THE ACTION OF PHENETHYL ALCOHOL AND PHENYLACETONITRILE ON BIOLOGICAL ACTIVITIES IN MICROORGANISMS

By

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CHAPTER I

INTRODUCTION

The germination of bacterial endospores involves the breaking of a dormant state. This dormancy is characterized by resistance to various chemical agents, extremes of heating, and to simple staining procedures. Germination has been followed by the loss of refractility as observed by phase contrast microscopy (Pulvertaft and Haynes, 1951), or spectrophotometry (Powell, 1950), and also by the loss of heat resistance (Hills, 1950). The ability to absorb a simple stain, such as methylene blue or crystal violet has also been employed (Powell, 1950; Powell, 1951). Certain physiological phenomena have been observed during germination. The loss of dipicolinic acid (Powell, 1953), calcium, and a nondialyzable polypeptide accompanies germination (Powell and Strange, 1953) and may account for about 30% of the total spore weight.

Hills (1949a; 1949b) reported that germination could proceed in a defined medium containing L-alanine, L-tyrosine, and adenosine. The defined system allowed for a reasonable investigation of specific triggering mechanisms involved in germination.

The refractive nature of spores and the lack of convincing evidence for a measurable metabolic rate in free spores (Crook, 1952) roused doubts as to the presence of active enzymes. Stewart and Halvorson (1953) found a heat stable alanine racemase in spores which was 3-16 times more active in spores than vegetative cells and suggested the enzyme functioned as a negative feed-back regulator to prevent germination of spores in the presence of L-alanine. Other enzymes found to be active in spores were a diaphorase-like enzyme (Spencer and Powell, 1952) and catalase (Lawrence and Halvorson, 1954). These investigators found germination could occur without L-alanine in Bacillus megaterium which possesses the racemase. They also found L-alanine could induce germination in Bacillus subtilis morphotype globigii spores lacking racemase activity. Thus, it was suggested that L-alanine may serve a catalytic role.

The stereospecificity of the L-alanine binding site was an early observation (Hills, 1949a). Woese, Morowitz, and Hutchinson (1958) carried out a detailed analysis and suggested that D-isomers and analogues bind at the same site as do the initiators. They found a need for electronegativity from the carboxyl group, but not for ionization. Further, an alteration of the steric properties of the amino group diminished binding.

The first indication that alanine may serve as a substrate for metabolic germination was proposed by Halvorson

and Church (1957). They found an active hexose monophosphate pathway and key enzymes for the Entner-Doudoroff pathway in spore extracts. L-Alanine was oxidatively deaminated to ammonia and pyruvate during germination. Pyruvate was suggested as the common intermediate and a prerequisite for germination since BEP (bis-1:3-beta-ethylhexyl-5-methyl-5-aminohexahydrophrimidine), a specific inhibitor of pyruvate oxidation, inhibited germination.

O'Connor and Halvorson (1959), used labeled L-alanine and confirmed that some exogenous L-alanine was utilized during the initial stage of germination. However, the major source of the metabolite came from endogenous reserves, thus suggesting deamination of L-alanine does play a role in triggering L-alanine induced germination.

A major contribution was the isolation and characterization of L-alanine dehydrogenase (AlD) in bacterial endospores (O'Connor and Halvorson, 1960). This enzyme catalyzes the following reaction:

$$\text{L-alanine} + NAD^+ + H_2O \longrightarrow \text{Pyruvate} + NH_3 + NADH + H^+$$

They found the activity of AlD is specific for NAD^+ and is inhibited by sulfhydryl binding agents. The pH optimum for deamination is 9.8 and for amination, 8.8. The $K_{eq.}$ of 1.3×10^{-14} strongly favors amination. Thus, the recycling of NADH by NADH oxidase and concomitant pyruvate oxidation appears necessary to drive the reaction.

The specificity profiles of AlD indicated that it binds with aliphatic L- and D-alpha-amino acids containing less

than seven carbons (O'Connor and Halvorson, 1961b). Glycine, sarcosine, and structural analogues of alanine having alkyl, hydroxymethyl or thiomethyl substitutions on the alpha-carbon also bind with the enzyme. The L-isomers serve as substrates, whereas the D-isomers, glycine, and sarcosine are competitive inhibitors.

O'Connor and Halvorson (1961a) suggested that AlD was the specific binding site involved in L-alanine induced germination and compared the specificity of AlD with amino acid induced germination. The binding constants (K_m) indicated AlD as the initial binding site in heat-activated spores. This type of germination was suggested to be limited by the number of AlD molecules per spore. Induction was accomplished with L-alanine, L-alpha-NH_2-n-butyric acid, and L-cysteine. AlD activity is essential for L-alanine induced germination since, (1) alanine deamination is essential for germination, (2) complexants of the enzyme inhibit L-alanine deamination and germination, and (3) heat activation enhances the enzyme expression and L-alanine-stimulated germination.

Other observations make it difficult to conclude that AlD is the sole trigger mechanism. McCormick and Halvorson (1963) found that elevated levels of AlD in spores lead to decreased germination rates with L-alanine. Further, it was noted that an increase in AlD concentration decreased the activity of NADH oxidase. Thus, the NADH oxidase activity may serve as an effective trigger mechanism.

Freese and Cashel (1965) found that AlD$^-$ mutants could still be induced to germinate with L-alanine but were inhibited by D-alanine. However, these mutants may have been slightly "leaky" thereby allowing sufficient AlD synthesis for the initiation of germination. Also, the AlD$^-$ spores may be physically different than AlD$^+$ spores and the noted variation in refractility of the AlD$^-$ spores (20%) versus the AlD$^+$ (70%) would support this assumption (Freese and Cashel, 1965).

Recent investigations have indicated a dual metabolic role for L-alanine during germination and it was suggested that the initiation process involved active remnants of normal metabolic reactions (Wax, Freese, and Cashel, 1967).

The NADH oxidase, mentioned earlier as being closely linked to the deamination of L-alanine (O'Connor and Halvorson, 1961a), has been reported by Doi and Halvorson (1961b) to be stimulated by dipicolinic acid (DPA). DPA is a normal constituent of spores, contributing up to 3-6% of the total spore weight (Perry and Foster, 1956). The stimulation was not due to chelation, but DPA apparently served as a cofactor for or with flavin mononucleotide (FMN) since atabrine, a flavin analogue, inhibited the FMN and DPA stimulation of the NADH oxidase. The rate of NADH oxidase activity is greater with FMN alone, than a combination of FMN and DPA or with DPA alone. DPA could be substituted for FMN because both were competitively inhibited by atabrine. Therefore, DPA is acting on or near the site

of FMN action. The proposed model (Figure 1) demonstrates the competition for a common enzyme site of FMN and DPA and the flavin requirement for DPA oxidation (Doi and Halvorson, 1961b). DPA may therefore serve as an initiator of germination via an electron accepting mechanism which could explain why spores with low DPA content react slowly to L-alanine induction (Keynan, Murrell, and Halvorson, 1962).

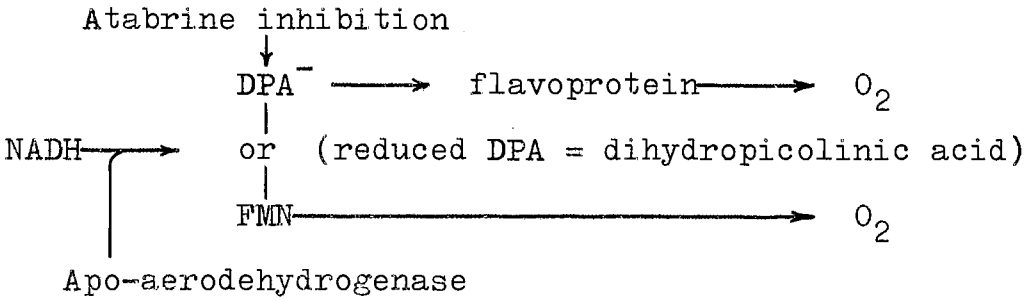


Figure 1. Proposed Function of NADH Oxidase in Germination of Bacterial Spores

Doi and Halvorson (1961a) reported the existance of a soluble flavoprotein oxidase system for terminal oxidation in spores. This type of system contrasts with vegetative cells which have a particulate cytochrome system. The soluble flavoprotein is relatively insensitive to cyanide, whereas the particulate cytochrome system is very sensitive.

The electron transport system of spores has been summarized by Halvorson (1962). He proposed a soluble NADH oxidase, stimulated by DPA and giving sufficient NAD^+ to

drive the deamination of L-alanine by AlD. DPA could also serve as an electron acceptor. This would explain the increase in respiratory activity during sporulation, the rise in respiration following activation and germination of spores, and the germination of spores under anaerobic conditions.

It is important to understand the significance of independent events to fully understand germination, however the development of a model may be useful to depict the various stages involved in germination (Figure 2).

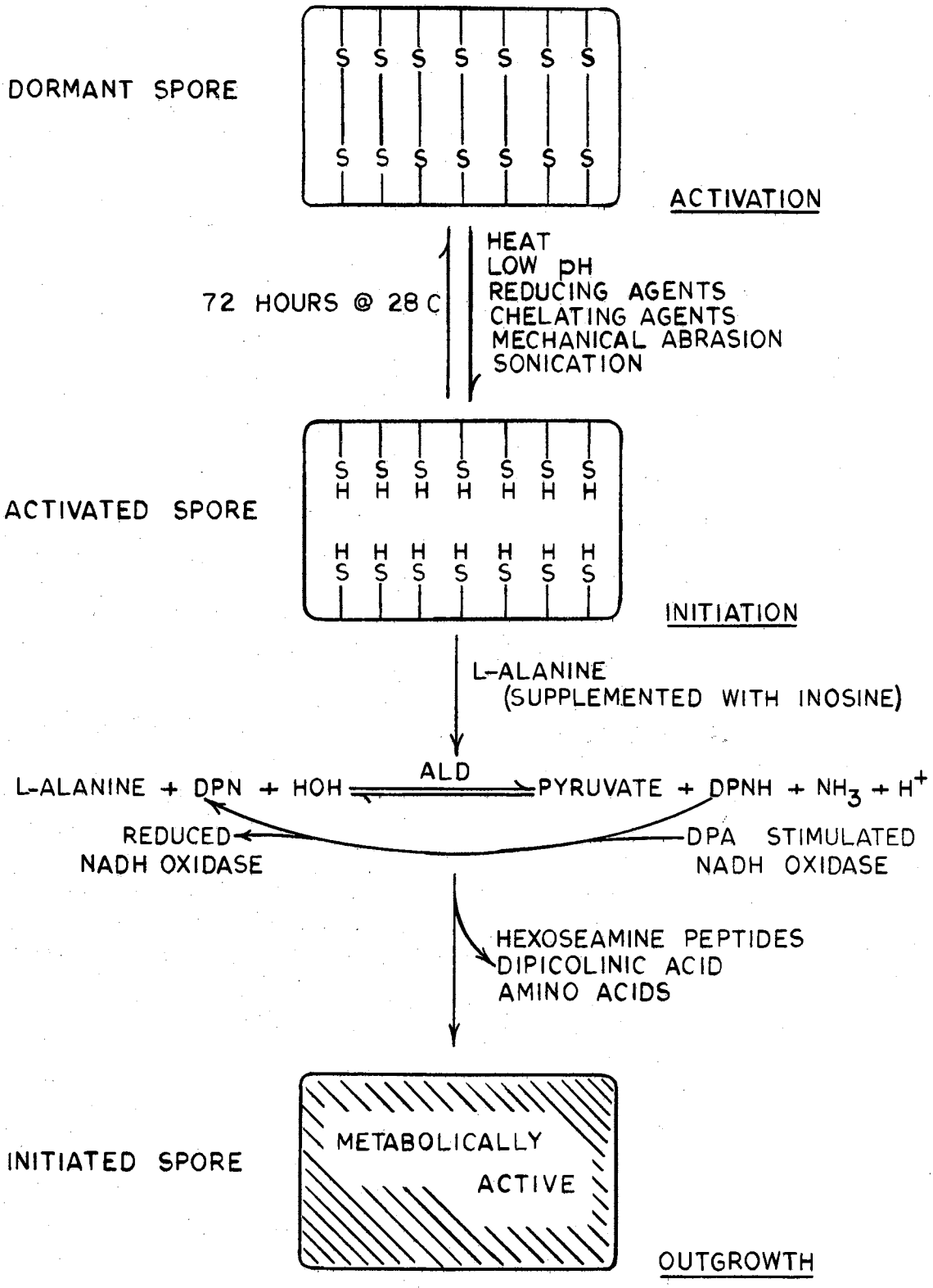
Germination has recently been described as involving three different kinds of sequential events. These are the activation phase, the germination or initiation phase, and the outgrowth phase. This thesis deals primarily with the first two phases with emphasis on initiation.

The process of activation is regarded as a reversible process involving no metabolic reactions. It is best explained as a change in a structural protein that poises the spores for germination when confronted with appropriate germinating agents. The high activation energy might suggest denaturation of macromolecules. Also, activation is dependent on the history and type of spore and not all spores are activated. (Keynan and Halvorson, 1965)

Heat activation increases the germination rate, activates dormant enzymes (Church and Halvorson, 1957), and changes the requirement for induction of germination (Powell and Hunter, 1955). Vinter (1961) proposed that disulfide

Figure 2

Proposed Model for Spore Germination


DORMANT SPORE
S S S S S S S
S S S S S S S
ACTIVATION
72 HOURS @ 28 C
HEAT
LOW pH
REDUCING AGENTS
CHELATING AGENTS
MECHANICAL ABRASION
SONICATION
ACTIVATED SPORE
SH SH SH SH SH SH SH
HS HS HS HS HS HS HS
INITIATION
L-ALANINE
(SUPPLEMENTED WITH INOSINE)
L-ALANINE + DPN + HOH
ALD
PYRUVATE + DPNH + NH3 + H+
REDUCED
NADH OXIDASE
DPA STIMULATED
NADH OXIDASE
HEXOSEAMINE PEPTIDES
DIPICOLINIC ACID
AMINO ACIDS
INITIATED SPORE
METABOLICALLY
ACTIVE
OUTGROWTH

linkages protected the spore from irradiation and heat. The spore coat was observed to possess a cystine rich protein. The protective role of disulfide linkages was strengthened when Gould and Hitchins (1963) found that agents capable of reducing disulfide linkages would render the spore susceptible to lysozyme or H_2O_2 and also caused a loss of refractility and DPA. Based on these data they predicted a model for the spore structure which consisted of an outer spore protein coat rich in disulfide bonds. Below this layer was a lysozyme sensitive mucopeptide and under this or directly associated with it was the DPA. The spore coat was located beneath these protecting layers.

Keynan, Evenchik, Halvorson, and Hastings (1964) have suggested a mechanism for germination of endospores based on data indicating activation by reducing agents, such as beta-mercaptoethanol and thioglycollate, or by a pH of 4.5 or less. Activation using these agents was reversible and dependent on the temperature. They proposed that since the spore coat appears to be five times more rich in cystine than vegetative cells (Vinter, 1961), the tertiary structure of the protein in the spore coat may contribute to the dormant state. Thus, activation results in a reversible denaturation of the disulfide rich protein. The tertiary structure could be altered by reduction of the disulfide linkages to sulfhydryl groups. Reversal would involve reoxidation of the disulfide bond with concomitant restoration of the tertiary structure (Keynan, et al., 1964).

This concept was supported by the observations that optimal heat activation occurred at pH 2-3 (Curran and Evans, 1945; Keynan, Issahary-Brand, and Evenchik, 1965). The loss of charge by the carboxyl groups and enhanced repulsive forces of the amino groups at the low pH could lead to a change in the tertiary structure. An irreversible activation was observed after short exposures to pH 1.0. This could be due to extensive denaturation which prevents recognition of native components or blocking of groups essential for maturation. Oxidation of sulfhydryl groups to sulfonic acid or sulfhydryl exchange reactions which occur at a low pH may also cause irreversible activation.

Rowley and Levinson (1967) have cautioned against the disulfide reduction theory by pointing out that reducing agents also cause an exchange of spore calcium.

The direct involvement of disulfide linkages was recently studied by following the quenching of fluorescein mercuric acetate (Blankenship and Pallansch, 1966). This technique demonstrated the disulfide linkage in the dormant state and sulfhydryl groups in the activated state.

Activation by chemical or physical means with calcium-DPA (Ca-DPA) (Lee and Ordal, 1963; Freese and Cashel, 1965) suggests participation of ions during the early stages of germination. The individual ions of calcium or DPA may be made unavailable to the spores by forming Ca-DPA complexes. Freese and Cashel (1965) believed this would expose more receptor sites for L-alanine induction. Lee and Ordal

(1963) indicated that the permeability of spores could be increased by Ca-DPA which was dependent on pH and temperature. Also, various ions inhibited the germination of activated spores, but this could be reversed by sodium-DPA. It should be mentioned that in some strains of spores Ca-DPA serves to initiate germination rather than activation (Riemann and Ordal, 1961; Keynan, Murrell, and Halvorson, 1961).

The initiation phase is the actual transition of the spore to a cell poised for outgrowth and vegetative cell division. At this time, the cell is metabolically active, heat labile, stainable, and nonrefractile (Powell, 1950). Initiation may be accomplished chemically, with Ca-DPA (Riemann and Ordal, 1961; Keynan, Murrell, and Halvorson, 1961), physiologically, with L-alanine and adenosine (Hills, 1949a; Hills, 1949b), or mechanically, by abrasive agitation (Rode and Foster, 1960) and sonic oscillation (Wolgamott and Durham, unpublished results).

Levinson and Hyatt (1966) using Bacillus megaterium have demonstrated sequential events during initiation. First, the spores lost their resistance to heat and toxic materials (3.0 minutes). This was followed by the loss of DPA (4.7 minutes), stainability (5.5 minutes), phase contrast darkening (7.0 minutes) and a loss of turbidity (7.2 minutes). According to Campbell (1957) the loss of turbidity can be correlated to dye uptake, heat lability, and phase contrast darkening.

The phenomena associated with initiation appear to be the rapid depolymerization of spore structures containing DPA, calcium, and diaminopimelic acid. This degradation appears to be enhanced, physiologically, by the AlD enzyme system. However, many other compounds exert initiator effects either at the same site or by different mechanisms, depending on the history and strain of spores employed. Foerster and Foster (1966) indicated several amino acids capable of initiating physiological germination.

Macromolecular synthesis during initiation has not been detected or is nonexistant. Spores readily lose refractility in the presence of chloramphenicol, actinomycin D, puromycin (Keynan and Halvorson, 1965; Kobayashi, Steinberg, Higa, Halvorson, and Levinthal, 1965), mitomycin C, and vancomycin (Wolgamott and Durham, unpublished results).

Woese and Forro (1960) have suggested that messenger-ribonucleic acid (m-RNA) synthesis begins about 20 minutes after the germination process begins. The synthesis of deoxyribonucleic acid (DNA) begins approximately 80-120 minutes after germination has been initiated.

Kobayashi, et al. (1965) suggested that total RNA synthesis does not start until five minutes after initiation. They found no evidence for a stable m-RNA, but did find new RNA synthesis must occur before protein synthesis and DNA replication. The spores not only lacked functional RNA, but appeared to have a defective protein synthesizing system. Vinter (1965) found no detectable macromolecular

synthesis during initiation. Thus, macromolecular synthesis must begin after the initiation phase.

The initiation phase is essentially mediated by receptor sites, exposed by activation, and the generation of energy potential from the reduction of NAD^+ by AlD (Halvorson, 1962). This energy may facilitate the extrusion of degradative spore products and encourage the genesis of metabolic reactions.

During the second phase of this thesis, the action of phenethyl alcohol (PEA) and phenylacetonitrile (PAN) was studied in an effort to elucidate a specific mechanism to account for their inhibitory action on bacterial growth.

Lilley and Brewer (1953) used PEA as a selective agent to allow growth of gram positive organisms in mixed bacterial flora. This unusual selectivity lead Berrah and Konetzka (1962) to study the mechanism of PEA's action. They found PEA was bacteriostatic at a concentration of 0.25% for most gram negative organisms. The bacteriostatic effect could be reversed by washing and resuspending the cells in a fresh medium. PEA (0.25%) inhibited DNA synthesis without inhibiting protein or RNA synthesis. However, this effect was concentration-dependent since greater concentrations inhibited RNA and protein synthesis. In addition, they noted filamentous forms of Escherichia coli in the presence of PEA, similar to those observed during thymine deprivation (Barner and Cohen, 1954) and incubation with mitomycin C (Shiba, Terawaki, Taguchi, and Kawamata, 1959). However,

here the analogy ended, because PEA is bacteriostatic while thymine deprivation is lethal, and the effect of PEA is reversible while inhibition by mitomycin C is not. (Berrah and Konetzka, 1962) Thus, Berrah and Konetzka (1962) concluded that PEA was a selective, reversible inhibitor of DNA synthesis.

Evidence supporting the specificity of PEA for DNA synthesis was reported by Konetzka and Berrah (1962). They found PEA (0.34%) inhibited the replication of T_2 bacteriophage by approximately 98-100%. However, phage enzymes and coats were present in the cells treated with PEA. Roizman (1963) reported the reversible inhibition of herpes simplex, a DNA virus, in HEp-2 cells by 0.1-0.2% PEA.

Treick and Konetzka (1964) studied different phases of growth and suggested that PEA inhibited the initiation of replication, but did not impede replication of DNA in progress, since replication could be controlled by intermittent addition and removal of PEA.

Folsome (1963) reported that PEA inhibited DNA synthesis, recombination, and heterozygote formation. He noted that phage DNA could serve in transcription but not replication and proposed that replication was necessary for genetic recombination. Also, since transformation could occur without DNA synthesis via a single strand of transforming DNA, he proposed PEA prevented the formation of DNA single strands.

The implications of early data using PEA suggested a specific inhibition of DNA replication (Treick and Konetzka, 1964). The blockage of conjugation by PEA lead to a model suggesting replication was necessary for the transfer of genetic markers (Jacob, Brenner, and Cuzin, 1963; Roeser and Konetzka, 1964).

Slepecky (1963) reported the initiation phase of germination was completely inhibited by PEA (0.05%). He postulated that PEA was inhibiting the synthesis of new m-RNA. This was the first report which indicated that PEA might affect something other than DNA synthesis.

Nonoyama and Ikeda (1964) observed that PEA (0.3%) was inhibitory for the synthesis of the RNA phages, Beta and MS-2. PEA was inhibitory and reversible, but not destructive. However, when PEA and the phage were added simultaneously the effect of PEA was not reversible.

Rosenkranz, Carr, and Rose (1964) reported that a concentration of 0.04% and 0.2% PEA inhibited the synthesis of alkaline phosphatase and beta-galactosidase, respectively. Since induction of enzymes requires the synthesis of a specific m-RNA followed by synthesis of the specific enzyme protein, but does not require DNA synthesis, they concluded that PEA specifically inhibited the synthesis of m-RNA.

Rosenkranz, Carr, and Rose (1965a) found no effect by PEA on the physical properties of DNA or on the in vitro synthesis of poly-phenylalanine, but they observed that

inducible enzyme synthesis was very sensitive to PEA. Thus, they again concluded that PEA acts specifically on m-RNA synthesis, and it is the m-RNA role in metabolic regulation that prevents cell division. Additional proof was offered when it was demonstrated that PEA (0.5%) would halt the death of cells undergoing thymineless death (Rosenkranz, Carr, and Rose, 1965b). Since m-RNA synthesis is essential for thymineless death (Hanawalt, 1963), the inhibition of m-RNA synthesis by PEA was deduced to be responsible for arresting the thymine deprived cells from exponential death.

Recently, Prevost and Moses (1966) reported that PEA acted by inhibiting total RNA synthesis; PEA had no specific inhibitory action on m-RNA synthesis. They found PEA inhibited RNA synthesis and enhanced breakdown of previously formed RNA. They observed no preferential inhibition of beta-galactosidase synthesis, since a 98% inhibition of protein synthesis produced only a 25% inhibition of beta-galactosidase synthesis. Thus, they suggested that PEA could interfere with RNA synthesis and catabolite repression.

