Compounds



COMPOUND	R	R′	
METHOXYTHIAZOLE HCL	$-OCH_3$	$-H$	(HCL SALT)
METHOXYTHIAZOLE	$-OCH_3$	$-H$	
DIMETHOXYTHIAZOLE	$-OCH_3$	$-OCH_3$	
HEXYLOXYTHIAZOLE	$-O(CH_2)_5CH_3$	$-H$	
HEXYLOXYTHIAZOLE HCL	$-O(CH_2)_5\ CH_3$	$-H$	(HCL SALT)

Compound Preparation for Protozoan Testing

A stock solution of the compound was prepared as described for the bacterial experiments.

Radioisotope Precursors

Glucose-1-^{14}C (specific activity 3.0 mCi/mmole), uracil-2-^{14}C (specific activity 40.6 mCi/mmole), DL-alanine-1-^{14}C (specific activity 30.0 mCi/mmole) and 2-amino-[1-^{14}C]-isobutyric acid (specific activity 60 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) and DL-leucine-1-^{14}C (specific activity 20 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 stored at -15 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.

Microscopy

Visible light photomicrography was performed on a Wild M-20 research microscope equipped for dual-illumination of darkfield, bright-field and phase contrast observations. Illumination was provided by a 12 volt, 100 watt tungsten lamp and an HBO-200 watt high pressure mercury burner.

Photographs were taken with a Wild type MEL-13 photoautomat fitted with a Wild 35 mm film back. Kodak Panatomic-X slide film was utilized for all photography.

Microorganisms

Bacillus subtilis W23 was obtained from the culture collection of Dr. F. R. Leach, Oklahoma State University. *Bacillus megaterium*, *Staphylococcus aureus* and *Escherichia coli* were from the culture collection of the Department of Microbiology, Oklahoma State University. A strain of *Pseudomonas*, tentatively identified (Ferguson, 1967) as *Pseudomonas fluorescens* NND, was obtained from the culture collection of Dr. N. N. Durham, Oklahoma State University. The pseudomonad was characterized as a Gram-negative, motile rod which forms smooth raised colonies on nutrient agar and gives a negative reaction for hydrogen sulfide production, indole production and nitrate reduction. In addition, it does not produce gas in glucose but does produce 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 5 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 0.5 percent glucose. The medium was prepared by dissolving the NaCl, $(NH_4)_2SO_4$, KH_2PO_4, and K_2HPO_4 in deionized distilled water and adjusting the pH to 7.0 with KOH. The medium was 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. A stock solution of 10 percent glucose and minimal salts solution was prepared and autoclaved separately. 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. The basal medium was supplemented with 0.1 ml of the minimal salts solution for each 100 ml of medium and glucose was added to give the desired concentration. 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 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 to 16 hours at 35 C. The cells were harvested from the slants by adding 2 ml of 0.01 M potassium phosphate buffer to the slants, scrapping the cells off the agar surface and pipetting the liquid into a sterile test tube. Growth studies were performed in tubes (15 x 150 mm) containing 5 ml of glucose or succinate medium and the test compound or water and dimethyl sulfoxide (control). The tubes were inoculated with cells from the harvested slants to an absorbance of 0.05 at 540 nm. The tubes were incubated at 37 C with constant shaking in trays. Growth was determined by measuring the change in optical density at 540 nm using a Bausch and Lomb Spectronic 20 (18 mm light path).

Viable Cell Determinations

Glucose-grown B. subtilis cells were inoculated into 20 ml of glucose minimal medium to an absorbance of 0.10. The side arm flasks were incubated at 37 C until the culture reached an absorbance of 0.13. Aliquots (1.0 ml) were removed, diluted in 99 ml saline blanks and plated in duplicate using nutrient agar pour plates. This sample served as the initial control. The test compounds or water (control) were added to the cultures, the absorbance recorded, samples (1.0 ml) removed and nutrient pour plates made at indicated times. The plates were allowed to solidify, then inverted and incubated at 37 C for 15-18 hours. Those plates with between 30 and 300 colonies were counted using a Quebec colony counter.

Compound-Antibiotic Potentiation

Studies were conducted to see if noninhibitory concentrations could enhance the action of antibiotics at low concentrations or could reverse the action of these same antibiotics at inhibitory concentrations. The antibiotics used in this phase of the study were actinomycin D from Merck, Sharp and Dohme Research Laboratories and mitomycin C from National Biochemical Corporation.

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

The non-inhibitory concentrations of both the compounds and the antibiotics were determined by measuring change in optical density similar to the growth studies. The maximum concentration that gave

control growth were those used in the second phase of the experiment. Different combinations of inhibitory-non-inhibitory concentrations were tested, and the resulting growth inhibition was determined when compared to identical cultures containing water, compound or antibiotic as control.

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 harvested from 12-16 hour slants. The test compounds, or water, were added to desired concentrations prior to, simultaneously or following addition of the labeled substrate. The labeled compound (0.3 ml of 5 μCi/ml) along with the appropriate carrier (0.5 ml of 1 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. The filters were placed in 25 x 45 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 done on a Nuclear Chicago Model 720 scintillation counter with a counting efficiency of 40 percent or a Beckman LS-3133P with a counting efficiency of 99.5 percent.

Experiments to measure leakage of radioactive substrates were conducted similar to the procedures described above except that the 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 the test compounds or 2 percent Triton X-100 (Sigma Chemical Company). Samples were removed and counted as above.

Reversal Studies

The reversal of growth inhibition was determined for the methoxy-thiazole hydrochloride by exposing the cells to the compound for the indicated period of time, centrifuging the cells at low speed (5,900 x g for 10 minutes), washing the cells with 0.01 M potassium phosphate buffer, resuspending the cells to the initial optical density, and following the growth as in the growth experiments. The control was exposed to water and DMSO for the same period of time, centrifuged, washed and resuspended.

The other four compounds had varying degrees of water insolubility which made it impossible to remove the compounds by centrifugation. For these compounds 0.5 ml samples, removed from the test tubes containing the compounds and the cells, were inoculated into 4.5 ml of minimal medium. Growth was followed as in the earlier reversal experiment.

Enzyme Induction

P. fluorescens cells were grown in succinate medium for 12 to 15 hours, harvested by centrifugation (5,900 x g for 10 minutes) and washed twice with 0.01 M potassium phosphate buffer. The washed cells resuspended in the phosphate buffer were used to inoculate the succinate medium plus acetamide and the appropriate combinations of the compounds to an initial O.D. of 0.2 at 540 nm. Samples (0.25 ml) were removed at

the specified time intervals and immediately stored at -15 C for subsequent enzyme determinations.

Assay Method

The assay for enzyme synthesis was based on the formation of hydroxamic acids from acyl phosphates and hydroxylamines at pH 6.5 to 7.0 (Lipmann and Tuttle, 1945). The hydroxamic acids will react with ferric salts to produce red to violet complexes which can be quantitated spectrophotometrically.

Preparation of the Standard Curve

A hydroxamic acid stock solution was prepared by dissolving 0.5 g of succinic anhydride in 20 ml of freshly neutralized 2.0 M hydroxylamine hydrochloride. The solution was allowed to stand 10 minutes and water was added to give a final volume of 50 ml. A standard solution was prepared by diluting 1 ml of the stock solution to a final volume of 40 ml with distilled water. Two ml of the standard solution was added to 1.0 ml of freshly neutralized hydroxylamine hydrochloride and allowed to stand for 10 minutes. Three ml of ferric chloride reagent (6.0 percent w/v in 2.0 percent HCl v/v) was added and the absorbance read at 540 nm. The reading obtained from this solution was equivalent to 4.0 micromoles of acetohydroxamic acid. Different dilutions of the stock solution were prepared in a total volume of 40 ml to give varying concentrations of the acetohydroxamate. The absorbance of each dilution was measured, and a standard curve for acetohydroxamate was prepared.

Amidase Activity

The Brammer and Clarke (1964) modification of the Lipmann and Tuttle (1945) method for hydroxamic acid determination was used in this study. The modification is based on the quantitative determination of acetohydroxamate, the end product of the translocase reaction of the amidase enzyme. Acylhydroxamates are formed when the amidase enzyme transfers the acyl group of the substrates amides to hydroxylamine (Kelly and Kornberg, 1962). The reaction is as follows:

$$CH_3C(O)NH_2 + NH_2OH \longrightarrow CH_3C(O)NHOH + NH_3$$

The substrate mixture was prepared by mixing equal volumes of 0.4 M acetamide solution, 2.0 M freshly neutralized hydroxylamine hydrochloride, and 0.1 M 2-amino-2(hydroxymethyl)-1,3-propanediol (Tris) buffer (pH 7.2). The frozen samples which were taken from the culture flasks were thawed and 0.75 ml of the substrate mixture was added to each. The tubes were incubated at 37 C for 15 minutes, and the reaction was stopped by adding 2.0 ml of $FeCl_3$. The absorbance was then read at 540 nm and the amount of acetohydroxamate produced was calculated using the standard curve. The specific activity was measured, and one unit of amidase was defined as the amount of enzyme that would produce one micromole of acetohydroxamate per milligram dry cell weight per 15 minutes.

Deoxyribonucleic Acid Isolation

Growth of Cells

Deoxyribonucleic acid (DNA) was isolated from either Bacillus subtilis or P. fluorescens using the Saito and Miura (1963) modification

of the procedure of Marmur (1961). Cells from 12 to 15 hour old cultures were used to inoculate 3 liters of either succinate or glucose medium distributed in 6 Fernback flasks. After 5 to 6 hours growth at 37 C on a reciprocal shaker, the cells were harvested by centrifugation (8,000 x g for 10 minutes). Usually 12 g of packed cells were obtained by this method. The cells were divided into two 6 g masses and stored at -15 C until needed.

Extraction of P. fluorescens DNA

Six grams of packed cells were mixed with 12 mg lysozyme dissolved in 6 ml saline-disodium ethylene diamine tetracetate (EDTA) (0.15 M NaCl-0.01 M EDTA). The suspension was then incubated at 37 C. After 45 minutes of incubation, 50 ml of tris-SDS buffer (0.01 M tris buffer, 1.0 percent SDS, 0.10 M NaCl, pH 9.0) was added, followed by the addition of an equal volume of redistilled phenol saturated with water prior to use. This mixture was placed in a glass stoppered round bottom flask and shaken for 20 minutes in an ice bath (below 4 C). The emulsion was separated into two layers by slow speed centrifugation (650 x g for 10 minutes). The upper phase was clarified by centrifugation (16,300 x g for 10 minutes) to remove any remaining cell debris. The nucleic acids were precipitated by gently mixing the clarified suspension with two volumes of cold ethanol (95 percent). The thread-like precipitate was collected on a glass rod and dissolved in 20 ml of dilute saline-citrate (1:10 dilution of 0.15 M NaCl-0.015 M trisodium citrate, pH 7.0), followed by the addition of 2.2 ml of acetate-EDTA (3.0 M sodium acetate-0.001 M EDTA, pH 7.0). Ribonuclease (600 µg/ml), previously heated for 10 minutes at 80 C, was added to the dissolved nucleic acid to give

final concentration of 50 μg/ml. The suspension was placed at 37 C for 30 minutes. The digest was cooled and mixed with an equal volume of water-saturated phenol and shaken for 10 minutes in an ice bath (below 4 C). The suspension was again separated by centrifugation (650 x g for 10 minutes) and the upper phase clarified (16,300 x g for 10 minutes). DNA was precipitated by gently mixing two volumes of cold ethanol (95 percent) with the suspension; the DNA was collected on a glass rod and dissolved in 20 ml of the dilute saline-citrate and 2.2 ml of acetate-EDTA for the complete elimination of RNA. While the solution was rapidly stirred, 0.54 volumes of isopropanol was slowly added, and the DNA precipitate was collected with a glass rod. The RNA elimination procedure was repeated once more using only one-half of the required volumes in the precipitation. DNA was then stored in ethanol (95 percent) until used.

Stock solutions of DNA were prepared by dissolving DNA in saline-citrate and storing at 4 C. DNA concentration was determined by using a nomograph (distributed by California Corporation for Biochemical Research) and the DNA absorbance ratio at 260-280 nm.

Extraction of B. subtilis DNA

Six grams of packed cells were mixed with 12 mg lysozyme dissolved in 6 ml saline-EDTA as in the extraction of the P. fluorescens DNA. The mixture was incubated for 20 minutes at 37 C; the pH of the cell suspension was monitored during this period and adjusted to pH 8.0 by the addition of dilute NaOH. The cell suspension was quickly frozen in an acetone-dry ice bath and kept at -20 C. Fifty ml Tris-SDS buffer (pH 9.0) was added to the frozen cells and the cells were suspended by

stirring with a glass rod. The suspension was warmed in a 60 C water bath for complete thawing. The presence of the SDS resulted in the lysis of the cells and also suppressed the action of DNases. While the lysis was still incomplete, the freezing and thawing were repeated two more times. The rest of the extraction procedure parallels the earlier procedure beginning with the addition of equal volume of redistilled phenol.

DNA Thermal Denaturation Studies

Tubes containing DNA and DNA plus the test compounds were prepared in a total volume of 2.5 ml of saline-citrate (0.15 M NaCl-0.015 M trisodium citrate, pH 7.0). After incubation for 30 minutes at 37 C, an equal volume of methanol (2.5 ml) was added to each tube which was shaken thoroughly and the tube was plugged with a rubber stopper. Thermal denaturation was carried out in a heated water bath. The tubes were allowed to equilibrate for 7 minutes at each temperature, the denaturation was temporarily stopped after each step by placing the tubes in an ice bath for 5 minutes. Absorbancies were read on the Beckman DU Spectrophotometer at 260 nm, and an absorbance ratio was calculated by dividing the absorbance at 260 nm of each temperature by the initial absorbance 260 nm at 37 C.

DNA Dialysis Procedure

DNA plus the appropriate combinations of compounds to be tested were prepared in saline-citrate buffer. These mixtures were then placed in dialysis tubing (1/4 in inner diameter) and dialyzed against 4 liters of saline-citrate buffer for the prescribed time intervals at 4 C with

constant stirring and with the buffer changed at 24 hour intervals. After the dialysis was complete, the samples were removed from the tubing for use in denaturation studies.

EDTA Treatment of Cells

P. fluorescens was grown for 12 hours in succinate minimal medium, harvested, washed once in 0.12 M tris(hydroxymethyl)aminomethane (Tris) buffer, pH 8.0, and suspended in Tris buffer to an absorbancy of 0.5 at 540 nm. The cells were treated with an equal volume of EDTA (2×10^{-4} M, pH 7.0; final concentration 1×10^{-4} M) for 2 minutes at 37 C. The action of EDTA was stopped by making 1:10 dilutions into fresh medium. In some experiments the cells were packed by centrifugation and resuspended in minimal medium. This new suspension was used to inoculate tubes for enzyme assay. In other experiments the 1:10 dilution was used to inoculate culture tubes containing the appropriate medium and growth in the presence or absence of the test compounds was measured spectrophotometrically. Control cells were treated with Tris buffer. This procedure is based on a similar study by Leive (1968).

Protozoan Test System

Tetrahymena pyriformis, syngen 1 was obtained from the collection of Dr. D. L. Nanney, University of Illinois. Stock cultures were maintained in test tubes containing 5 ml of proteose peptone medium and stored at room temperature (25 C). To perpetuate the cell system, 1 ml of the stock solution was transferred to 4 ml of the medium every 3 to 4 days.

Protozoan Culture Media

The proteose peptone medium used in this study was composed of 2.0 percent proteose peptone (Difco), 0.2 percent dextrose and 0.1 percent yeast extract. The medium was made up at 10 times the above concentration, centrifuged at 7,000 x g for 30 minutes to remove any undissolved matter and stored at -15 C in 25 ml aliquots until needed. The concentrate was diluted with 225 ml distilled water and autclaved (115 psi for 15 to 30 minutes) prior to use.

Protozoan Growth Experiments

A 72-hour culture of Tetrahymena pyriformis was standardized to an absorbancy of 0.5 at 540 nm using a Spectronic 20. This was used to inoculate a second proteose peptone medium to a final concentration of 7.5 percent (v/v) with the previous stock solution. Test tubes (15 x 150 mm) were inoculated with 2.7 ml of the second stock solution. To this were added 0.3 ml of various concentrations of the test compounds to give final concentrations of 300 µg/ml, 50 µg/ml, 25 µg/ml, 12.5 µg/ml and 6.25 µg/ml. The tubes were incubated at room temperature (25 C) and absorbancy at 540 nm was read at 24 and 48 hours. Controls were inoculated with 0.3 ml of distilled water.

Reverse-Phase Thin-Layer Chromatography

This procedure is adapted after one used by Boyce and Milborrow (1965). Glass plates (20 x 20 cm) were coated with Silica gel G (Merck). The plates were allowed to air dry and then were impregnated with octanol by placing the plates in a chromatographic chamber containing 200 ml of 5 percent octanol in ether. The plates were removed from the

chamber after 12 hours and were again air dried. The test compounds were dissolved in acetone to a final concentration of 1 mg/ml and 10 µl was spotted 2 cm from the bottom of the plate. All five compounds were run at one time. The plates were developed by the ascending technique; the mobile phase, 50 percent acetone, was allowed to migrate 12 cm, giving a total migration of 10 cm for the spots. The plates were again air dried and the spots located by using UV light.

CHAPTER III

RESULTS

Physical Data

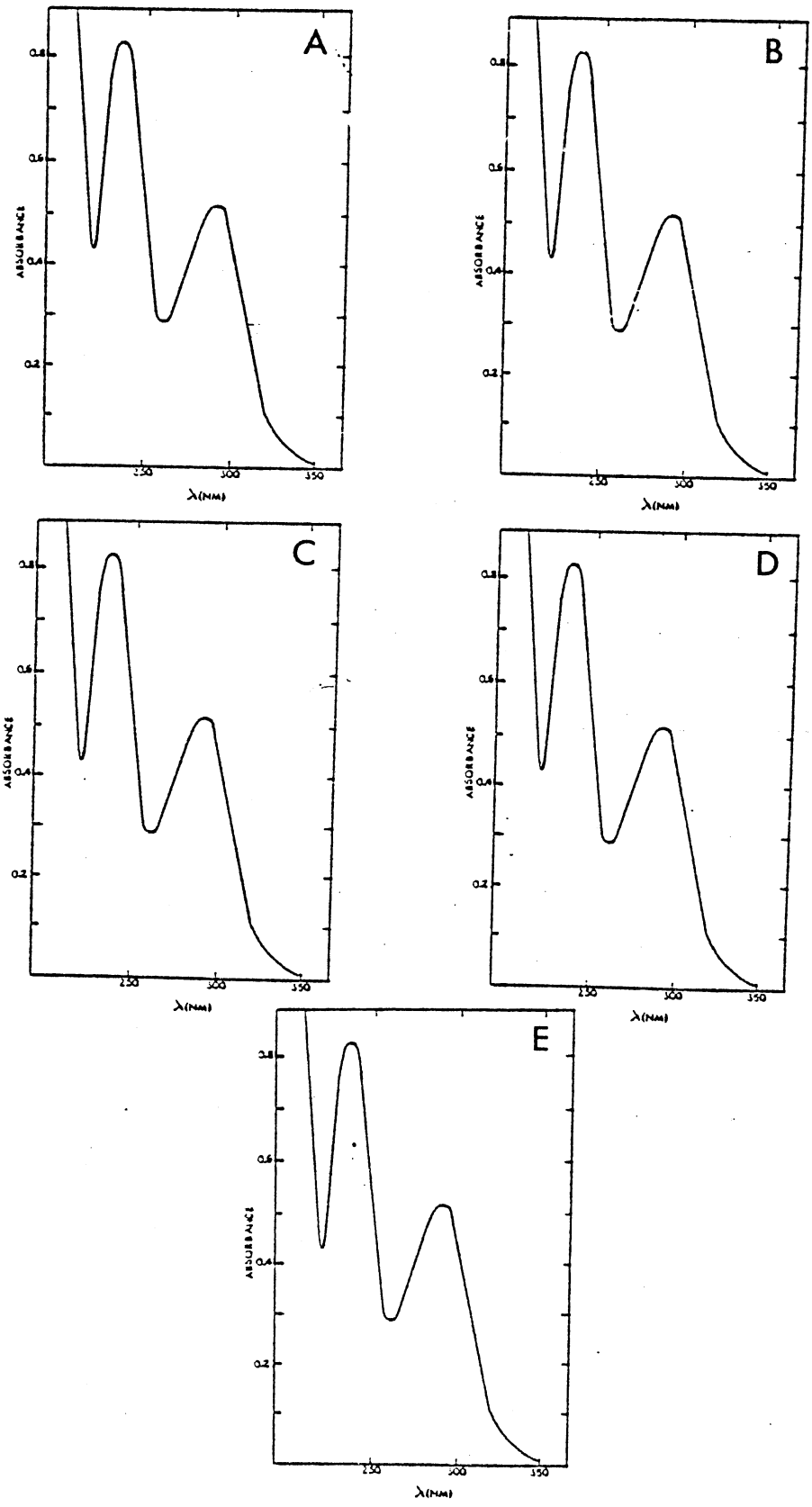
Ultraviolet Absorption Spectra and Standard Curves

The purpose of determining the physical properties of the novel thiazoles was two-fold: (1) to determine those properties which might aid in the detection of the molecules during biological testing, (2) to determine those properties necessary to quantitate the lipophilicity of the compounds using the Hansch analysis. The ultraviolet absorption spectra were examined using the Cary 14 recording spectrophotometer at room temperature for all the compounds utilizing thiazole dissolved in either minimal medium (pH 7.0), water, methanol or octanol.

Because some of the compounds were relatively insoluble in either water or minimal medium an initial spectrum was determined using thiazole suspended in methanol (Figure 4). All the compounds have a strong absorption peak at 241 nm and a second smaller peak at 295 nm. Although there is a slight difference in relative peak intensities, the general shape and distribution of the spectra was indicative of the thiazoles close structural relationship.

It was assumed that the compounds would always have similar spectra regardless of the medium in which the compounds were dissolved.

Figure 4. UV Spectra of Thiazoles Dissolved in Methanol. A. Methoxythiazole Hydrochloride (5.96×10^{-5} M); B. Methoxythiazole (6.3×10^{-5} M); C. Dimethoxythiazole (5.8×10^{-5} M); D. Hexyloxythiazole (5.3×10^{-5} M); and E. Hexyloxythiazole Hydrochloride (4.95×10^{-5} M)


A
B
C
D
E
ABSORBANCE
λ(NM)
250
300
350

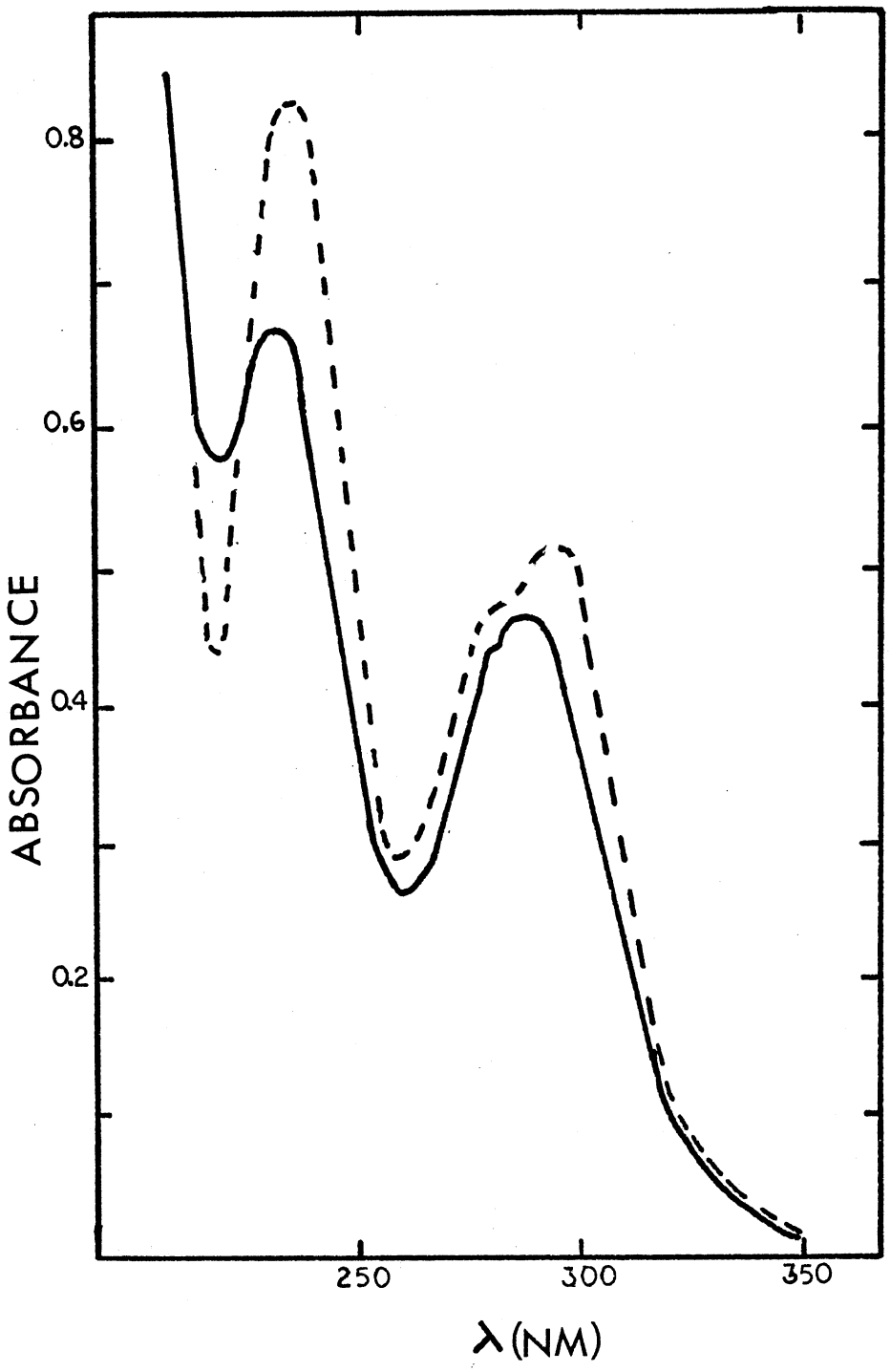
Therefore, though only the methoxythiazole hydrochloride was able to be completely dissolved in water, or minimal medium, the spectrum is thought to be representative of all the compounds. The spectrum of the methoxythiazole hydrochloride in both water and minimal medium is very similar to the spectrum in methanol with the major peaks being shifted to 236 nm and 285 nm from 241 nm and 295 nm, respectively. There was also a slight shoulder at 280 which had not been present before. When the methoxythiazole hydrochloride was suspended in octanol there was a peak at 294 nm and a shoulder at 275 nm and another major peak at 241 nm (Figure 5).