White and White (1964) implied that PEA may have an effect on the electron transport chain. This conclusion was based on enhanced growth inhibition of E. coli by mitomycin C or streptonigrin in the presence of cyanide, 2, 4-dinitrophenol (DNP), or PEA. PEA was also shown to reduce the tetrazolium compound MTT,

[3-(4,5-dimethylthiozalyl-2)-2,5-diphenylmonotetrazolium bromide]. Likewise, MTT could reverse the synergistic influence of PEA on mitomycin C activity. Since cyanide prevents electrons from passing to oxygen, it was suggested that PEA may have a similar action (White and White, 1964).

Mendelson and Fraser (1965) could not detect changes in the physical properties of DNA treated with PEA. However, they did note a shrinkage of the T_2 bacteriophage heads in the presence of PEA. They proposed that PEA affected a protein-DNA interaction or possibly a protein-protein interaction. They also quoted a personal communication with H. V. Rickenberg who found that PEA inactivated purified beta-galactosidase from E. coli.

The involvement of the bacterial membrane was suggested by Treick and Konetzka (1964) as a means of blocking the initiation of DNA replication. They felt that the replication cycle may be affected by an alteration of the membrane which prevents genome attachment. Genome attachment may be a prerequisite for initiating replication according to the "replicon" model of Jacob, Brenner, and Cuzin (1963). Remsen, Lundgren, and Slepecky (1966) supported this contention by reporting that PEA inhibited sporulation of Bacillus cereus by preventing the formation of a spore septum or forespore membrane.

Silver and Wendt (1967) also suggested that PEA directs its attack toward the cell's permeability barrier.

They found that 0.2-0.4% PEA sufficiently altered cell permeability so acriflavine could be taken up and presumably be bound to nucleic acids. The ability of the cell to admit acriflavine was lost when PEA was removed. The presence of cyanide did not alter the repair of the permeability barrier. The flux of potassium ions (^{45}K) also indicated alteration of the cell's permeability system. It was concluded that PEA brought about a breakdown of the cell's permeability barrier. Lester (1965) also suggested PEA altered the permeability of Neurospora crassa.

This thesis is concerned with the initiation process of endospore germination and the effect of various compounds on receptor sites responsible for initiation. The second phase of the thesis attempts to elucidate the inhibitory influence of PEA and simultaneously find a mechanism for inhibition by PAN. The studies on PAN evolved from an original discovery that PAN inhibited initiation of germination and vegetative cell growth.

CHAPTER II

MATERIALS AND METHODS

Organisms

The organisms used have been previously identified and were obtained from stock cultures. Pseudomonas fluorescens and Bacillus cereus were obtained from the stock cultures of Dr. N. N. Durham, Oklahoma State University. The pseudomonad was tentatively identified by Montgomery (1966). Escherichia coli B was obtained from Dr. E. T. Gaudy, Oklahoma State University. Escherichia coli thymine$^-$ arginine$^-$ uracil$^-$ ($T^-A^-U^-$) was received from Daniel Billen and T. Lapthesophon of M.D. Anderson Hospital and Tumor Institute, Houston, Texas. Micrococcus lysodeikticus was donated from the stock culture of Dr. E. A. Grula, Oklahoma State University.

Growth Studies

The comparison of growth in the presence and absence of various inhibitors was performed with cells growing in tryptic soy broth (TSB) (DIFCO). Growth was followed by measuring the increase in absorbance at 540 mμ with a Beckman DU spectrophotometer. Aliquots of 1.5 ml were withdrawn

at specified intervals and the absorbance determined. Microcuvettes, with a one centimeter light path, were used to chamber the aliquots. Some growth assays were performed with 10 ml medium in 16 mm Spectronic 20 tubes or with 250 ml side arm flasks employing 80-100 ml quantities.

For more exacting studies, chemically defined media were used. P. fluorescens was grown in succinate salts (Kirkland and Durham, 1965) and the E. coli strains in M-9 medium (Adams, 1959). The M-9 was supplemented with 20 µg per ml thymine and uracil, and 28 µg per ml arginine for growth of the auxotrophic strain (T. Lapthesophon, personal communication).

Viable cell counts were made from 80 ml growth systems in 250 ml Erlenmeyer flasks incubated at 37 C on a reciprocal shaker. One tenth ml samples were withdrawn at regular intervals and diluted in triple distilled water and plated on the surface of nutrient agar plates. The plates were dried 5-7 days before use. The cells were spread over the surface with a sterile glass rod, and were incubated at 37 C for 18-24 hours prior to colony enumeration.

Inhibitors

Compounds used as inhibitors were purchased from various commercial sources. Different activities were evident from each company.

Beta-phenethyl alcohol (2-phenylethanol, PEA) and beta-phenethylamine (2-phenylethylamine) were purchased from

Matheson, Coleman, and Bell, division of Matheson Co., Inc., Norwood, Ohio.

Beta-phenethylamine, DL-alpha-phenethylamine, and phenylacetonitrile (benzyl cyanide, PAN) were purchased from K & K Laboratories, Plainview, New York.

Phenylacetonitrile was also obtained from Eastman Organic Chemicals, division of Eastman Kodak, Co., Rochester 3, New York.

Concentrations are indicated in the text as % (v/v).

Radioactive Compounds

L-Arginine-U-^{14}C (173 mc per mMole), uracil-2-^{14}C (58 mc per mMole), and thymine-methyl-^{3}H (11 c per mMole) were purchased from Schwartz Bioresearch, Inc., Orangeburg, New York.

D-Glucose-U-^{14}C (55 mc per mMole) was purchased from Calbiochem, Los Angeles, California.

Radioactivity Determination

The procedure used for determining radioactivity employed filtering 0.1-0.2 ml cells on Millipore filters (10 mm in diameter with a pore size of 0.45 μ) under vacuum on a three filter vacuum train. The cells were washed twice with 1 ml NaCl (0.15 M). The filters were removed, placed in scintillation vials and dissolved in 0.5 ml 1,4-dioxane. After dissolution, 9.5 ml of scintillation fluid

was added to the vial. The scintillation fluid was composed of 43 ml "Liquiflor" (Nuclear Chicago Corp.) in one liter of 1,4-dioxane. The final concentration was 4 g diphenyloxazole (PPO) and 50 mg 1,4-bis-2-(5-phenyl oxazole) benzene (POPOP) per liter. Radioactivity was determined using a Nuclear Chicago Corp. liquid scintillation, ambient spectrometer.

Hydrogen Flame Gas Chromatography

The inhibitors used in this study were analyzed by hydrogen flame gas chromatography, with the generous assistance of Dr. E. M. Hodnett.

Samples were analyzed on an Aerograph with a HY FI oven, Model A 550 (Wilkens Instrument and Research, Inc.). Twenty minute equilibration periods were allowed after temperature changes were made. Vapor injections were made with a small syringe previously cleaned with carbon tetrachloride.

Mass Spectrometry

Mass spectrographs were obtained for phenylacetonitrile from both commercial sources by Dr. F. R. Leach. The spectrographs were determined at 70 ev, 1.7 kv, I. S. of 200 C with the sample temperature at 20 or 30 C.

Variation from the parent peak (117) and free radicals (90 and 77) were assumed impurities (105 and 122 of PAN from K & K Laboratories).

Spore Preparation

Spores of B. cereus were obtained by growing the cells in G-medium (Church, Halvorson, and Halvorson, 1954) supplemented with 100 mg per ml $CaCl_2$. Five ml of an 18 hour culture were used to inoculate seven liter quantities of G-medium in Microferm vats. The cells were grown in a Microferm table top fermentor, Model MF-214, New Brunswick Scientific Co. The growth temperature was 30 C and aeration was accomplished by rotary stirring at 400 rpm and forced air injection of 3,000 cc per minute at 21 C and 14.7 psia. Excessive foaming was controlled with Dow Corning Antifoam A. The extent of sporulation was periodically checked using a Petroff-Hauser slide and a Wild phase contrast microscope. Sporulation terminated after 36 hours under the aforementioned conditions.

The spores were harvested with a Sharples centrifuge and suspended in seven liters of sterile G-medium from which glucose and yeast extract were omitted. These spore suspensions were placed on the Microferm unit for 30 minutes at 25 C with stirring at 700 rpm and forced air injection of 8,000 cc per minute to permit autolysis of the remaining vegetative cells and to facilitate the endotropic sporulation of previously committed forespores.

The spores were then collected with the Sharples centrifuge and washed immediately using seven liters of potassium phosphate buffer containing 14 g of K_2HPO_4 and

7 g of KH_2PO_4 per liter (pH 7.0). This was followed by two washings in seven liter quantities of sterile, triple-distilled water. All washings were performed on the Microferm unit employing the same conditions as used for autolysis. The spores were collected, suspended in 1.5 liters of sterile, triple-distilled water, and stored at 4 C to allow the spores to settle. Samples were withdrawn from the sediment and lyophilized on a Thermovac Industries Corp. freeze-dryer. The remaining suspended spores and the lyophilized spores were examined using a Wild phase contrast microscope and contained less than 0.1% nonrefractile or methylene blue stainable cells. The procedure for spore preparation is summarized in Figure 3.

Germination System

Germination was determined by measuring the decrease in absorbance at 625 mμ using matched pyrex or quartz cuvettes (1 cm light path) and a total liquid volume of 3 ml. The spores were routinely washed 3 times in sterile, triple-distilled water before use. Activation was accomplished by heating the spores at 60 C for 1 or 2 hours. After activation the spores were washed twice in 0.066 M potassium phosphate buffer, pH 7.0. The spore concentration was adjusted to give an absorbance of 0.4-0.5 at 625 mμ when 1 ml was added to 2 ml of a germination mixture (including the proper germinants) at time zero.

Figure 3

Procedure for the Preparation of Bacillus Cereus Spores

Five ml of an 18 hour culture of B. cereus were inoculated into a seven liter volume of G-medium supplemented with 100 mg per ml $CaCl_2$ in a 14 liter Microferm vat

The cells were grown on a Microferm at 30 C. Aeration was accomplished with a rotary stirrer (400 rpm) and air injection (3,000 cc per minute at 70 F and 14.7 psia)

The extent of sporulation was determined with a Wild phase contrast microscope using a Petroff-Hauser slide

Sporulation was virtually complete after 36 hours and the spores were harvested with a Sharples centrifuge

The cells were suspended in 7 liters of G-medium lacking glucose or yeast extract. This suspension was placed on the Microferm for 30 minutes at 25 C and stirred at 700 rpm with air injection of 8,000 cc per minute

The spores were harvested with a Sharples centrifuge and suspended in 7 liters of 0.1 M potassium phosphate buffer, pH 7.0

This suspension was placed on the Microferm and subjected to the same conditions of agitation used for autolysis

The spores were harvested with a Sharples centrifuge

The spores were suspended in 7 liters of sterile, triple-distilled water and washed with the Microferm for 30-45 minutes (same conditions as for autolysis). This was performed twice

The spores were suspended in 1.5 liter of sterile, triple-distilled water, allowed to settle for several days at 4 C, and samples were removed from the sediment and lyophilized

The suspended spores were stored at 4 C and the lyophilized spores were placed in a dessicator and kept at 0 C. Both means of storage maintained refractile spores

The purity of the spores used throughout these experiments was ascertained by two methods. First, the supernatant solution of the spore washings was checked for 280 mμ-absorbing material. Spores with any 280 mμ-absorbing material would lose some of their refractility in the presence of inosine. Thus, the second method employed a control with inosine as the only challenging germinant. When this control gave no indication of changing phase, the spore preparation was considered relatively free from extraneous material.

It was noted that unknown components were released during heat activation that would allow a phase change in response to inosine. This made it necessary to wash the heat activated spores before checking germination.

The loss of heat resistance was determined by plating the survivors of a germinated suspension and comparing to a control suspension of ungerminated spores. During the course of normal germination, 16-20% of the spores lost their resistance to 80 C (20 minutes).

Germination was also indicated by the release of ninnydrin positive compounds. These compounds were detected by separation on paper chromatograms (Whatman No. 1), with methanol: water: pyridine (80:20:4, v/v). The chromatograms were sprayed with 0.25% ninhydrin (in acetone) and developed at 100 C for 3-4 minutes.

The release of dipicolinic acid (DPA) and other components extruded during spore germination were measured in the supernatant solution using ultraviolet absorption. In assaying for such release, inosine was excluded from the systems. Spectra were obtained using a Cary recording spectrophotometer.

Thymineless Death

The multiple auxotroph, E. coli $T^-A^-U^-$, was used to study the effect of certain inhibitors on thymineless death. When thymine auxotrophs are deprived of thymine, in an otherwise complete medium, they undergo exponential death (Barner and Cohen, 1954).

The cells were grown to the exponential phase in M-9 medium supplemented with 20 µg per ml of thymine and uracil and 28 µg per ml arginine. At an absorbance of 0.5 (540 mµ), the cells were washed twice in 0.15 M NaCl and readjusted to an $A_{540\ m\mu} = 0.5$ (about 5×10^8 cells per ml). The cells were then divided into 10 ml aliquots, sedimented by centrifugation, and 10 ml of prewarmed M-9 medium (devoid of thymine) was added to all tubes except the one which had M-9 with thymine. The addition of the medium was regarded as time zero. Dilutions and platings were made every 30 minutes for 3-4 hours. Two techniques were used after the addition of the medium. The first was used when various inhibitor concentrations were employed and consisted of a series of individual 10 ml systems.

The second, used when inhibitors were added at various intervals, started with 80 ml (eight 10 ml aliquots pooled in a 250 ml flask) with 10 ml withdrawn and inhibitor added at designated intervals. During the period of thymine starvation the cells were kept at 37 C on a reciprocal shaker.

Viable cell counts were obtained by diluting cell samples in NaCl (0.15 M) and plating on the surface of nutrient agar plates (1.5% agar). Each sample was plated in duplicate or triplicate and only the plates with 30-300 colonies were considered statistically valid. Colonies were counted after 18-24 hours incubation at 37 C.

Manometric Assay

Manometric assays were performed using E. coli B. Nutrient agar plates were spread with cells from a 12-15 hour culture and incubated at 37 C for 20 hours. The cells were harvested with 0.01 M potassium phosphate buffer (pH 7.0), which was also used to wash the cells twice. The suspension density was adjusted so a 1:24 dilution gave an absorbance of 0.5 at 540 mμ on a Bausch and Lomb Spectronic 20. Glucose (1.0 mMole and 2.0 mMole) was used as substrate in a total volume of 3 ml. All measurements were made at 37 C in a Warburg manometer with air as the gas phase. Carbon dioxide was absorbed on fluted filter paper saturated with 0.2 ml potassium hydroxide (20%).

Glucose Utilization

E. coli $T^-A^-U^-$ cells were obtained by growing in TSB for 6 hours and washed twice with NaCl (0.15 M). The cell suspension was adjusted so a 1:10 dilution gave an absorbance of 0.580 at 540 mμ on the Beckman DU. The cells were divided into 5 ml aliquots and sedimented by centrifugation. The pellets were resuspended in fresh M-9 medium. The compounds to be checked for an effect on glucose uptake were added at time zero. The glucose remaining at designated intervals was ascertained by the Glucostat assay (Worthington Biochemical Co.). This employs the following reaction:

$$\text{Glucose} + O_2 + H_2O \longrightarrow H_2O_2 + \text{gluconic acid}$$

$$H_2O_2 + \text{reduced chromagen} \longrightarrow \text{oxidized chromagen}$$

To check the glucose concentration, 0.02 ml was withdrawn and added to 4 ml Glucostat reagent plus 8.98 ml of triple-distilled water. After 10 minutes, the reaction was stopped by adding 1 drop 6 M HCl. The acid also served to stabilize the color which was measured at 400 mμ.

Synthesis of Protein, RNA, and DNA

The synthesis of these macromolecules was determined by following incorporation of L-arginine-U-^{14}C, uracil-2-^{14}C, and thymine-methyl-^{3}H. Glucose incorporation was followed with glucose-U-^{14}C. The auxotroph, *E. coli* $T^-A^-U^-$, was employed since the specific requirement for these

compounds makes it a useful tool for following the specific synthesis of protein, RNA, and DNA.

The cells were grown in M-9 medium until they reached late exponential phase ($A_{540\ m\mu} = 0.50$). The cells were then washed twice with NaCl (0.15 M), suspended in fresh, prewarmed M-9 medium to an absorbance of 0.50 at 540 mμ (Beckman DU). Growth recovery occurred during a 20 minute incubation at 37 C. The cells were divided into ten 5 ml aliquots. To each pair, one an inhibited and one a control, a specific isotope was added. The amount of isotope added is listed in Table I. At 30 minute intervals 0.5 ml samples were withdrawn and immediately frozen in an acetone-dry ice bath. These samples were thawed and 0.2 ml samples were filtered and counted, as previously described.

Rapid Metabolite Uptake

E. coli $T^-A^-U^-$ was grown to an absorbance of 0.4 at 540 mμ in M-9 supplemented with the auxotrophic nutrients. The cells were harvested by centrifugation and washed twice with NaCl (0.15 M) at room temperature. The last wash was performed on 3.1 ml aliquots and these pellets were suspended in 3.1 ml of 0.01 M potassium phosphate buffer (pH 7.0) five minutes prior to the addition of the isotope. The inhibitors were added 2 minutes before the isotope to assure their solubilization. Samples (0.1 ml) were withdrawn, filtered, and radioactivity measured as described

TABLE I

ISOTOPES FOR RAPID UPTAKE

Isotope	Amount of Carrier	Specific Activity
Arginine-U-^{14}C	1.7×10^3 pmole	2.8×10^8 cpm/μmole
Glucose-U-^{14}C	1.1×10^3 pmole	4.3×10^{10} cpm/μmole

previously. The isotopes and the quantity employed are listed in Table II.

TABLE II

GROWTH INCORPORATION ISOTOPES

Isotope	Amount of Carrier	Specific Activity
Thymine-methyl-^3H	1.6×10^2 μmole	1.4×10^2 cpm/μmole
Arginine-U-^{14}C	1.6×10^2 μmole	3.0×10^3 cpm/μmole
Uracil-2-^{14}C	1.8×10^2 μmole	7.3×10^3 cpm/μmole
Glucose-U-^{14}C	2.2×10^4 μmole	2.1×10^2 cpm/μmole

Anaerobic Growth Assay

Anaerobic growth was accomplished by growing *E. coli* $T^-A^-U^-$ at 37 C in Brewer's modified Bacto thioglycollate medium without glucose (DIFCO). Anaerobic adaptation was accomplished by 16 hours growth in this medium under nitrogen. Inoculations for growth studies were made by placing 0.1 ml of a 16 hour thioglycollate culture below the surface of 10 ml of thioglycollate broth. These systems were flooded with nitrogen, capped with Parafilm and aluminum foil. No oxidation of methylene blue was apparent during the 12 hours that growth was followed.

Isolation of Particulate Cell Fraction

Particulate cell fractions of *E. coli* $T^-A^-U^-$, *P. fluorescens*, and *M. lysodeikticus* were isolated according to the following scheme, modified from Salton (1964).

1. Cells were disintegrated by sonic oscillation; 10-20 thirty second bursts, in the cold with maximum power setting on a Raytheon sonicator.
2. The whole cells and debris was sedimented by centrifugation at 2,500 x g for 7.5 minutes.
3. The resulting supernatant solution was centrifuged at 7,500 x g for 20 minutes for gram positive cells and 34,800 x g for 30 minutes for gram negative cells.
4. The pellet was washed with 1 M NaCl, heated for 30 minutes at 50 C, and digested with trypsin (3 mg per ml for 2 hours at 37 C for gram positive and 10 mg per ml for 2 hours for gram negative cells).

5. After centrifugation, the fractions were washed twice with triple-distilled water.

6. The pellet after the second wash was resuspended in triple-distilled water and centrifuged for 20 minutes at 2,500 x g to remove whole cells and other particulate matter.

7. The particulate fraction remained in suspension.

The preparations were suspended to an absorbance of 0.8-0.9 at 380 mμ on the Cary spectrophotometer. The effect of PEA and PAN on the particulate fraction spectrum (380 mμ to 640 mμ) was checked at one hour intervals for up to five hours.

Cell Membrane Isolation and Reaggregation

The isolation and reaggregation of M. lysodeikticus membranes was performed according to the technique devised by Butler, Smith, and Grula (1967). The exception to the Butler, Smith, and Grula (1967) technique, employed for membranes assayed for lipid content, involved growing cells to an absorbance of 0.6 at 540 mμ (late log phase) in TSB. The cell yield was approximately twice that obtained using defined medium. The cells were washed in Beta buffer (Razin, Morowitz, and Terry, 1965) and treated with 2 mg per ml lysozyme in 0.15 M NaCl.

For systems that were reaggregated in the presence of PEA and PAN, the compounds were added to the dialyzing solution at the desired concentrations and incubated at 4 C.

Lipid Profile of Reaggregated Membrane

Membrane subunits (10 ml) were reaggregated by dialysis against Beta buffer, Beta buffer plus PEA, and Beta buffer plus PAN. Ten days were allowed for reaggregation. Lipid material was extracted from the reaggregates by heating for 30 minutes at 55 C in the presence of 5 ml methanol under a nitrogen atmosphere. After heating, two volumes of chloroform were added and the system was again flushed with nitrogen. This was held at 25 C for about 12 hours. The fraction was washed twice with equal volumes of 2.0 M KCl and the aqueous phase was removed by aspiration. The chloroform fraction was concentrated by flushing with nitrogen. The concentrate was solubilized in iso-octane and the absorbance adjusted to about 0.9-1.0 at 205 mμ on the Cary spectrophotometer. The ultraviolet profile (205 to 340 mμ) of the lipids was measured. Also, the visible spectrum (340 to 640 mμ) was measured to ascertain the effect of PEA and PAN on the extracted carotenoids. The technique employed for lipid analysis was adapted from Salton (1967).

NADH Oxidase Assay

NADH oxidase was obtained from E. coli $T^-A^-U^-$ cell wall and membrane preparations (Salton and Ehtisham-Ud-Din, 1965). The cells were grown to an absorbance of 0.6 at

540 mμ in TSB, washed twice in 0.05 M tris buffer (pH 7.5) and subjected to sonic oscillation at 4 C. The standard treatment was 2-3 ten second bursts with the cells cooled in ice for 5-10 minutes between sonications. The whole cells and debris were removed by centrifugation in a refrigerated Sorvall RC-2 at 2,445 x g for 10 minutes. The insoluble material was decanted and sedimented by centrifugation at 34,800 x g for 30 minutes. This material was washed with 0.05 M tris buffer (pH 7.5) and sedimented at 34,800 x g (30 minutes). The particulate material was held in ice prior to examination. The absorbance was adjusted to about 0.95 at 340 mμ using the Cary spectrophotometer. The assay system consisted of 1.0 ml 0.05 M tris buffer (pH 7.5), 1.0 ml wall-membrane material, and 1.0 ml NADH (300 μg per ml in 0.05 M tris buffer, pH 7.5). The oxidation of NADH was followed spectrophotometrically for 2-3 minutes by the decrease in absorbance at 340 mμ. The individual systems were prepared in 3 ml quartz cuvettes (1 cm light path). The inhibitors were added 1-2 minutes prior to the addition of substrate (NADH) to facilitate solubilization.

CHAPTER III

RESULTS AND DISCUSSION

Requirements for Amino Acid Induced Germination

To limit the parameters involving amino acid induced germination, studies were performed to ascertain the minimum requirements necessary to germinate spores of Bacillus cereus. Germination, as used in this thesis, will refer to the initiation phase and will be used synonymously with initiation.

Ribosides were suggested by Lawrence (1955) to function during germination as a source of free ribose. In our system, no release of reducing groups from inosine was detected by Nelson's (1944) arsenomolybdate assay. However, inosine did act synergistically with certain amino acids to give maximum germination rates. Therefore, the inosine concentration was held constant in the germination system, and the function of specific amino acids was made the primary concern of this investigation.

It was found that L-alanine (Hills, 1949a) and L-phenylalanine induced germination of the spores of the B. cereus investigated. Other amino acids gave partial initiation, but this finding was not pursued.

The minimum concentration of L-alanine or L-phenylalanine necessary for maximum germination was about 3.7×10^{-3} M. Maximum germination is defined as the germination rate achieved in the presence of excess amino acid. Under these conditions the receptor sites are saturated and the addition of more amino acid does not facilitate greater or faster germination. The minimum amino acid requirements were determined so inhibitor interference would be maximal.

Additive Effect of Amino Acids on Induction

To determine if L-alanine and L-phenylalanine had different receptor sites, the effect of the two amino acids, separate and in combination, on germination was investigated. It had previously been established by paper chromatography that the L-alanine and L-phenylalanine were free of other ninhydrin-positive compounds. About 30-40% germination was obtained with 8.26 µg per ml L-alanine or 16.5 µg per ml L-phenylalanine. When L-phenylalanine was added to the L-alanine system, additional induction was observed (Figure 4). This additive effect suggested that L-alanine and L-phenylalanine have separate receptor sites for germination induction.

The specificity of the germination receptor site reportedly involves the alpha-amino, the carboxyl, and the alpha-carbon of the inducer (Woese, Morowitz, and Hutchison, 1958). It is also stereospecific for the L- and D-isomers (Hills, 1949a). Support for this three point

Figure 4

The Additive Effect of L-Alanine and L-Phenylalanine on Initiation

Quantities of amino acids giving partial initiation were tested simultaneously to depict the additive effect of amino acid induced germination: Δ L-Alanine, 8.26 µg per ml; O L-Phenylalanine, 16.5 µg per ml; ● L-Alanine, 8.26 µg per ml plus L-Phenylalanine, 16.5 µg per ml.

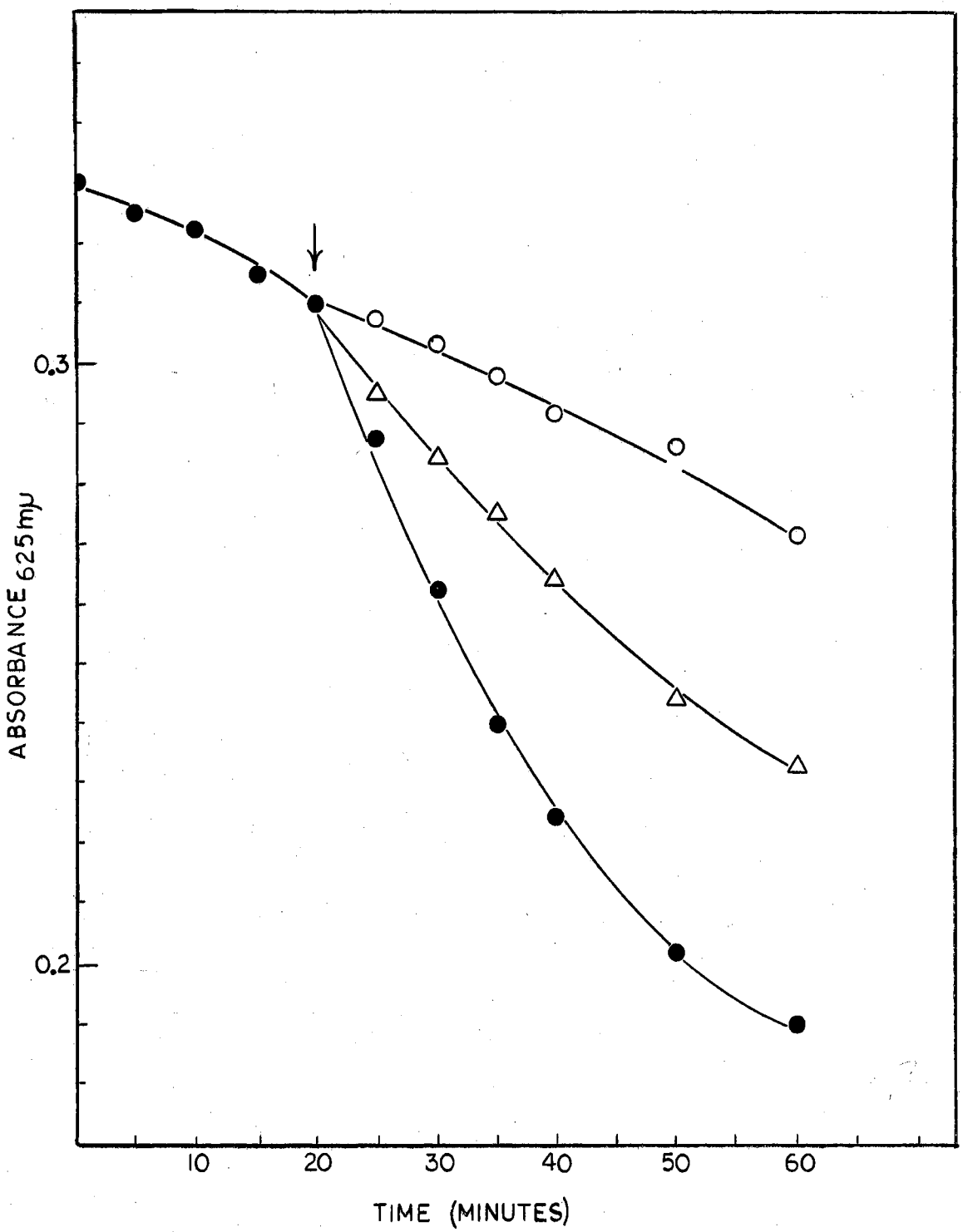
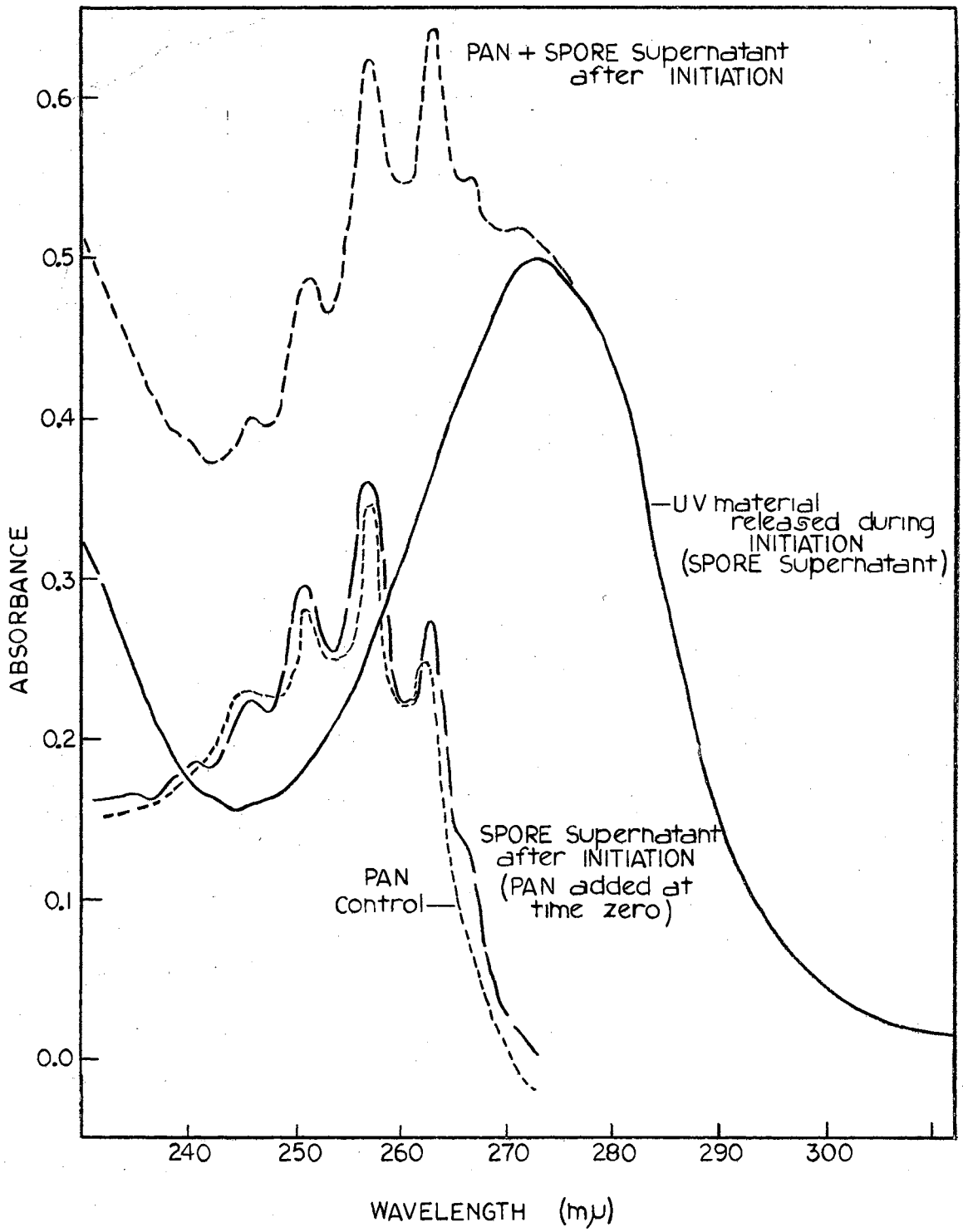

0.3
0.2
ABSORBANCE 625mμ
10
20
30
40
50
60
TIME (MINUTES)

Figure 5

The Ultra-Violet Profile of Material Released During Initiation and Inhibition of Initiation by PAN


PAN + SPORE Supernatant after INITIATION
UV material released during INITIATION (SPORE Supernatant)
SPORE Supernatant after INITIATION (PAN added at time zero)
PAN Control
ABSORBANCE
0.0
0.1
0.2
0.3
0.4
0.5
0.6
240
250
260
270
280
290
300
WAVELENGTH (mμ)

specificity evolves from our finding that L-phenylalanine will serve with L-alanine to induce germination. Also, we found that p-fluorophenylalanine and thienylalanine were functional as initiators. Use of these analogs resulted in only a slight modification of the germination kinetics.

Inhibition of Initiation

The inhibition of initiation by phenethyl alcohol (PEA) was first reported by Slepecky (1963). He suggested PEA prevented m-RNA synthesis, but no indication of any macromolecular synthesis during initiation has been reported (Kobayashi, et al., 1965; Vinter, 1965).

Related compounds were also found to block initiation. Beta-phenethylamine, benzyl alcohol, and phenylacetonitrile (PAN) were effective inhibitors. Even high concentrations of methanol and ethanol blocked initiation. When these compounds were added to germination systems they prevented the loss of ninhydrin-positive materials, as determined by paper chromatography, and the release of dipicolinic acid (DPA) and other ultraviolet absorbing materials (Figure 5). The spores retained heat resistance and refractility, so these compounds apparently inhibit one of the initiating reactions during germination (Figure 2).

Antibiotics, known to block certain macromolecular synthesis, such as RNA and protein, were not effective as inhibitors of initiation (Table III). These antibiotics and other compounds were effective, however, in preventing

Figure 6

The Alleviation of Beta-Phenethylamine Inhibition by the Addition of L-Phenylalanine

L-Phenylalanine (100 μg per ml, final concentration) was added at the arrows: ● Control, L-alanine (100 μg per ml) and inosine (100 μg per ml); ▲ L-alanine and inosine plus beta-phenethylamine (0.05%); △ L-phenylalanine added at 12.5 minutes; ○ L-phenylalanine added at 27.5 minutes.

TABLE III

THE EFFECT OF VARIOUS COMPOUNDS ON L-ALANINE INDUCED GERMINATION*

Compound	Concentration	Per Cent Germination
Actinomycin D	50 μg per ml	97%
Vancomycin	50 μg per ml	98%
Mitomycin C	50 μg per ml	100%
Chloramphenicol	50 μg per ml	93%
Phenethyl alcohol	0.05%	8%
Beta-phenethylamine (MCB)	0.05%	36%
Phenylacetonitrile	0.01%	5%
Alpha-phenethylamine	0.05%	88%
Toluene	0.02%	24%
Benzyl alcohol	0.03%	58%
Sodium cyanide	2×10^{-3} M	100%
	4×10^{-3} M	98%

*Germination was induced with L-alanine (100 μg per ml) and inosine (100 μg per ml). The per cent germination was determined by the absorbance ratio with the compound to that of the system without the compound after 60 minutes, at pH 7.0.

the outgrowth of spores. A functional cytochrome system is apparently not required for initiation since cyanide was not inhibitory (Table III).

Reversal of Inhibition by Additional Substrate

When L-alanine induction was blocked by beta-phenethylamine, either L-phenylalanine or L-alanine could alleviate the inhibition (Figure 6). The extent of alleviation was dependent on the ratio of amino acid to inhibitor (Figure 7). This suggests a competition between inhibitor and substrate for a common receptor site.

Induction Sensitivity Variation

L-Alanine and L-phenylalanine initiation were found to have differing sensitivities to beta-phenethylamine (Figure 8). Using a concentration of 3.7×10^{-3} M (both inducers), it was observed that L-alanine induction was more sensitive to beta-phenethylamine than spores germinated with L-phenylalanine. Conversely, the induction by L-phenylalanine was more sensitive to PEA than the L-alanine induced spores (Figure 9).

The additivity and variation in sensitivity of the initiation receptor site suggests that it could be allosteric. The receptor site may bind one or the other, but not both of the amino acids. Also, beta-phenethylamine and PEA appear to act as allosteric effectors, each having a greater effect at one site than the other.

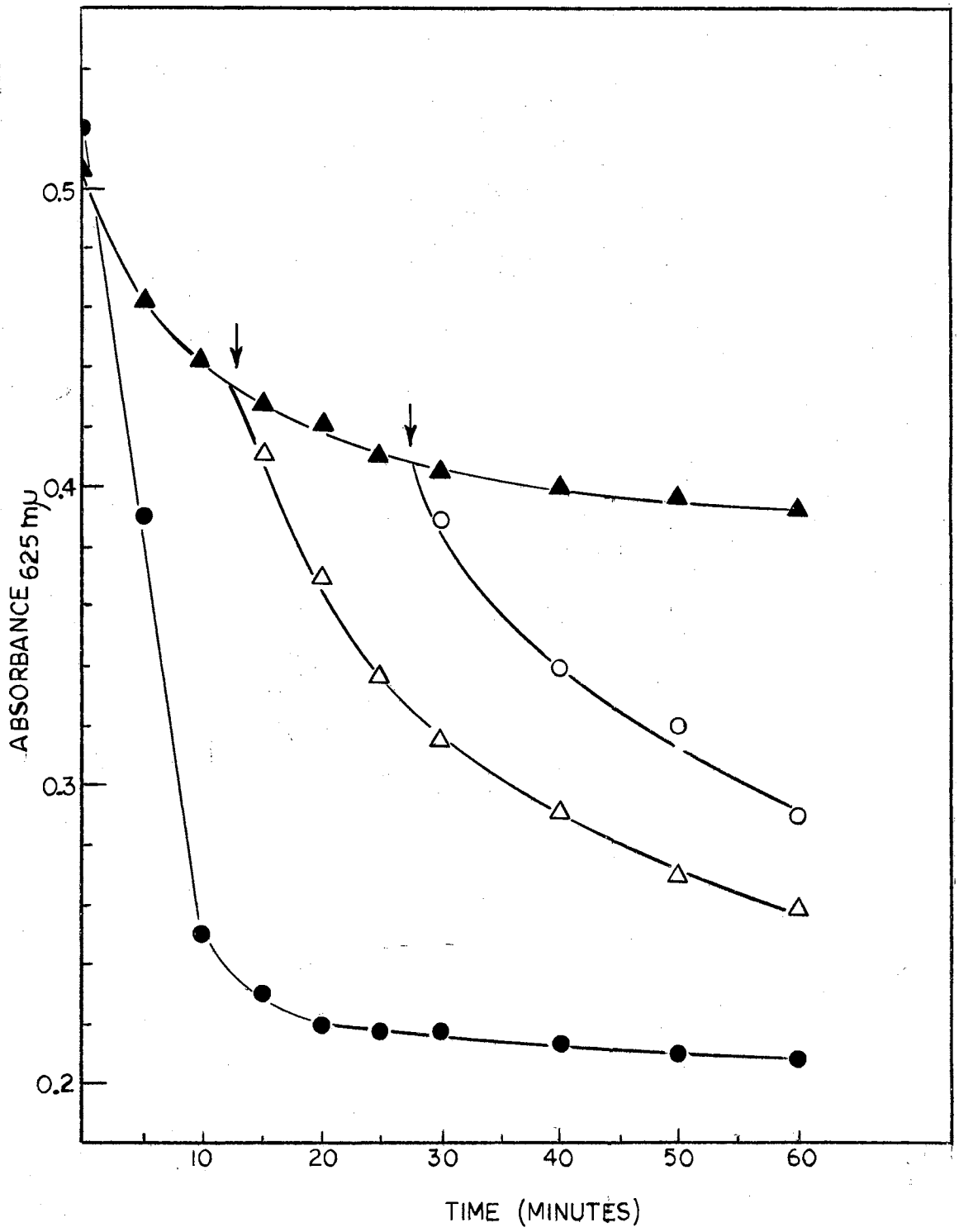

0.5
0.4
0.3
0.2
ABSORBANCE 625 mμ
10
20
30
40
50
60
TIME (MINUTES)

Figure 7

The Alleviation of Beta-Phenethylamine Inhibition with Various Concentrations of L-Phenylalanine

Various concentrations of L-phenylalanine were added at the onset of initiation: ● Control, L-alanine (100 µg per ml) and inosine (100 µg per ml); ○ beta-phenethylamine (0.05%) plus L-alanine and inosine; △ L-phenylalanine (8.25 µg per ml) added to inhibited system; □ L-phenylalanine (82.5 µg per ml) added to inhibited system.

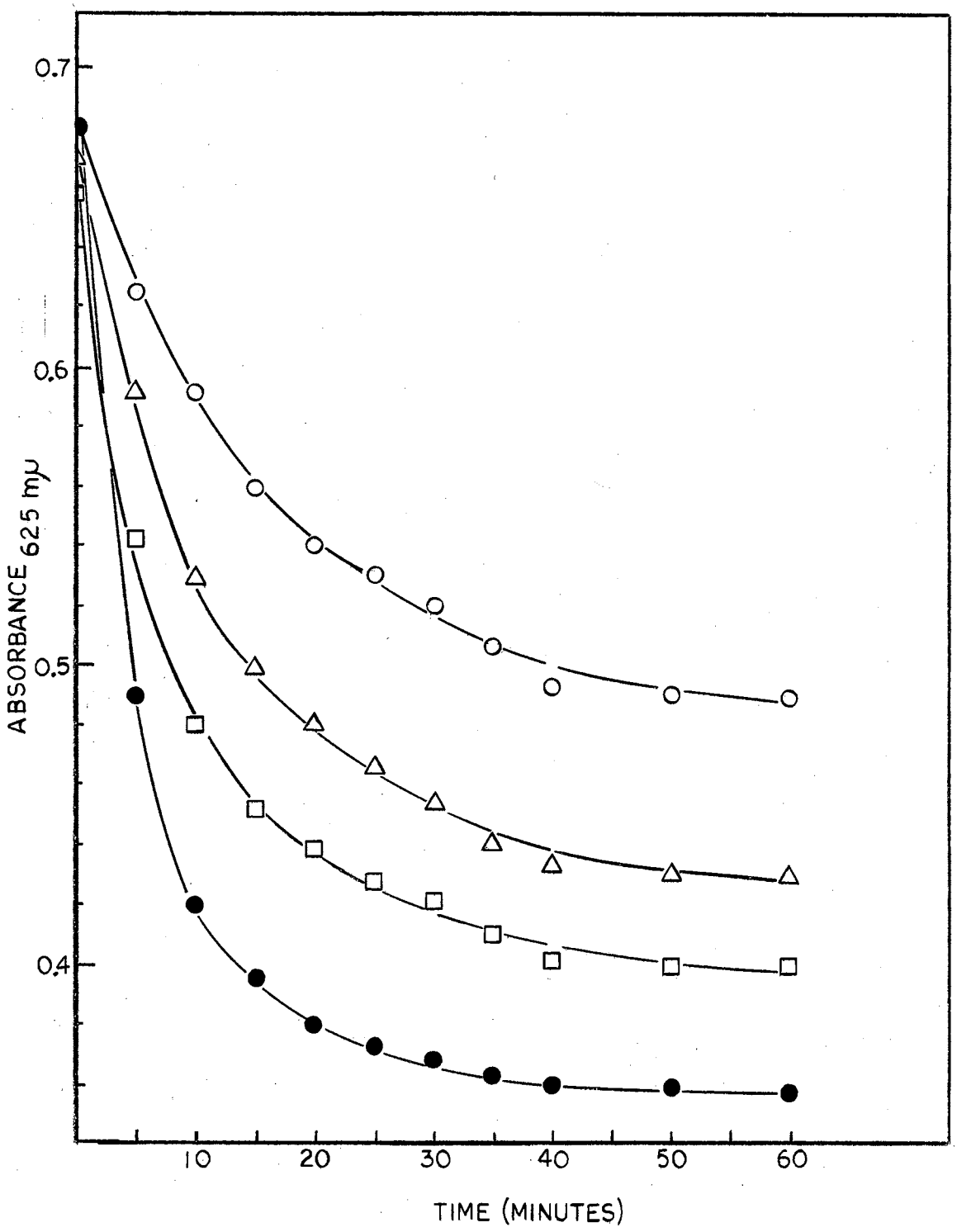

0.7
0.6
0.5
0.4
ABSORBANCE 625 mμ
10
20
30
40
50
60
TIME (MINUTES)

Figure 8

Sensitivity of L-Alanine and L-Phenylalanine Induced Germination to Beta-Phenethylamine Inhibition

Amino acids and inhibitors were added at time zero:

● L-alanine (0.0037 M); o L-phenylalanine (0.0037 M);

▲ L-alanine plus beta-phenethylamine (0.01%); △ L-phenylalanine plus beta-phenethylamine (0.01%).