To facilitate quantitation, standard curves were constructed for the spectral data obtained for the major peaks in the different solvents. A linear relationship was always obtained notwithstanding the medium used or the peaks measured within the concentrations ranged used (1.98×10^{-5} M to 3.57×10^{-5} M).

Hansch Analysis

Initially it had been planned to quantitate the relative lipophilicity of the compounds by using the standard Hansch analysis. Spectral data on the compounds in both octanol and water had been obtained for that purpose. In the original experimental design, Hansch had pelleted his compounds, suspended the pellet between the octanol and water phase and measured the appearance of the compound in both phases. Because of the limited amount of compound available and the predicted water insolubility of some of the compounds, it was decided to suspend the compounds in the phase in which it was most soluble and then measure its disappearance from the one layer and its appearance in the other.

Figure 5. UV Spectra of Methoxythiazole Hydrochloride (5.96×10^{-5} M) in Octanol (— — —) and Water (——)


0.8
0.6
0.4
0.2
ABSORBANCE
250
300
350
λ (NM)

Being soluble in both octanol and water, the methoxythiazole hydrochloride proved to be an excellent initial test system for the proposed modifications. The methoxythiazole hydrochloride was dissolved in the water layer and the octanol phase was then added. Two-ml aliquots were taken every 5 minutes over 30 minutes and also after 45 minutes, 60 minutes and 24 hours. The water samples were read at 236 nm and the octanol samples were read at 241 nm. Figure 6 shows the resultant curves. There is a sharp drop in the concentration of the methoxythiazole hydrochloride in the water phase concommitant with a sharp rise in the octanol phase. Both curves level off between 25-30 minutes and remain stable for 24 hours. The final molar ratio of the methoxythiazole hydrochloride in the water layer to the octanol layer is 1:15.

Next the same approach was used with the hexyloxythiazole. Since investigation showed that this compound was more soluble in octanol than in water, it was first dissolved in octanol and the water phase was then layered on. Data are shown in Figure 7. Though a slight drop in the octanol concentration can be measured, a corresponding rise in the water layer was not apparent. Further work with the Hansch analysis was abandoned when it became apparent that the remaining compounds exhibited activity similar to hexyloxythiazole.

Boyce and Milborrow had first proposed in 1965 that partitioning between two phases correlating structure and biological activity could be accomplished using thin-layer chromatography. The lipophilicity of the compound is quantitated by using the constant R_m defined as the log $(1/R_f - 1)$. Bate-Smith and Westall (1950) had shown that R_m values were linearly related to log P. This method offered three advantages over the traditional Hansch analysis: (1) the lipophilicity of the five

Figure 6. Hansch Analysis of Methoxythiazole Hydrochloride. ●, concentration of methoxythiazole in octanol layer; ▲, concentration of methoxythiazole in water layer.

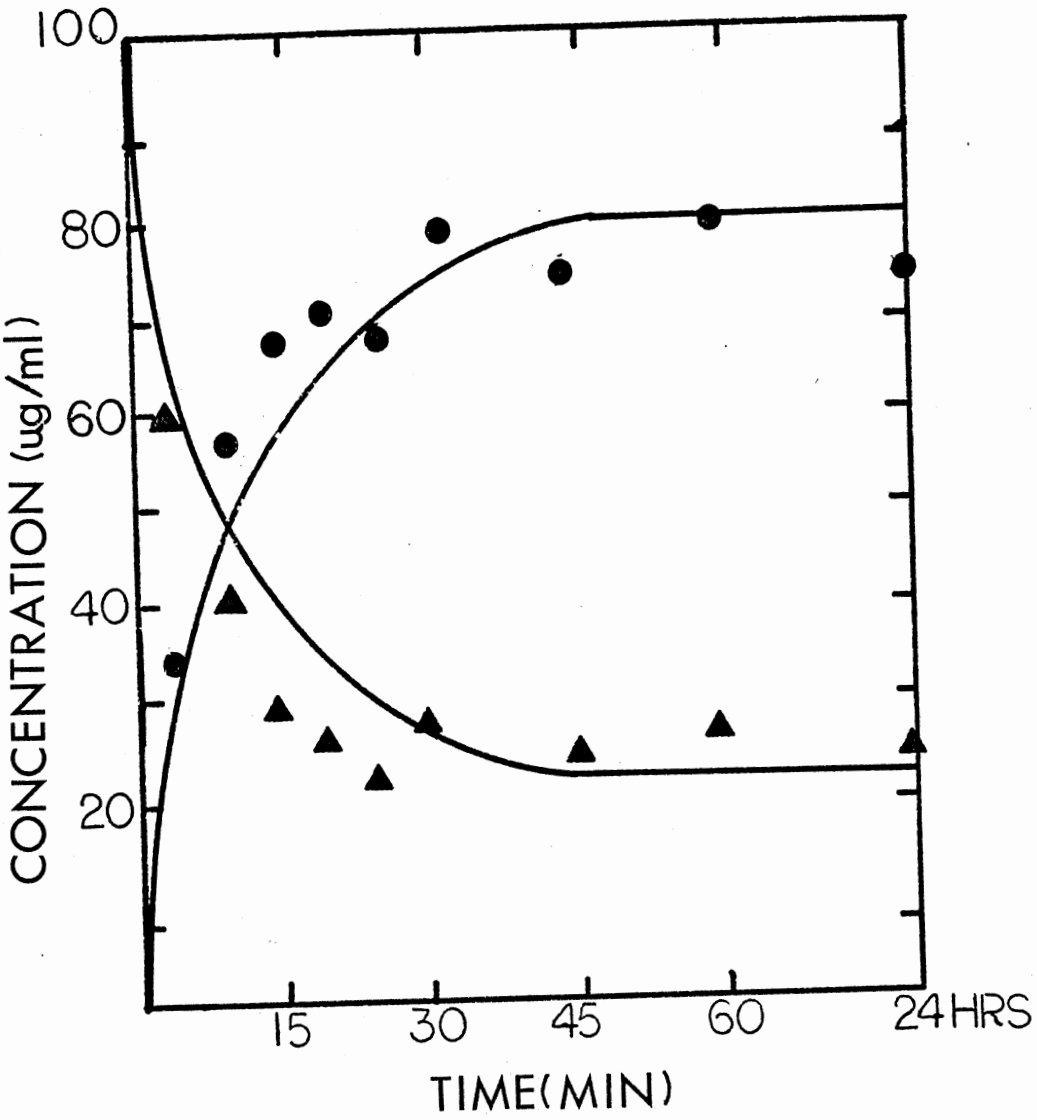
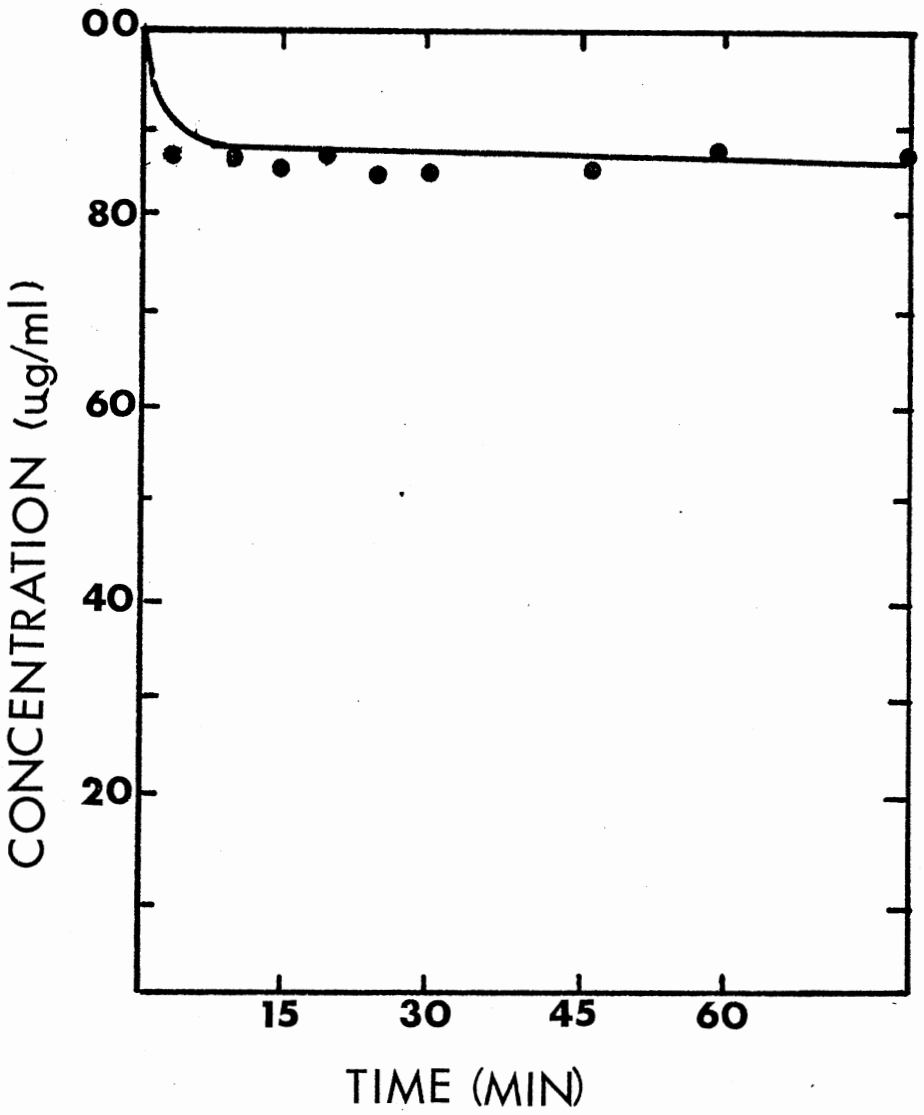

100
80
60
40
20
CONCENTRATION (ug/ml)
15
30
45
60
24HRS
TIME(MIN)

Figure 7. Hansch Analysis of Hexyloxythiazole.
●, concentration of hexyloxythiazole
in octanol layer.


00
80
60
40
20
CONCENTRATION (ug/ml)
15
30
45
60
TIME (MIN)

compounds could be quantitated simultaneously, (2) very small quantities of the compounds were required, and (3) the solubility of the compound in either phase would not prevent the quantitation of its lipophilicity.

Chromatography was carried out on glass plates impregnated with 5 percent octanol in ether. The mobile phase was made up of aqueous acetone. Trial plates were run to determine the concentrations of aqueous acetone that would give the best separation of the compound spots. Figure 8 shows the R_f values plotted against percent acetone. With concentrations of acetone below 30 percent the spots for the more lipophilic compounds remained at the point of origin. The methoxythiazole hydrochloride spots migrated away from the origin but migrated the same distance. At higher concentrations all the compounds migrated with the solvent front. The optimum concentration for separation of the compound spots was therefore between 40 and 50 percent.

To ascertain that the difference in R_f values was a result of octanol impregnation of the plates, plates without octanol were run using 50 percent aqueous acetone as the mobile phase (Table I). Without octanol impregnation the three methoxythiazoles migrated very close to the solvent front and had the same R_f value, 0.095. The two hexyloxythiazoles also migrated quite far, showing very little separation with R_f values of 0.820 and 0.850. In constrast with octanol impregnated plates, there was separation of all the compounds with the least lipophilic compound migrating almost half way.

Using the data obtained from the octanol impregnated plates, the relative lipophilicity-hydrophilicity of the compounds was quantitated by calculating the R_m values for each compound (Table I). The higher or more positive the R_m value the more lipophilic the compound, the

Figure 8. Effect of Acetone Concentration on R_f Values of Thiazoles. ▲, methoxythiazole hydrochloride; ■, methoxythiazole; ●, dimethoxythiazole; Δ, hexyloxythiazole; O, hexyloxythiazole hydrochloride.

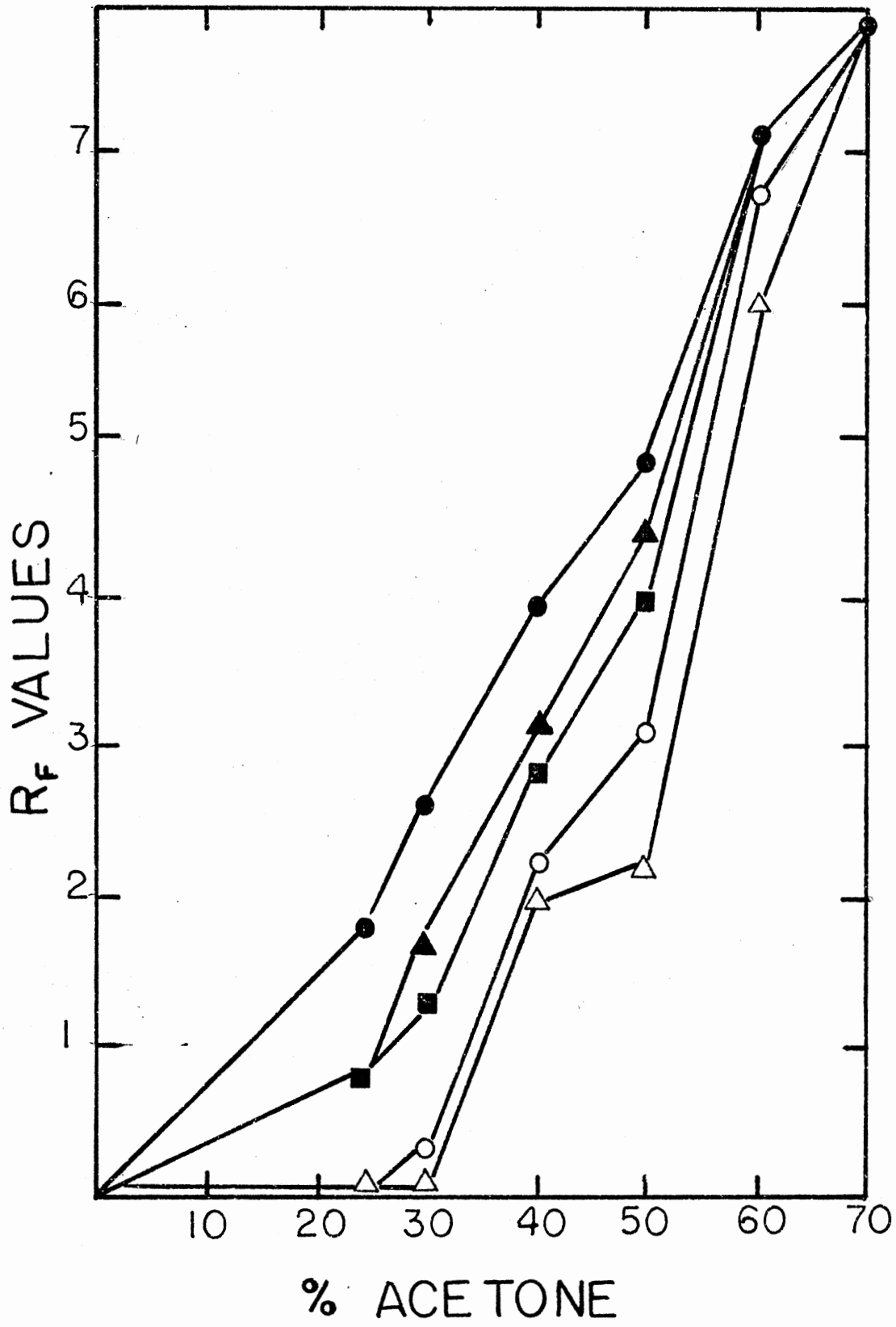

R_F VALUES
1
2
3
4
5
6
7
10
20
30
40
50
60
70
% ACETONE

TABLE I

R_F AND R_m PARTITION VALUES OF THIAZOLES SUSPENDED IN 50 PERCENT ACETONE[a]

Compound	Octanol Impregnation		No Octanol Impregnation	
	R_F	R_m	R_F	R_m
Methoxythiazole Hydrochloride	0.350	+0.0735	0.950	-1.27
Methoxythiazole	0.407	+0.039	0.950	-1.27
Dimethoxythiazole	0.489	+0.019	0.950	-1.20
Hexyloxythiazole	0.250	+0.423	0.820	-0.65
Hexyloxythiazole Hydrochloride	0.260	+0.377	0.850	-0.75

[a] R_m is defined as log $(1/R_F - 1)$.

lower or more negative, the more hydrophilic the compound. By this analysis the dimethoxythiazole is the least lipophilic compound with an R_m value of +0.019, followed by the methoxythiazole at +0.039 and the methoxythiazole hydrochloride at +0.0735. The two hexyloxythiazoles were more lipophilic with R_m values of +0.423 and +0.377. These results correspond well with the data obtained from the attempts to carry out the standard Hansch analysis.

Characterization of Antibacterial Activity

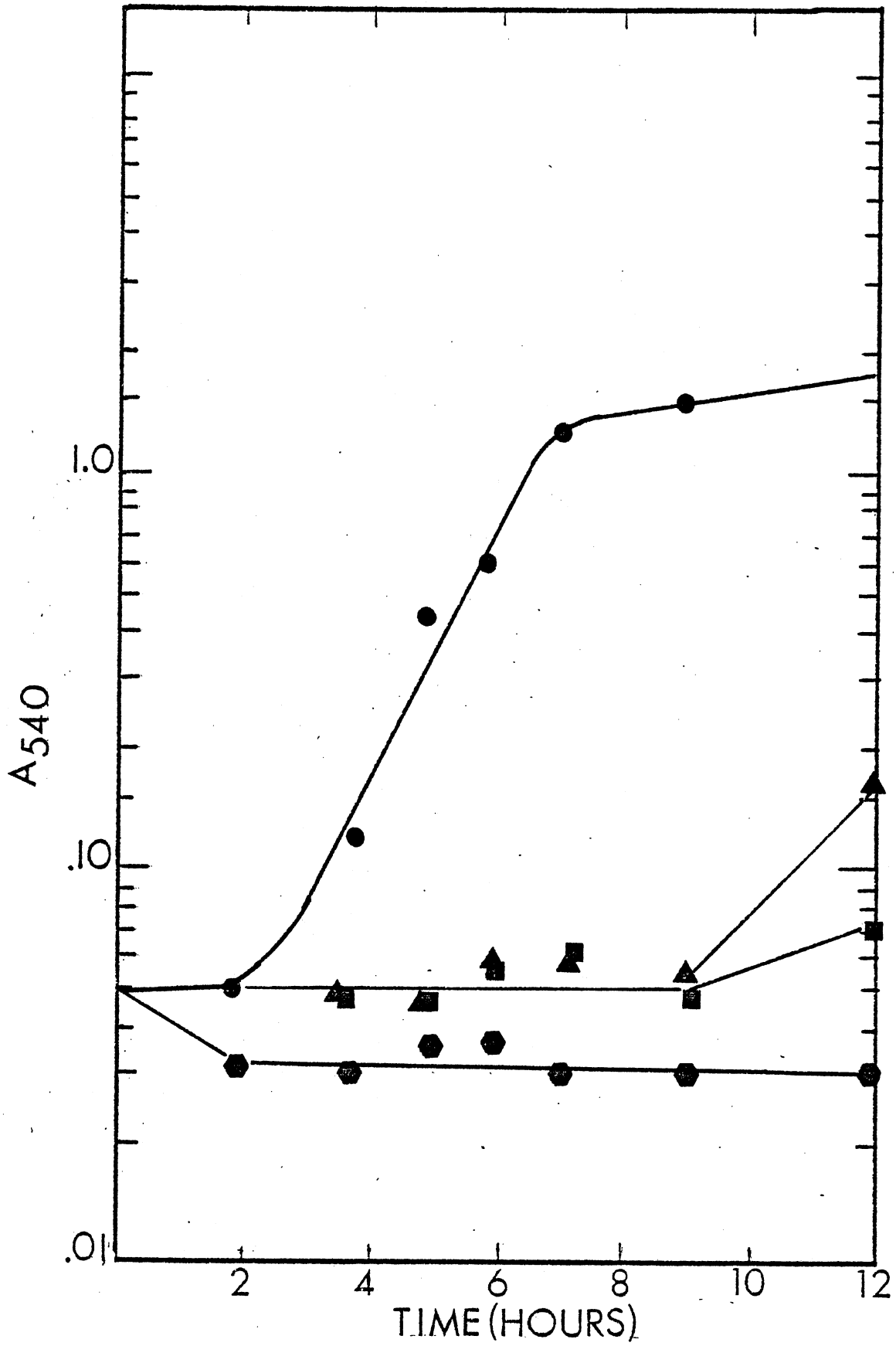
Thiazole Inhibition of Bacterial Growth

The antibacterial activity of the thiazoles was determined by adding the test compounds to cultures of *B. subtilis* W23, *S. aureus*, *B. megaterium*, *E. coli* and *P. fluorescens* NND. The growth response was followed by measuring the change in absorbance at 540 nm.

The growth of *B. subtilis*, *B. megaterium* and *S. aureus* was substantially reduced in the presence of all the methoxythiazoles and the hexyloxythiazole but not the hexyloxythiazole hydrochloride. Figures 9 and 10 and Table II show the results obtained with both control and thiazole containing cultures of *B. subtilis*.

The hexyloxythiazole hydrochloride (5.9×10^{-5} M) and the hexyloxythiazole (6.6×10^{-5} M) showed no inhibition of growth at either 8 or 24 hours. A five-fold increase in the concentration of the hexyloxythiazole (3.3×10^{-4} M) completely inhibited *B. subtilis* for 24 hours. The hexyloxythiazole exhibited rather anomalous behavior in growth studies due to its high degree of insolubility in the minimal medium used in the studies. In some experiments a forty-fold decrease in concentration (1.1×10^{-6} M) of the hexyloxythiazole showed total inhibition at

Figure 9. Effect of the Methoxythiazoles on B. subtilis Growth in Glucose Minimal Medium. ●, control; ⬢, methoxythiazole hydrochloride (7.9×10^{-5} M); ■, methoxythiazole (8.62×10^{-5} M); ▲, dimethoxythiazole (7.6×10^{-5} M).


1.0
.10
.01
A540
2
4
6
8
10
12
TIME (HOURS)

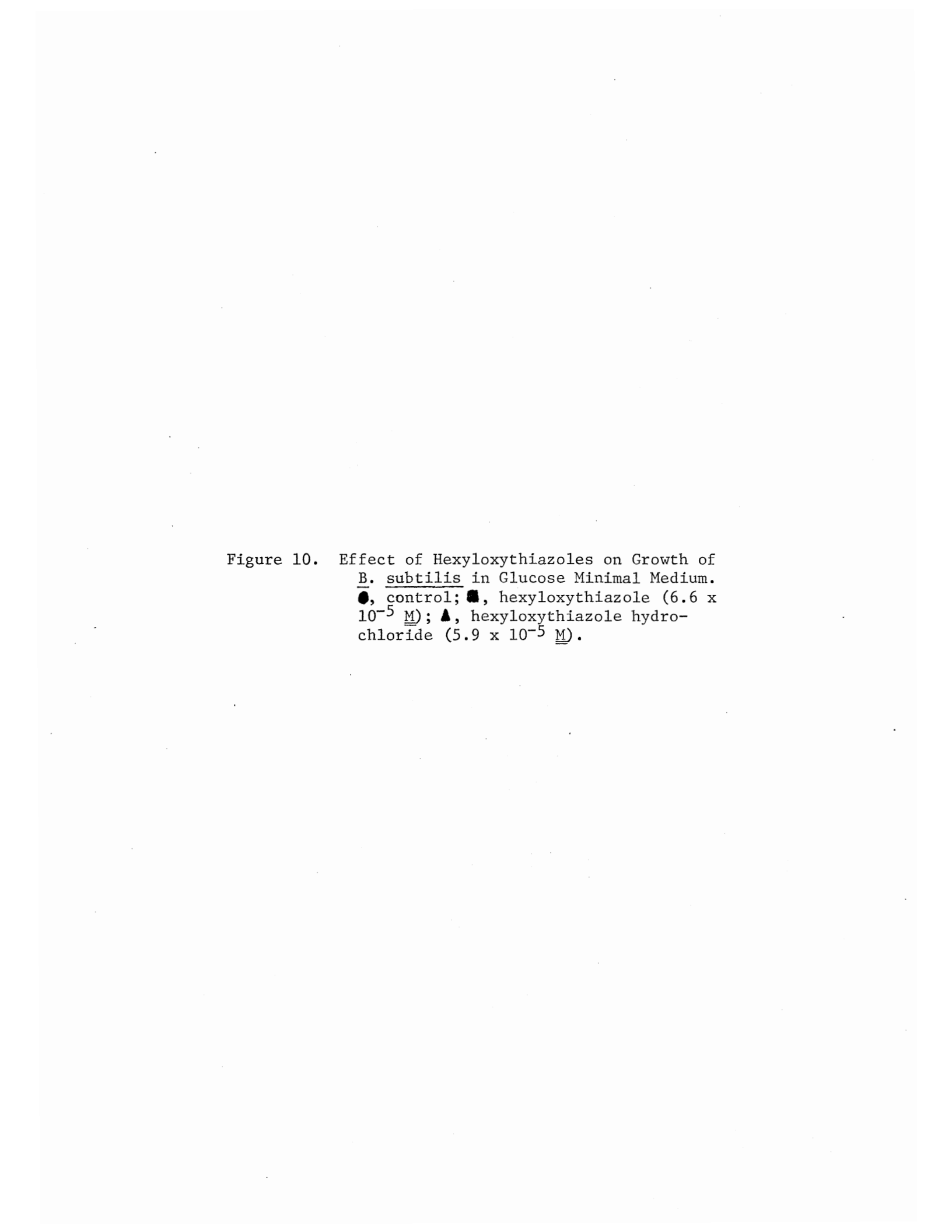


Figure 10. Effect of Hexyloxythiazoles on Growth of B. subtilis in Glucose Minimal Medium. ●, control; ■, hexyloxythiazole (6.6×10^{-5} M); ▲, hexyloxythiazole hydrochloride (5.9×10^{-5} M).

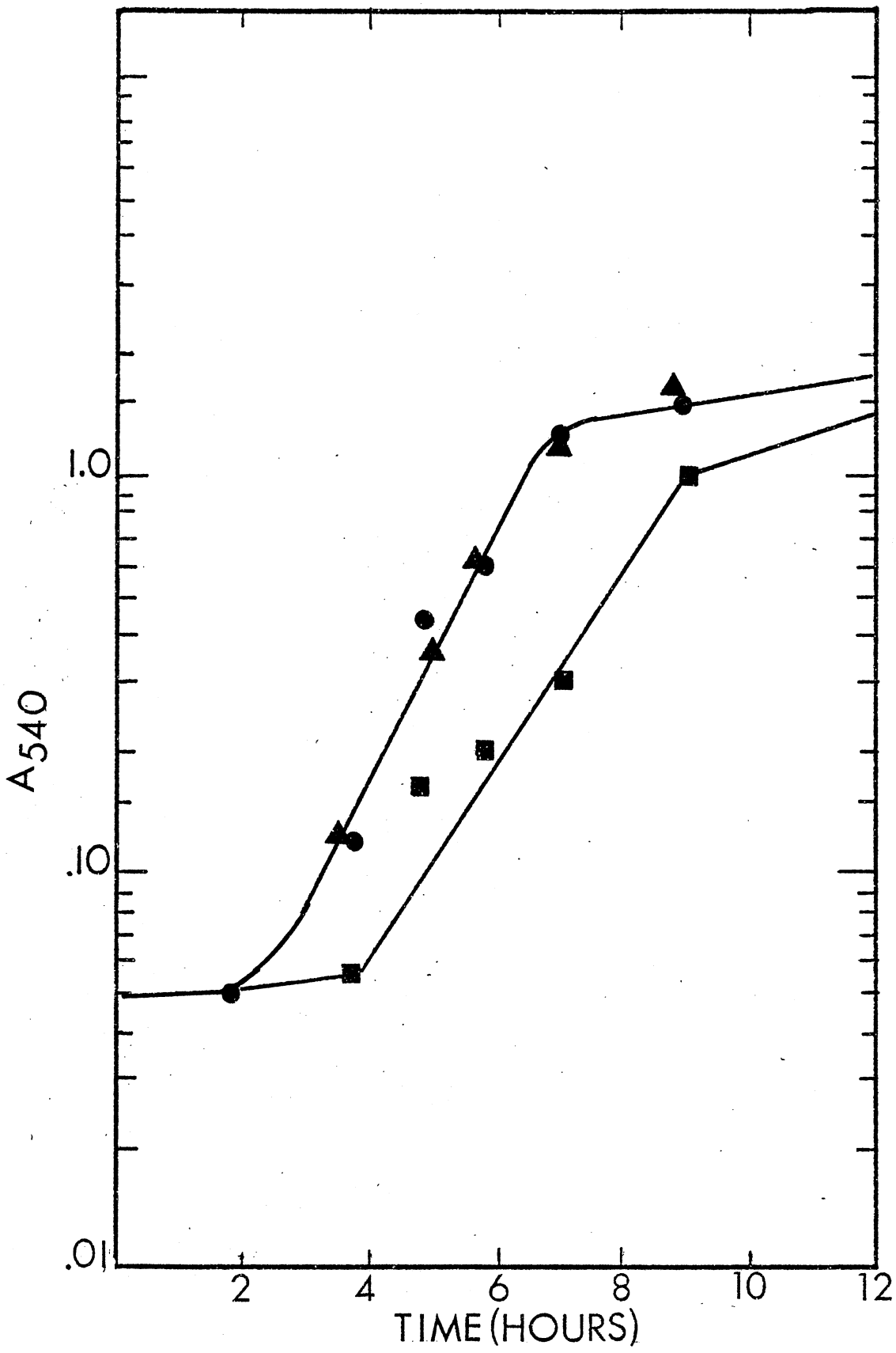

1.0
.10
.01
A540
2
4
6
8
10
12
TIME (HOURS)

TABLE II

THIAZOLE-INDUCED GROWTH INHIBITION OF GRAM-POSITIVE BACTERIA

Compound	Concentration (M)	Percent Inhibition of Growth[a]					
		B. subtilis		B. megaterium		S. aureus	
		8 hours	24 hours	8 hours	24 hours	8 hours	24 hours
Methoxythiazole Hydrochloride	3.9×10^{-4}	100	100	100	100	100	100
	1.58×10^{-4}	100	100	100	100	100	100
	7.9×10^{-5}	100	100	100	100	100	100
Methoxythiazole	4.3×10^{-4}	100	100	100	100	100	100
	1.72×10^{-4}	100	75	100	76	100	75
	8.62×10^{-5}	100	25	100	22	100	31
Demethoxythiazole	3.8×10^{-4}	100	100	100	100	100	100
	1.52×10^{-4}	100	53	92	46	100	51
	7.6×10^{-5}	100	0	100	0	100	0
Hexyloxythiazole[b]	3.3×10^{-4}	100	100	100	100	100	100
	1.32×10^{-4}[c]	75	0	62	0	83	0
	6.6×10^{-5}[d]	0	0	0	0	0	0
Hexyloxythiazole Hydrochloride	2.9×10^{-4}	0	0	0	0	0	0
	1.1×10^{-4}	0	0	0	0	0	0
	5.9×10^{-5}	0	0	0	0	0	0

[a] Tubes containing glucose minimal medium and either thiazole at the indicated concentration or water were inoculated to initial absorbancy (540 nm) of 0.05. Growth was followed spectrophotometrically and percent inhibition was determined by comparing absorbancy of control tubes with thiazole-containing tubes.

[b] Unless otherwise noted all experiments gave same results.

[c] Results from two experiments, third experiment showed 100 percent inhibition at both 8 hours and 24 hours for all organisms.

[d] Results from four experiments, a fifth and sixth experiment showed 100 percent inhibition at 8 hours and 24 hours for all organisms; a seventh experiment showed from 50-60 percent inhibition at 8 hours for all organisms and control growth at 24 hours.

24 hours. The hexyloxythiazole hydrochloride, though it manifested the same solubility problems, never exhibited growth inhibition in any of the experiments performed. Both compounds exhibited the same activity against both S. aureus and B. megaterium that they had against B. subtilis.

The methoxythiazole and the dimethoxythiazole exhibited intermediate activity against all the Gram-positive organisms. They both displayed total inhibition of bacterial growth at concentrations of 4.3×10^{-4} M and 3.8×10^{-4} M, respectively, for 8 hours. At a five-fold decrease in the concentration (8.6×10^{-5} M and 7.6×10^{-5} M) both compounds still gave almost total inhibition of growth at 8 hours but at 24 hours the dimethoxythiazole gave control readings while the methoxythiazole showed only 25 percent inhibition of control growth. At even lower concentrations (2.2×10^{-5} M and 1.91×10^{-5} M), the dimethoxythiazole showed 25 percent inhibition at 8 hours while the methoxythiazole showed 63 percent inhibition during the same period. At lower concentrations tested both compounds gave control growth.

Of the five compounds tested, the methoxythiazole hydrochloride showed the greatest activity against the Gram-positive test organisms. At concentrations of 7.9×10^{-5} M, the methoxythiazole gave complete inhibition during the 24 hours of incubation. At concentrations as low as 3.96×10^{-5} M there was still complete inhibition at 8 hours with 50 percent inhibition at 24 hours. Table II summarizes the activities of all five compounds against the Gram-positive organisms showing percent inhibition of various concentrations of the compounds at 8 and 24 hours.

The Gram-negative microorganisms, E. coli and P. fluorescens, were less sensitive to the thiazoles.

E. coli showed about 50 percent inhibition when incubated with 3.9 x 10^{-4} M methoxythiazole hydrochloride. Concentrations of 7.9 x 10^{-5} M showed no inhibition (Table III). The methoxythiazole and dimethoxythiazole both showed about 15 percent inhibition at 4.3 x 10^{-4} M and 3.8 x 10^{-4} M, respectively. Neither of the hexyloxythiazoles showed any inhibition at the concentrations tested.

P. fluorescens was the least sensitive bacterium tested. Concentrations in the range of 2.9 x 10^{-4} M to 4.3 x 10^{-4} M of the five compounds showed no inhibition (Figure 11). The low sensitivity of the pseudomonad might be expected because this group of organisms is typically resistant to many inhibitor molecules perhaps in part as 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, Doorehbos 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.

Factors Affecting Thiazole Induced Inhibition

The degree of thiazole-induced inhibition could be affected by three things: (1) the concentration of the test compounds, (2) the initial mass of the cells, and (3) the time of addition of the test compounds.

In studies on the inhibition of microorganisms, it had been noted that the inhibition was dependent on the concentration of the thiazole (Table II). Figure 12 shows how the stepwise inhibition of B. subtilis

TABLE III

THIAZOLE-INDUCED GROWTH INHIBITION OF E. COLI

Compound	Concentration (M)	Percent Inhibition of Growth[a] 8 Hours	24 Hours
Methoxythiazole Hydrochloride	3.9×10^{-4}	50	0
	7.9×10^{-5}	0	0
Methoxythiazole	4.3×10^{-4}	15	0
	8.62×10^{-5}	0	0
Dimethoxythiazole	3.8×10^{-4}	14	0
	7.6×10^{-5}	0	0
Hexyloxythiazole	3.3×10^{-4}	0	0
	6.6×10^{-5}	0	0
Hexyloxythiazole Hydrochloride	2.9×10^{-4}	0	0
	5.9×10^{-5}	0	0

[a]Growth inhibition determined as in Table II.

Figure 11. Effect of Thiazoles on Growth of P. fluorescens in Glucose Minimal Medium. ●, control; ▲, methoxythiazoles (3.8 to 4.3 x 10^{-4} M); ■, hexyloxythiazoles (2.9 to 3.3 x 10^{-4} M).

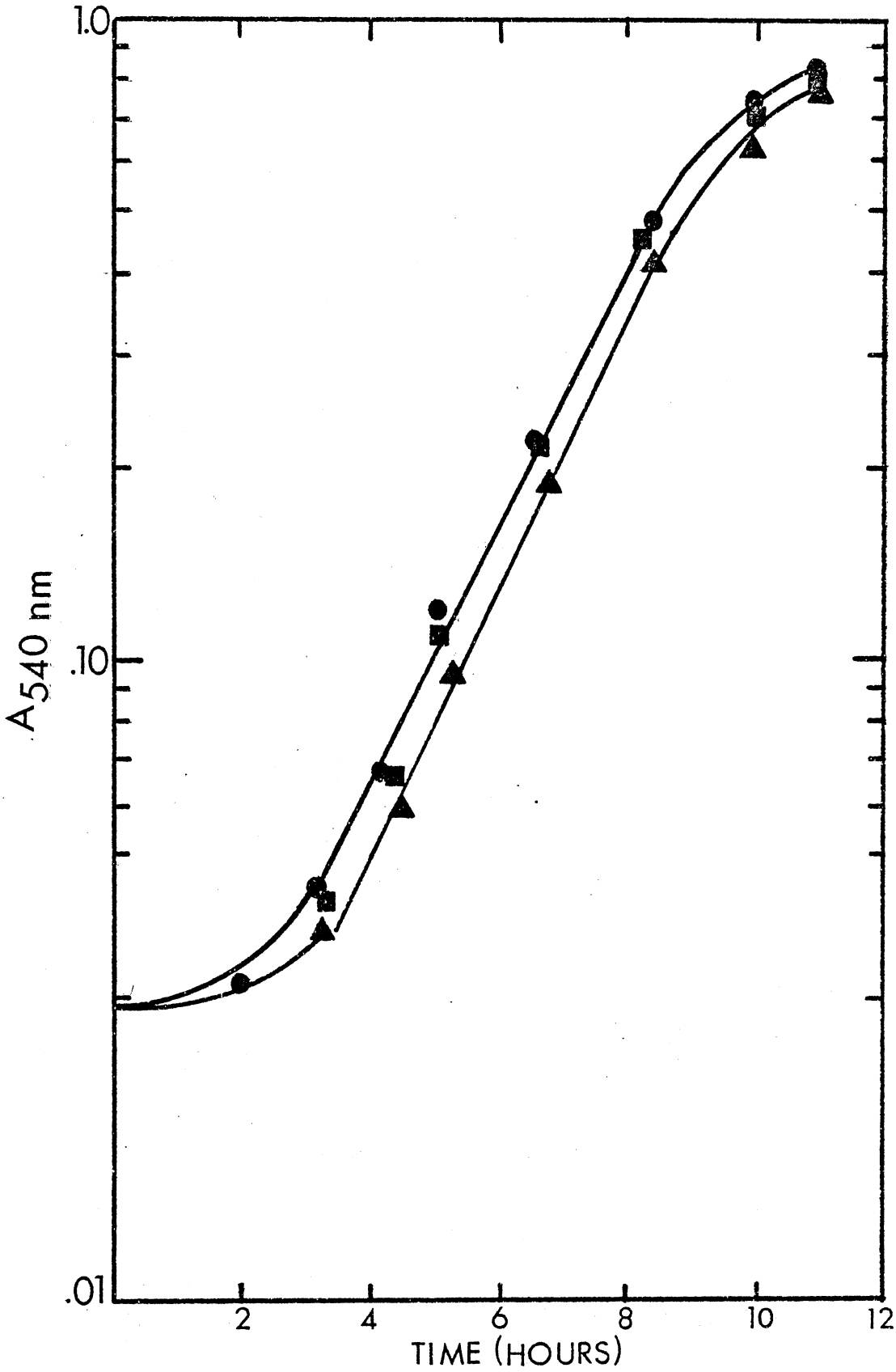
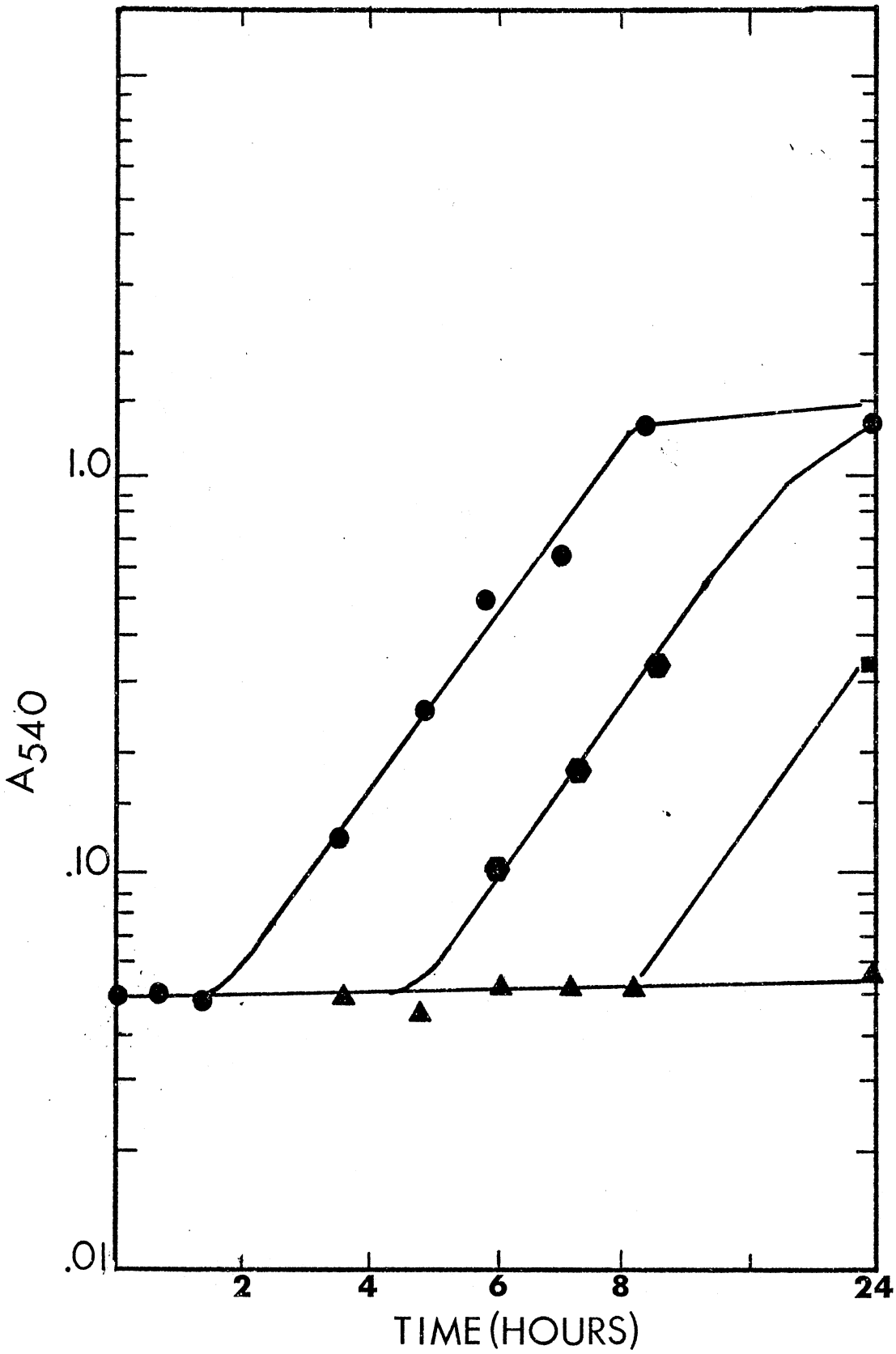

1.0
.10
.01
$A_{540\ nm}$
2
4
6
8
10
12
TIME (HOURS)

Figure 12. Effect of Methoxythiazole Hydrochloride Concentration on Thiazole Induced Growth Inhibition of B. subtilis in Glucose Minimal Medium. ●, control; ▲, methoxythiazole hydrochloride (3.9×10^{-4} M); ■, methoxythiazole hydrochloride (3.96×10^{-5} M); ◆, methoxythiazole hydrochloride (1.98×10^{-5} M).


1.0
.10
.01
A540
2
4
6
8
24
TIME (HOURS)

is related to the concentration of the methoxythiazole hydrochloride. At concentrations of 3.9×10^{-4} M, there is total inhibition of B. subtilis during the 24 hours of incubation, at concentrations of 3.96×10^{-5} M there is only 50 percent inhibition at 24 hours, and at still lower concentrations (1.98×10^{-5} M), there was control growth at 24 hours and 50 percent inhibition at 8 hours. As the concentration was continually decreased, the amount of inhibition during the specified time also decreased; inhibition being manifested as an extension of the lag period with cultures exhibiting control growth rate once they were in the exponential phase of growth. Though this phenomenon was more marked in the studies with the methoxythiazole hydrochloride, it could be observed with all the compounds that exhibited inhibitory activity against B. subtilis.

The size of the initial innoculum of B. subtilis also affected the inhibitory activity of the thiazole compounds (Table IV). As the size of the inoculum increased from 0.25 mg dry weight to 1.1 mg dry weight, the concentration needed to give total inhibition also increased. With the methoxythiazoles at concentrations between 3.8×10^{-4} M and 4.3×10^{-4} M, there was 100 percent inhibition for 8 hours. When the dry weight of the initial inoculum was increased three-fold (0.75 mg dry weight), both the methoxythiazole and the dimethoxythiazole caused only 75 percent and 20 percent inhibition, respectively, at the same concentrations. The most active compound, the methoxythiazole hydrochloride, could maintain 100 percent inhibition at 0.75 mg dry weight initial inoculum but when the inoculum was increased to 1.1 mg dry weight the same concentration gave only 67 percent inhibition.

TABLE IV

EFFECT OF SIZE OF INITIAL INOCULUM ON THIAZOLE-INDUCED GROWTH INHIBITION OF B. SUBTILIS

Compound	Concentration (M)	Percent Inhibition at 8 Hours Initial Inoculum[a] (mg dry weight)			
		<0.25	0.25	0.75	1.1
Methoxythiazole Hydrochloride	3.9×10^{-4}	100	100	100	67
	1.58×10^{-4}	100	100	75	0
	7.9×10^{-5}	100	67	20	0
Methoxythiazole	4.3×10^{-4}	100	100	75	15
	1.72×10^{-4}	100	30	0	0
	8.62×10^{-5}	100	0	0	0
Dimethoxythiazole	3.8×10^{-4}	100	85	20	0
	1.5×10^{-4}	100	30	0	0
	7.6×10^{-5}	100	0	0	0
Hexyloxythiazole[b]	3.3×10^{-4}	100	72	0	0
	1.32×10^{-4}[c]	75	30	0	0
	6.6×10^{-5}[d]	0	0	0	0

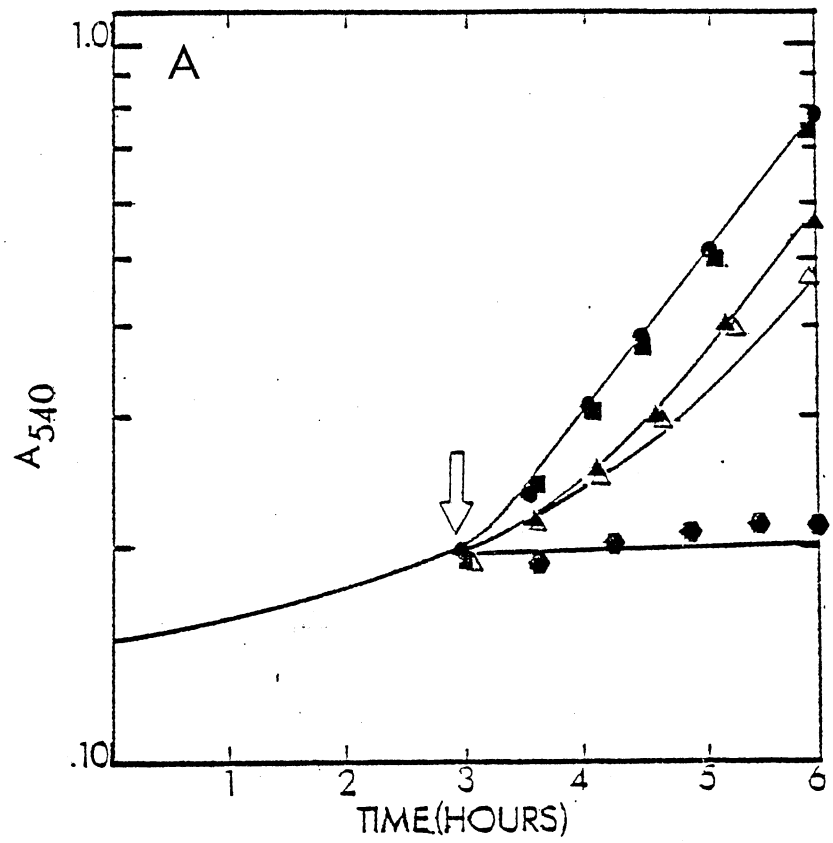
[a] Percent inhibition determined as in Table II.
[b] Average of at least three tests unless otherwise noted.
[c] Results of three experiments, fourth experiment showed 100 percent inhibition at initial inoculum of <0.25, 0.25 and 0.75.
[d] Results from four experiments, three other experiments showed 100 percent inhibition.

The effect of the time of addition of the thiazoles on their inhibition was tested by suspending the B. subtilis cells to a standard optical density and adding the test compounds at either zero time or at a time when the cultures were actively growing (3 hours after zero time). Figure 13a and b shows the effect of four concentrations added at the different times on the degree of inhibition of the methoxythiazole hydrochloride. At concentration of 1.5×10^{-4} M or higher, the methoxythiazole hydrochloride completely inhibited the growth of B. subtilis when added at zero time; a concentration of 7.9×10^{-5} M gave 67 percent inhibition. When the compound was added to actively growing cultures, none of the concentrations were able to totally inhibit growth. Growth inhibition was 70 percent in medium containing methoxythiazole hydrochloride at a concentration of 3.9×10^{-4} M and, as the concentration of the compound was lowered, the degree of inhibition also decreased; the lowest concentration tested (7.9×10^{-5} M) exhibiting control growth. Table V shows similar results for the other active compounds.

Reversal of Thiazole Induced Growth Inhibition

The thiazole induced growth inhibition of B. subtilis was tested for reversibility following removal of the test compound from the growth medium. B. subtilis cells were suspended in glucose minimal medium containing water (control) or the methoxythiazole at the following concentrations: 7.9×10^{-5} M, 1.5×10^{-4} M and 3.9×10^{-4} M. The cells were pelleted, washed with medium and suspended to the same absorbance in

Figure 13. Effect of Methoxythiazole Hydrochloride on B. subtilis. ●, control; ◆, methoxythiazole hydrochloride (3.9×10^{-4} M); Δ, methoxythiazole hydrochloride (2.4×10^{-4} M); ▲, methoxythiazole hydrochloride (1.5×10^{-4} M); ■, methoxythiazole hydrochloride (7.9×10^{-5} M). (A) Thiazole added to an actively growing culture. (B) Thiazole added to lag phase culture.


A
1.0
.10
A_{540}
1
2
3
4
5
6
TIME(HOURS)

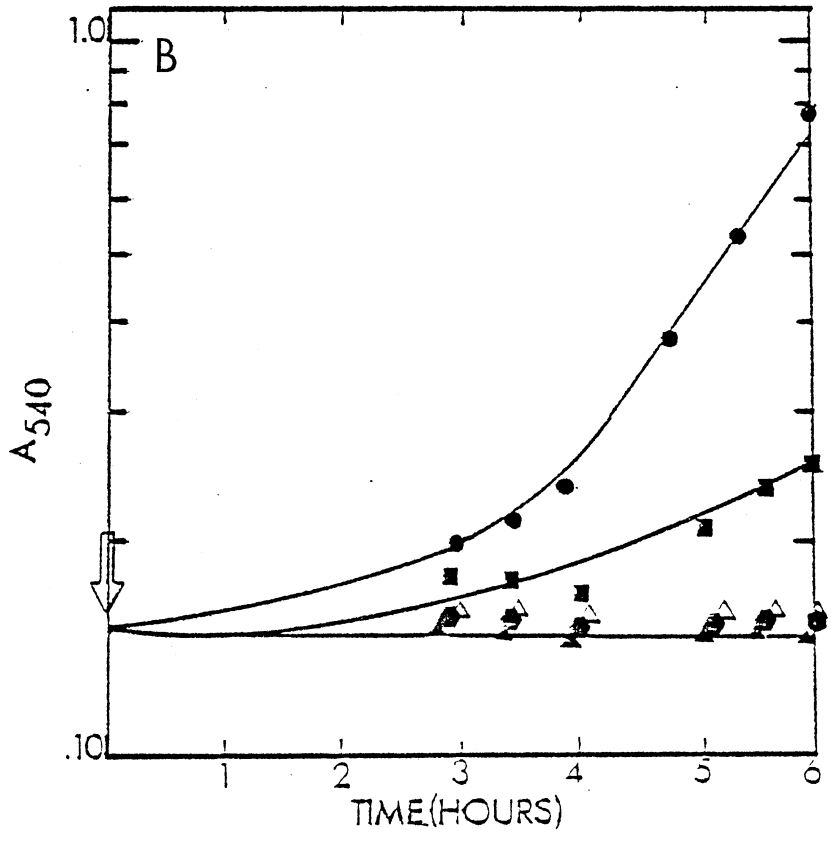

B
1.0
.10
A_{540}
1
2
3
4
5
6
TIME(HOURS)

TABLE V

EFFECT OF TIME OF ADDITION ON THIAZOLE-INDUCED GROWTH INHIBITION OF B. SUBTILIS

Compound	Concentration (M)	Percent Inhibition of Growth at 8 Hours	
		Time of Addition (Hour)	
		0[a]	0 + 3[b]
Methoxythiazole	4.3×10^{-4}	100	67
	1.72×10^{-4}	30	0
	8.62×10^{-5}	0	0
Dimethoxythiazole	3.8×10^{-4}	85	42
	1.52×10^{-4}	30	0
	7.6×10^{-5}	0	0
Hexyloxythiazole	3.3×10^{-4}	72	0
	1.32×10^{-4}	30	0
	6.6×10^{-5}	0	0

[a]Tubes containing glucose minimal medium containing thiazole or water (control) were inoculated to initial absorbancy (540 nm) of 0.15 from an overnight culture of B. subtilis percent inhibition was determined as in Table II.