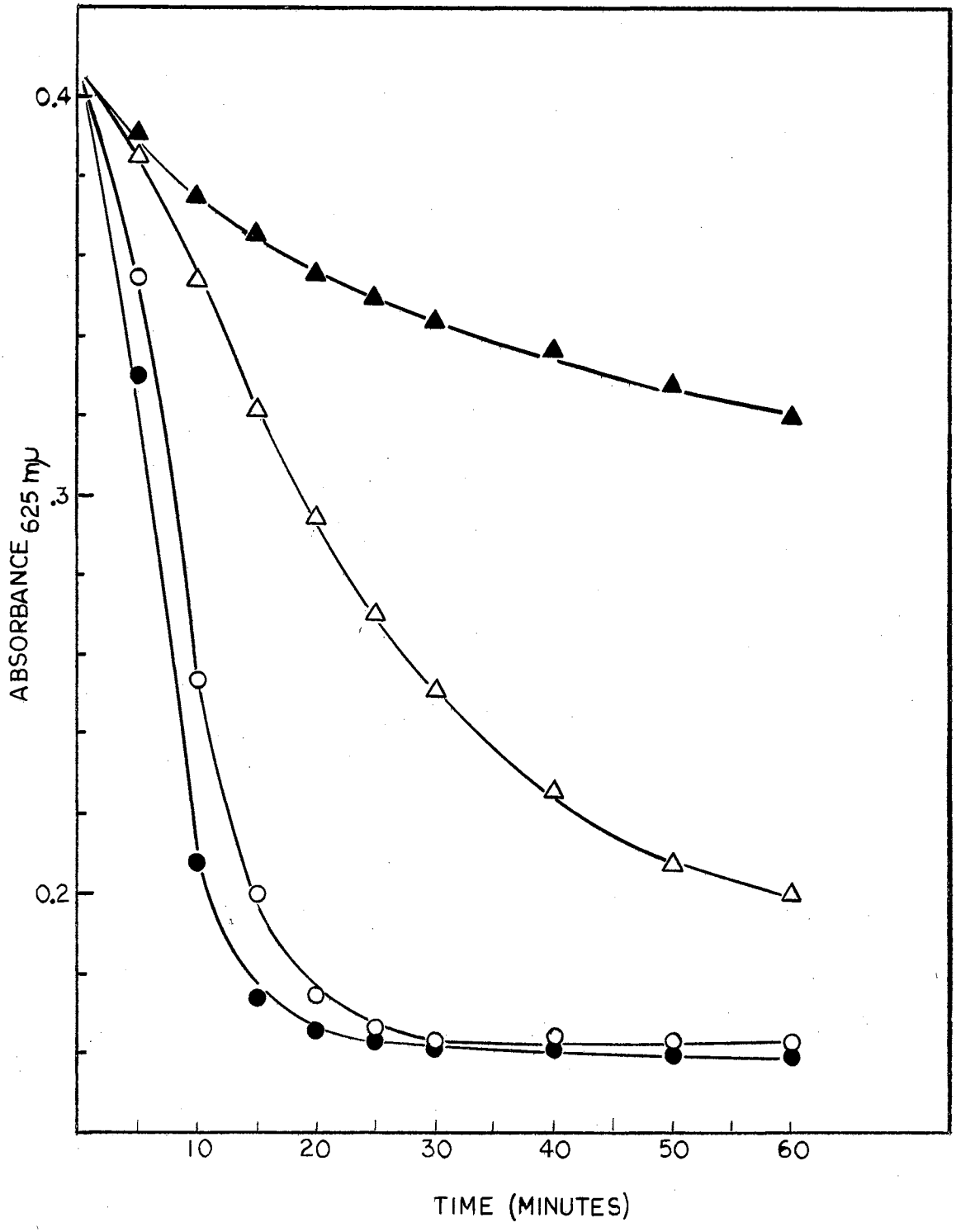

0.4
.3
0.2
ABSORBANCE 625 mμ
10
20
30
40
50
60
TIME (MINUTES)

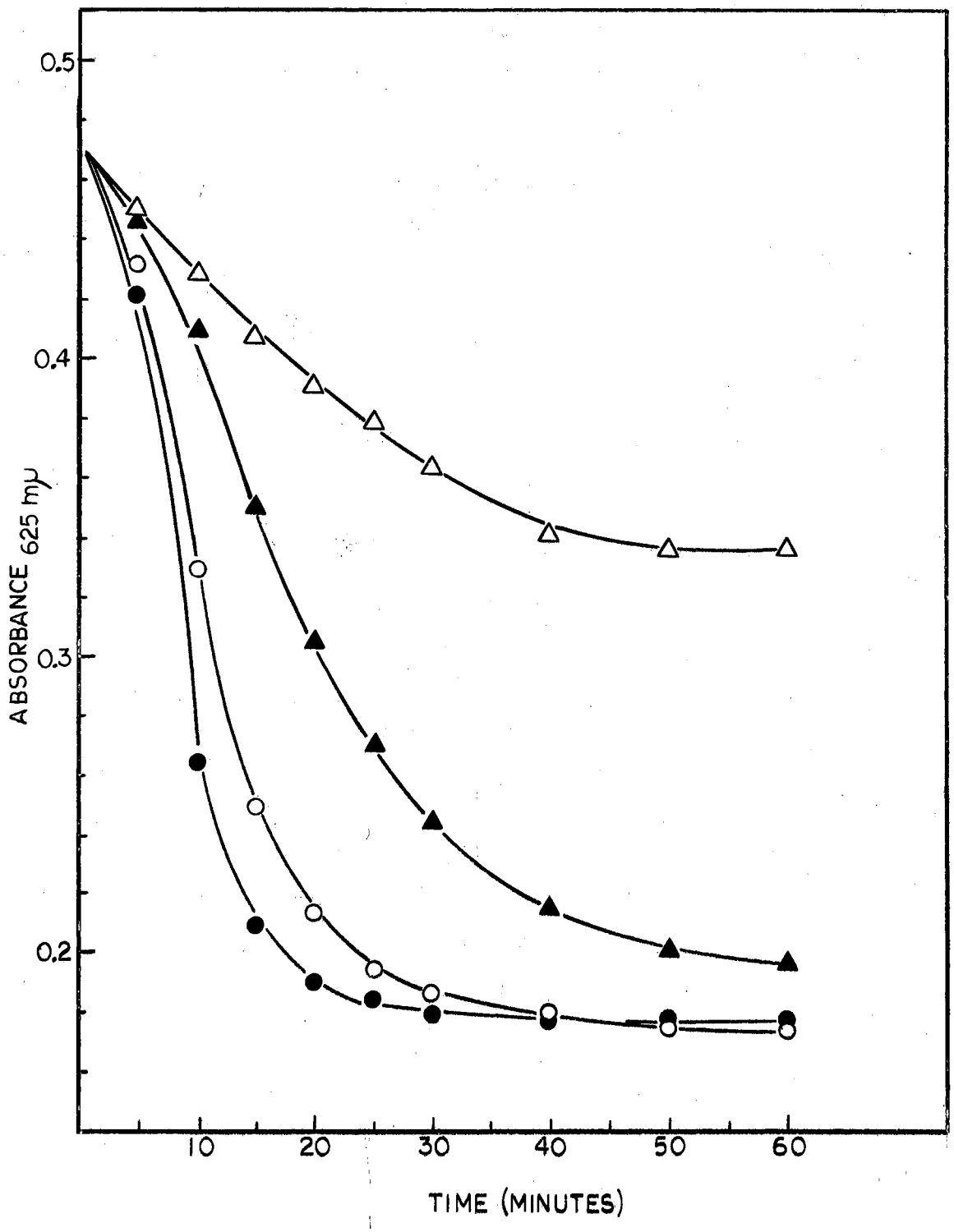
Figure 9

Sensitivity of L-Alanine and L-Phenylalanine Induced Germination to Phenethyl Alcohol Inhibition

Amino acids and inhibitors were added at time zero:

● L-alanine (0.0037 M); O L-phenylalanine (0.0037 M);

▲ L-alanine plus PEA (0.025%); △ L-phenylalanine plus PEA (0.025%).


0.5
0.4
0.3
0.2
ABSORBANCE 625 mμ
10
20
30
40
50
60
TIME (MINUTES)

Effect of pH on Germination

The pH of the medium was found to influence the L-alanine induction mechanism as well as inhibition by beta-phenethylamine and PEA, but little difference in germination was observed at pH 7.0 or 9.0 (Figure 10). All previous studies were performed at pH 7.0. The pH optimum of 9.4 for amino acid induced germination has been reported to correspond to the optimum of L-alanine dehydrogenase (AlD) (O'Connor and Halvorson, 1961a).

A concentration of PEA giving about 50% inhibition (0.03%) at pH 7.0 was checked at pH 9.0. At this higher pH, PEA was no longer inhibitory (Figure 11). This could be explained if the high pH enhanced the stability of the L-alanine receptor site and/or increased the affinity of the receptor for the substrate.

Opposite results were obtained with beta-phenethylamine (0.03%). Using this inhibitor, only partial inhibition was observed at pH 7.0, but at pH 9.0, considerably greater inhibition of spore induction was obtained (Figure 12). This effect was observed with beta-phenethylamine from two commercial sources, and is also stereospecific since alpha-phenethylamine was not inhibitory at either pH (7.0 or 9.0). The effect of alpha-phenethylamine at pH 7.0 is synonymous with the L-alanine control.

The greater activity of beta-phenethylamine from K & K Laboratories, compared to that obtained from the Matheson,

Figure 10

The Effect of pH on L-Alanine Induced Germination

Germination was induced with L-alanine (44.5 μg per ml) at: □ pH 5.0; ○ pH 7.0; ● pH 9.0; ▲ pH 11.0.

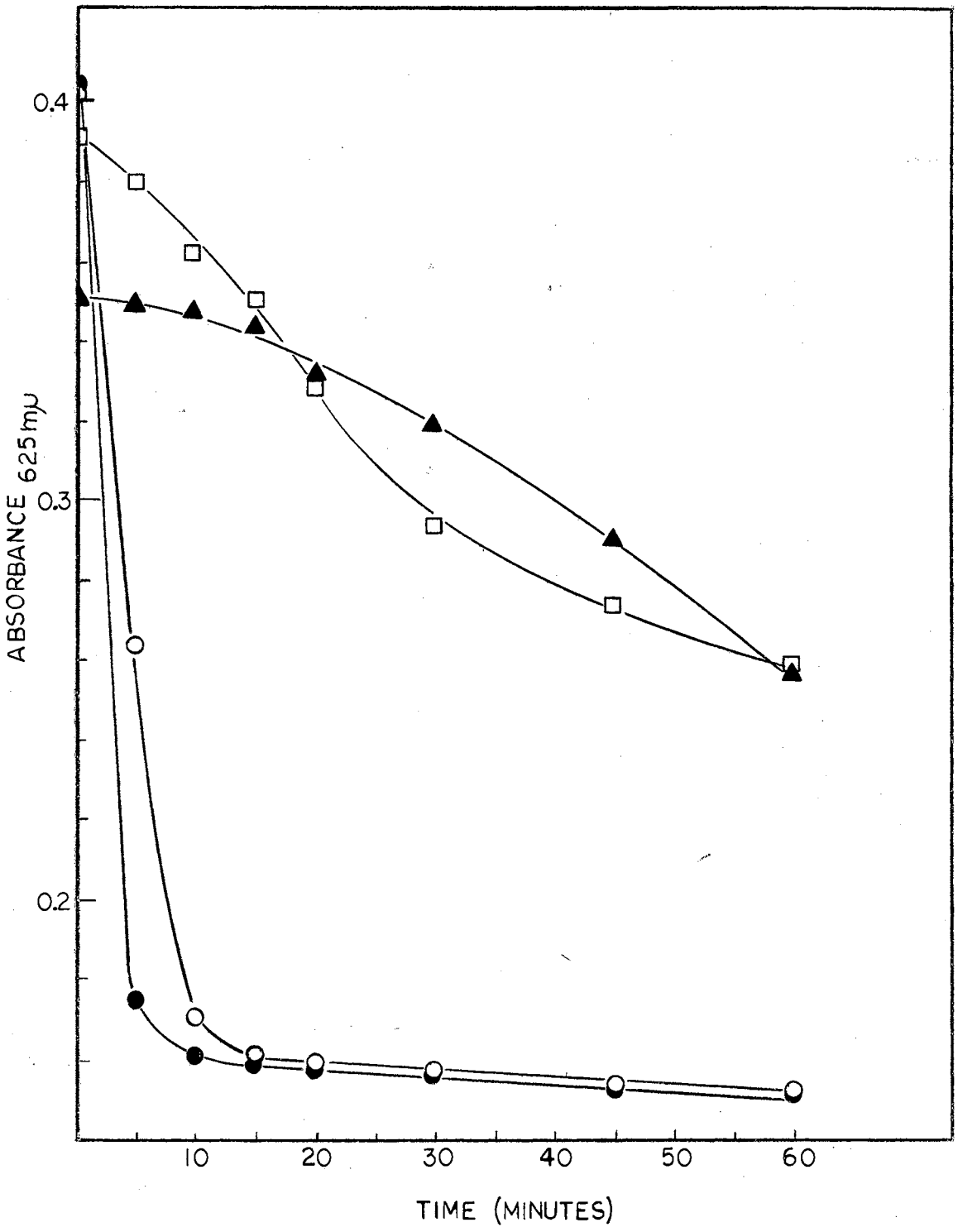

0.4
0.3
0.2
ABSORBANCE 625 mμ
10
20
30
40
50
60
TIME (MINUTES)

Figure 11

The Effect of pH on Phenethyl Alcohol Inhibition

Germination was induced with L-alanine (44.5 µg per ml) and the effect of PEA (0.03%) was determined at different pH values: O pH 7.0; ● pH 9.0. both with PEA. The control without PEA was analogous to the pH 9.0 curve with PEA.

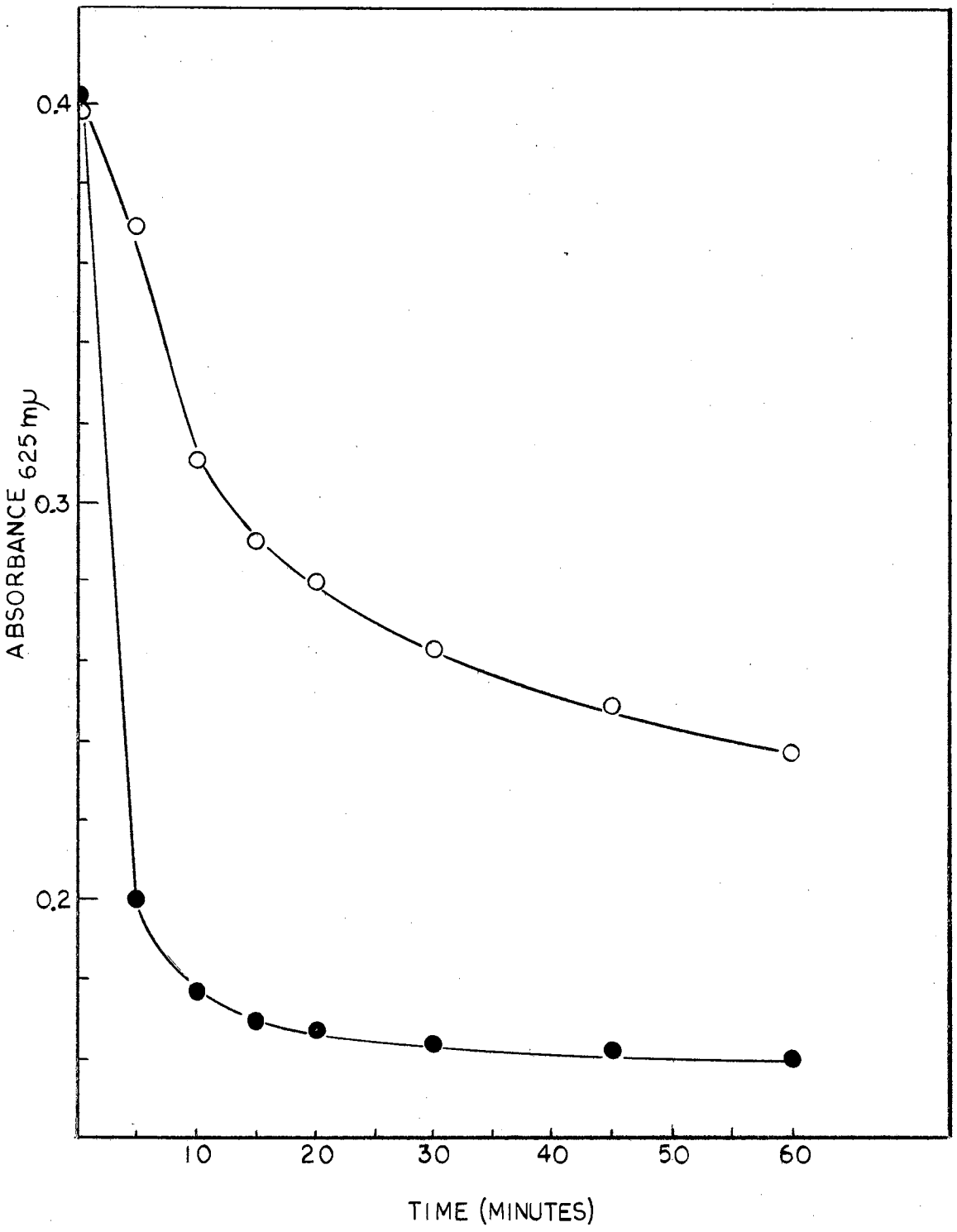
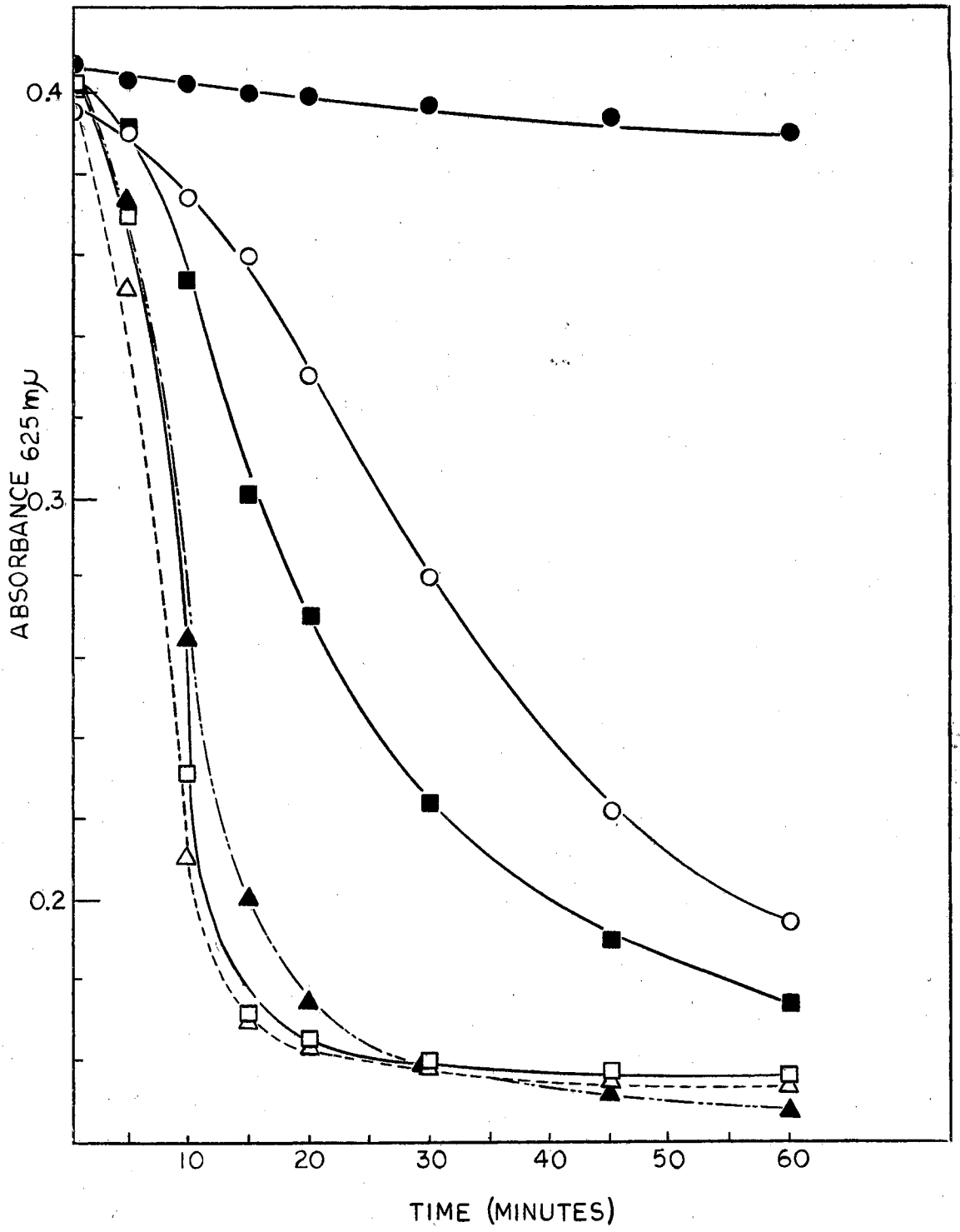

0.4
0.3
0.2
ABSORBANCE 625 mμ
10
20
30
40
50
60
TIME (MINUTES)

Figure 12

The Effect of pH on Inhibition by Alpha- and Beta-Phenethylamine from Two Commercial Sources

Germination was initiated by L-alanine (44.5 µg per ml) and the effect of different pH values was determined: △ alpha-phenethylamine (0.03%) at pH 7.0, and L-alanine control; ▲ alpha-phenethylamine (0.03%), pH 9.0; □ beta-phenethylamine (0.03%), from MCB at pH 7.0; ■ beta-phenethylamine (0.03%), from MCB at pH 9.0; ○ beta-phenethylamine (0.03%) from K & K at pH 7.0; ● beta-phenethylamine (0.03%), from K & K at pH 9.0.


0.4
0.3
0.2
ABSORBANCE 625 mμ
10
20
30
40
50
60
TIME (MINUTES)

Coleman, & Bell product, was thought to be due to some material carried over from an early stage of its synthesis. For this reason, PAN, which forms beta-phenethylamine upon reduction (Johnson and Guest, 1909), was tested for its effect on this system. PAN (0.01%) inhibited initiation and was not affected by pH changes.

The varied response of L-alanine induced germination to this class of inhibitors might indicate an allosteric receptor site. At pH 9.0, beta-phenethylamine is the most potent inhibitor. This corresponds to the observation that L-alanine induction is more sensitive to inhibition by beta-phenethylamine than L-phenylalanine induction. Also, the alleviation of inhibition of PEA, at pH 9.0, correlates with the finding that L-alanine induction is more resistant to inhibition by PEA. These observations indicate that initiation depends on the inducer, its concentration, the inhibitor and its concentration, as well as the pH.

The receptor site responsible for initiation is probably AlD, which is responsible for providing the activated spore with a potential reducing source. Thus, reaction at this receptor site is necessary for amino acid induction. Our data suggest the receptor site is allosteric and may be induced using either L-alanine or L-phenylalanine. The inhibitors, PEA and beta-phenethylamine might act as allosteric effectors causing a conformational modification at areas on or near the binding site for a specific amino

acid. A model of such a receptor site, based on allosteric proposals by Monod, Wyman, and Changeux (1965), is presented in Figure 13.

The suggestion that PEA acts as an allosteric effector, possibly altering protein configuration, does not conform to its reported mechanism. This discrepancy provided the basis for further investigation of the mechanism of PEA, while simultaneously studying the effect of PAN.

Analysis of Inhibitors

The previously noted difference in inhibition by beta-phenethylamine from K & K Laboratories and Matheson, Coleman, and Bell Corporation, coupled with an obvious color difference in the two products, made it imperative to determine which inhibitor contained impurities. No difference in the boiling points could be detected, but trace impurities of related compounds may not alter boiling points.

Hydrogen flame gas chromatography was employed to check for trace impurities. There was a slight shoulder on the gas chromatograms of compounds from K & K Laboratories, but resolution was not fine enough to detect impurities.

The presence of impurities in PAN was confirmed with mass spectrometry. In the sample from K & K there were definite peaks, unrelated to the parent, parent plus one, and parent plus two peaks (Figure 14). It is also

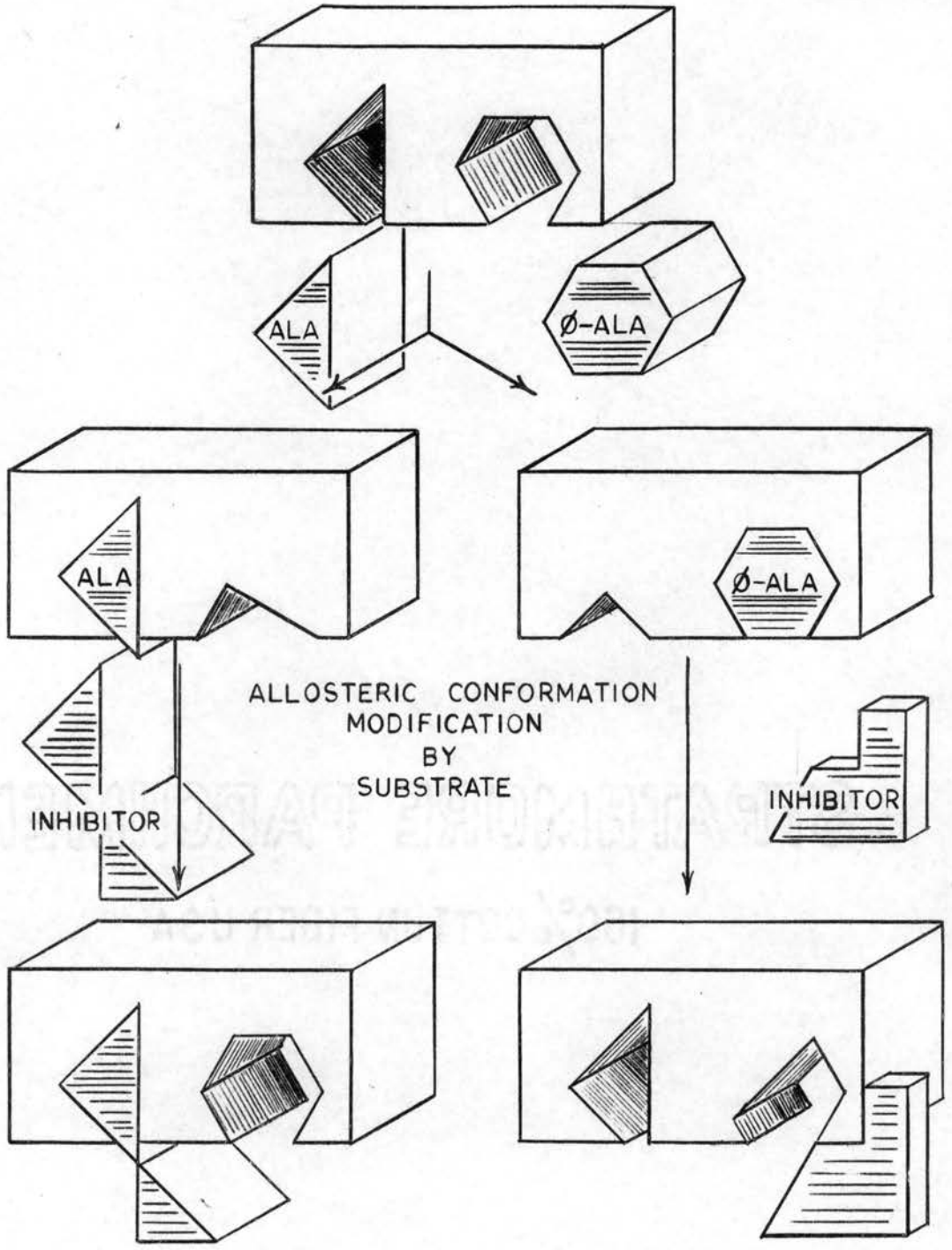
Figure 13

Model Receptor Site

The model illustrates how each amino acid can alter the conformation after binding, thereby preventing additional binding.