[b]Same as above, except glucose minimal medium inoculated with overnight culture of B. subtilis was incubated three hours before addition of thiazoles or water (control).

fresh medium supplemented with water or methoxythiazole hydrochloride, incubated and growth measured.

The results (Figure 14) show that the control cells suspended in medium lacking the methoxythiazole hydrochloride quickly resumed growth and reached stationary phase at about 10 hours. In the reversal system where cells treated with 3.9×10^{-4} M methoxythiazole hydrochloride were resuspended in medium lacking the thiazole, growth was not resumed during the 24 hours of incubation. Where growth was only partially inhibited during the first phase of the study, the resuspended cells quickly resumed growth and reached the stationary phase at the same time as the control culture (10 hours).

The other compounds that showed activity posed a problem when trying to assess the reversibility of their inhibition; their insolubility in minimal medium meant that washing the cells would not remove them from the presence of the compounds. Instead, B. subtilis cells were suspended in minimal medium containing either water or the compounds (3.8×10^{-4} M to 4.3×10^{-4} M) and incubated under growth conditions for 2.5 hours. Samples (0.5 ml) were removed and suspended in fresh medium without thiazole, incubated and growth measured (Figure 15). Though the lag period for the compounds differed, once initiated, growth of cells in the reversal system proceeded at a slightly reduced rate and did attain the maximum cell growth as noted in the control.

These results indicate that the thiazoles with intermediate growth inhibition are reversible following removal of the steroid from the medium. The reversibility of the methoxythiazole hydrochloride is concentration dependent; at higher concentrations it is not reversible whereas at lower concentrations it is readily reversible.

Figure 14. Reversal of Methoxythiazole Hydrochloride Induced Growth Inhbition of B. subtilis. Cells were grown for two hours in the presence of the thiazole then washed and resuspended in glucose minimal medium lacking thiazole. ●, control; ◆, methoxythiazole hydrochloride (3.9×10^{-4} M); ■, methoxythiazole hydrochloride (1.58×10^{-4} M); ▲, methoxythiazole hydrochloride (7.9×10^{-5} M).

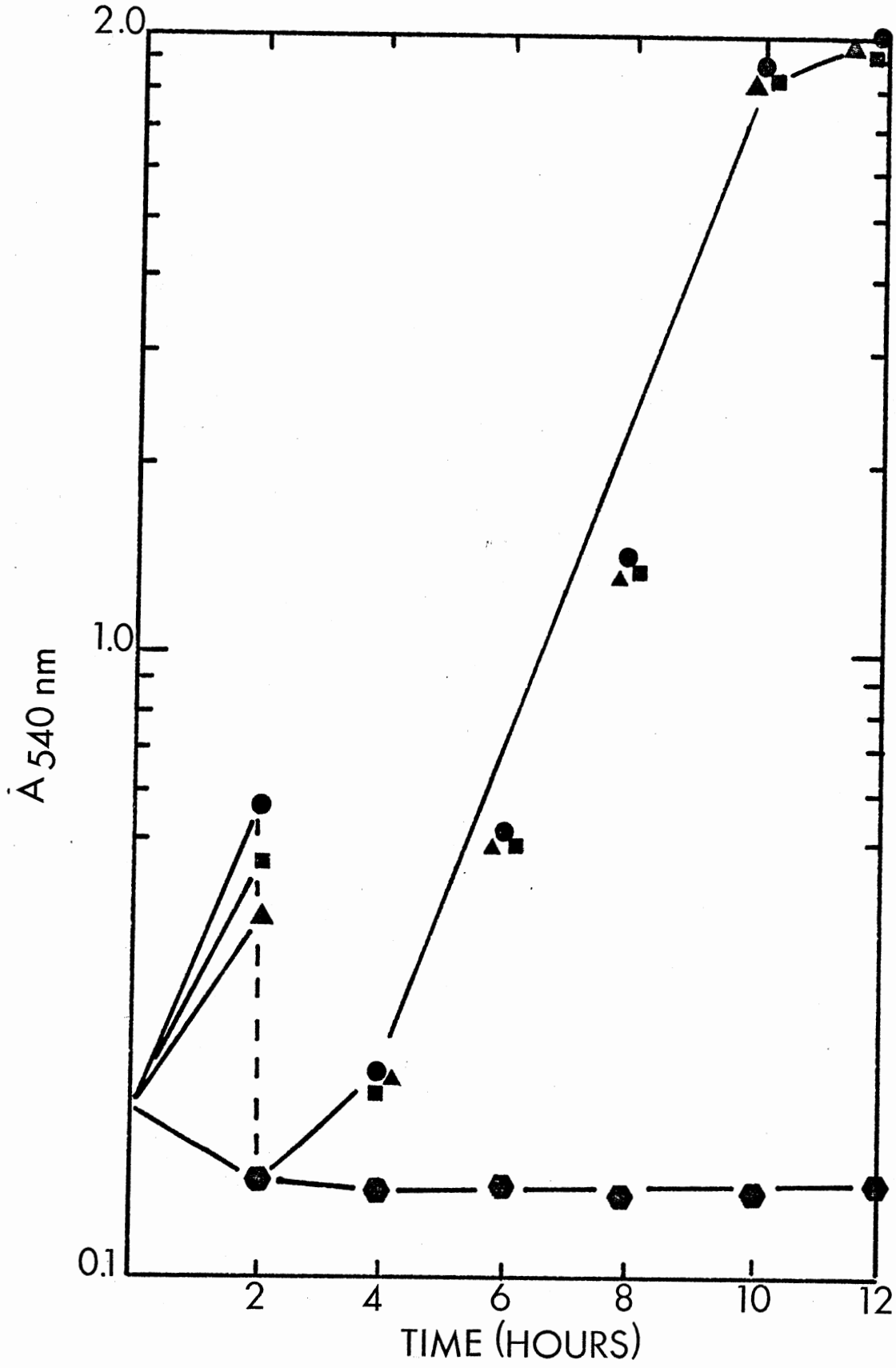
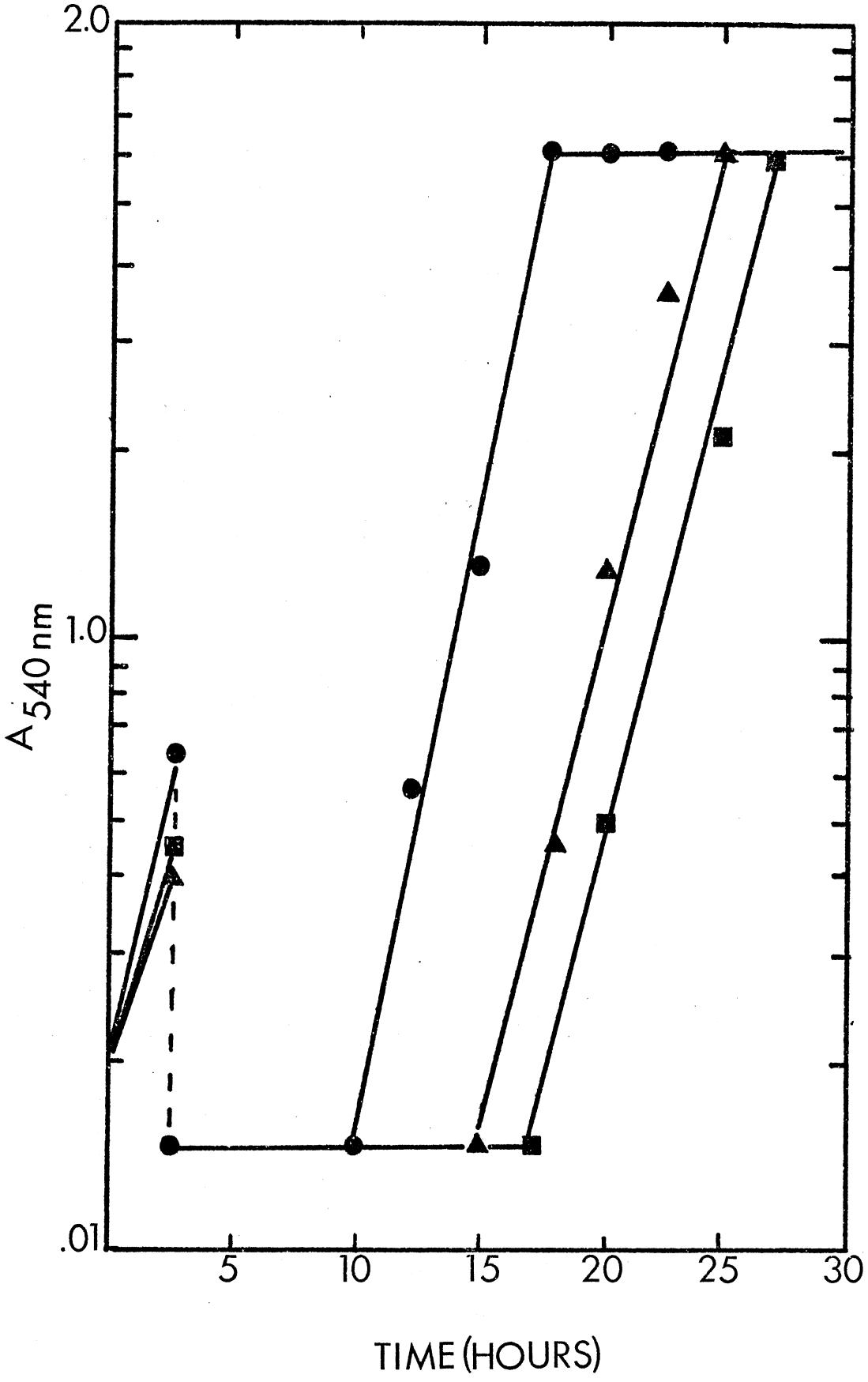

2.0
1.0
0.1
Å 540 nm
2
4
6
8
10
12
TIME (HOURS)

Figure 15. Reversal of Dimethoxy- and Methoxythiazole Induced Growth Inhibition of B. subtilis. Cells were suspended in medium containing the thiazole, after two hours .5 ml samples were inoculated into fresh medium without thiazole. ●, control; ▲, dimethoxythiazole (3.8×10^{-4} M); ■, methoxythiazole (4.3×10^{-4} M).


2.0
1.0
.01
$A_{540\,nm}$
5
10
15
20
25
30
TIME (HOURS)

Thiazole Stability Under Growth Conditions

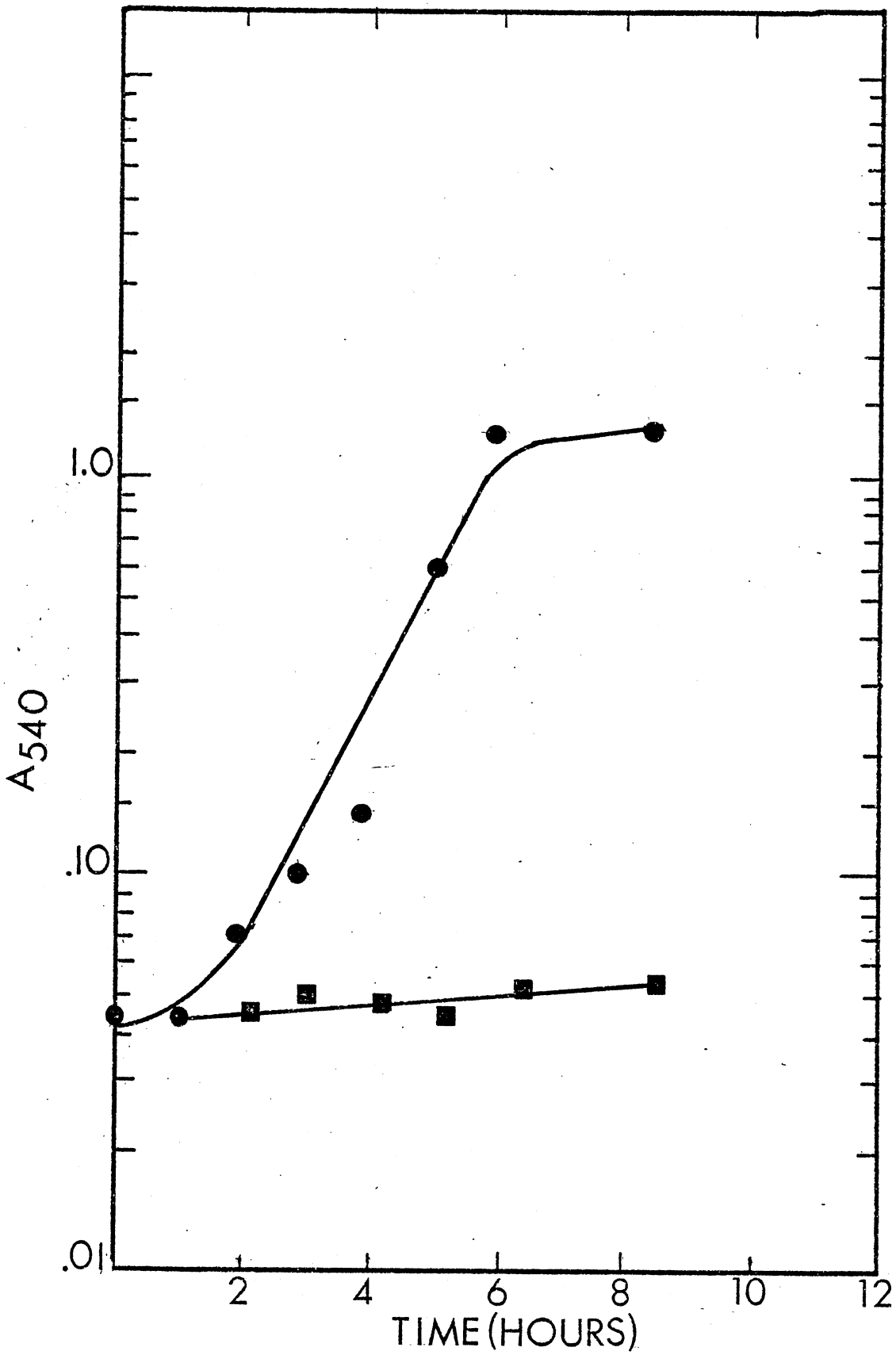
The stability of a compound within the experimental environment can be a limiting factor in its inhibition; both the degree and reversibility of inhibition can be a reflection of its breakdown under growth conditions. To learn more about the effects of the medium and incubation conditions on thiazoles, the stability of the methoxy- and hexyloxy-thiazoles was determined by both ultraviolet absorption spectroscopy and microbiological growth assay.

When the methoxythiazole hydrochloride was incubated in glucose minimal medium under growth conditions, the concentration remained the same during the 24 hours measurements were made. Because of their insolubility in glucose minimal medium, the stability of the other compounds could not be determined spectrophotometrically.

To complement the spectrophotometric studies of the methoxythiazole hydrochloride and to determine the stability of the insoluble compounds, a biological assay was utilized in which B. subtilis, the most sensitive microorganism tested, was inoculated into: (1) medium containing the thiazole which had been incubated for 24 hours, (2) fresh thiazole containing medium, and (3) incubated medium plus "fresh" unincubated steroid. Figure 16 shows the results with the methoxythiazole hydrochloride. The inhibition of the thiazole at all three concentrations (3.9×10^{-4} M, 1.58×10^{-4} M and 7.9×10^{-5} M) was the same for incubated and fresh thiazole. Similar results were obtained for all the thiazoles that exhibited activity against B. subtilis.

From these experiments it is obvious that the reversal of growth inhibition observed when the cultures began growing after a long lag period was not a result of the breakdown of the compound. The varying

Figure 16. Stability of Methoxythiazole Hydrochloride Under Growth Conditions. B. subtilis cells were suspended in glucose minimal medium containing either incubated or fresh methoxythiazole hydrochloride and growth followed. ●, glucose minimal medium control; ■, incubated or fresh methoxythiazole hydrochloride (7.9×10^{-5} M, 1.58×10^{-4} M and 3.9×10^{-5} M).


1.0
.10
.01
A_{540}
2
4
6
8
10
12
TIME (HOURS)

degrees of inhibitory activity of the different thiazoles was not a result of their varying instability under growth conditions.

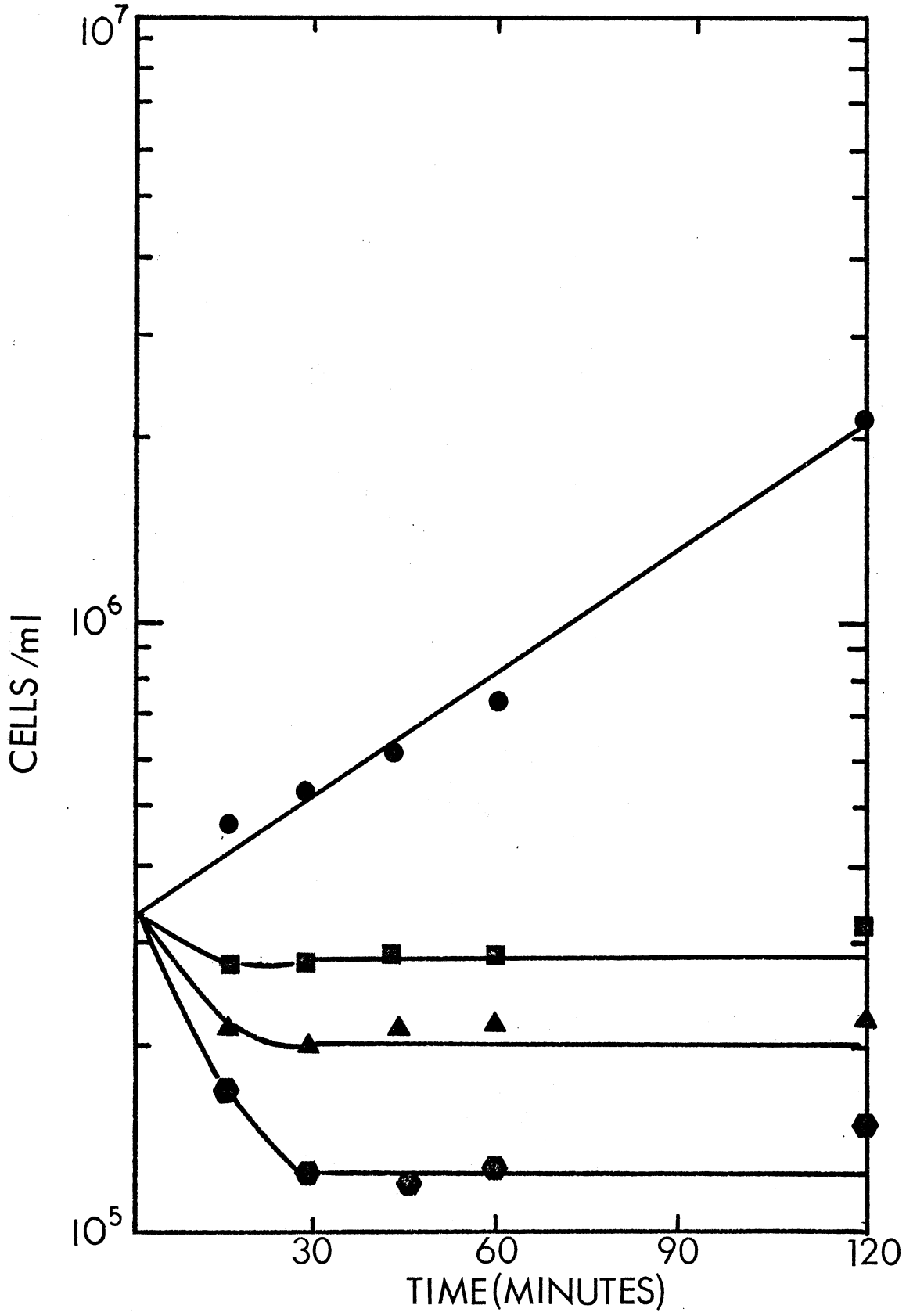
Effect of Thiazole on Cell Viability

Studies were conducted to determine if the thiazole inhibition was bacteriostatic or bacteriocidal. The test compounds (3.8 to 4.3 x 10^{-4} M) were added to actively growing cultures, samples removed, diluted, plated in nutrient agar and the colonies counted (Figure 17).

While the control cells steadily increased in numbers, the count in the treated cultures declined during the first hour and then leveled off for the last hour the counts were taken. Although addition of the methoxy- and dimethoxythiazole did show an initial increase in the number of viable organisms, these results indicate that at the concentrations tested the compounds are not bacteriocidal since a large number of microorganisms did remain viable.

It could be postulated that the viable cells after two hours of treatment with the thiazole represented a naturally occurring portion of the original cell population (before thiazole treatment) which was less sensitive to thiazole action. To test this hypothesis, cells exposed to thiazole or water (control) for two hours were washed, 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 17, indicating that no thiazole-resistant cell population predominated in the surviving culture.

Figure 17. Effect of Methoxythiazoles on B. subtilis Cell Viability. ●, control; ⬢, methoxythiazole hydrochloride (3.9×10^{-4} M); ▲, methoxythiazole (4.3×10^{-4} M); ■, dimethoxythiazole (3.8×10^{-4} M).


10^7
10^6
10^5
CELLS/ml
30
60
90
120
TIME (MINUTES)

Thiazole Inhibition of Substrate Accumulation

To help elucidate a possible site or mechanism of action, the effect of the test compounds on the accumulation of various labeled substrates was determined. The substrates were selected in order to focus on different cellular processes which could be primary sites of action: ^{14}C-alanine for protein synthesis, ^{14}C-uracil for RNA synthesis, ^{14}C-thymine for DNA synthesis, ^{14}C-glycerol for membrane synthesis, ^{14}C-diaminopimelic acid for cell wall mucopeptide synthesis, and ^{14}C-glucose for membrane permeability.

Figure 18 shows the results with ^{14}C-uracil when the B. subtilis cells were treated with methoxythiazole hydrochloride added to an actively growing culture. When uracil and a non-inhibitory concentration of the methoxythiazole hydrochloride (7.9×10^{-5} M) were added simultaneously, there was no inhibition of accumulation of the substrate. As the concentration was increased, there was a decreased incorporation of uracil that mirrored the increase in growth inhibition.

When the methoxythiazole hydrochloride (3.96×10^{-4} M) was added 3 hours before ^{14}C-uracil, at zero time, to a culture of B. subtilis, there was total inhibition of accumulation of uracil (Figure 19). Decreasing the concentration of methoxythiazole hydrochloride caused a slight increase in incorporation for 2 hours but then incorporation declined. At the intermediate concentrations (2.4×10^{-4} M and 1.68×10^{-4} M) growth inhibition was 100 percent. At the lowest concentration growth inhibition was 70 percent.

Table VI summarizes the effect of methoxythiazole, dimethoxythiazole and hexyloxythiazole on uracil incorporation. Whether added

Figure 18. Effect of Methoxythiazole Hydrochloride Concentration on ^{14}C-Uracil Incorporation in B. subtilis When the Thiazole is Added to an Actively Growing Culture. ●, control; □, methoxythiazole hydrochloride (3.9×10^{-4} M); ○, methoxythiazole hydrochloride (2.4×10^{-4} M); △, methoxythiazole hydrochloride (1.58×10^{-4} M); ▪, methoxythiazole hydrochloride (7.0×10^{-5} M).

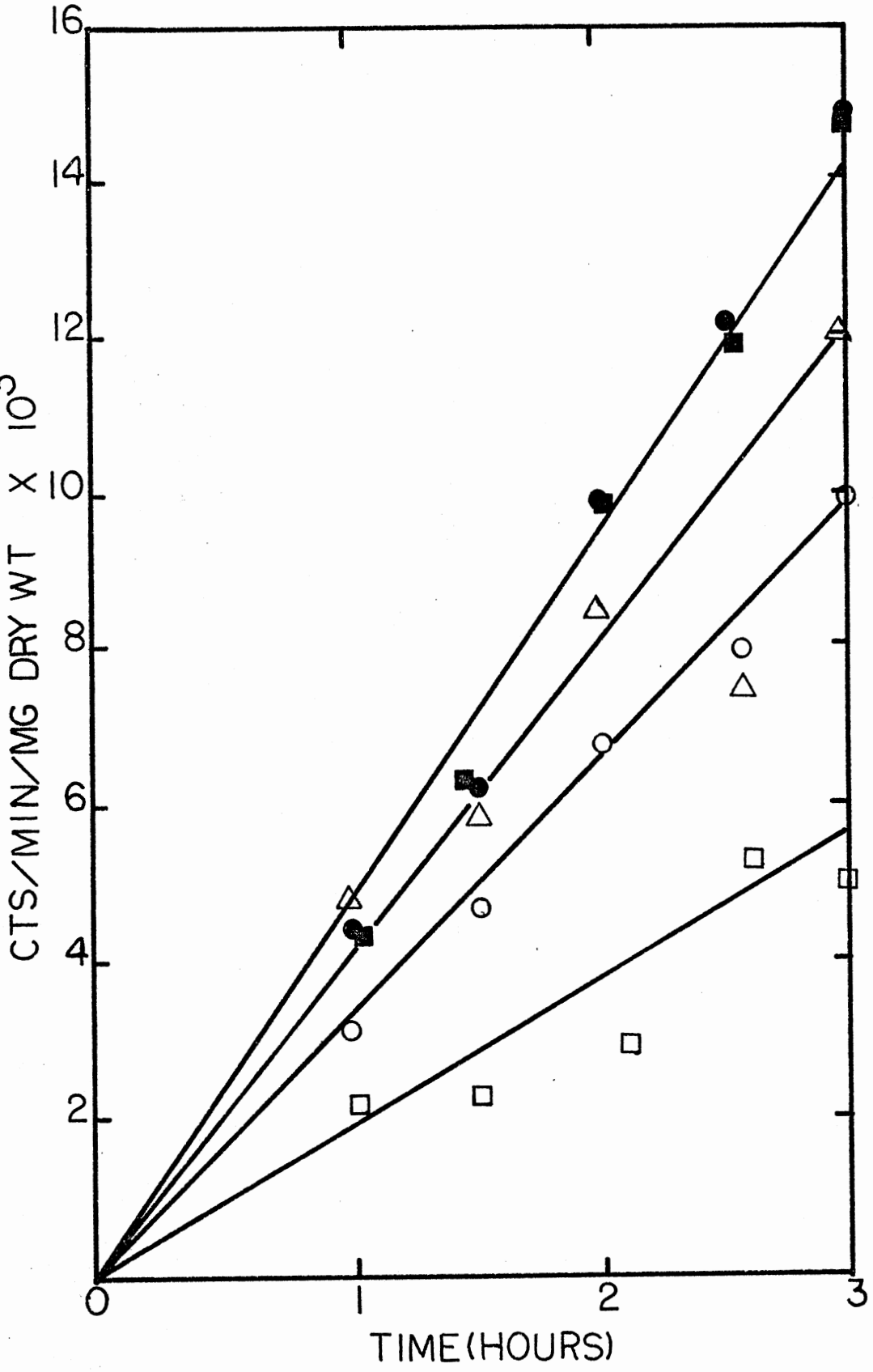

16
14
12
10
8
6
4
2
0
1
2
3
CTS/MIN/MG DRY WT X 10^3
TIME (HOURS)

Figure 19. Effect of Methoxythiazole Hydrochloride on ^{14}C-Uracil Incorporation of B. subtilis When Thiazole is Added at Zero Time. ●, control; □, methoxythiazole hydrochloride (3.9×10^{-4} M); ○, methoxythiazole hydrochloride (2.4×10^{-4} M); Δ, methoxythiazole hydrochloride (1.58×10^{-4} M); ■, methoxythiazole hydrochloride (7.9×10^{-5} M).

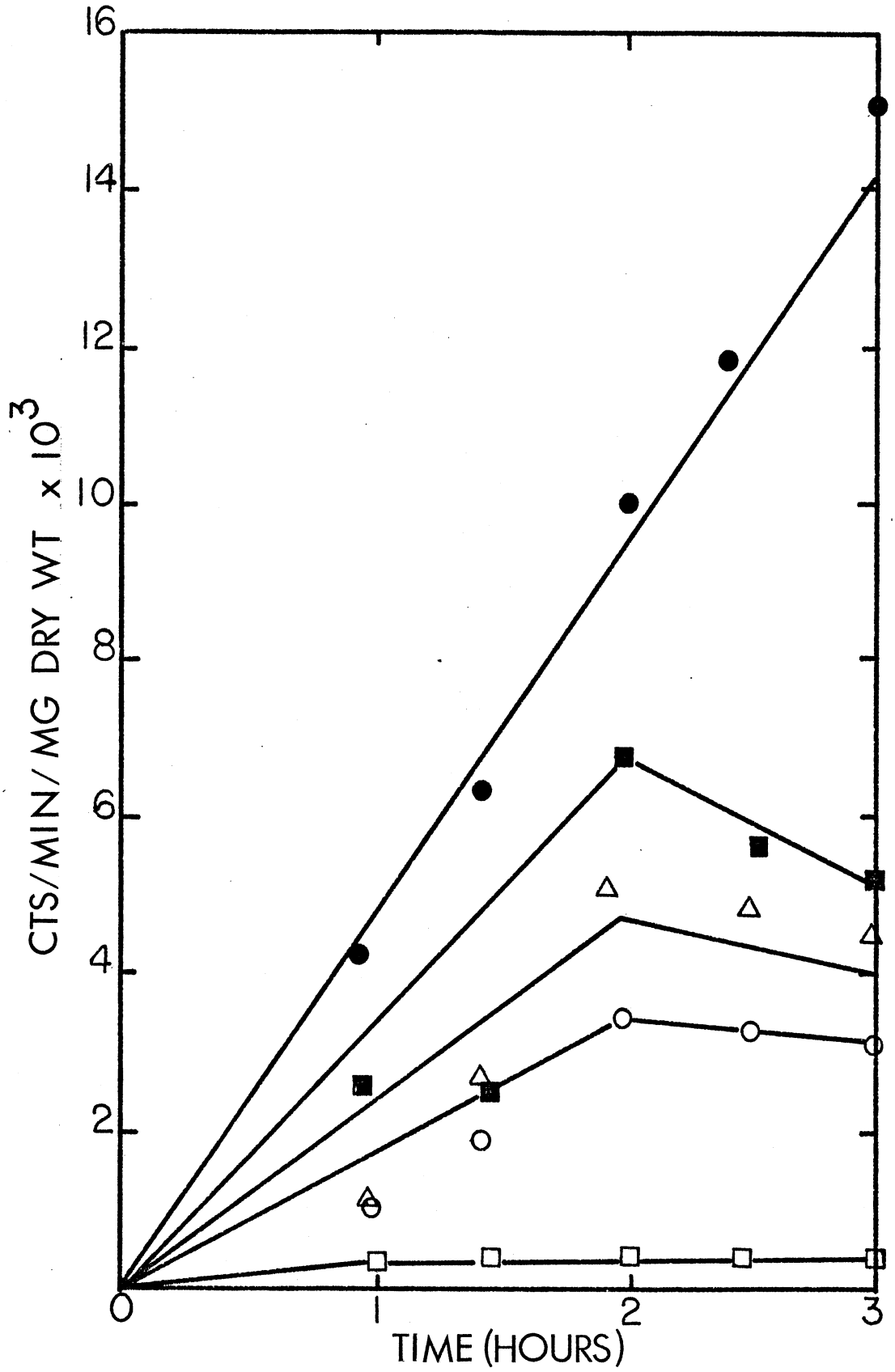

16
14
12
10
8
6
4
2
0
1
2
3
CTS/MIN/MG DRY WT x 10^3
TIME (HOURS)

TABLE VI

EFFECT OF THIAZOLES ON ^{14}C-URACIL INCORPORATION OF *B. SUBTILIS*

Compound	Concentration (M)	CPM/mg Dry Weight at 3 Hours Time of Addition (Hour) 0	0 + 3
Control	--	14,206	14,100
Methoxythiazole	4.3×10^{-4}	1,253	8,000
	2.45×10^{-4}	4,547	12,507
	1.72×10^{-4}	6,326	14,272
	8.62×10^{-5}	12,503	14.350
Dimethoxythiazole	3.8×10^{-4}	3,124	10,717
	2.3×10^{-4}	4,212	11,364
	1.5×10^{-4}	9,372	14,506
	7.6×10^{-5}	14,518	14,650
Hexyloxythiazole	3.3×10^{-4}	6,627	9.754
	2.0×10^{-4}	8,234	11,471
	1.32×10^{-4}	14,505	13.987
	6.6×10^{-5}	14,106	14,361

to growing cultures or at zero time, an increase in growth inhibition caused by an increase in test compound concentration was reflected in a decrease in accumulation.

All test compounds exhibited a similar effect on alanine, thymine, diaminopimelic acid and glucose incorporation.

Thiazole Inhibition of Substrate Uptake

It seemed plausible, based on the foregoing data, that thiazole inhibition of substrate accumulation was a result of a general change in membrane permeability. To test this hypothesis, the effect of thiazole on short-term uptake of labeled substrate was measured by adding radioactive material to growing B. subtilis cultures followed by addition of the steroid or water (control). Samples were removed at the indicated times, washed, counted, and the uptake of radioactivity was determined.

In the absence of test compound, B. subtilis rapidly concentrated ^{14}C-glucose (Figure 20). The addition of methoxythiazole hydrochloride (2.4×10^{-4} M) immediately inhibited uptake, an effect which continued throughout the 5 minutes of testing. In addition to glucose, an inhibition of uptake was observed using alanine (Figure 21), serine, lysine, leucine and uracil in the presence of methoxythiazole hydrochloride.

The dimethoxy- and methoxythiazole showed an effect similar to the methoxythiazole hydrochloride on the uptake of the various substrates tested but at higher concentrations (Figures 20 and 21). At the concentrations measured, the hexyloxythiazole hydrochloride showed no effect on uptake while the hexlyonythiazole showed very little effect (Figures 20 and 21).

Figure 20. Effect of Thiazoles on Uptake of ^{14}C-Glucose by B. subtilis When the Thiazole is Added to an Actively Growing Culture. ●, hexyloxythiazole hydrochloride (1.6×10^{-4} M) and control; Δ, methoxythiazole hydrochloride (2.4×10^{-4} M); ■, methoxythiazole (2.6×10^{-4} M); □, dimethoxythiazole (3.1×10^{-4} M); O, hexyloxythiazole (1.9×10^{-4} M).

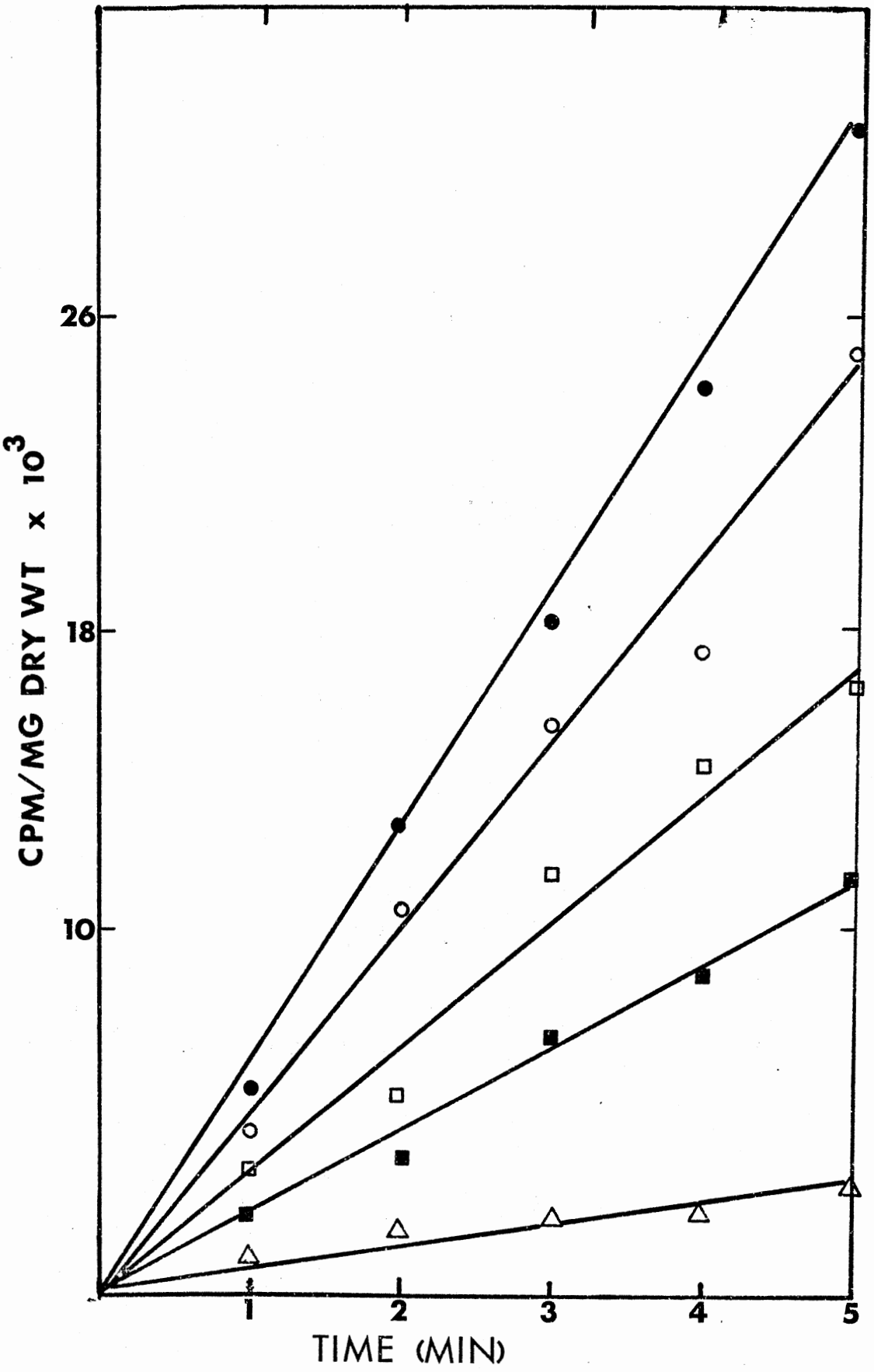
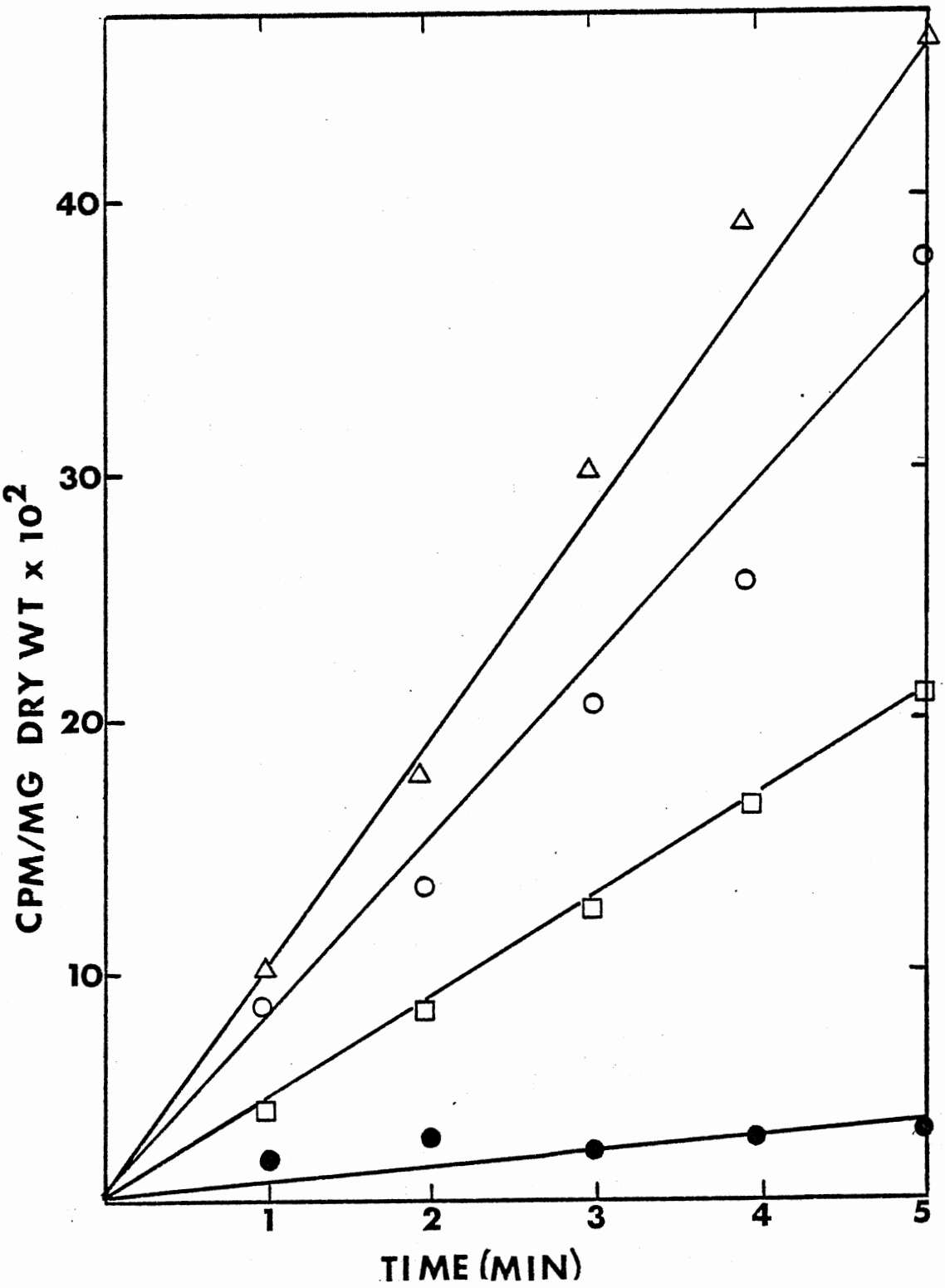

CPM/MG DRY WT x 10^3
26
18
10
1
2
3
4
5
TIME (MIN)

Figure 21. Effect of Thiazoles on ^{14}C-Alanine Uptake in B. subtilis When the Thiazole is Added to an Actively Growing Culture. Δ, hexyloxythiazole hydrochloride (1.6×10^{-4} M) and glucose minimal medium control; ●, methoxythiazole hydrochloride (2.4×10^{-4} M); □, methoxythiazole (2.6×10^{-4} M); ○, dimethoxythiazole and hexyloxythiazole (1.9×10^{-4} M).


40
30
20
10
CPM/MG DRY WT x 10^2
1
2
3
4
5
TIME (MIN)

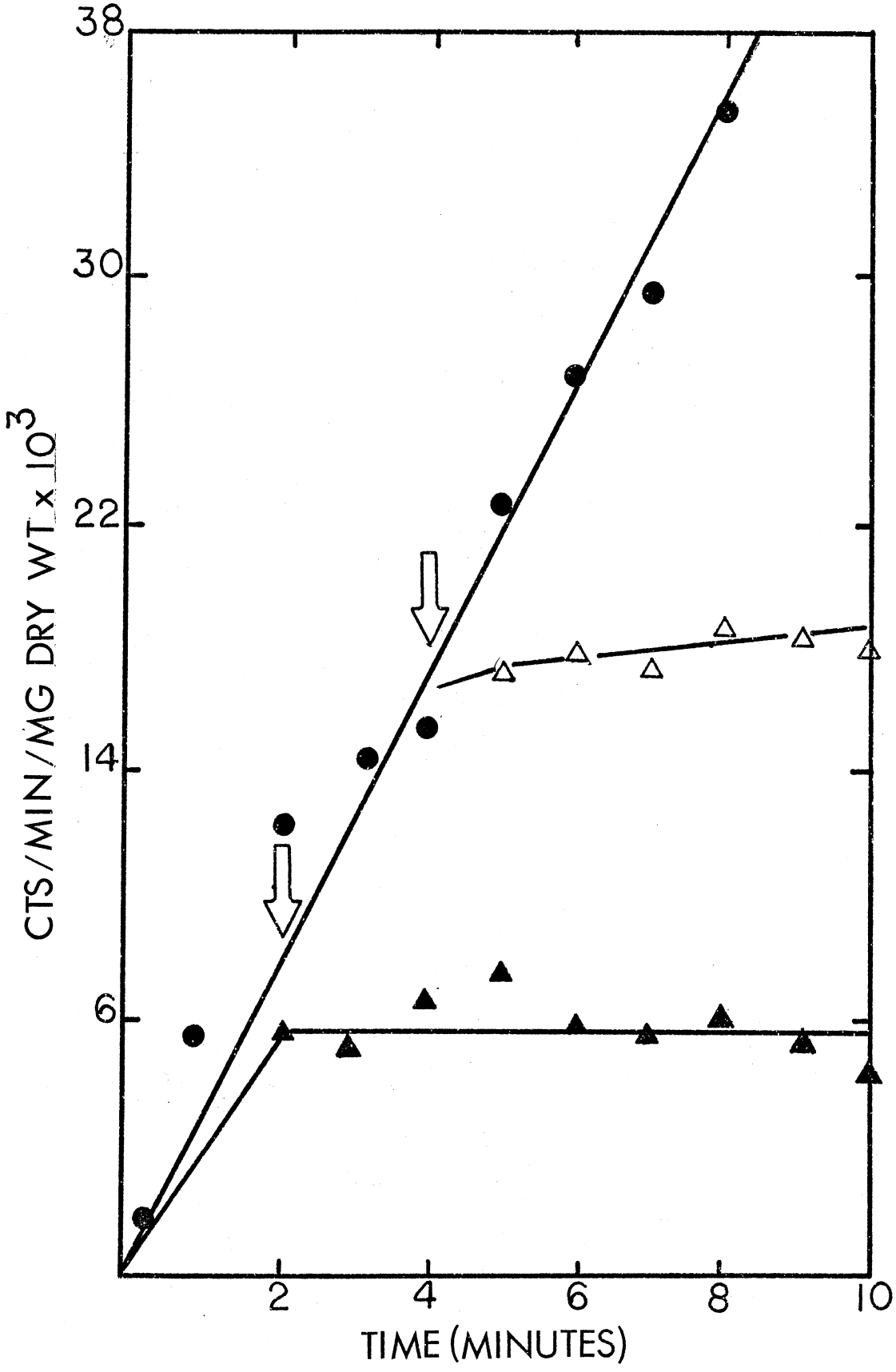
When the effect of similar concentration of the five compounds on short-term uptake was compared, the compounds showed the same degree of activity as they had in the growth studies (Figures 20 and 21). The methoxythiazole hydrochloride (2.4×10^{-4} M) showed the greatest activity by totally inhibiting glucose and alanine uptake. The methoxythiazole (2.58×10^{-4} M) and the dimethoxythiazole (2.3×10^{-4} M) showed intermediate activity. The methoxythiazole inhibited glucose uptake 70 percent and the alanine uptake 63 percent during the 5 minutes measurements were taken. The dimethoxythiazole inhibited glucose uptake 50 percent and alanine uptake 15 percent. The hexyloxythiazole showed the least inhibition of the active compounds giving only 10 percent inhibition of glucose uptake. The hexyloxythiazole hydrochloride exhibited no inhibition of glucose or alanine uptake.

The Speed of Thiazole Induced Inhibition of Substrate Uptake

To determine how quickly the thiazoles could inhibit substrate uptake, labeled substrate was added to growing cultures of *B. subtilis* and the test compound or water (control) was added either 2 or 4 minutes after the label. Samples were taken at indicated times, washed, and counted and the uptake of radioactivity was determined.

Untreated cells rapidly accumulated ^{14}C-glucose (Figure 22). When the methoxythiazole hydrochloride (2.4×10^{-4} M) was added 2 minutes after the label, there was a 69 percent reduction in accumulation within 1 minute and an 80 percent reduction after 8 minutes. When the compound was added 4 minutes after the label, there was a reduction in accumulation of 16 percent after 2 minutes and a 42 percent reduction at the end

Figure 22. Speed of Methoxythiazole Hydrochloride-Induced Inhibition of ^{14}C-Glucose Uptake in B. subtilis. ●, control; ▲, methoxythiazole hydrochloride (2.4×10^{-4} M) added at 2 minutes; Δ, methoxythiazole hydrochloride (2.4×10^{-4} M).


38
30
22
14
6
CTS/MIN/MG DRY WT x 10^3
2
4
6
8
10
TIME (MINUTES)

of the 10 minute counting period. To achieve the same results with the methoxythiazole and the dimethoxythiazole required higher concentrations, 4.3×10^{-4} M and 3.81×10^{-4} M, respectively.

These results demonstrate the immediate inhibition of labeled substrate accumulation caused by the active thiazole compounds and suggest an interaction of the thiazole with the cell membrane.

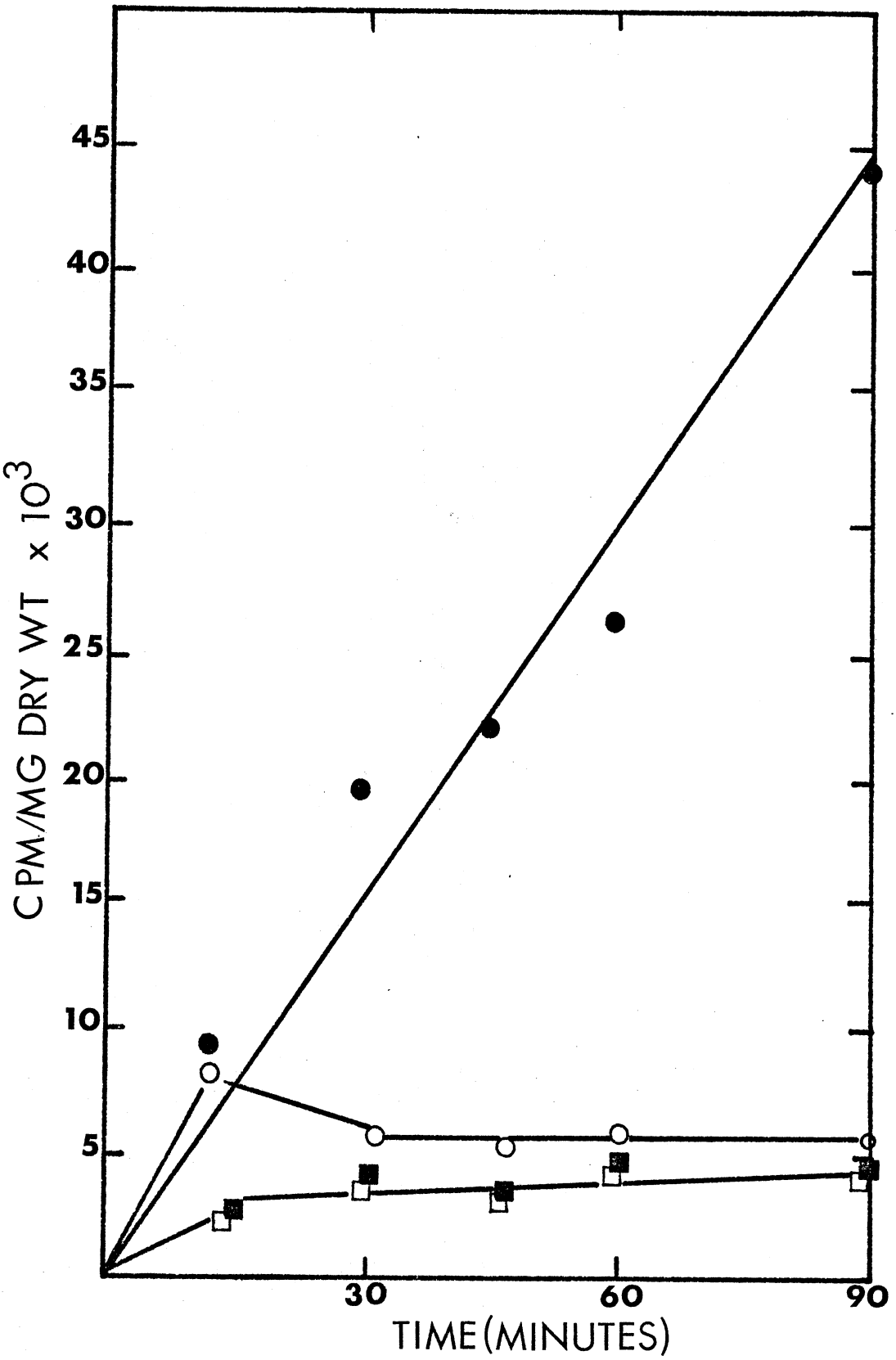
Reversibility of Accumulation Inhibition

The reversibility of thiazole-induced accumulation inhibition was determined using B. subtilis cells suspended in glucose minimal medium containing either methoxythiazole hydrochloride (3.96×10^{-4} M) or water (control) for 2 hours. After 2 hours, the cells were pelleted, washed with glucose minimal medium and the pellet resuspended in fresh medium with labeled glucose. For the next 3 hours samples were removed, washed, counted and glucose accumulation determined. The results (Figure 23) show that inhibition of glucose accumulation was not reversed by the removal of the methoxythiazole hydrochloride from the medium.