It also depicts the possible affect the inhibitors may have at or near the binding site.

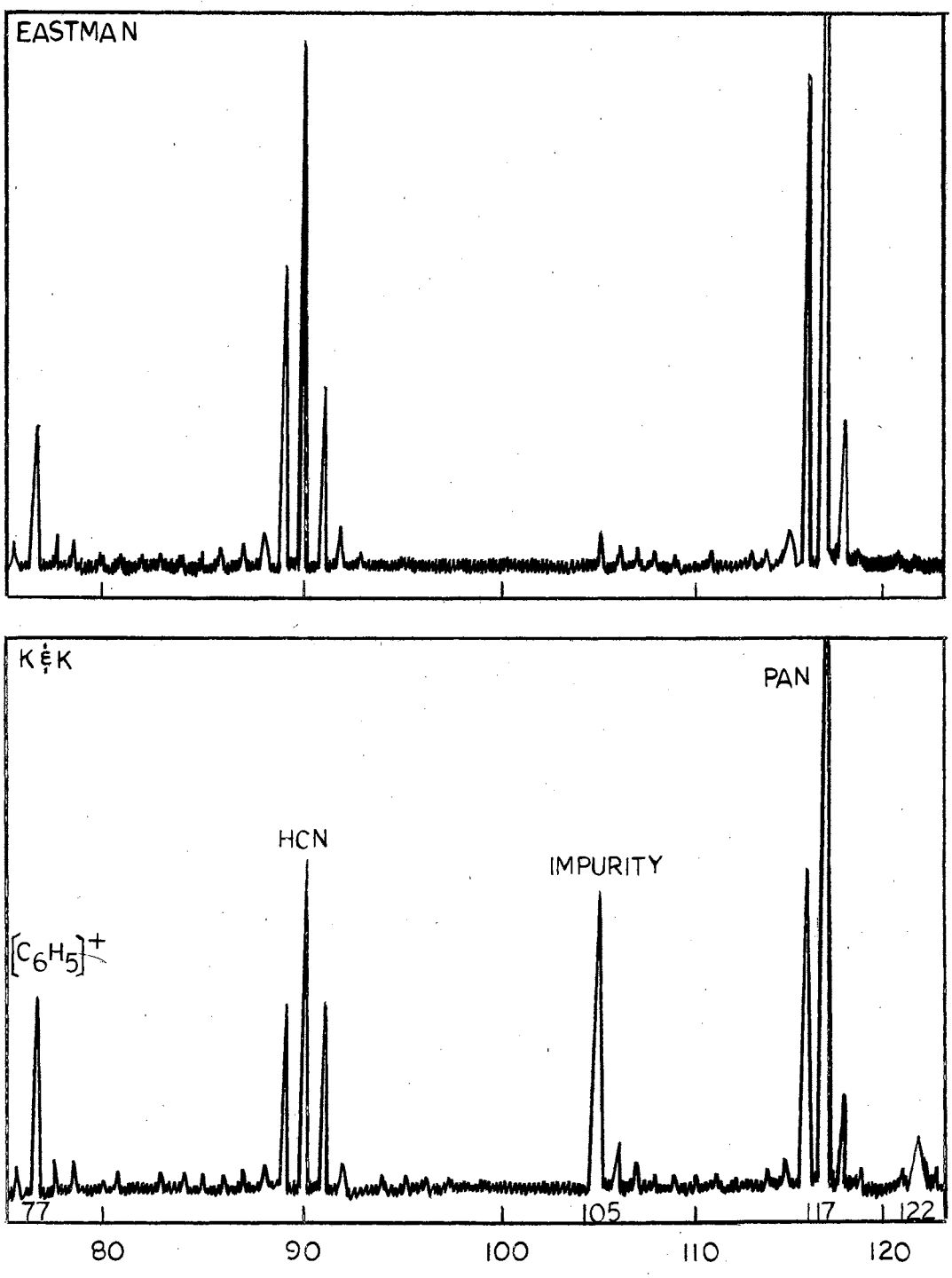
MODEL RECEPTOR SITE



DIRECT BLOCKAGE ——OR——SURFACE ALTERATION (INDIRECT)
BY INHIBITOR ——(β-PENH_2 OR PEA)

Figure 14

Tracings of Mass Spectrographs of Phenylacetonitrile (PAN) From Two Commercial Sources


EASTMAN
K & K
PAN
HCN
IMPURITY
$[C_6H_5]^+$
77
105
117
122
80
90
100
110
120

illustrated that these peaks were not apparent in the sample from Eastman Kodak and Co. Consequently, chemicals obtained from K & K Laboratories were not employed in this investigation.

Effect of PAN on Vegetative Growth

The finding that PAN inhibited initiation of germination led to an investigation of its effect on vegetative cell growth. Like PEA (Berrah and Konetzka, 1962), PAN was more effective against gram negative organisms. As shown in Figures 15 and 16, about twice as much PAN was required to obtain complete inhibition of B. cereus as compared with E. coli. PAN could be added at various intervals during exponential growth and substantially increase the generation time (Figure 17).

Viable cell counts, made with E. coli B, suggested that PAN was bacteriostatic at concentrations employed to stop cell division (Figure 18). The inhibitory effect of PAN could be overcome by suspending the cells in a fresh growth medium (Figure 18). Similar results with PEA were reported by Berrah and Konetzka (1962).

Effect of PAN on Macromolecular Synthesis

The multiple auxotroph, E. coli $T^{-}A^{-}U^{-}$, was used to determine the effect of PAN on macromolecular synthesis. The uptake of each auxotrophic metabolite is a criterion

Figure 15

The Effect of PAN on the Growth of Escherichia coli B in Tryptic Soy Broth

E. coli was incubated at 37 C in TSB (80 ml) under the following conditions: ● Control; O PAN (0.075%); Δ PAN (0.1%).

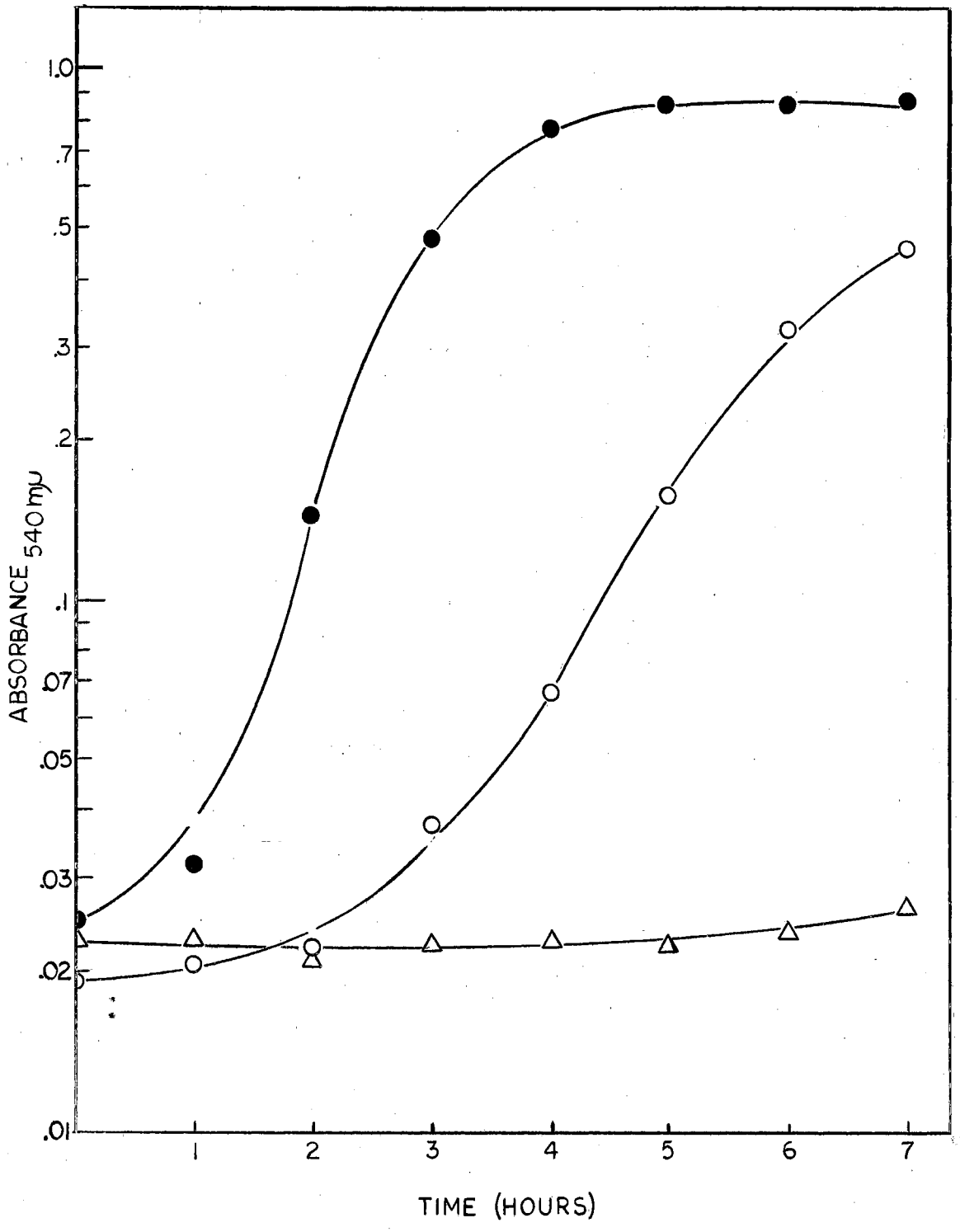

1.0
.7
.5
.3
.2
.1
.07
.05
.03
.02
.01
ABSORBANCE 540mμ
1
2
3
4
5
6
7
TIME (HOURS)

Figure 16

The Effect of PAN on the Growth of Bacillus cereus in TSB

Bacillus cereus was incubated at 37 C in TSB (80 ml) with the following concentrations of PAN: ● control; ○ PAN (0.15%); △ PAN (0.2%).

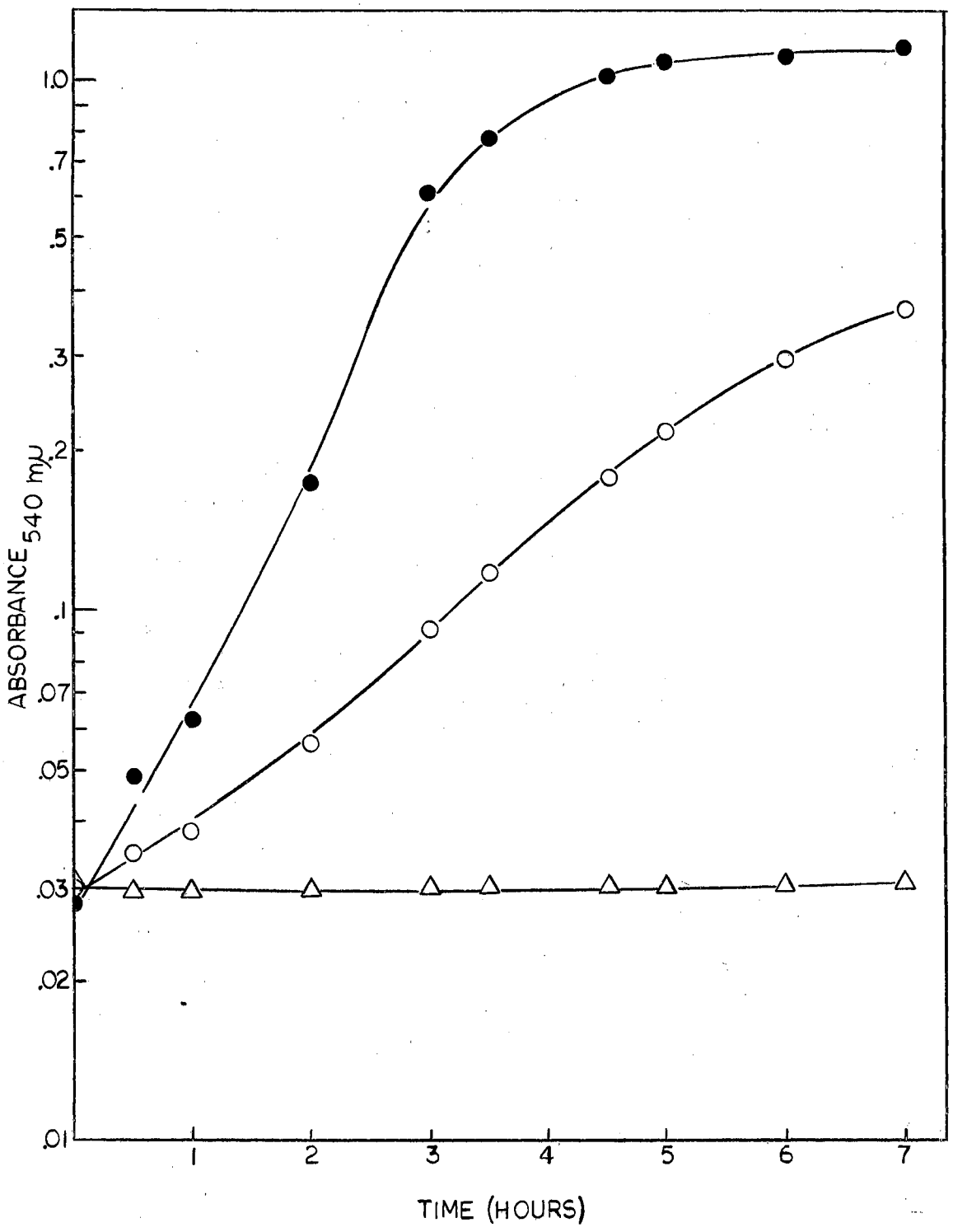

ABSORBANCE 540 mμ
1.0
.7
.5
.3
.2
.1
.07
.05
.03
.02
.01
1
2
3
4
5
6
7
TIME (HOURS)

Figure 17

The Effect of PAN Added at Intervals During Growth of *E. coli* B in TSB

PAN (0.125%) was added at: ● no time, control; ▽ 2.5 hours; □ 3 hours; △ 3.5 hours; ○ 4 hours.

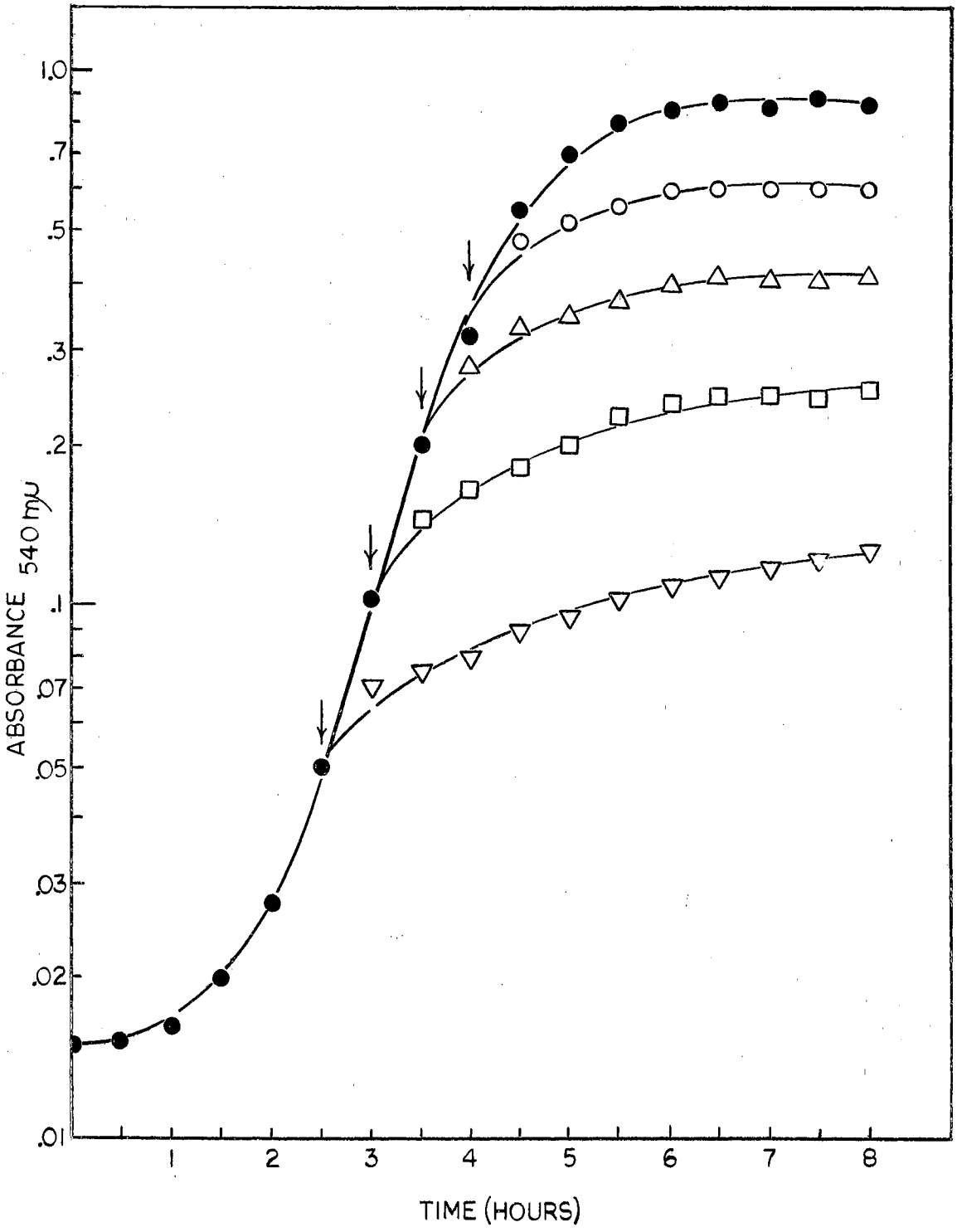
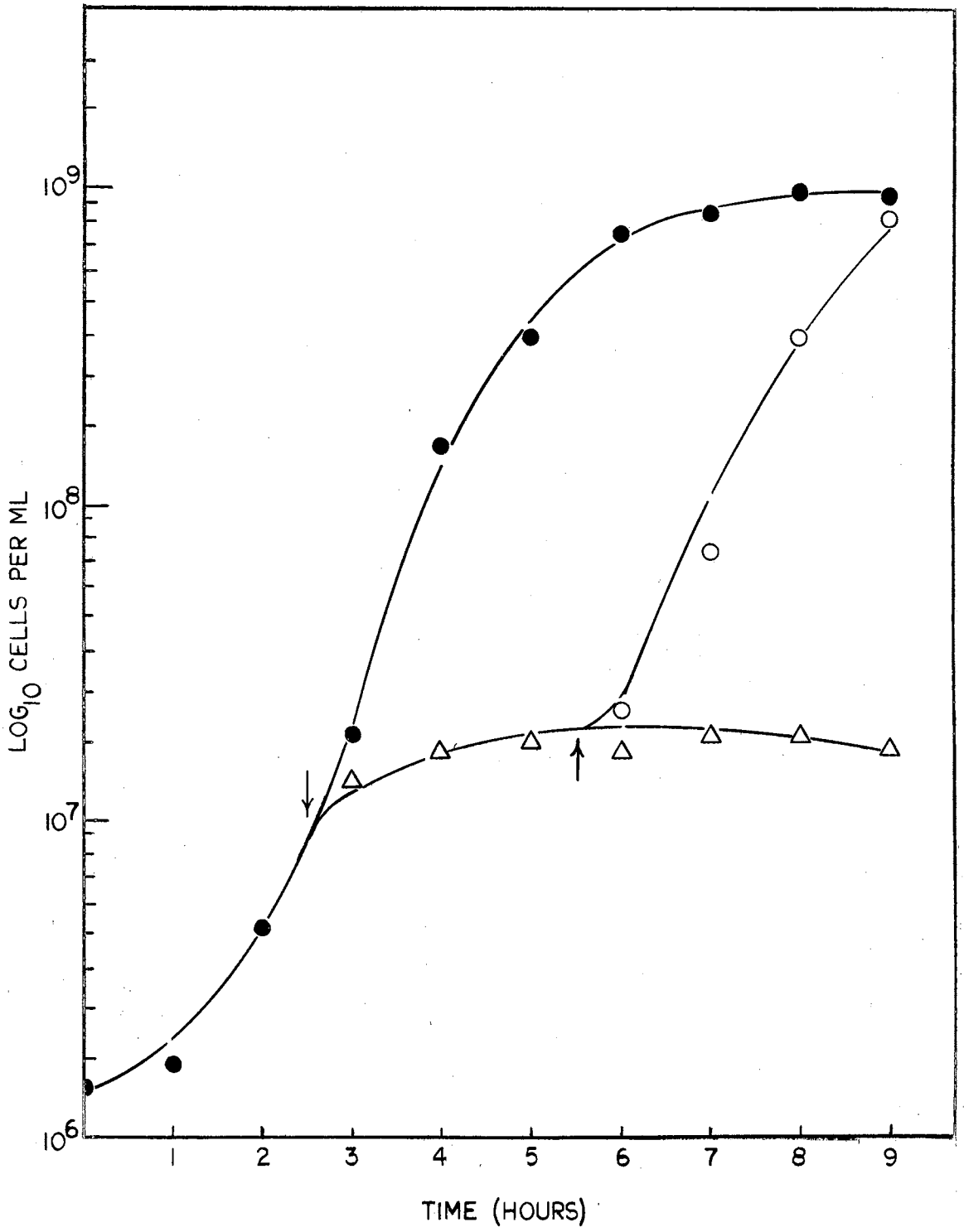

1.0
.7
.5
.3
.2
.1
.07
.05
.03
.02
.01
ABSORBANCE 540 mμ
1
2
3
4
5
6
7
8
TIME (HOURS)

Figure 18

The Bacteriostasis and Reversal of PAN's Inhibition on the Growth of *E. coli* B

E. coli B was incubated at 37 C in TSB (80 ml) and the viable cell counts were followed by diluting 0.1 ml aliquots. After 2.5 hours the control system (●) was divided and PAN (0.125%) was added to one half (△). The system with PAN was subdivided at 5.5 hours allowing one half to remain with the inhibitor. The second half was centrifuged and the cells suspended in fresh TSB prewarmed to 37 C (○).