Effect of Divalent Cations on Thiazole Induced Growth Inhibition

Earlier results from this study suggested that the thiazoles exert some action at the cell membrane. Since magnesium ions stabilize the bacterial membrane (Weibull,1956) and di- and multivalent cations may prevent membrane swelling by influencing conformation of membrane proteins (Brown, 1962), experiments were conducted to determine if the observed growth inhibition and the inhibition of substrate accumulation could result from thiazole chelation or competition with divalent

Figure 23. Reversal of Thiazole Induced Inhibition of ^{14}C-Glucose Incorporation in B. subtilis. ●, control untreated cells resuspended in fresh medium; □, methoxythiazole hydrochloride (3.96×10^{-4} M) treated cells resuspended in fresh glucose minimal medium without thiazole; ■, untreated cells suspended in medium supplemented with methoxythiazole hydrochloride (3.96×10^{-4} M); ○, methoxythiazole hydrochloride (3.96×10^{-4} M) treated cells resuspended in medium containing methoxythiazole hydrochloride (3.96×10^{-4} M).


45
40
35
30
25
20
15
10
5
CPM/MG DRY WT x 10^3
30
60
90
TIME (MINUTES)

cations. Cultures of B. subtilis were supplemented with 5.3×10^{-3} M magnesium chloride, manganese chloride or potassium chloride or 5.3×10^{-4} M ferric chloride and thiazole (2.95×10^{-4} M - 3.96×10^{-4} M). Neither the resulting growth inhibition nor the inhibition of substrate accumulation was affected by the presence of di- or multivalent cations in the growth medium (Table VII).

Thiazole Induced Change in Membrane Permeability

Studies were undertaken to determine if the thiazole inhibition of uptake could be related to a possible detergent-like action on the membrane resulting in leakage of metabolite precursors. To test this hypothesis, leakage of both radioactive and nonradioactive compounds was examined.

The leakage of nucleic acids from treated cells was measured by the change in absorbance of the supernatant at 260 nm and 280 nm. B. subtilis cells were incubated with the test compounds, periodically 5 ml samples were removed, centrifuged and the absorbance of the supernatant measured. The major problem in this study was that the test compounds also absorbed in the 260 to 280 nm range. Attempts were made to use a standard preparation of the test compounds as a blank but results from the experiments were inconclusive. The possibility exists that because the amount of compound adsorbed to the surface of the cell or actually taken up by the cell could not be taken into account more compound was blanked out than was actually present in the supernatant and leakage of small quantities of nucleic acids was masked.

To overcome this problem, small quantities (25 µl) of the supernatant were spotted on chromatography paper (Whatman #1), chromatographed

TABLE VII

EFFECT OF CATIONS ON THIAZOLE-INDUCED GROWTH AND UPTAKE INHIBITION ON B. SUBTILIS[a]

Compound	Cation[b]	Glucose Uptake at 5 Minutes (CPM/mg dry weight x 10^3)	Percent Inhibition of Control Growth
Control		37.0	--
Methoxythiazole Hydrochloride	--	2.7	100
	Mg^{++}	2.6	100
	Mn^{++}	2.4	100
	K^{++}	2.2	100
	Fe^{+++}	2.4	100
	Mg^{++}, Mn^{++}, K^{++}, Fe^{+++}	2.3	100
Methoxythiazole	--	7.2	100
	Mg^{++}	7.1	100
	Mn^{++}	7.4	100
	K^{++}	7.3	100
	Fe^{+++}	7.3	100
	Mg^{++}, Mn^{++}, K^{++}, Fe^{+++}	7.3	100
Dimethoxythiazole	--	17.1	100
	Mg^{++}	17.0	100
	Mn^{++}	17.0	100
	K^{++}	17.4	100
	Fe^{+++}	17.2	100
	Mg^{++}, Mn^{++}, K^{++}, Fe^{+++}	17.1	100

[a] Percent inhibition was determined as in Table II.

[b] Reversal studies were run with the following concentrations of thiazoles: 3.9×10^{-4} M methoxythiazole hydrochloride; 4.3×10^{-4} M methoxythiazole; 3.8×10^{-4} M dimethoxythiazole. Glucose minimal medium was supplemented with 5.3×10^{-3} M magnesium chloride, manganese chloride or or potassium chloride or 5.3×10^{-4} M ferric chloride.

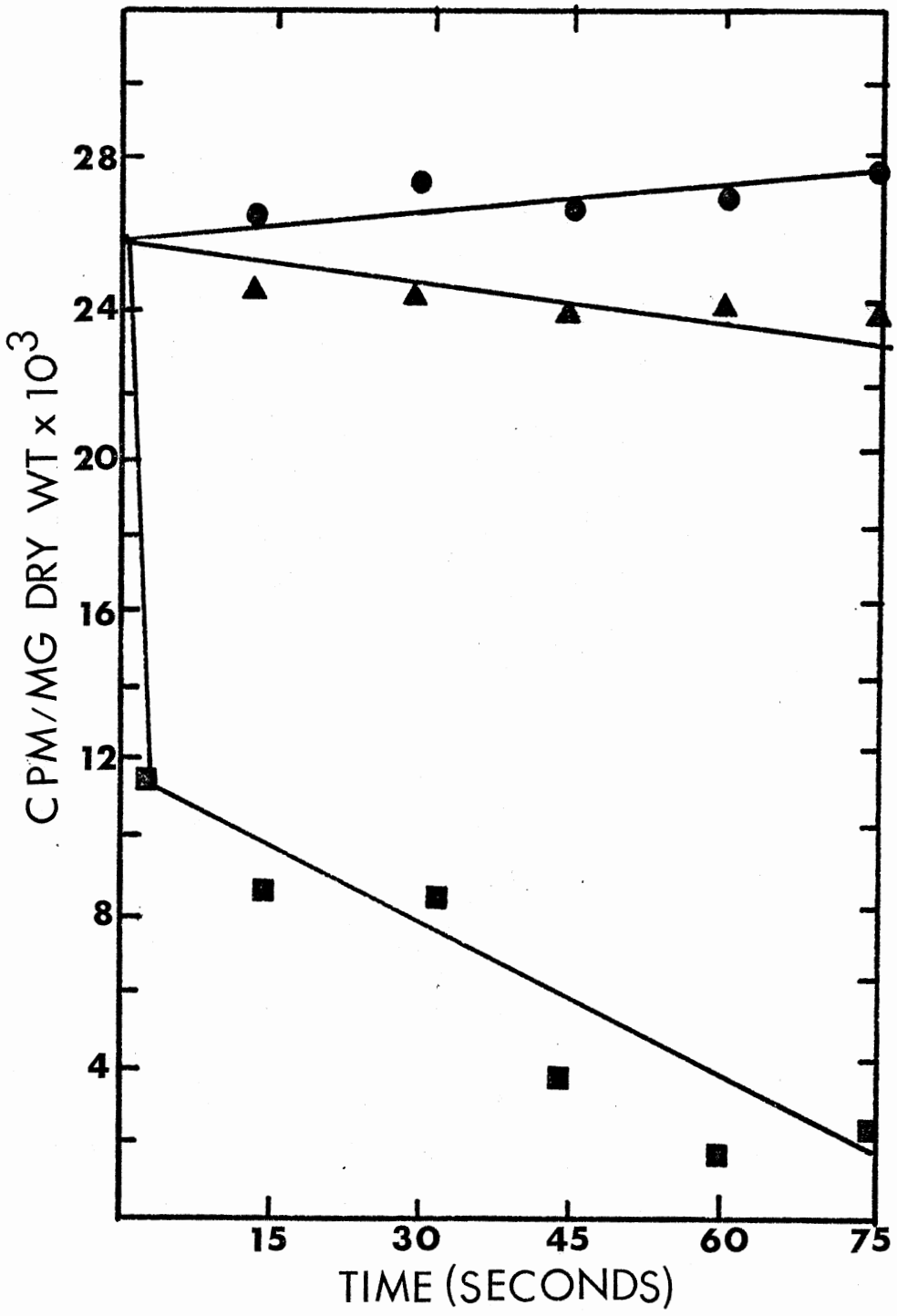
in one direction in the first solvent (isopropanol and formic acid), air dried, and chromatographed in the second direction in the second solvent (t-butanol and methylethylketone). The paper was either sprayed or exposed to reagents to locate either amino acids, nucleic acids or double-bonded compounds. Controls were run so that any spots due to the compounds could be located. The results again were rather inconclusive; the same spots were found on both chromatograms. It is still plausible that a subtle change in membrane permeability would be missed by this technique. Concentrating large quantities of supernatant could possibly resolve this question but this was prohibited by the small quantities of test compounds available.

Using a labeled, non-metabolizable substrate membrane permeability changes induced by the thiazole could be measured without interference from the thiazole. B. megaterium cells were labeled with ^{14}C-α-aminoisobutyric acid, a non-metabolizable substrate (Marquis and Gerhardt, 1964), and treated with water (control), 3.9×10^{-4} M methoxythiazole hydrochloride or 1 percent Triton X-100.

Initial studies were performed by labeling B. megaterium cultures suspended in glucose minimal medium under growth conditions with 1 ml (5 μCi/ml) of α-aminoisobutyric acid for 5 minutes. At time zero 6 ml aliquots of the labeled cells were added to 6 ml of medium containing water, 7.8×10^{-4} M methoxythiazole hydrochloride or 2 percent Triton X-100, samples were removed and the retention of radioactivity determined.

The results (Figure 24) indicated that in the control there was no leakage of labeled "pool" material but there does appear to be a slight leakage of the "pool" material in the methoxythiazole hydrochloride

Figure 24. Effect of Methoxythiazole on Leakage of ^{14}C-α-Aminoisobutyrate from B. megaterium. ●, control; ■, triton-X 100 (1 percent); ▲, methoxythiazole hydrochloride (3.9×10^{-4} M).


28
24
20
16
12
8
4
CPM/MG DRY WT x 10^3
15
30
45
60
75
TIME (SECONDS)

treated cells. In contrast, the non-ionic detergent, Triton X-100, caused complete release of the labeled precursor. The other methoxythiazoles and the hexyloxythiazole showed no leakage of substrate.

These observations give some support to the assumption that at least the methoxythiazole hydrochloride could cause a subtle perturbation of the membrane, but it does not have a detergent-like action which would result in major permeability changes.

Binding of Thiazole to Whole Cells

An initial attempt was made to quantitate the binding of the thiazole to whole cells by measuring the absorbance of the supernatant after various periods of incubation with the methoxythiazole hydrochloride. The results were quite erratic; there was an increase in the concentration of the compound from zero time indicating a greater concentration than was originally suspended in the medium. This could be accounted for if there was a leakage of compounds that absorbed in the UV range like the methoxythiazole hydrochloride.

To provide indirect proof for binding, three concentrations of methoxythiazole hydrochloride (7.9×10^{-5} M, 1.68×10^{-4} M, and 3.96×10^{-4} M) were incubated with B. subtilis for 2.5 hours, the cells were pelleted and the supernatant was inoculated with untreated cells. At the most inhibitory concentration (3.96×10^{-4} M), there was total inhibition of growth for 24 hours (Table VIII). The control culture for the intermediate concentration (1.68×10^{-4} M) gave 60 percent inhibition of growth at 6 hours while the incubated thiazole inhibited growth only 48 percent. At the lowest concentration (7.9×10^{-5} M), the control

TABLE VIII

BINDING OF METHOXYTHIAZOLE HYDROCHLORIDE BY *B. SUBTILIS*

Compound	Concentration (M)	Percent Inhibition of Growth[a] 6 Hours	24 Hours
Incubated:			
Methoxythiazole Hydrochloride	3.96×10^{-4}	100	100
	1.68×10^{-4}	48	0
	7.9×10^{-5}	0	0
Unincubated:			
Methoxythiazole Hydrochloride	3.96×10^{-4}	100	100
	1.68×10^{-4}	60	0
	7.9×10^{-4}	50	0

[a]Tubes of glucose minimal medium containing either thiazole that had been previously incubated with *B. subtilis* or fresh thiazole were inoculated to an initial absorbancy (540 nm) of 0.15 and growth inhibition determined as in Table II.

showed growth inhibition of 50 percent at 6 hours while the incubated thiazole showed control growth.

Though the amount of compound bound by the cell cannot be determined, the results suggest that a possible irreversible binding of some of the compound takes place.

Thiazole Inhibition of Cell-Free Systems

The original hypothesis under which this work was begun was that by changing the lipophilicity of a compound its interaction or passage across the membrane and therefore its degree of biological activity could be affected. It would thus be natural to assume that removal of the permeability barrier (i.e., the cell membrane) would negate differences based on permeability. Using a cell-free system, such as thermal denaturation of bacterial DNA, is one way in which studies could be carried out and possibilities explored.

Thermal Denaturation of DNA

DNA, isolated from both *P. fluorescens* and *B. subtilis*, at a concentration of 20 µg/ml was incubated with the test compounds (7.9×10^{-5} M) or water (control). A ratio of the changes in absorbancy at 260 nm at different temperatures was plotted and the "melting point", the temperature at which the steeply rising part of the curve reaches half the ultimate increase of UV absorbance (Marmur and Doty, 1959), was determined for each compound.

Using DNA isolated from *P. fluorescens*, the melting point for the control was 76 C. The melting point for none of the compounds could be determined since only a small change was observed at temperatures as

high as 85 C (Figure 25). The same results were found when DNA from B. subtilis was used. This stabilization is most probably the result of the compounds complexing with the DNA molecule though the significance of this complex to biological activity needs further study. These results lend support to the hypothesis that the degree of activity of different thiazoles is an expression of their relative lipophilicity.

Effect of Thiazole Concentration on Thermal Denaturation

The effect of varying the concentration of the thiazoles on the DNA "melting point" was determined. As with the growth inhibition studies, incremental changes in the concentration of the methoxythiazole hydrochloride were reflected in incremental decreases in the "melting point" (Figure 26). At a concentration of 7.9×10^{-5} M a melting point could not be determined since only a small change in optical density was observed at temperatures as high as 85 C. When the concentration was decreased by half (3.96×10^{-5} M), the percent inhibition of denaturation was decreased by 20 percent and the melting point was 82 C, a shift of 6 C from control. At the lowest concentration (1.98×10^{-5} M), the melting point of the methoxythiazole treated DNA (78 C) was shifted almost to that of the control (76 C). Similar results were found with the other four thiazoles against P. fluorescens DNA and with all five compounds against B. subtilis DNA (Table IX).

Reversibility of Thiazole Induced Inhibition of DNA Denaturation

The reversibility of the interaction of methoxythiazole

Figure 25. Effect of Thiazoles on Thermal Denaturation of P. fluorescens DNA. □, control, DNA (20 µg/ml); ○, methoxythiazole hydrochloride (7.9×10^{-5} M) and DNA (20 µg/ml); ●, dimethoxythiazole (8.62×10^{-5} M) or hexyloxythiazole hydrochloride (5.9×10^{-5} M) and DNA (20 µg/ml); Δ, hexyloxythiazole (6.6×10^{-5} M) and DNA (20 µg/ml).

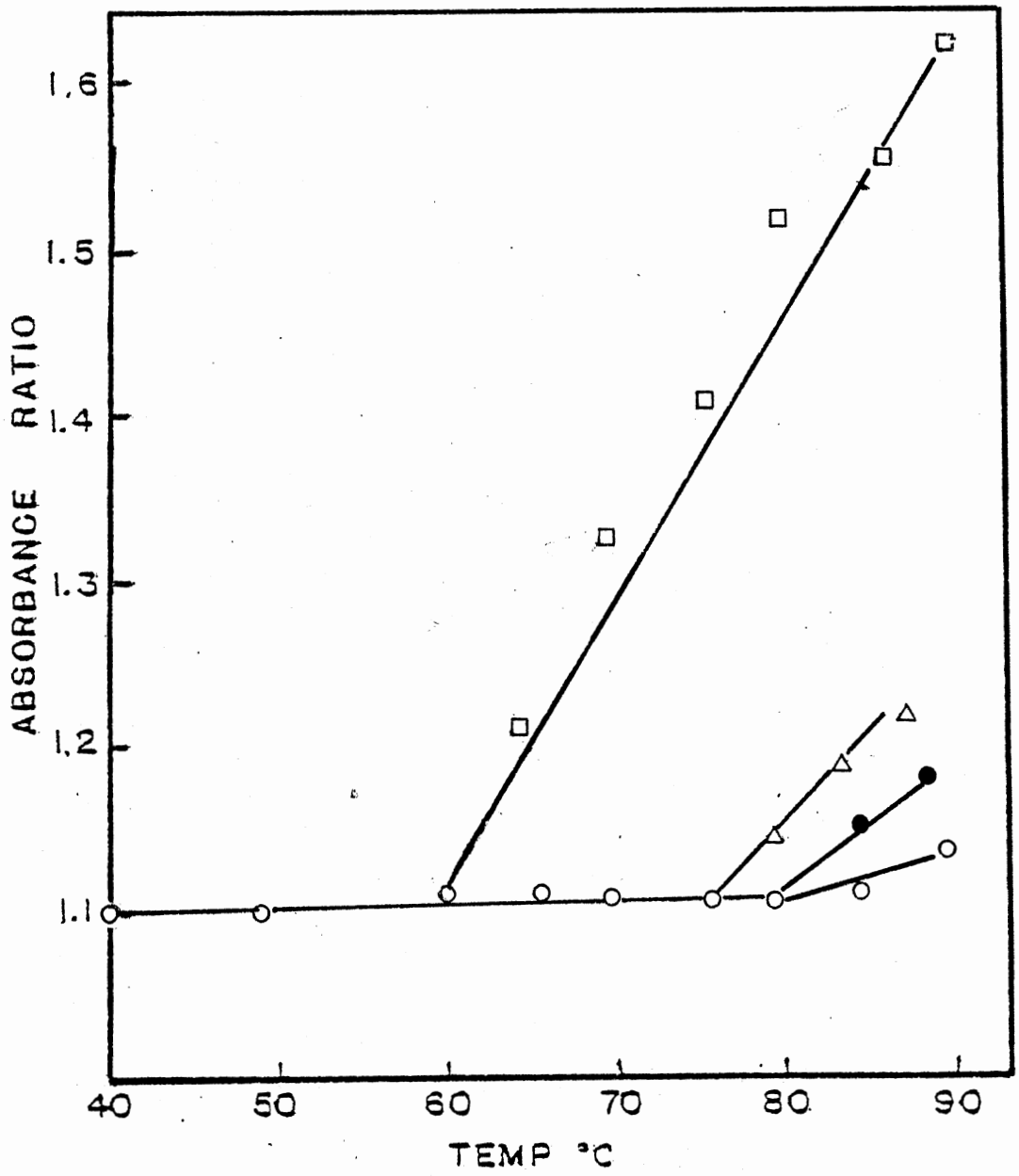

ABSORBANCE RATIO
1.6
1.5
1.4
1.3
1.2
1.1
40
50
60
70
80
90
TEMP °C

Figure 26. Effect of Varying Concentration of Methoxythiazole Hydrochloride on Thermal Denaturation of P. fluorescens DNA. ●, control DNA (20 µg/ml); ▲, methoxythiazole hydrochloride (7.9×10^{-5} M); Δ, methoxythiazole hydrochloride (3.96×10^{-5} M); O, methoxythiazole hydrochloride (1.98×10^{-5} M).

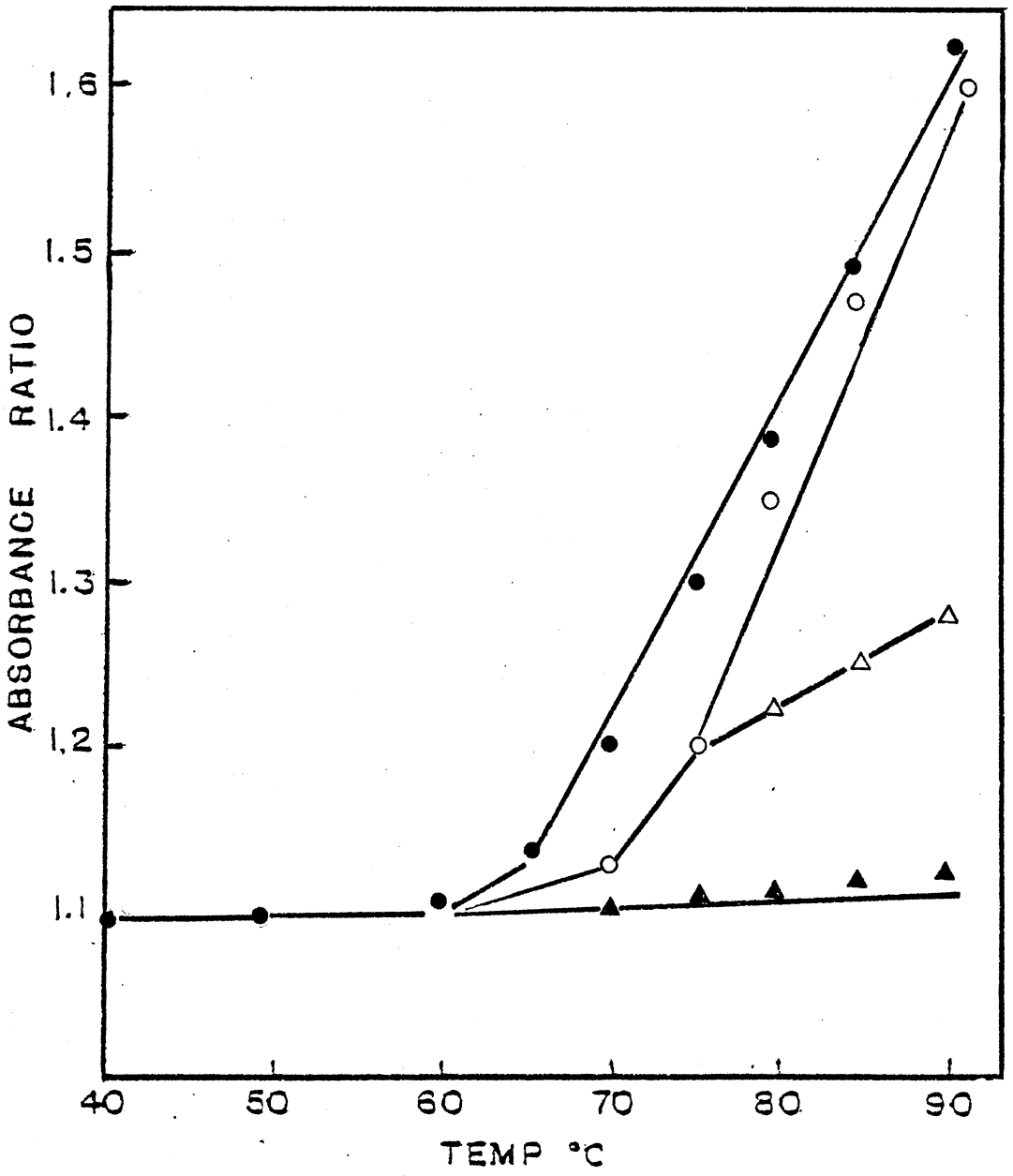

ABSORBANCE RATIO
1.6
1.5
1.4
1.3
1.2
1.1
40
50
60
70
80
90
TEMP °C

TABLE IX

THE EFFECT OF THIAZOLES OF THERMAL DENATURATION OF *P. FLUORESCENS* AND *B. SUBTILIS*

Compound	Concentration (M)	DNA Melting Point (°C)	
		B. subtilis	*P. fluorescens*
Control	--	76	75
Methoxythiazole Hydrochloride	7.9×10^{-5}	--	--
	3.96×10^{-5}	82	83
	1.98×10^{-5}	78	76
Methoxythiazole	8.6×10^{-5}	--	--
	4.3×10^{-5}	83	83
	2.15×10^{-5}	76	74
Dimethoxythiazole	7.6×10^{-5}	--	--
	3.8×10^{-5}	84	82
	1.9×10^{-5}	76	74
Hexyloxythiazole	6.6×10^{-5}	--	--
	3.3×10^{-5}	84	84
	1.65×10^{-5}	78	76
Hexyloxythiazole Hydrochloride	5.9×10^{-5}	--	--
	2.9×10^{-5}	83	83
	1.47×10^{-5}	78	77

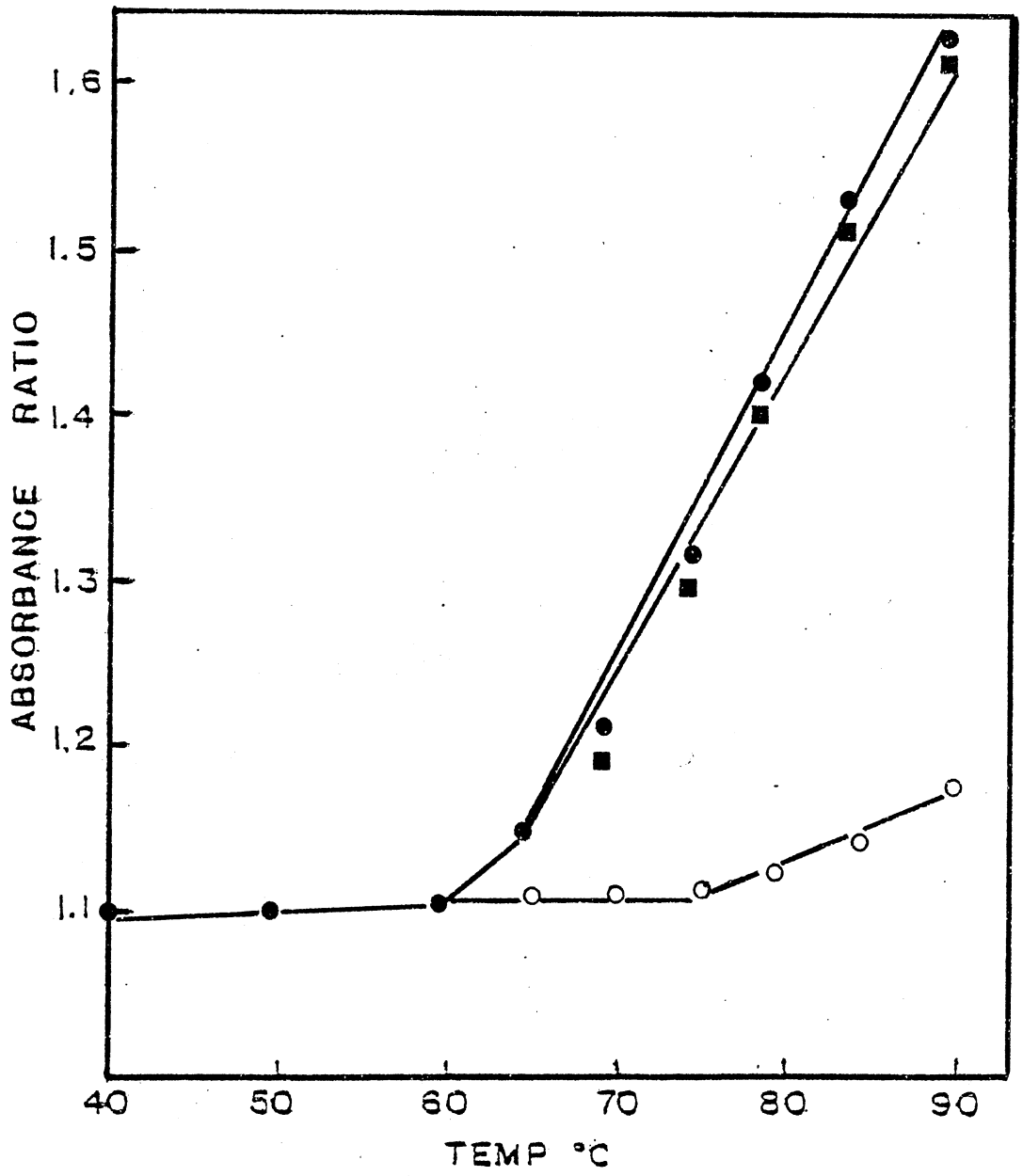
hydrochloride with DNA was investigated. The thiazole at the same concentration that inhibited thermal denaturation was suspended with B. subtilis or P. fluorescens DNA (20 µg/ml) in dialysis tubing dialyzed for 48 hours in saline-citrate buffer, pH 7.0. The suspension was then removed and used in thermal denaturation experiments. Figure 27 shows that enough of the thiazole was dialyzed out for the treated DNA to exhibit a normal denaturation curve. This would indicate that the binding or interaction of the methoxythiazole hydrochloride and the DNA is reversible. The same reversible interaction was demonstrated in experiments with the other thiazole compounds.