10^9
10^8
10^7
10^6
LOG_{10} CELLS PER ML
1
2
3
4
5
6
7
8
9
TIME (HOURS)

for the synthesis of DNA, protein, and RNA, respectively. The effect of PAN on DNA, protein, and RNA synthesis was concentration dependent. PAN, at 0.125%, the minimum needed to inhibit growth, caused a preferential inhibition of DNA synthesis (Figure 19). However, when the concentration was raised to 0.15%, all macromolecular synthesis was markedly affected. This could be interpreted to mean that PAN, like PEA, blocks cell division by inhibiting DNA synthesis. However, since PEA and PAN both serve to inhibit initiation, possibly by acting on a specific receptor, further investigation was warranted.

Effects of PEA and PAN on Glucose Oxidation

During the investigation of the effect of PAN on vegetative growth, it was observed that partially inhibited cells created a lower medium pH than cells which were either not inhibited or completely inhibited. This suggested that an altered metabolism, and probably an enhanced metabolic rate, accompanied PAN bacteriostasis. To determine the effects of PAN and PEA on glucose oxidation, E. coli B was assayed manometrically. Both inhibitors caused an increase in the rate of oxygen uptake, as well as an increased consumption of oxygen (Figures 20 and 21). These data suggested PEA and PAN were uncouplers of oxidative phosphorylation, however, the possibility existed that these compounds may merely facilitate the entry of glucose.

Figure 19

The Effect of PAN on Thymine, Uracil, Arginine, and Glucose Incorporation

The uptake by E. coli $T^-A^-U^-$ of thymine (^3H), uracil (^{14}C), arginine (^{14}C), and glucose (^{14}C) is compared: (●) control system, and (○) system with PAN (0.125%).

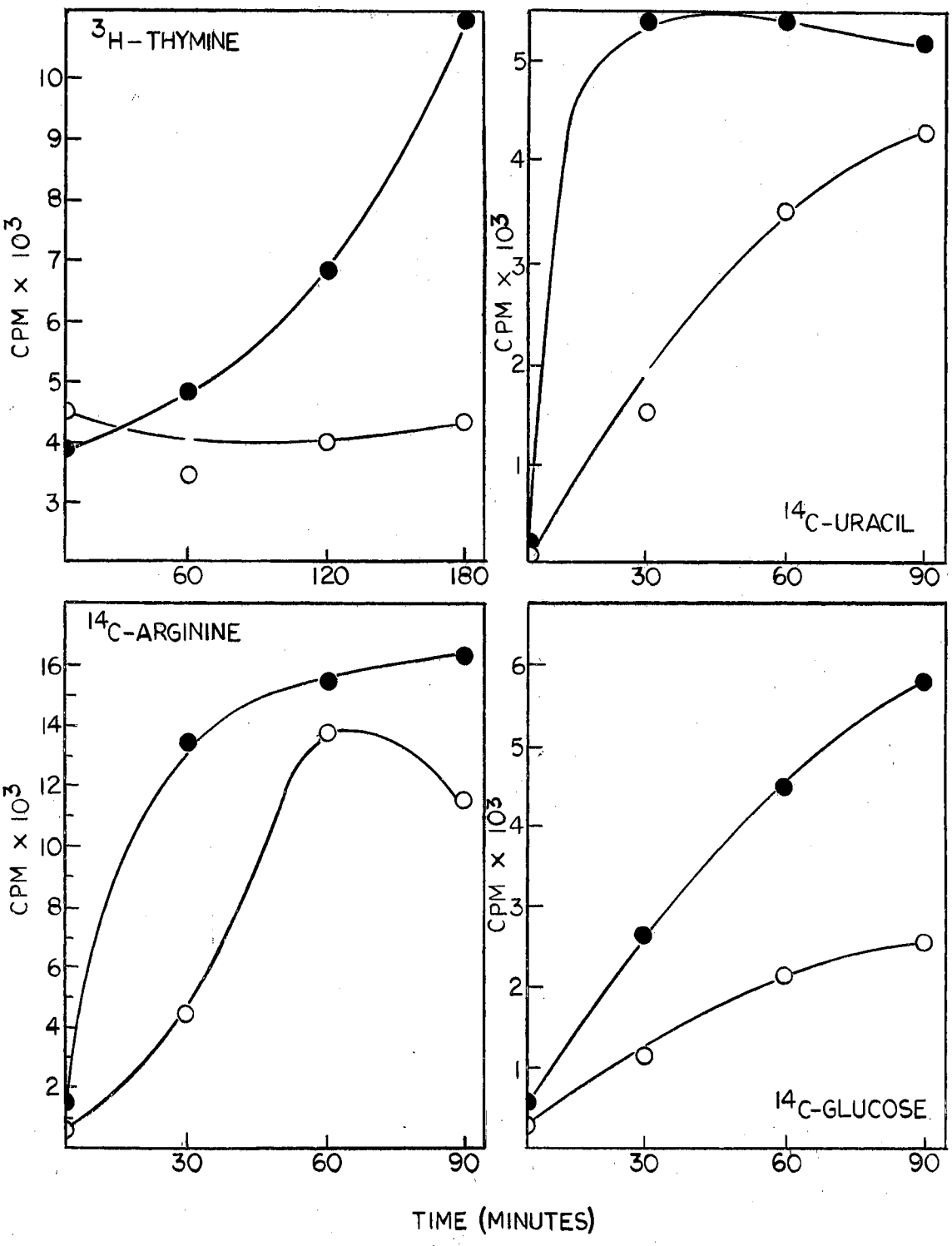

^{3}H-THYMINE
CPM x 10^{3}
^{14}C-URACIL
CPM x 10^{3}
^{14}C-ARGININE
CPM x 10^{3}
^{14}C-GLUCOSE
CPM x 10^{3}
TIME (MINUTES)

Figure 20

The Effect of PAN on Glucose Oxidation

Oxygen uptake of respiring E. coli B was determined manometrically: ● endogenous respiration rate; ○ control respiration rate; ▽ PAN (0.015%); □ PAN (0.075%); △ PAN (0.125%).

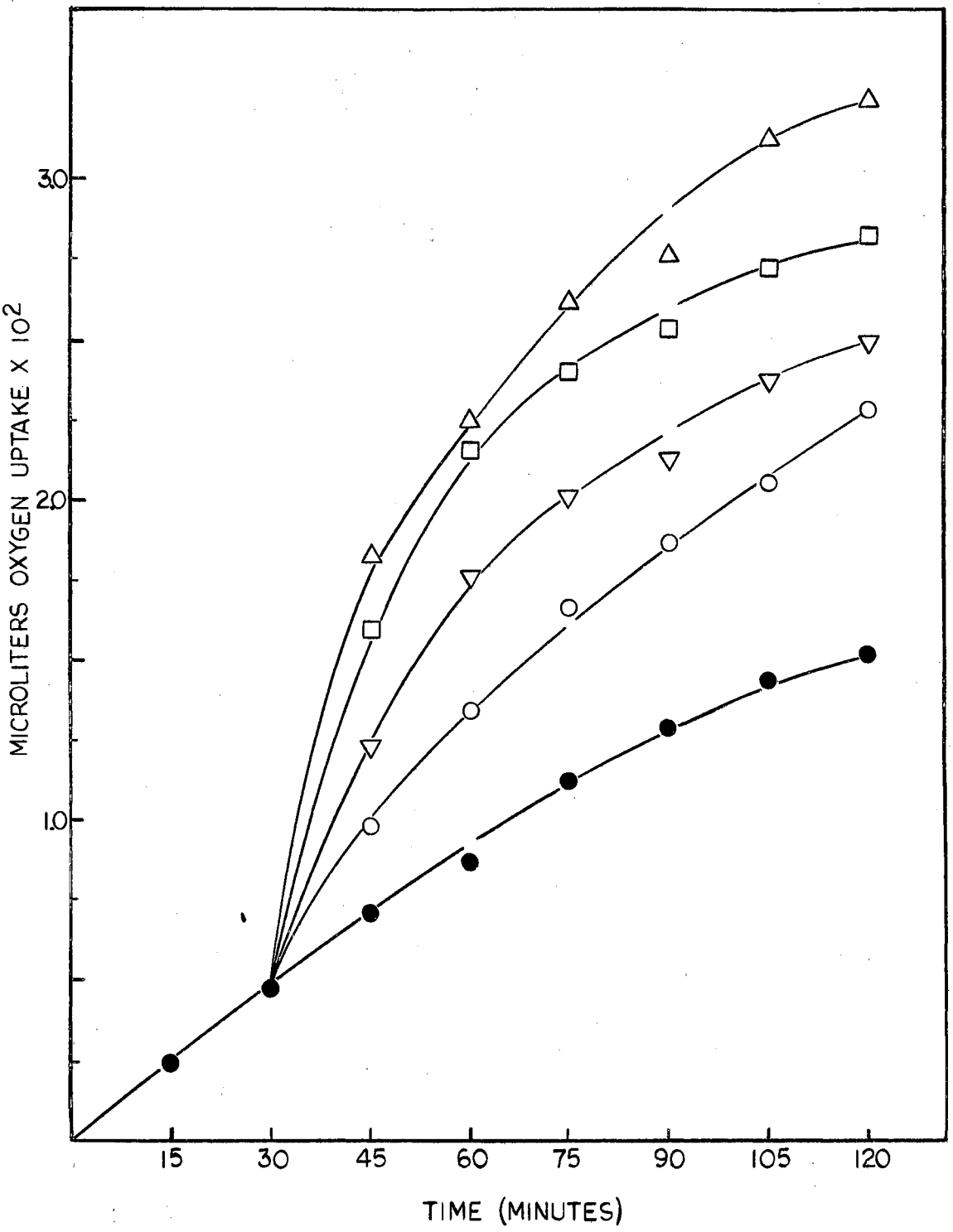
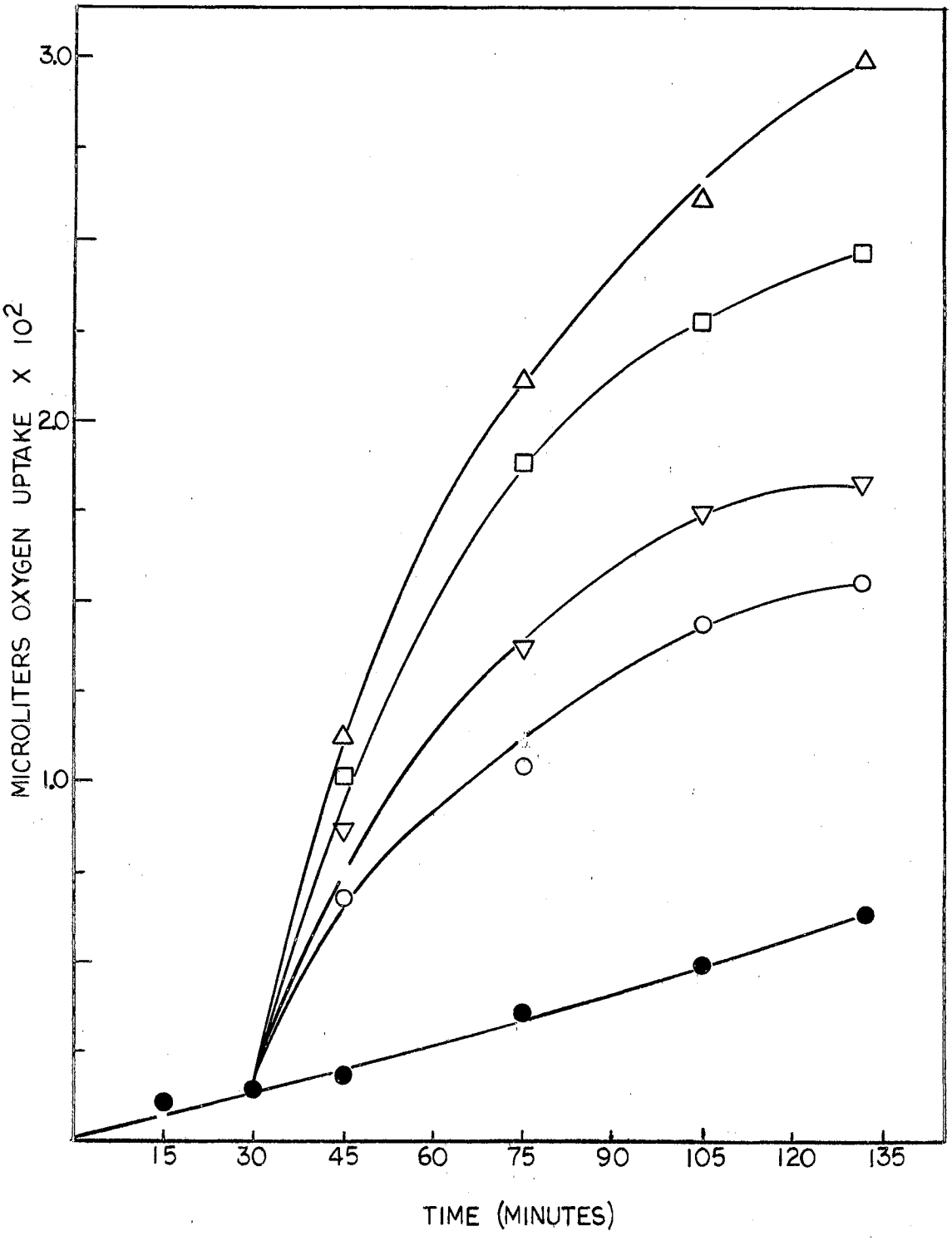

MICROLITERS OXYGEN UPTAKE X 10²
3.0
2.0
1.0
15
30
45
60
75
90
105
120
TIME (MINUTES)

Figure 21

The Effect of PEA on Glucose Oxidation

Oxygen uptake of *E. coli* B was determined manometrically: ● endogenous respiration rate; ○ control respiration rate; ▽ PEA (0.05%); □ PEA (0.2%); △ PEA (0.25%).


3.0
2.0
1.0
MICROLITERS OXYGEN UPTAKE X 10^2
15
30
45
60
75
90
105
120
135
TIME (MINUTES)

This possibility was eliminated by following glucose depletion with the Glucostat reagent. No significant change in the uptake of substrate by cells treated with PEA or PAN was detected. This agrees with the data of Rosenkranz, Carr, and Rose (1965a) which indicated growth inhibitory concentrations of PEA did not alter glucose uptake by E. coli.

With no evidence for an effect by PEA or PAN on glucose uptake, the most plausible explanation for the enhanced oxygen consumed would involve uncoupled oxidative phosphorylation. The resulting decrease in cellular free energy might also explain the reported inhibition of RNA phage production by PEA (Nonoyama and Ikeda, 1964) or thymineless death rescue by PEA (Rosenkranz, Carr, and Rose, 1965b).

Interference of Metabolite Uptake

Active transport is the movement of specific molecules across the cell membrane against a concentration gradient (Lehninger, 1965). Since the process requires energy, compounds such as 2,4-dinitrophenol (DNP) inhibit active transport by blocking the flow of energy. Since PEA and PAN may also be uncouplers, their effect on the rate of metabolite accumulation was investigated.

Two known respiratory poisons, cyanide and DNP, were used as controls. Cyanide complexes with metalloporphyrins and copper enzymes, thus, blocking the transfer

of electrons from cytochrome oxidase to nascent oxygen (Warburg, 1949). DNP inhibits energy production by blocking phosphorylation of adenosine diphosphate (Cooper and Lehninger, 1957a; 1957b).

The uptake of glucose-U-^{14}C was measured using E. coli $T^-A^-U^-$ suspended in 0.01 M potassium phosphate buffer (pH 7.0). This technique revealed that PEA and PAN, as well as cyanide and DNP retarded the uptake of glucose (Figure 22).

Additionally, the active accumulation of an auxotrophic metabolite was followed. L-Arginine-U-^{14}C uptake in the presence of the suspected uncouplers led to some unusual observations. PAN (0.125%) and cyanide (2×10^{-3} M) enhanced the uptake of arginine, while PEA (0.25%) and DNP (4×10^{-8} M) retarded arginine uptake (Figure 23). This variation may be due to an unique uptake mechanism for the auxotrophic metabolite or may be due to a similar mechanism of action for PAN and cyanide, whereby cyanide blocks respiration, but increases fermentation (Thimann, 1963). Another possibility has been suggested by Dr. F. E. Young (personal communication). He felt that a bifunctional mechanism, whereby the active transport assay measures the influx and efflux simultaneously, might be in evidence. Thus, PAN and cyanide could alter the efflux more than the influx, thereby increasing retention of the compound. The overall effect would appear as a stimulated incorporation. Pursuing this concept further, PEA and DNP

Figure 22

The Effect of PEA, PAN, Cyanide and DNP on Uptake of Glucose-U-^{14}C

The uptake of glucose, as influenced by these compounds, for *E. coli* $T^-A^-U^-$: ● control; ▽ cyanide (2×10^{-3}M); ○ PAN (0.125%); ■ PEA (0.25%); △ DNP (4×10^{-8}M).

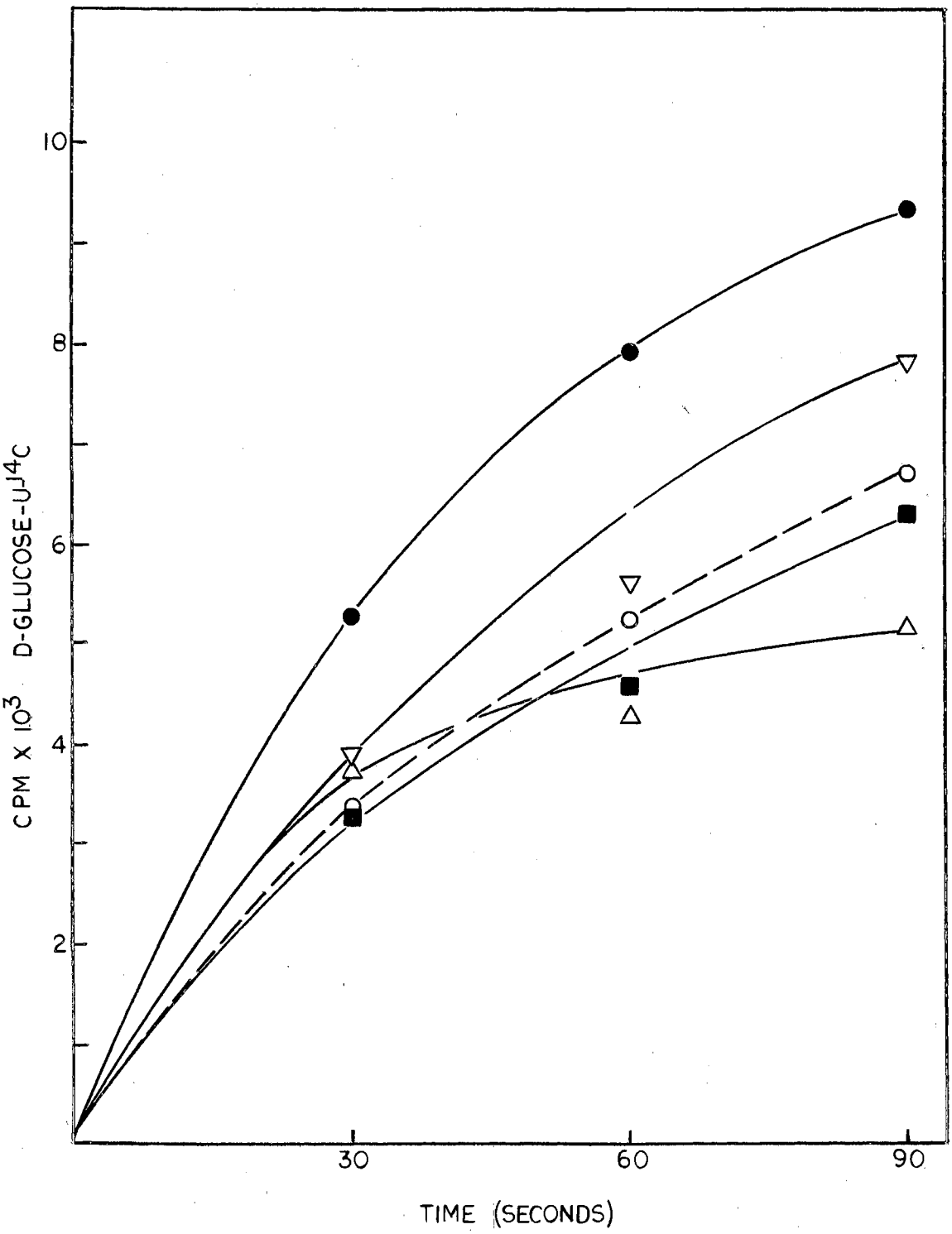
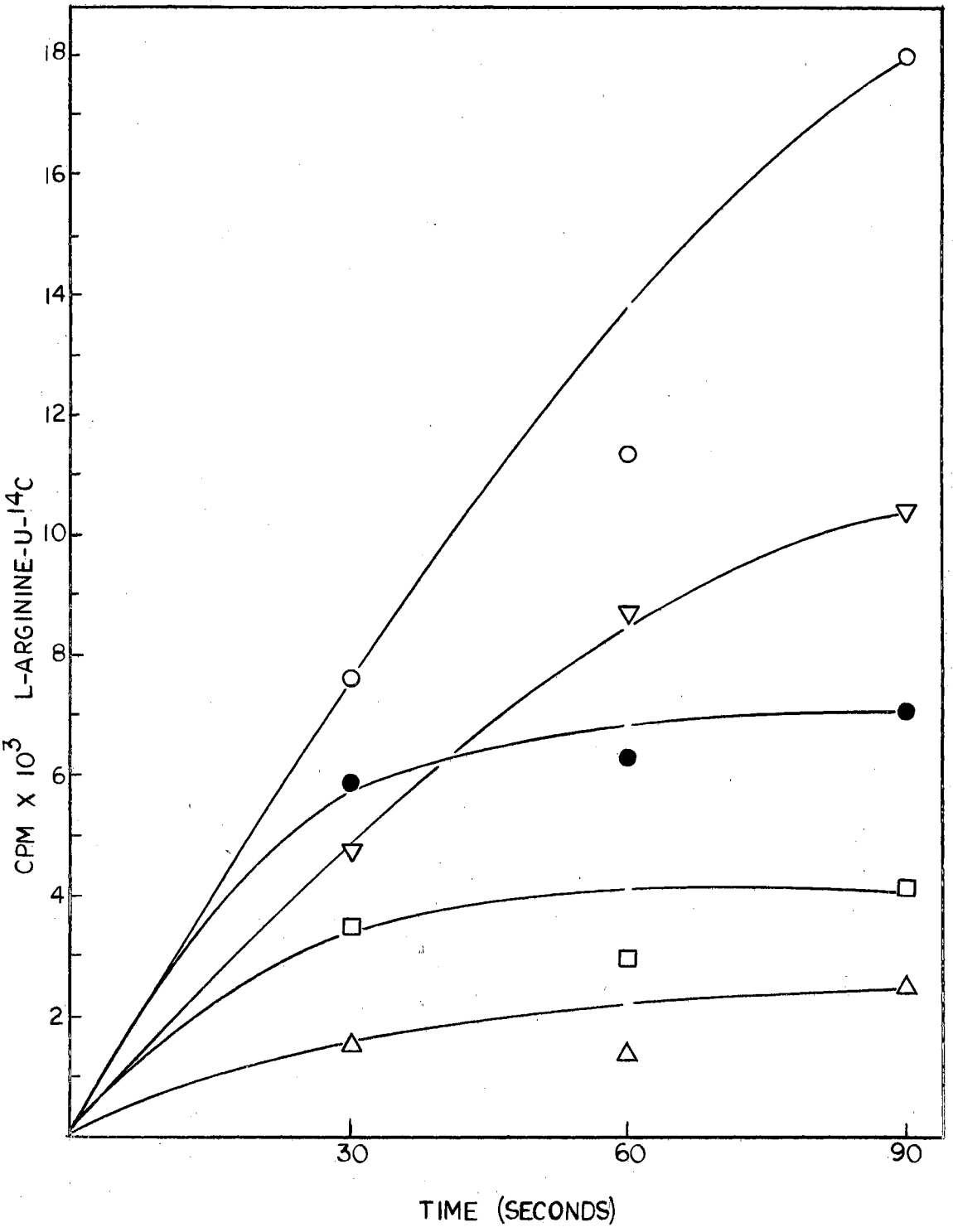

CPM X 10^3 D-GLUCOSE-U-^{14}C
10
8
6
4
2
30
60
90
TIME (SECONDS)

Figure 23

The Effect of PAN, PEA, Cyanide, and DNP on the Uptake of Arginine-U-^{14}C by *E. coli* T$^-$A$^-$U$^-$

The rapid accumulation of arginine in the presence of: ● control; ▽ cyanide (2×10^{-3}M); ○ PAN (0.125%); □ PEA (0.25%); △ DNP (4×10^{-8}M).