Correlation of Thermal Denaturation Inhibition and Biological Activity

Attempts were made to correlate the interaction of the various thiazoles with DNA and biological activity two ways: (1) by assaying for back mutation of two auxotrophic strains of B. subtilis and (2) by assaying for an inducible enzyme in P. fluorescens.

B. subtilis Br 151, a double auxotroph deficient in tyrosine and tryptophan synthesis, and B. subtilis EC 63, a triple auxotroph deficient in lysine, methionine and tryptophan synthesis, were obtained from the culture collection of Dr. F. R. Leach, Oklahoma State University. The two test organisms were exposed to non-inhibitory concentrations of the test compounds or water (control) for 2 hours. Appropriate dilutions were made and viable plate counts obtained using glucose minimal medium supplemented with one or more of the missing nutrients. No significant rate of back mutation was observed.

Figure 27. Reversal of Methoxythiazole Hydrochloride Induced Inhibition of DNA Denaturation. ●, control, DNA (20 µg/ml) dialyzed for 48 hours; O, methoxythiazole hydrochloride (7.9×10^{-5} M) and B. subtilis DNA (20 µg/ml); ■, methoxythiazole hydrochloride (7.9×10^{-5} M) and B. subtilis DNA (20 µg/ml) dialyzed for 48 hours.


ABSORBANCE RATIO
1.6
1.5
1.4
1.3
1.2
1.1
40
50
60
70
80
90
TEMP °C

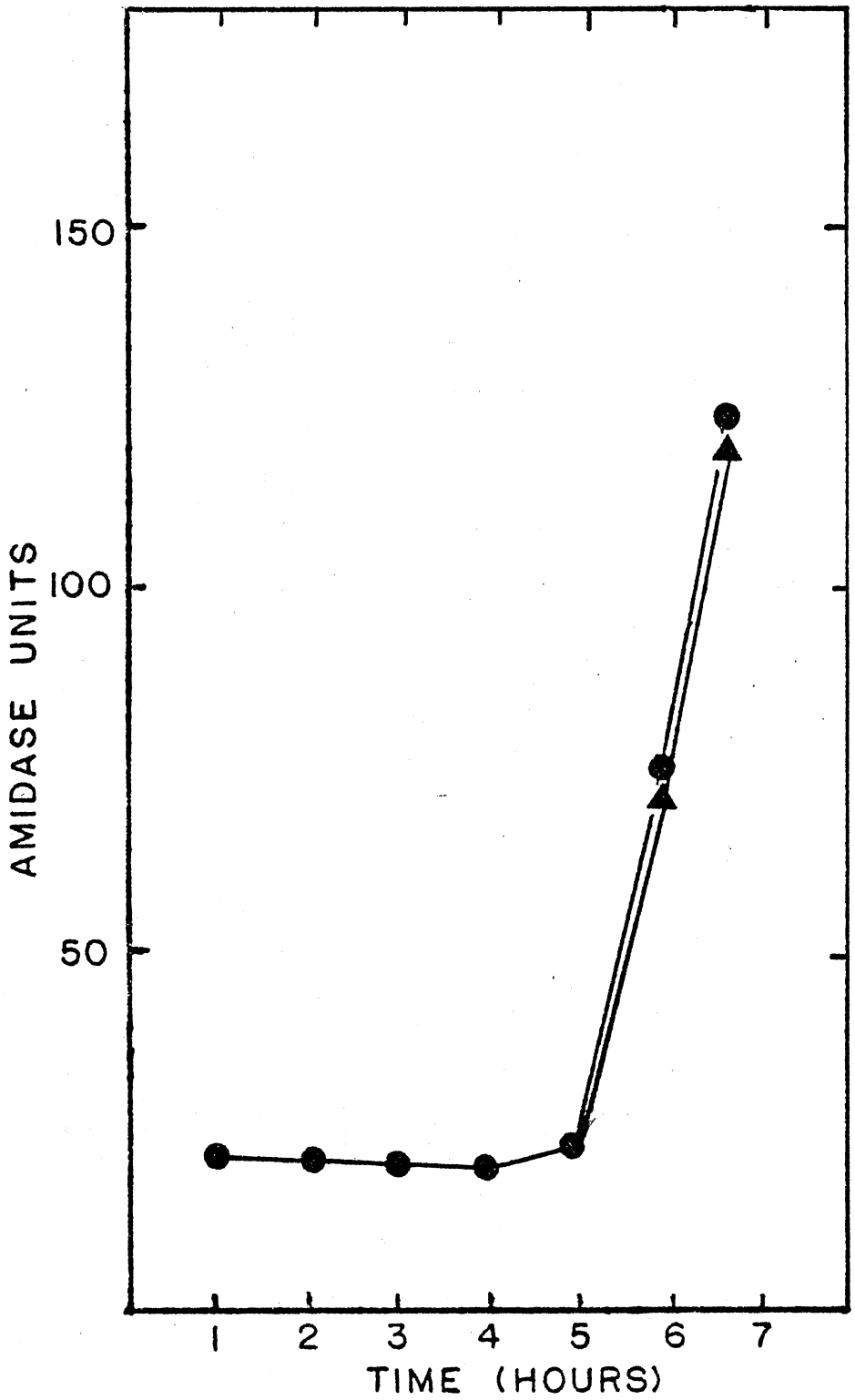
A second attempt was made to correlate DNA-thiazole interaction with a demonstrable biological effect. A change in DNA activity could be monitored by measuring a change in the synthesis of an inducible enzyme system. It was also postulated that the enzyme assay would be a more sensitive system to assay for biological activity of the thiazoles against P. fluorescens than the growth studies. The amidase enzyme was induced by growing the bacteria in succinate minimal medium containing acetamide. Samples were removed at specified time intervals and frozen for subsequent enzyme determinations. No effect could be observed by any of the compounds on enzyme production (Figure 28).

Effect of EDTA Treatment on Thiazole Inhibition

Removal of the cell membrane (by using cell-free systems) in B. subtilis and P. fluorescens caused a measurable change in the biological activity of the non-active thiazoles. Since (1) all the thiazoles were inactive against P. fluorescens and (2) P. fluorescens like all Gram-negative bacteria has an outer membrane that can act as a permeability barrier, it was postulated that removal of the outer membrane by EDTA treatment could enhance the activity of the thiazoles.

Cells were treated with EDTA for 2 minutes, washed with fresh succinate minimal medium and either (1) resuspended in succinate minimal medium plus thiazole for studies on growth inhibition or (2) inoculated into succinate medium supplemented with acetamide and thiazole for enzyme assays. In neither case were thiazoles (2.95×10^{-5} M to 4.3×10^{-5} M) able to exert any inhibitory effect. Removal of the outer membrane did not enhance thiazole activity against P. fluorescens.

Figure 28. Effect of Thiazoles on Induced Enzyme Synthesis in P. fluorescens. ●, control; ▲, thiazoles (1.8 to 2.5 x 10^{-4} M).


150
100
50
AMIDASE UNITS
1
2
3
4
5
6
7
TIME (HOURS)

Characterization of Activity Against *Tetrahymena Pyriformis*

Tetrahymena is an ubiquitous, free living freshwater protozoan; it has been widely used in diverse studies at the cellular level (Everhart, 1972) and has been sensitive in detecting anticancer agents (Frankel, 1965). The organism offers many advantages in chemotherapy studies: (1) it is an eucaryotic cell that can be grown in a completely chemically defined medium; (2) it has a short generation time; (3) its growth can be measured spectrophotometrically; and (4) it is a large cell (20 x 50 μ) which makes it favorable for studying individual cells. The cortex structure is a complex composed in many places of three membrane layers in close approximation to each other, making it an excellent system for studying possible effects of alterations in membrane permeability on drug activity.

Thiazole Induced Growth Inhibition

Tetrahymena pyriformis was incubated with various concentrations of the thiazoles or water (control), absorbancies were read at 540 nm at 24 and 48 hours and growth inhibition determined (Table XI). The methoxythiazoles showed the greatest activity, with between 54 and 65 percent inhibition of growth for 48 hours at a concentration of 1.2×10^{-3} M to 1.9×10^{-3} M. The hexyloxythiazoles showed growth similar to the control value at the concentrations employed.

Effect of Varying Concentrations on Thiazole Inhibition of Growth

As with the bacterial studies, an incremental decrease in the

TABLE X

THIAZOLE-INDUCED GROWTH INHIBITION OF T. PYRIFORMIS

Compound	Concentration (M)	Absorbancy 540 at 48 Hours	Percent Inhibition at 48 Hours
Control	--	0.86	--
Methoxythiazole Hydrochloride	1.9×10^{-3}	0.38	54
	2.0×10^{-4}	0.37	55
	1.0×10^{-5}	0.45	46
	1.5×10^{-5}	0.74	10
	2.5×10^{-5}	0.85	0
Methoxythiazole	1.29×10^{-3}	0.33	60
	2.15×10^{-4}	0.34	60
	1.07×10^{-4}	0.38	46
	5.4×10^{-5}	0.62	25
	2.7×10^{-5}	0.78	5
Dimethoxythiazole	1.14×10^{-3}	0.29	65
	1.9×10^{-4}	0.38	54
	9.5×10^{-5}	0.68	18
	4.7×10^{-5}	0.86	0
	2.4×10^{-5}	0.86	0
Hexyloxythiazole	9.9×10^{-4}	0.75	9
	1.7×10^{-4}	0.80	3
	8.2×10^{-5}	0.79	4
	4.12×10^{-5}	0.74	10
	2.06×10^{-5}	0.77	7
Hexyloxythiazole Hydrochloride	8.9×10^{-4}	0.74	10
	1.5×10^{-4}	0.85	0
	7.4×10^{-5}	0.64	12
	3.7×10^{-5}	0.83	0
	1.8×10^{-5}	0.83	0

concentration of the active thiazoles was reflected in an incremental decrease in growth inhibition (Table II). A six-fold decrease in the concentration of the dimethoxythiazole caused an 11 percent decrease in growth inhibition, cutting the concentration ten-fold caused a 36 percent decrease in inhibitory activity. No change in inhibitory activity was seen with a six-fold decrease in the concentration of the methoxythiazole but with a ten-fold decrease in concentration, there was a 14 percent drop in inhibitory activity. A further two-fold drop in concentration resulted in a 21 percent drop in inhibition. The methoxythiazole hydrochloride which had shown the greatest activity against B. subtilis, showed a decrease in growth inhibition of only 9 percent with a concommitant ten-fold decrease in concentration. A further decrease in inhibitory activity of 36 percent resulted from a further two-fold decrease in concentration.

Thiazole Induced Morphological Changes

T. pyriformis cells were examined microscopically for gross morphological changes during incubation with inhibitory concentrations of the methoxythiazoles (4×10^{-4} M). T. pyriformis' characteristic pear shape (Figure 29) and large size made it easy to determine changes in morphology which might be induced by the methoxythiazoles while the motility of the protozoan provided a second indicator of inhibitory activity.

Treatment of the protozoan with the methoxythiazoles caused a complete rounding of the cell (Figure 30) with a concommitant loss of motility. Not all cells underwent the rounding at the same time and an intermediate stage could be seen where the rounding process caused a

Figure 29. Tetrahymena pyriformis Exhibiting Characteristic Pear Shape

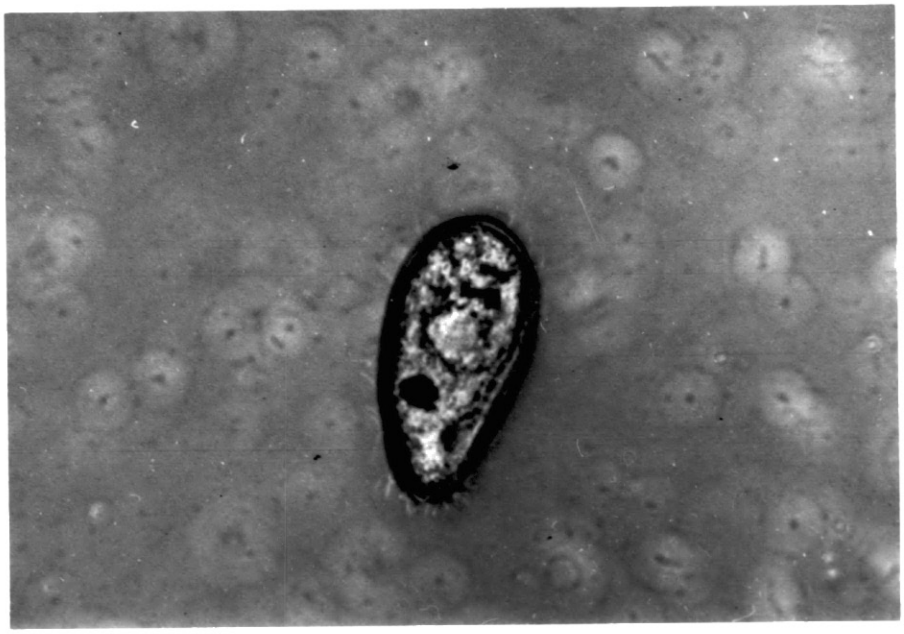
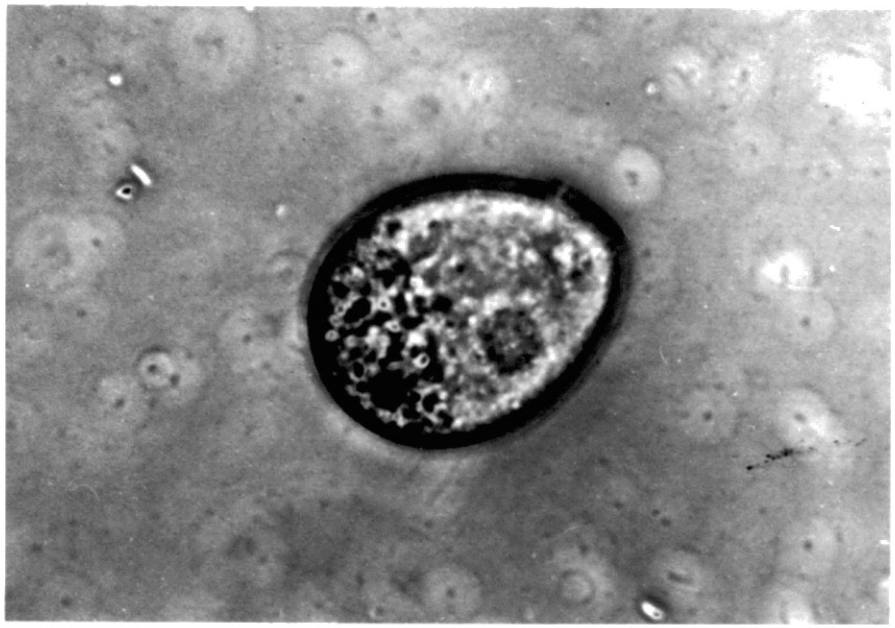
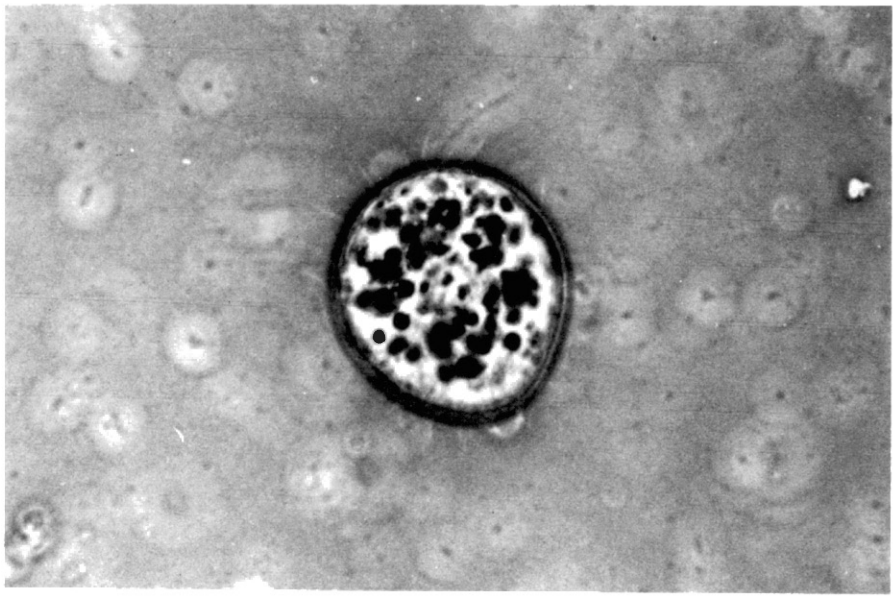


Figure 30. Tetrahymena pyriformis After 3 Hours of Treatment with Methoxythiazole Hydrochloride (3.96×10^{-4} M)

Figure 31. Tetrahymena pyriformis After 6 Hours of Treatment with Methoxythiazole Hydrochloride (3.96×10^{-4} M)





distortion of the normally slender pear shape to what could be called a "fat" pear (Figure 31). During the entire time that morphological changes were measured (48 hours), control cells exhibited few of the aberrant types that were observed in the treated cells. At all times that samples were taken, over 94 percent of the control cells exhibited the characteristic pear shape morphology as well as motility.

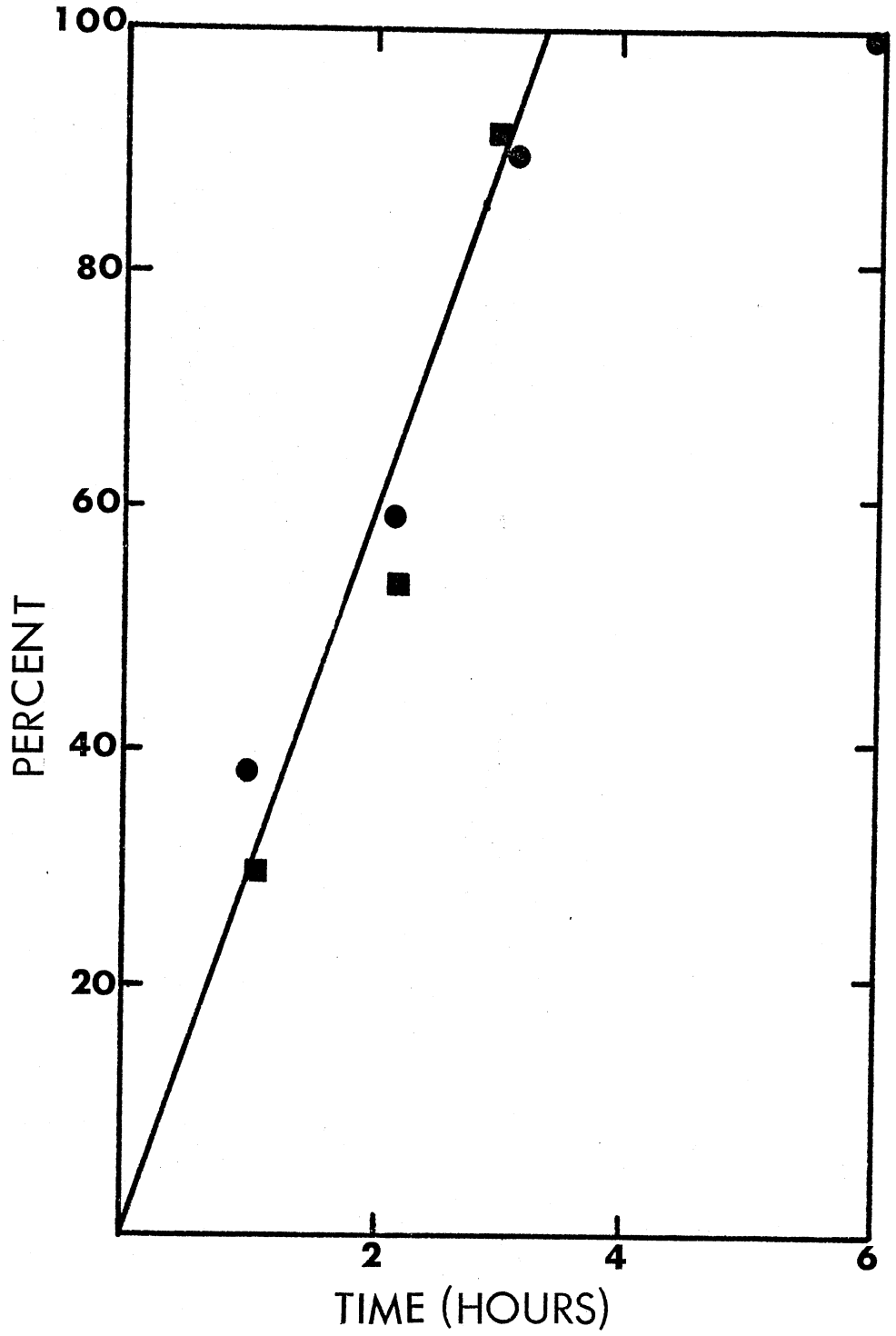
Experiments were run to determine how quickly the changes in morphology occurred and to determine what percent of the protozoan population was affected. Samples were taken from both treated or control cultures at indicated time intervals, were viewed microscopically, and the field was scanned. Exactly 100 cells were classified on the basis of morphology and motility. Several fields were examined and the results were the average of at least three counts.

After 1 hour of incubation with the methoxythiazole, 37 percent of the cells were non-motile and rounded (Figure 32). At three hours of incubation, 86 percent exhibited the abnormal morphology and the loss of motility and by six hours, all cells were completely rounded and non-motile. When the change in motility and shape are plotted separately (Figure 32), it appears that the loss of motility and shape may occur simultaneously.

Recovery from the Thiazole Induced Inhibition

Morphological and motility changes provided an excellent system for monitoring the reversal of thiazole induced inhibition. Cells were incubated with the thiazole for a minimum of 6 hours and for a maximum of 48 hours. At indicated times after incubation, the cells were

Figure 32. Percent of T. pyriformis Cells Exhibiting Observed Morphological Changes. ■, percent of population exhibiting rounding; ●, percent of population exhibiting loss of motility.


100
80
60
40
20
PERCENT
2
4
6
TIME (HOURS)

centrifuged and resuspended in fresh medium, samples were taken and counted and classified microscopically every 30 minutes for two hours.

After six hours of incubation with the methoxythiazole hydrochloride, the cells of T. pyriformis showed 70 percent recovery at the end of a two hour period (Figure 33). The recovery was gradual with cells going through the intermediate fat pear shape with a gradual increase in motility until they exhibited normal motility and shape.

The percent recovery depended on the time of incubation of the cells with the thiazole (Figure 34). Cells incubated for 9 hours had only 36 percent recovery after 2 hours while cells exposed 12 hours had only 24 percent recovery. Cells exposed in excess of 24 hours showed no recovery during the time monitored (48 hours).

During these experiments, it was noticed that lysed cells were present in the different samples. Since lysis could account for the failure to recover, new experiments were run to determine the effect of length of incubation on the percent lysis of the population. Figure 35 shows that with increased incubation time there is an increase in lysis of the cells.

Figure 33. Recovery of T. pyriformis Cells from Thiazole Induced Morphological Changes. Cells were exposed to methoxythiazole hydrochloride for 6 hours, centrifuged and resuspended in fresh medium without thiazole. ●, percent of population exhibiting normal morphology and motility after being resuspended in fresh medium.