18
16
14
12
10
8
6
4
2
CPM X 10^3 L-ARGININE-U-^{14}C
30
60
90
TIME (SECONDS)

may alter the total (influx and efflux) active transport, thus creating an equilibrium concentration below the norm. Thus, it would appear that these compounds interfere with metabolite accumulation.

Effect of PAN on Transformation

It has been shown that PEA inhibits the transformation of Bacillus subtilis 25 and Bacillus subtilis 168. (Richardson and Leach, 1967) It appears PEA may interfere with the uptake of transforming DNA (Dr. F. R. Leach and Arlan G. Richardson, personal communication). Since it has been reported that the uptake of DNA is energy dependent (Stuy, 1962; Anagnostopoulos and Spizizen, 1961), the effect of PAN on transformation was determined. Using the defined transformation system of Hall (personal communication), it was observed that PAN decreased transformation by about 4-fold at a concentration of 0.075%.

Thymineless Death

Barner and Cohen (1954) found that deprivation of thymine from thymine auxotrophs, in an otherwise complete medium, leads to exponential death. During thymineless death (TD) protein and RNA synthesis continue, but DNA synthesis is blocked. Death by this means has been called a consequence of cytoplasmic synthesis without DNA synthesis, or more specifically "unbalanced growth" (Cohen and Barner, 1954). The actual mechanism has not been

elucidated and several theories have been advanced. Maaløe and Hannawalt (1961) proposed TD was the result of "attempted DNA synthesis." Others have attributed the phenomenon to "single stranded nucleolytic scissions" of DNA (Pauling and Hannawalt, 1965), colicin induction (Mennigmann, 1964), and prophage induction (Rolfe, 1967). Recently, Luzzati (1967) proposed that defective transcription of the DNA template by RNA polymerase was responsible for TD.

This aspect of the investigation evolved from a report that PEA countered TD by specifically inhibiting m-RNA synthesis (Rosenkranz, Carr, and Rose, 1965b). Our intentions were to illustrate that an effect on cellular energetics could not be excluded as an explanation for other effects attributed to PEA.

First, the concentrations of PAN (0.1-0.125%) sufficient to arrest TD were determined (Figure 24). The amount of PEA necessary to arrest TD was 0.5% (Rosenkranz, Carr, and Rose, 1965b; Wolgamott and Durham, unpublished results), which is twice the concentration used to inhibit cell division. However, PAN arrested TD at the same concentration which inhibited cell division. PAN (0.1%) also arrested TD when added at designated intervals (Figure 25).

In an attempt to prove the necessity of normal energy flow for TD, cyanide and DNP were added at certain intervals. Both respiratory poisons inhibited TD and dramatized that the effect of PEA and PAN on energy flow could

Figure 24

The Effect of Various Concentrations of PAN on Thymineless Death

The thymine auxotroph, *E. coli* $T^-A^-U^-$, was deprived of thymine under the following conditions: ○ control with thymine; ● control without thymine; △ PAN (0.20%, added at 1 hour after removal of thymine); ■ PAN (0.125 %); ▲ PAN (0.10%); □ PAN (0.075%).

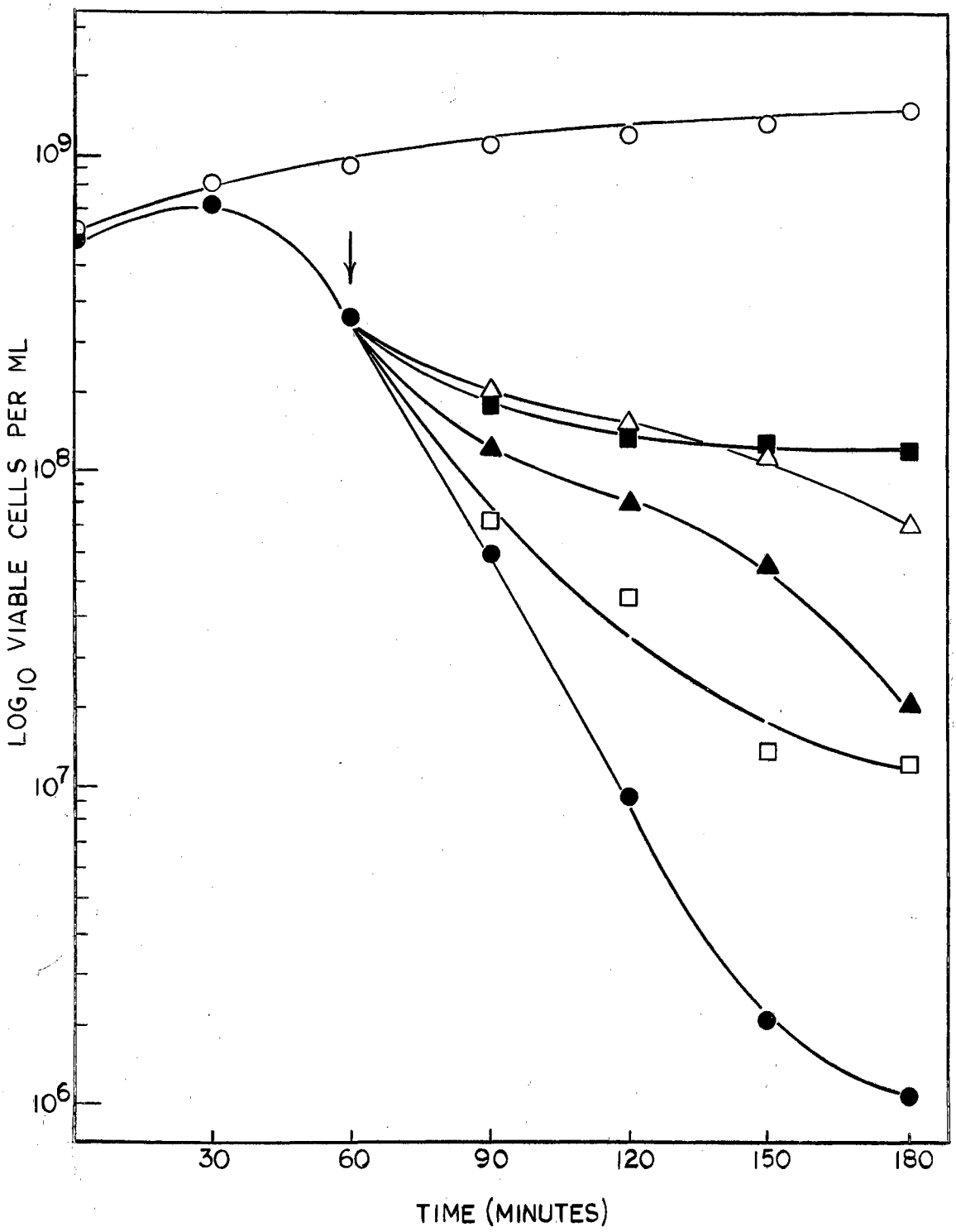
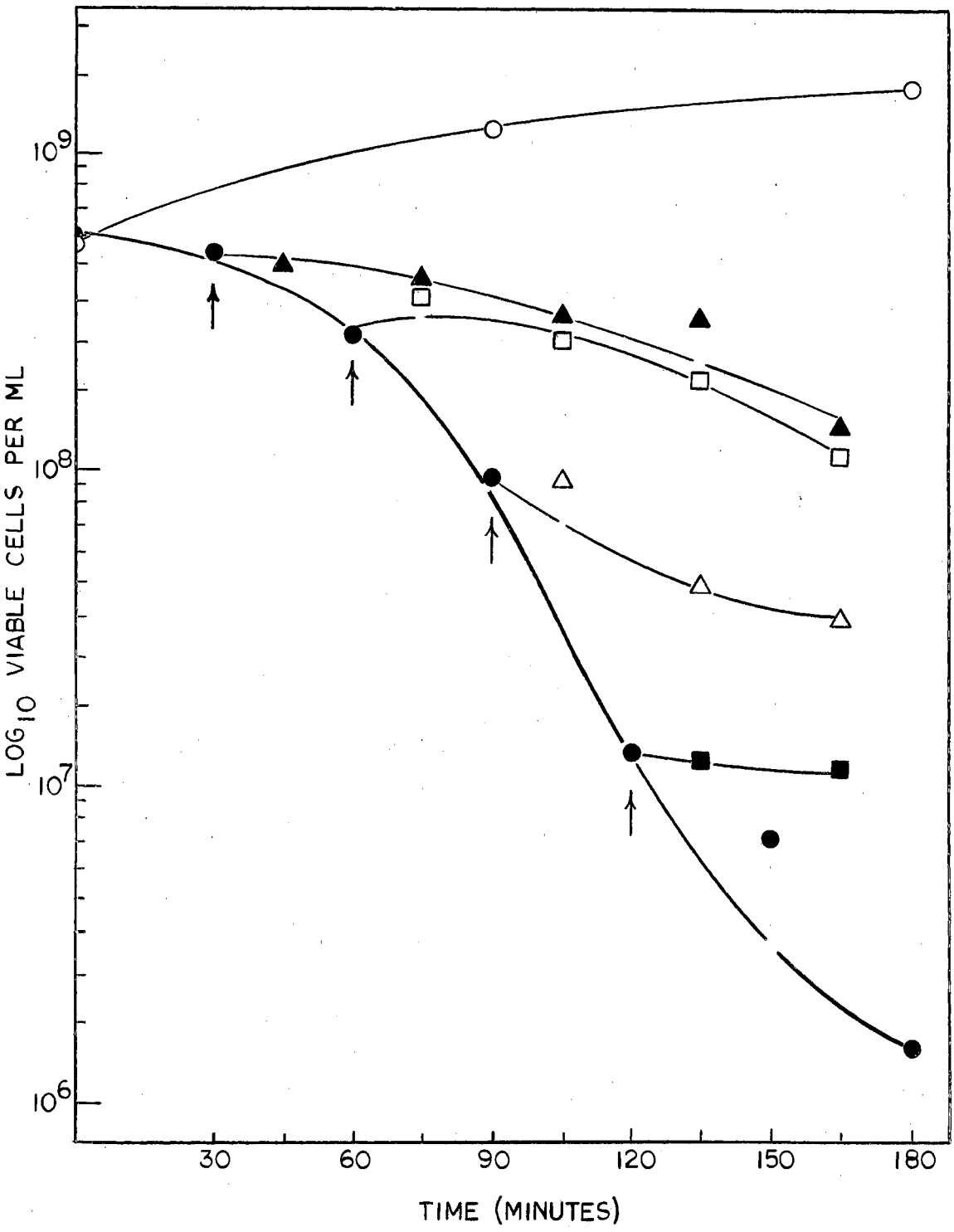

10^9
10^8
10^7
10^6
LOG$_{10}$ VIABLE CELLS PER ML
30
60
90
120
150
180
TIME (MINUTES)

Figure 25

The Effect of PAN Added at Intervals During Thymineless Death

PAN (0.1%) was added to E. coli $T^-A^-U^-$ cells during the exponential phase of TD: ○ control with thymine; ● control without thymine; ▲ PAN (0.1%, added 30 minutes after thymine removal); □ PAN, added at T_{-60}; △ PAN added at T_{-90}; ■ PAN added at T_{-120}.


10^9
10^8
10^7
10^6
LOG$_{10}$ VIABLE CELLS PER ML
30
60
90
120
150
180
TIME (MINUTES)

not be excluded as a mechanism for inhibiting TD. The effect of cyanide (2×10^{-3} M) is depicted in Figure 26. Similar results were obtained with DNP (4×10^{-8} M). Freifelder and Maaløe (1964) were the first to suggest oxidative metabolism was a requisite for TD. They arrested TD by flushing with nitrogen.

Effect on Anaerobic Growth

The observation that PEA and PAN affected the uptake of metabolites under fermentative conditions (Wolgamott and Durham, unpublished results), prompted an investigation to determine the involvement of an aerobic mechanism. If the inhibitors were active under anaerobic conditions, uncoupling of oxidative phosphorylation would not solely explain the action of PEA and PAN.

Growth studies with E. coli $T^-A^-U^-$ in thioglycollate medium indicated PEA and PAN also inhibited cell growth under anaerobic conditions (Figure 27). Thus, the evidence for uncoupling under aerobic conditions may not completely account for the mechanism of inhibition by PEA and PAN. However, both inhibitors could inhibit growth by an effect on some metabolic process invoked by cells growing anaerobically which would not invalidate another mechanism of inhibition under aerobic conditions.

Figure 26

The Effect of Cyanide Added at Intervals During Thymineless Death

Sodium cyanide (2×10^{-3}M) was added to E. coli $T^-A^-U^-$ cells during phases of TD: ○ control with thymine; ● control without thymine; ▲ cyanide (2×10^{-3}) added at T_{-0}; □ cyanide added at T_{-30}; △ cyanide added at T_{-60}; ■ cyanide added at T_{-90}.

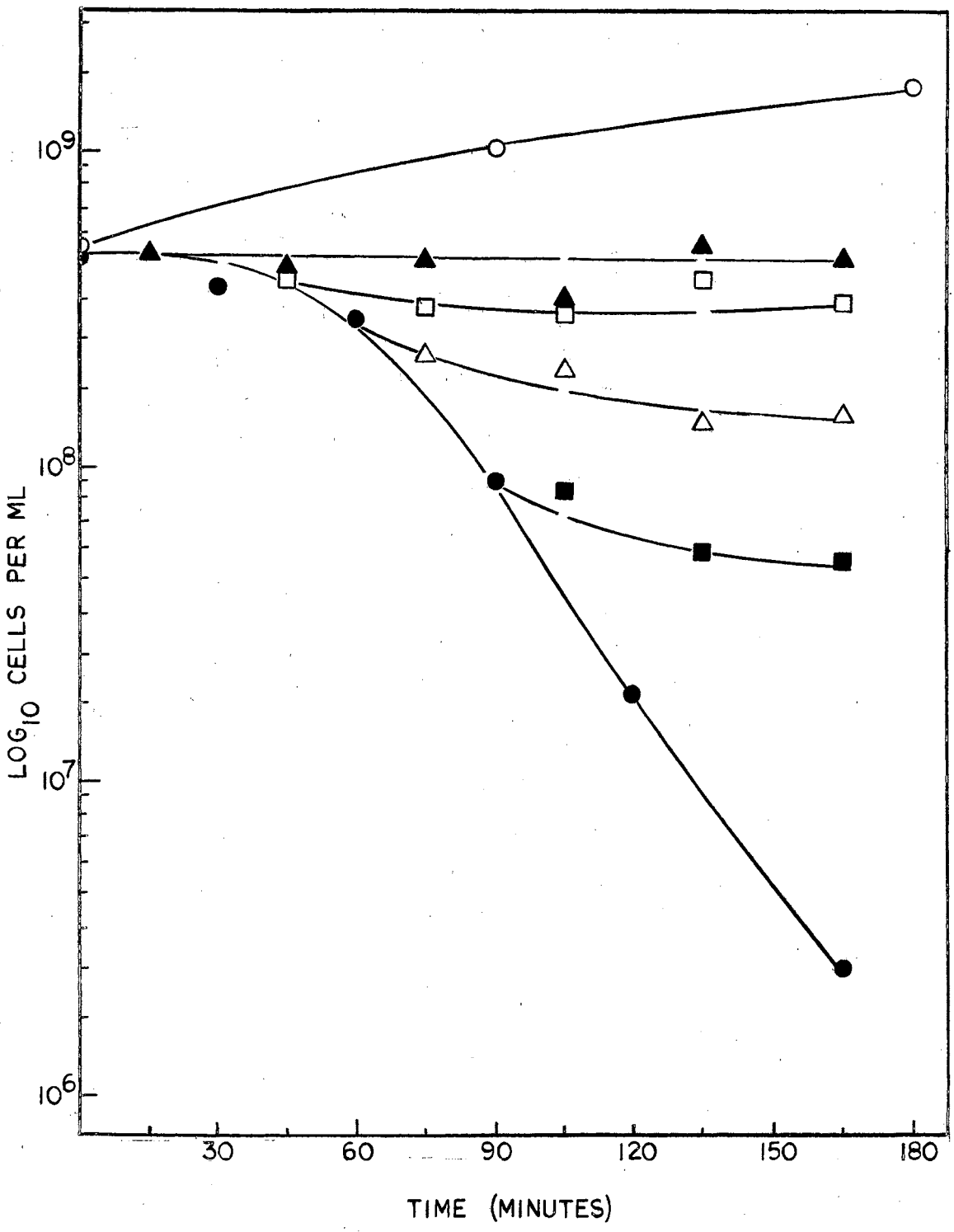
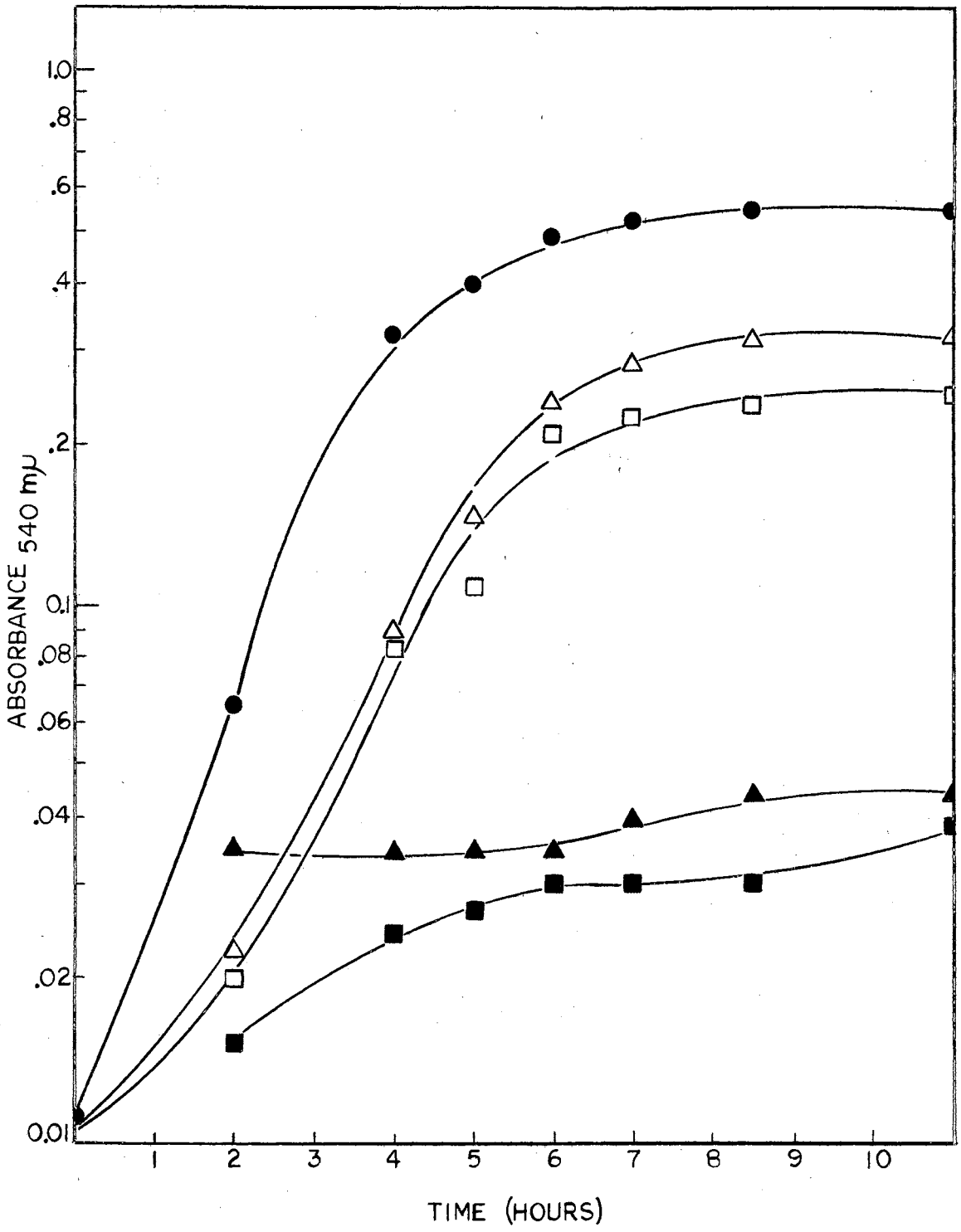

10^9
10^8
10^7
10^6
LOG$_{10}$ CELLS PER ML
30
60
90
120
150
180
TIME (MINUTES)

Figure 27

The Effect of PEA and PAN on Anaerobic Growth of *E. coli* $T^-A^-U^-$

E. coli $T^-A^-U^-$ was grown anaerobically under the following conditions: ● control; △ PAN (0.1%); □ PEA (0.15%); ▲ PAN (0.15%); ■ PEA (0.25%).


ABSORBANCE 540 mμ
1.0
.8
.6
.4
.2
0.1
.08
.06
.04
.02
0.01
1
2
3
4
5
6
7
8
9
10
TIME (HOURS)

PEA and PAN: Enzyme Inactivators

To determine if PEA and PAN exert a general, nonspecific toxic effect, a typical enzyme system was analyzed. The NADH oxidase system was chosen for three reasons. First, the enzyme is directly involved in transferring electrons to the flavoproteins of the respiratory chain. Secondly, it is associated with the membrane-wall of gram negative organisms (Salton and Ehtisham-Ud-Din, 1965). Also, the reduction of certain electron transporting compounds involved in metabolism made it a preferred system for study.