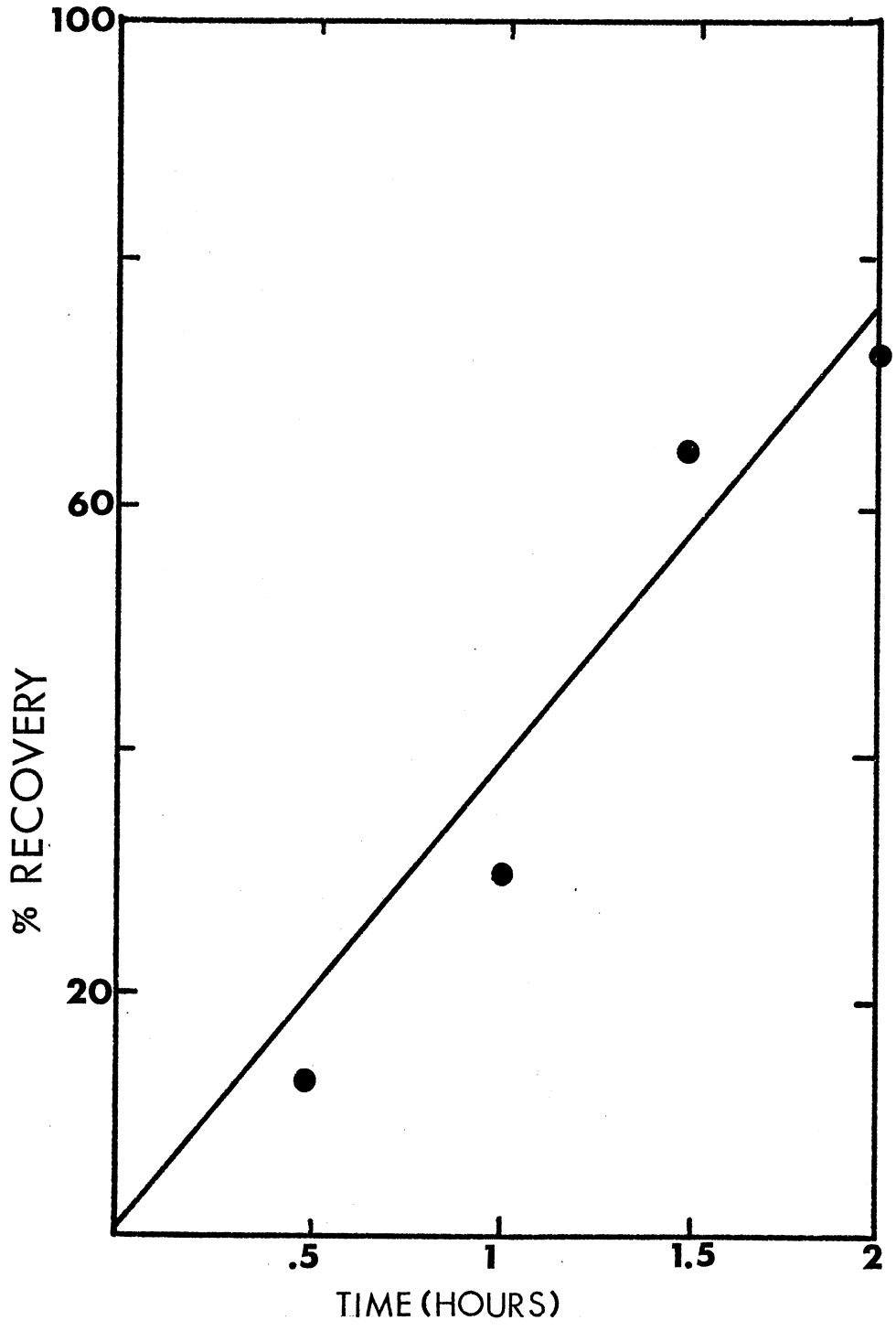

100
60
20
% RECOVERY
.5
1
1.5
2
TIME (HOURS)

Figure 34. Effect of Time of Exposure to the Thiazoles on the Recovery of T. pyriformis

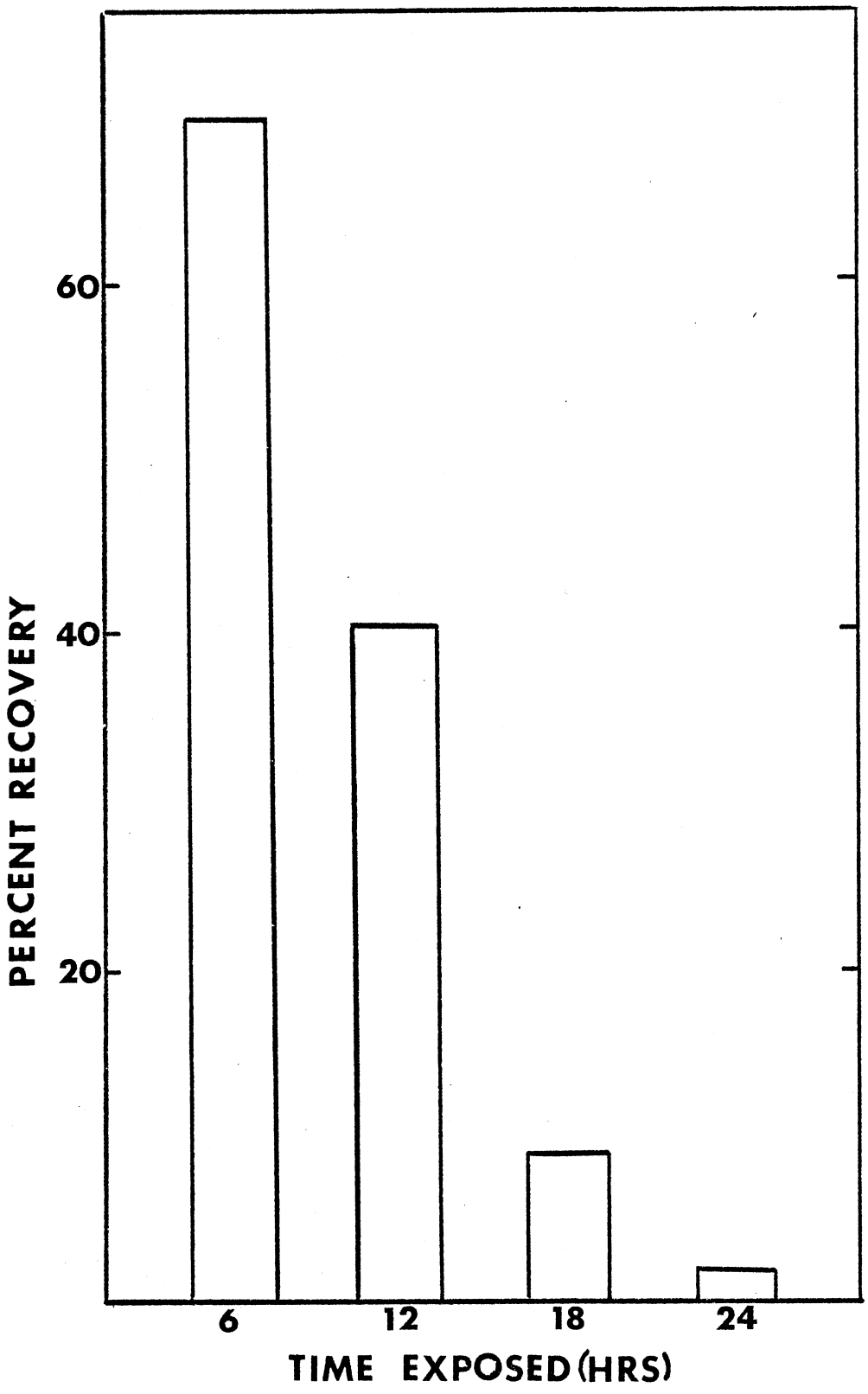
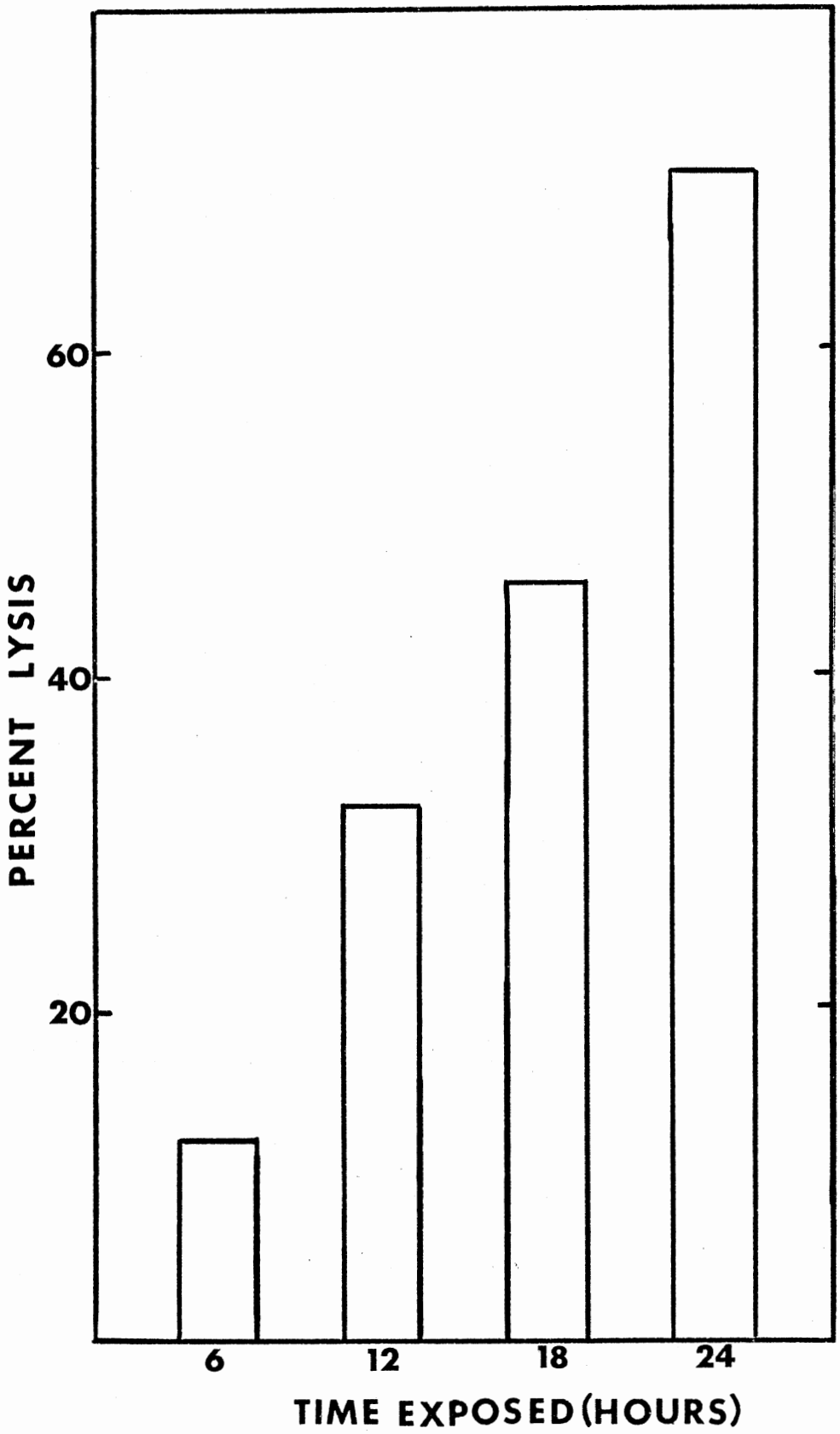

PERCENT RECOVERY
60
40
20
6
12
18
24
TIME EXPOSED (HRS)

Figure 35. Effect of Time of Exposure of T. pyriformis to the Thiazoles on Cell Lysis


PERCENT LYSIS
60
40
20
6
12
18
24
TIME EXPOSED (HOURS)

CHAPTER IV

SUMMARY AND CONCLUSIONS

Hansch and Fujita (1964) proposed that a biologically active molecule makes its way through cellular material by a "random walk" process accumulating at an active site at a concentration necessary to produce an inhibitory response. The passage of the molecules from the external millieu to the active site would be by passive permeation. The passage would depend more on the general physical properties of the molecule than on small differences, such as structure, stereochemistry, size and charge which are crucial in active transport (Penniston et al., 1969). The current concept of the cell membrane as a fluid lipid bilayer (Singer and Nicolson, 1972) indicates the importance of a compounds lipophilicity in mediating biological activity. Lipophilic substances would be unable to penetrate aqueous layers and would accumulate in the lipid region (Parker and McRobbie, 1974) while hydrophilic compounds would be unable to cross the lipophilic barriers. Compounds with an optimum balance between hydrophilicity and lipophilicity would exist and would be predicted to have greater biological acitivity (Hansch and Fujita, 1964). The purpose of this study was to determine which of a group of lipophilic and hydrophilic substituents when added to novel thiazole compounds might help the compound achieve such an optimal balance and therefore enhance its biological activity.

A study of the five novel thiazoles by reverse-phase thin-layer chromatography divided the compounds into two groups: the more lipophilic hexyloxythiazoles and the less lipophilic methoxythiazoles. Of the three methoxythiazoles, the dimethoxy- and the methoxythiazole were the least lipophilic with R_m values of +0.039 and +0.019, respectively. The methoxythiazole hydrochloride appeared to have intermediate hydrophilic-lipophilic properties. This assumption was based not only on the compounds R_m value (+0.073), the highest of the three methoxythiazoles, but also on its behavior during the standard Hansch analysis. The methoxythiazole hydrochloride dissolved in the water layer quickly partitioned into the octanol layer. The hexyloxythiazoles were more lipophilic with R_m values of +0.423 and +0.377. The changes in lipophilicity are a result of substituents added at positions 7 and 8 on the benzene ring. The increased lipophilicity is the result of the addition of hexyloxy group while there is decreased lipophilicity as a result of the addition of either one or two methoxy groups.

Using inhibition of bacterial growth as an indication of biological activity, all the thiazoles, with the exception of the hexyloxythiazole hydrochloride, exhibited the same activity at high concentrations (3.3 to 4.3 x 10^{-4} M) against Gram-positive organisms (B. subtilis, B. megaterium and S. aureus). When lower concentrations (6.6 to 8.6 x 10^{-5} M) were used, the methoxythiazole hydrochloride showed the greatest activity (100 percent inhibition of all Gram-positives at 24 hours), the dimethoxy- and methoxythizole showed intermediate activity (75 percent inhibition at 24 hours) while the hexyloxythiazole exhibited no activity. The hexyloxythiazole, because of its high degree of insolubility in glucose minimal medium, often gave erratic results in all the studies

performed. It is not clear how to rank the hexyloxythiazole with regard to biological activity and any interpretation of experiments involving it must remain rather tentative. The bulk of the evidence seems to indicate that the hexyloxythiazole possesses a limited amount of biological activity and for the purpose of this study it had been classified as being very close to or below the activity of the two intermediate-activity thiazoles when activity is measured on the basis of growth studies.

The five compounds showed a decrease in inhibitory effect against the Gram-negative organisms. There was only slight inhibition of E. coli and no inhibition of P. fluorescens. These results are thought to be indicative of the increased complexity and impermeability of the Gram-negative cell envelope.

All three of the methoxythiazoles inhibited growth of the eucaryotic organism T. pyriformis. The hexyloxythiazoles showed no inhibitory activity at the concentration measured. In bacterial studies, the methoxythiazole hydrochloride exhibited much greater activity than the other methoxythiazoles. In Tetrahymena studies no significant differences in biological activity of the three methoxythiazoles could be extrapolated from the present data.

The relative lipophilicity of the five thiazoles can be correlated to their biological activity. The less lipophilic compounds, the methoxythiazoles, showed greater activity against all Gram-positive organisms and the eucaryotic organism, T. pyriformis. The more lipophilic compounds exhibited no inhibitory activity against T. pyriformis and the hexyloxythiazole had limited activity against the Gram-positives. These results indicate that biological activity could be a result of the relative permeability of the compound in the aqueous and lipid layers that

it must transverse to reach its active site. This would be consistent with Hansch's theory of passive transport of a drug molecule. The greater activity of the methoxythiazole hydrochloride could be because it possessed the optimum balance between hydrophilicity and lipophilicity for traversing the cell barriers. The decreased activity of the dimethoxy- and methoxythiazole could result from a decreased ability to be taken up by the cell membrane and therefore reach its site of action either at the cell membrane or inside the cell. The lack of activity of the lipophilic compounds could then be explained in terms of their inability to be extracted from the lipid phase of the cell membrane by the aqueous phase inside the cell, as well as decreased ability to reach the cell membrane.

Incubation of the test compounds under growth conditions prior to the addition of the cells did not effect their degree of inhibition. The differences in relative activity was not then a reflection of the compounds stability under growth conditions.

Inhibition of accumulation or uptake of labeled substrate can be a more specific means for judging biological activity. Studies revealed that at high concentrations the active thiazoles inhibited the uptake of substrates by B. subtilis. The results were also consistent with the results from growth inhibition studies using thiazoles at lower concentrations: the less lipophilic methoxythiazoles were most inhibitory, with the methoxythiazole hydrochloride having the greatest inhibitory effect, while the more lipophilic hexyloxythiazoles showed little or no activity. Though the hexyloxythiazole continued to exhibit anomalous behavior, the range of its responses was decreased.

The thiazoles had similar inhibitory effects on the accumulation

and/or short term uptake of all the labeled compounds: glucose, alanine, thymine, diaminopimelic acid, uracil, serine, lysine and leucine. Because of the number of different compounds whose incorporation or uptake was affected, it was postulated that the inhibition was a result of a generalized effect on membrane permeability. Data to further prove this hypothesis was inconclusive. Studies on the leakage of a labeled non-metabolizable substrate indicated that the thiazoles do not mimic a detergent-like action on the membrane which would indicate a major permeability change. However, these experiments do not rule out the possibility of subtle permeability changes that could account for the observed inhibition of uptake and accumulation.

Exposure of T. pyriformis to the methoxythiazoles resulted in a rounding and shrinkage of the cell with a concommitant loss of motility. Though the cause of the rounding was not determined, similar effects have been reported for Tetrahymena suspended in starvation medium to induce synchronous growth (Tamura et al., 1969). Rounding and shrinkage of cells as a result of starvation would conform to the theory that the thiazoles induce subtle membrane changes that result in decreased uptake of nutrients, i.e., starvation.

The kinetics of both growth and uptake inhibition on bacteria were affected in the same way by two variables: concentration of inhibitory compound and the time of addition of the inhibitory compound. A step-wise decrease in the concentration of the thiazoles is reflected in a step-wise decrease in the degree of growth and uptake inhibition. The same concentration of thiazole added at zero time shows greater inhibition than when added to an actively growing culture. Growth inhibition can also be affected by the size of the initial inoculum; an increase in

the size of the initial inoculum results in a decrease in the percent inhibition. This could be another way of expressing the concentration dependence of the compounds; increasing the size of the initial inoculum would in effect decrease the number of molecules per cell. The affect of inoculum size on uptake inhibition was not determined but it could be surmised that the affect would be similar to that seen in growth inhibition.

Both growth and uptake inhibition showed similarities in their reversibility. Both inhibitions were reversible but the reversibility was concentration dependent; at the highest concentration tested the inhibition of the methoxythiazole hydrochloride could not be reversed while at the lower concentrations there was complete reversal as soon as the compounds were removed from the medium. The growth inhibition caused by the dimethoxy- and methoxythiazole could be reversed at the concentrations tested. The reversibility of their uptake inhibition was not determined. Finally, neither growth nor uptake inhbition could be reversed by the addition of divalent or multivalent cations.

Studies with thiazoles showed that at high concentrations of the compound the inhibition of uptake took place within one minute of addition of the test compound while measurable growth inhibition of actively growing cultures was not observed for several hours. This indicates that a subtle permeability change and the resultant uptake inhibition preceeds growth inhibition.

The similarities of factors that affect the inhibitions combined with the sequence of events (uptake preceeding growth inhibition) indicate that initial growth inhibition could be a result of the inhibition of uptake which is, in turn, a result of a generalized change in membrane

permeability. This proposed relationship could explain a number of the observed effects. Addition of the compounds at zero time would result in increased inhibition since the uptake of required nutrients would be blocked during the lag phase before cell division could take place. The same concentration of compound added to an actively growing culture would have decreased inhibition for two reasons: (1) an increase in cell number would occur before the inhibitory effect of nutrient depletion could be expressed; and (2) the increase in cell number would have the same affect as decreasing the number of molecules per cell, i.e., reducing the amount of compound available both at the membrane and the amount of compound that could be accumulated at a second active site.

There is no direct evidence to show binding of the thiazoles to B. subtilis cells. The results from experiments measuring the amount of compound present in the supernatant after incubation with the cells indicated an increase in the original concentration of the compound rather than a decrease. This could be accounted for if there was a leakage of compounds that absorbed in the same UV range as the thiazoles, i.e., nucleic acids, proteins, etc. The testing of the thiazole-containing supernatant for inhibitory activity did provide indirect support for the theory that the thiazoles are bound and/or accumulated by the cell. B. subtilis cells were inoculated into glucose minimal medium containing thiazole that had been previously incubated with the bacteria. The same concentration of fresh thiazole was inhibitory while the incubated thiazole showed control growth. Using increased concentrations of the thiazole caused an increase in growth inhibition but the incubated thiazole never matched the inhibition of the unincubated thiazole. These data would indicate that incubation with the cells decreased the amount

of thiazole available in the medium. Though instability of the thiazole under growth conditions has been eliminated as a possible cause of this decreased inhibition, the ability of the cells to inactivate the compound has not. However, breakdown of the compound by the cell should result only in a lag in cell growth and not the observed periods of growth inhibition.

It has been accepted that many known antimicrobial agents have multiple sites of action. Though generalized inhibition of uptake could result in the observed growth inhibition, it does not negate the existence of a secondary or even tertiary site of action. The interpretation of some of the data supports the existence of a second active site inside the cell proper. This internal site would explain the observed affect of compound concentration on both degree of growth inhibition and reversibility of the inhibition. Increasing the concentration of the thiazole would increase the accumulation of the compound at a second internal site and result in increased inhibition. At lower concentrations, the thiazoles would exert their affect only on the membrane. This inhibition at lower concentrations would require the presence of the compound and removal of the compound would result in the reversal of the inhibition.

The second site theory would also be consistent with the observed effect of time of addition on the thiazoles' degree of inhibition. The time between addition of the compound and observed inhibition would be proportional to the time it took for the compound to reach its site of action. In the case of zero addition, this time would be less than the lag phase, therefore, no growth would be observed; in the case of addition to an actively growing culture, this time would be the same as the time between addition of the compound and observed growth inhibition.

The concept of two sites is also consistent with the effect of increasing the initial inoculum. As mentioned previously, increasing the inoculum would be the same as decreasing the number of molecules per cell causing a decrease in the accumulation of the compound at the second site.

Thiazole-induced growth inhibition of T. pyriformis was also reversable. The thiazole-induced morphological changes provided an excellent system for studying the kinetics of both inhibition and reversal. As with the bacterial system the morphological changes preceeded the observed growth inhibition; morphological changes could be observed as early as one hour after exposure to the thiazole while observable growth inhibition was not apparent for 12 hours.

The reversal of the thiazole-induced morphological changes was time dependent; as the time of exposure to the thiazole increased there was a decrease in the number of cells that reverted to the normal pear-shape after the removal of the compound. Cells exposed for 24 hours had been irreversibly inhibited. The decrease in the reversibility of morphological changes and growth inhibition was proportional to an increase in cell lysis; this indicates a possible cause and effect relationship.

The interpretation of the data with Tetrahymena is not as clear cut as the bacterial data. It does lend support to the theory that (1) changes in membrane permeability could be the first event in the thiazole-induced growth inhibition but that these changes are reversible; (2) there is a possible secondary site inside the cell that results in irreversible inhibition; and (3) differences in the relative inhibitory activity of the thiazoles can be correlated to their lipophilic-hydrophilic properties which are an indication of their ability to

interact with the cell membrane and be accumulated at an interior active site.

The hypothesis put forth so far states, in part, that bacterial and protozoan growth inhibition is dependent upon a minimum number of the compound molecules reaching first the membrane and then any secondary site and that increasing the concentration will increase that number and therefore facilitate growth inhibition. According to Hansch's proposed model, the number of molecules reaching either the membrane or an internal active site would be dependent on ability of a molecule to pass through a series of aqueous and lipid layers. Based on these assumptions, differences in the observed degree of growth inhibition of the different thiazoles could be accounted for by differences in their relative lipophilicity.

Substances of low water solubility (i.e., the lipophilic hexyloxythiazoles) would be unable to penetrate the aqueous layers and it would accumulate in the lipid region. This would assume: (1) that the hexyloxythiazoles never reach the cell membranes or do not reach the cell in sufficient number to exert an effect, (2) any accumulation at the cell membrane does not have a deleterious effect on it, and (3) there is no accumulation at an interior active site.

The methoxythiazoles would represent the opposite case; compounds of low lipid solubility which would be unable to cross the lipophilic region of the cell (i.e., the cell membrane). Though the measured differences in relative lipophilicity (+0.039, +0.019 and +0.073) may appear to be slight, they may actually indicate a critical range of R_m values where slight changes in measured lipophilicity reflect a significant decrease in the interaction with the cell membrane. This slight

decrease in lipophilicity which would cause a decrease in the rate of both interaction with and crossing of the cell membrane would result in a modification of observable biological activity because: (1) a decrease in the number of molecules interacting with the cell membrane would result in a decrease in permeability changes as measured by a decrease in uptake inhibition of small metabolites, and (2) there would a decrease in the number of molecules crossing the membrane to be accumulated at an internal active site.

Of the five compounds tested, the methoxythiazole hydrochloride appeared to have the greatest biological activity in bacterial systems. This increased activity could be a result of its optimum balance of lipophilic and hydrophilic properties. Though its R_m value of +0.073 does not indicate this possibility, its behavior in the standard Hansch analysis does. Though it is soluble in water, it quickly partitions into the octanol layer. This would mean that the methoxythiazole could quickly interact or pass through the various aqueous and lipid phases that compose the cell. Once dissolved in water, it would reach the cell membrane and because of its lipophilic characteristics, it would be extracted by the lipid portion of the cell membrane and could exert its initial effect, a change in cell permeability. After interacting and/or traversing the cell membrane, the compound would partition into the internal aqueous phase and accumulate at the second active site.

The studies with the bacteria divided the compounds into three groups: the high-activity methoxythiazole hydrochloride, the immediate-activity dimethoxy- and methoxythiazole, and the inactive hexyloxy-thiazoles. The studies with the protozoan divided the compounds into two

groups: the highly active methoxythiazoles and the inactive hexyloxythiazoles. It had been proposed that differences in biological activity caused by differences in the compounds' membrane permeability would be magnified in the protozoan system rather than decreased. Failure to do so indicates that either: (1) factors other than membrane permeability mediate biological activity in the protozoan and the bacteria, or (2) the compounds' biological activity is dependent on more than its physical properties.

All five compounds stabilized bacterial DNA, preventing its unwinding at temperatures as high as 90 C. The inhibition of thermal denaturation, which was observed with both P. fluorescens and B. subtilis DNA, was concentration dependent (decreasing the concentration decreased the inhibition of unwinding) and was expressed only in the presence of the compounds. These results from cell-free experiments indicate (1) that in bacterial systems, differences in compound permeability can account for differences in biological activity in whole cells, (2) that lack of activity in Pseudomonas could be a result of impermeability of the compounds, and (3) that a possible second active site (DNA) may exist.

The inhibition of DNA thermal denaturation in cell-free systems has not been correlated to biological activity in whole cells. No increased back-mutation was observed when B. subtilis auxotrophs were exposed to any of the test compounds. A second test measured for changes in activity of the inducible amidase enzyme in P. fluorescens. Though there were no measurable changes in activity, the possibility existed that the complex structure of the pseudomonad cell envelope prevented the compounds from being concentrated at the internal active site. To test the plausibility of this statement, P. fluorescens cells were treated with

EDTA prior to the addition of the test compounds and induction of enzyme synthesis. Lieve (1968) has shown that EDTA treatment partially removes the lipopolysaccharide layer of the Gram-negative cell resulting in increased activity of known antimicrobial agents. These tests showed no measurable effect of the compounds on the enzyme synthesis after EDTA treatment. However, it is known that the cells quickly repair EDTA-induced damage; the short-term removal of the LPS may not have allowed for an inhibitory concentration to be accumulated at an internal active site.

There are a number of possible explanations for a thiazole-induced change in membrane permeability. Mitchell (1967) has proposed that active transport requires an energized membrane which is a result of the extrusion of protons during either electron transport, breakdown of ATP or photosynthesis. Active transport is coupled with the pulling of the protons back across the membrane. Anything that would affect the extrusion of protons (electron transport or ATPase) or that would increase the leakage of protons across the membrane should result in the proposed generalized membrane change.

Further investigation would involve: (1) measuring the binding, uptake and accumulation of labeled thiazole to determine possible sites of action; (2) investigating the thiazoles effect on electron transport or ATPase as a possible cause of the membrane permeability changes; and (3) correlating the thermal denaturation with either inhibition of protein synthesis or mutagensis.

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VITA[2]

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