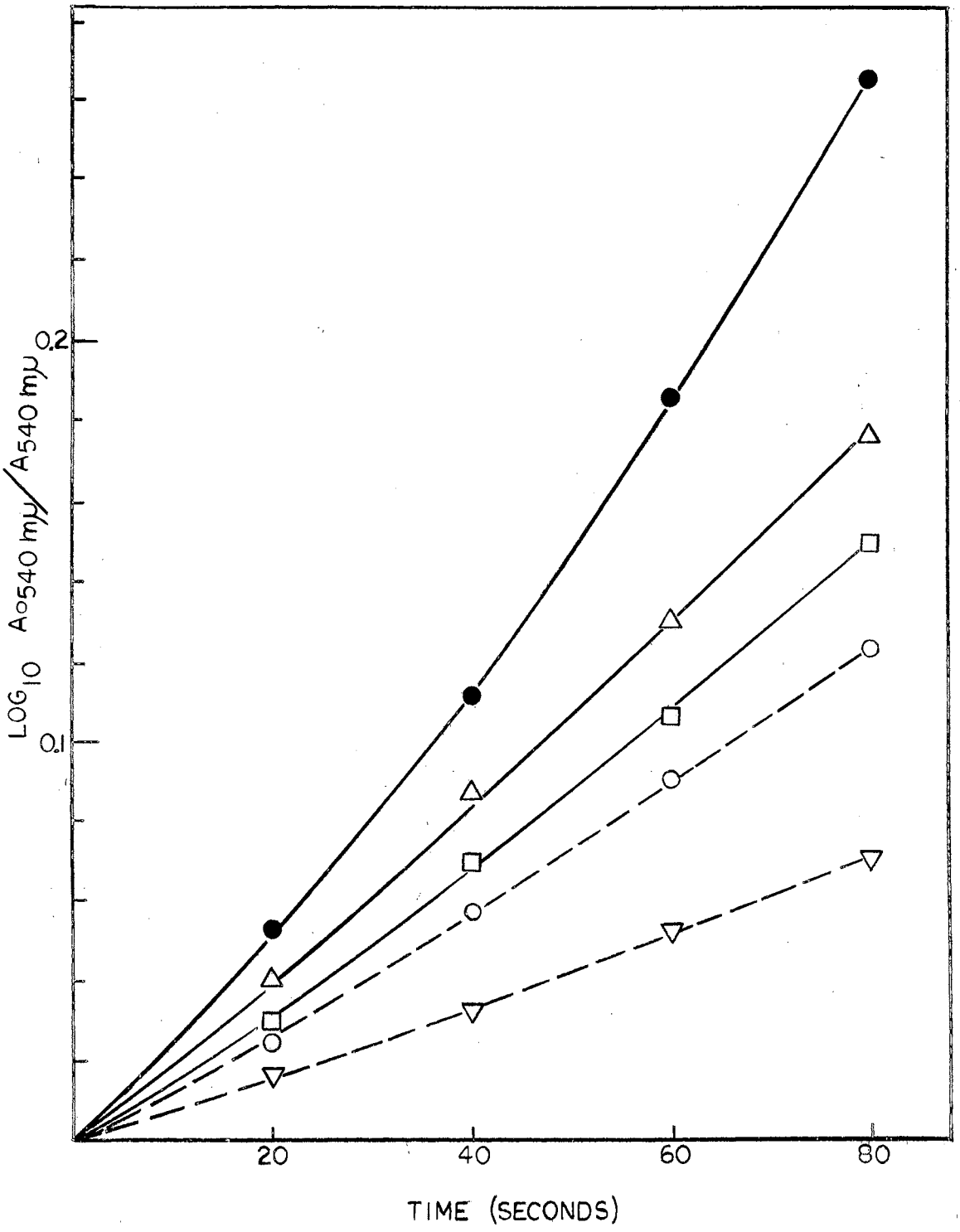
The activity of the NADH oxidase was followed spectrophotometrically as a decrease in absorbance at 340 mμ (Haas, Horecker, and Hogness, 1940). This decrease accompanies the oxidation of NADH to NAD^+. Both PEA (0.125-0.25%) and PAN (0.05-0.1%) retarded the oxidation of NADH (Figure 28). The inhibitors appeared to inactivate the enzyme, since activity decreased as the time of contact between inhibitor and enzyme increased.

The interference of PEA with protein-protein interactions was initially reported by Mendelson and Fraser (1965). They found that PEA caused the "shrinkage" of phage heads. They also, reported a personal communication with Dr. H. V. Rickenberg who found that PEA inactivated purified beta-galactosidase of E. coli. PEA may have a nonspecific action on enzymes and certain cell structures

Figure 28

The Effect of PEA on Nicotinamide Adenine Dinucleotide (NADH) Oxidase Activity

The enzyme, NADH oxidase, was exposed to the following conditions: ● control; △ PEA (0.125%) added at time zero; □ PEA (0.25%) added at time zero; ○ PEA (0.25%) added at ($T_{+10\ minutes}$); ▽ PEA (0.25%) added at ($T_{+60\ minutes}$) or 60 minutes prior to time zero.


LOG10 A°540 mμ/A540 mμ
0.1
0.2
20
40
60
80
TIME (SECONDS)

which would help explain the varied reports on its primary mechanism. These reports make it increasingly apparent that general structures instead of specific systems are primarily affected by PEA, and possibly PAN.

Effects of PEA and PAN on the Cytoplasmic Membrane

Triek and Konetzka (1964) postulated that PEA may act on the bacterial membrane, but little evidence was presented. Silver and Wendt (1967) supported this suggestion by reporting that PEA enhanced the uptake of acriflavine and altered the ionic flux of ^{45}K. Our investigation was, therefore, directed toward illustrating a direct effect of PEA and/or PAN on isolated membranes and their components.

The recently established technique of Butler, Smith, and Grula (1967) was employed for membrane isolation, disaggregation, and reaggregation. Whole membranes isolated from M. lysodeikticus were treated with various concentrations of PEA and PAN. The effect of these compounds on the visible absorption spectra (340-640 mμ) of the membranes was found to depend on the inhibitor concentration. The decrease in absorbance at 640 mμ (0.25% PEA for 2 hours) from 0.34 to 0.26 indicated a change in the membrane particle size or partial disaggregation. The same compounds and conditions were employed on isolated cell walls of E. coli $T^-A^-U^-$, P. fluorescens, and B. cereus; however,

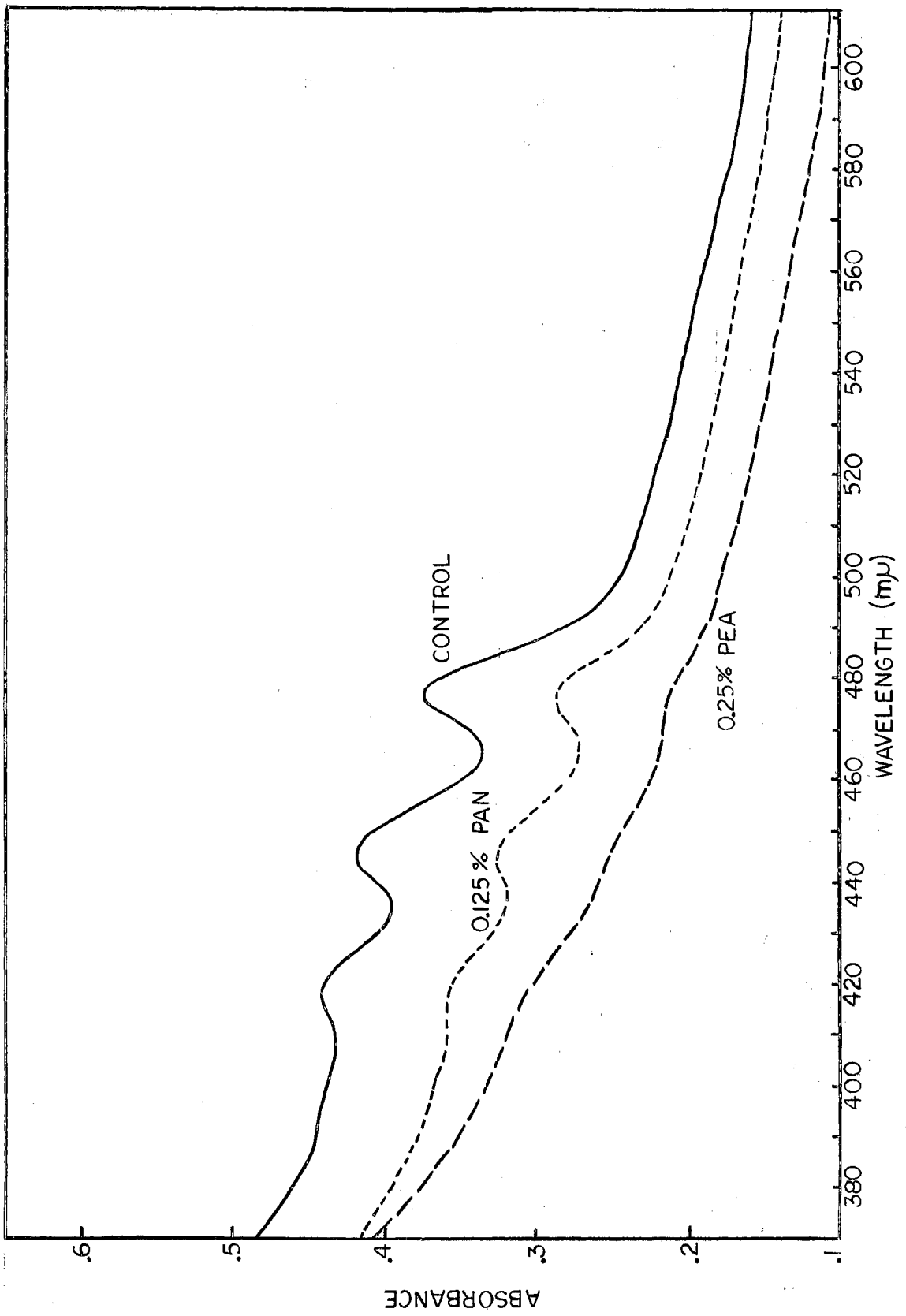
no decrease in absorption was detected. Thus, these compounds structurally altered the isolated membrane.

PEA's alteration of the permeability barrier was reported to be nonmetabolic, since it occurred in the presence of cyanide (Silver and Wendt, 1967). Thus, reversal of PEA inhibition may involve a physical reassociation of disaggregated membrane structures. To study this possibility we sought to determine the effect of PEA and PAN on physical reaggregation of membrane subunits. The membrane subunits have been found to reaggregate at 4 C in a dialysis system (Butler, Smith, and Grula, 1967). The effect of PEA and PAN on reaggregation was determined by allowing the compounds to remain in contact with the subunits during reaggregation. Reaggregation was terminated by centrifugation of reaggregated membrane particles and spectra of the sedimented material were obtained. The presence of PEA and PAN on membrane reaggregation is depicted in Figure 29. Using the carotenoids as markers, it was possible to note a reduction of the total membrane absorption. These results suggest PEA and PAN interfere with the physical reaggregation of membrane subunits.

Another method used to show a retardation of physical reaggregation of membrane subunits was quantitative analysis of the lipid in sedimented membrane material. The reaggregated material was collected and extracted with chloroform and methanol under nitrogen. The extracted material was concentrated under nitrogen and suspended

Figure 29

The Effect of PEA (0.25%) and PAN (0.125%) on the Reaggregation of Micrococcus lysodeikticus Membrane Subunits


ABSORBANCE
.6
.5
.4
.3
.2
.1
380
400
420
440
460
480
500
520
540
560
580
600
WAVELENGTH (mμ)
CONTROL
0.125% PAN
0.25% PEA

in iso-octane. The spectrum (205-640 mμ) was determined with a Cary recording spectrophotometer. The lipid profiles (Figure 30) suggested less extractable lipid material in the systems dialyzed in the presence of PEA and PAN. Also, a definite decrease in extractable carotenoids was noted (Figure 31).

The effect of PEA and PAN on subunits formed by disaggregation with sodium lauryl sulfate was also studied. PEA caused a decrease in carotenoid absorption (Figure 32). This effect was dependent on the concentration of PEA and the length of time PEA was in contact with the subunits. PAN did not alter the carotenoid absorption at the concentrations tested. The alteration of the absorption of the carotenoids by PEA may be due to reduction of the unsaturation along the poly-isoprenoid chain. This explanation is preferred over a possible structural modification because PEA has been reported to reduce the tetrazolium compound MTT [3-(4,5-dimenthylthiozalyl-2)-2,5-diphenyl-monotetrazolium bromide] (White and White, 1964).

These data suggest that PEA and PAN have a direct effect on the structural integrity of the bacterial membrane. The affinity these compounds have for the membrane is understandable. The membrane has a higher lipid concentration than other major cell structures. It would therefore have a greater affinity for water insoluble compounds such as PEA and PAN. Consequently, the hydrophobic areas of the membrane would concentrate and therefore

Figure 30

The Effect of the Presence of PEA (0.25%) and PAN (0.125%) During Membrane Reaggregation on Chloroform-Methanol Extractable Material of Membrane Reaggregates

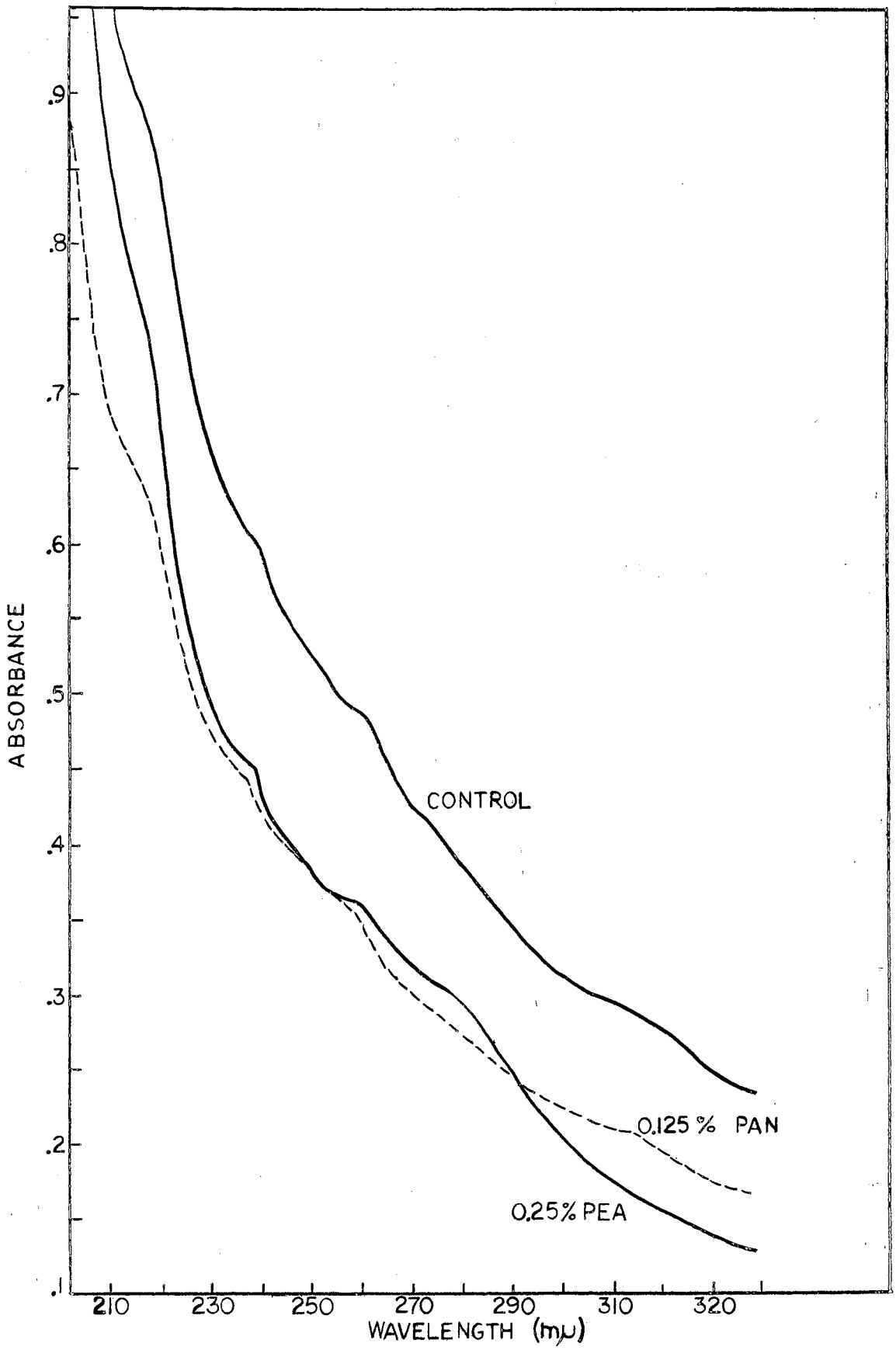

ABSORBANCE
.9
.8
.7
.6
.5
.4
.3
.2
.1
210
230
250
270
290
310
320
WAVELENGTH (mμ)
CONTROL
0.125 % PAN
0.25% PEA

Figure 31

The Effect of PEA (0.25%) and PAN (0.125%) on the Carotenoid Markers Extracted from Reaggregated Membranes by Chloroform-Methanol. (This is the visible spectrum of the same material depicted in Figure 30.)

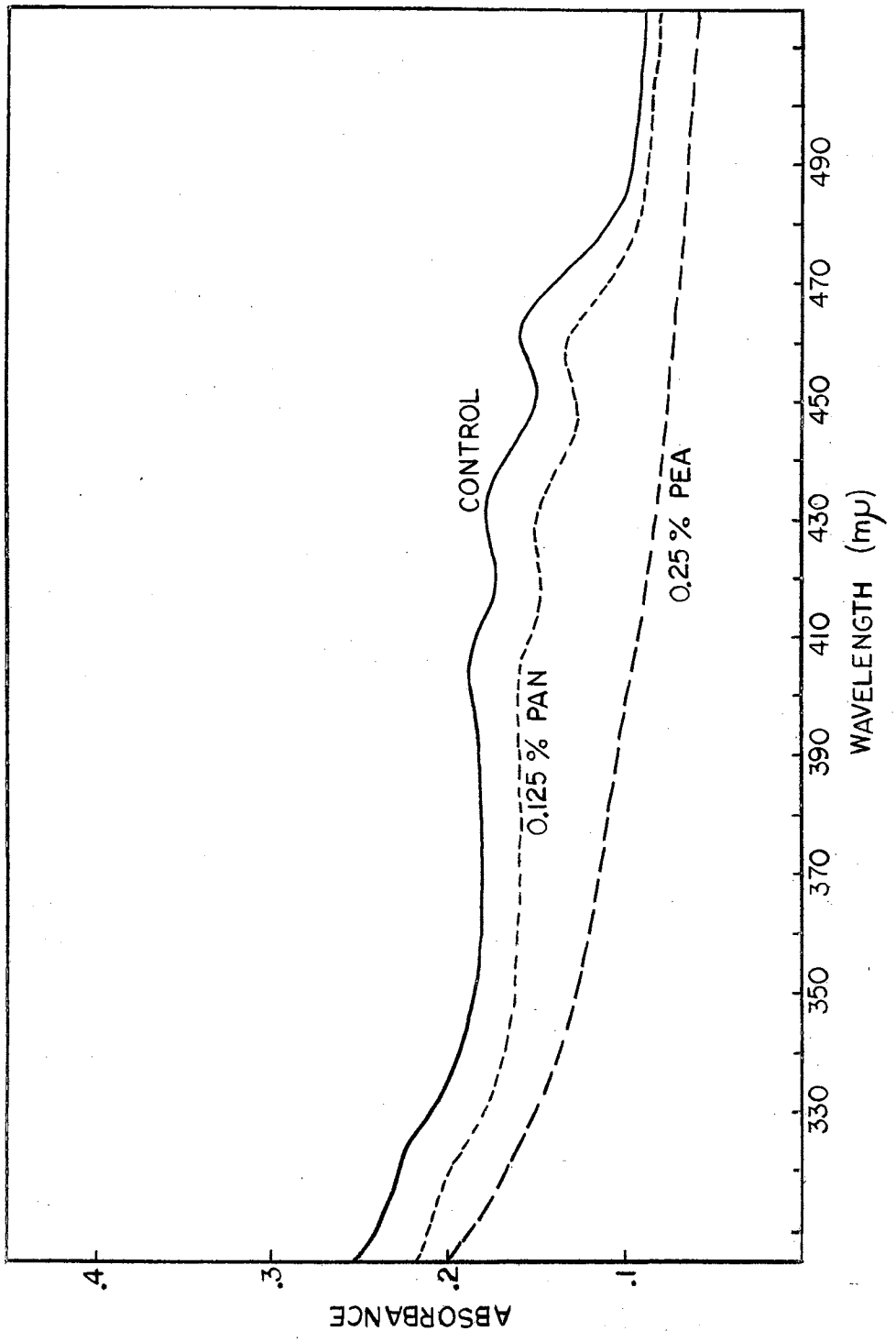
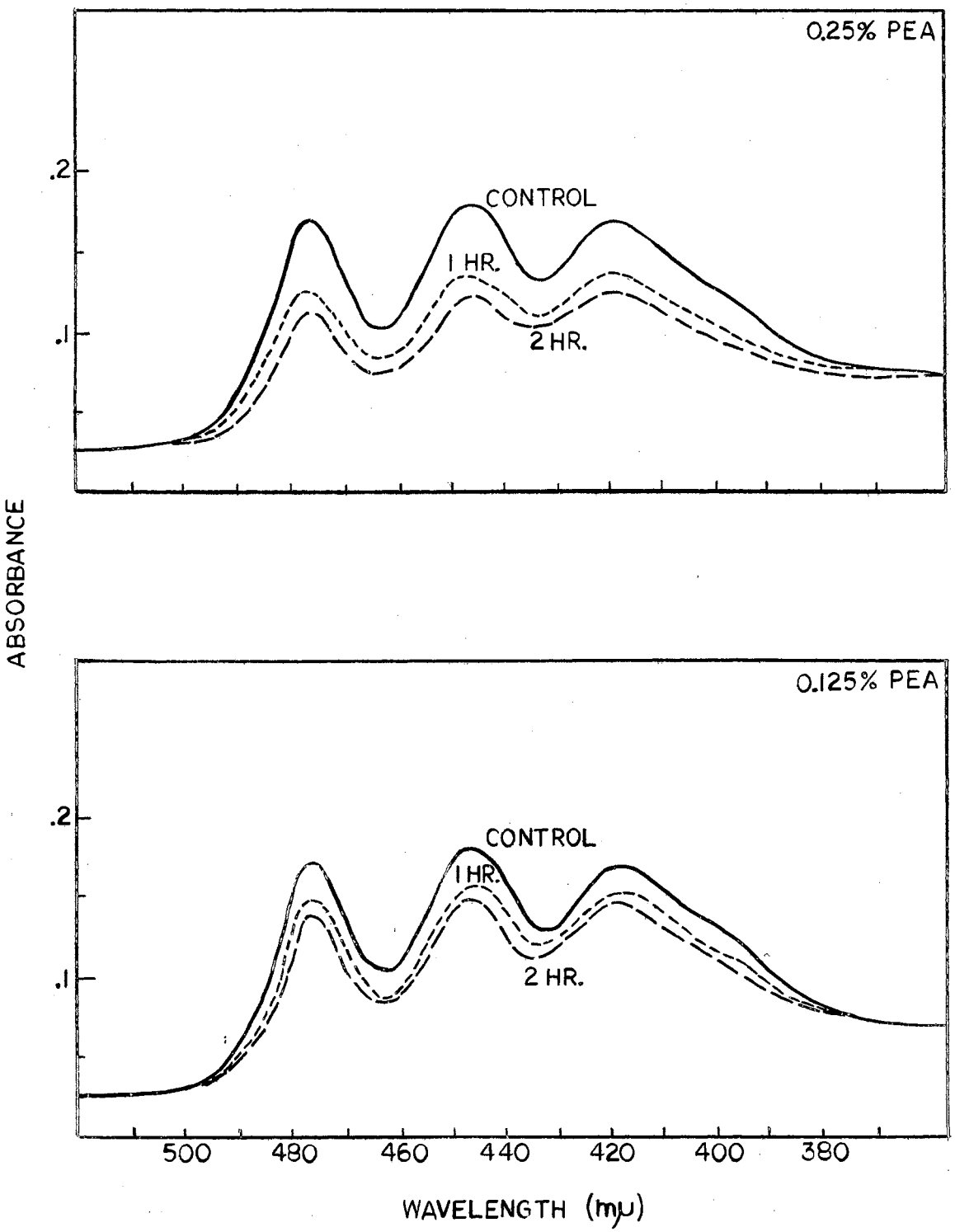

CONTROL
0.125 % PAN
0.25 % PEA
ABSORBANCE
.4
.3
.2
.1
330
350
370
390
410
430
450
470
490
WAVELENGTH (mμ)

Figure 32

The Effect of PEA on Isolated Membrane Subunits

PEA, at different concentrations and for different times, was incubated with the subunits at 27 C and the spectra were periodically determined.


0.25% PEA
.2
.1
CONTROL
1 HR.
2 HR.
ABSORBANCE
0.125% PEA
.2
.1
CONTROL
1 HR.
2 HR.
500
480
460
440
420
400
380
WAVELENGTH (mμ)

localize apolar compounds. This type of "target theory" would also account for the greater effect of PEA and PAN on the gram negative organisms, which have a higher lipid content in their cell wall than do gram positive organisms.

CHAPTER IV

SUMMARY AND CONCLUSIONS

Beta-phenethylamine, phenethyl alcohol, and phenylacetonitrile retard the extrusion of compounds normally released during initiation and inhibit the physical changes associated with germination. These compounds appear to act at one of the primary sites responsible for inducing germination. The receptor site for amino acid induced germination has been proposed to be the L-alanine dehydrogenase (O'Connor and Halvorson, 1961a). Our germination system could be induced by L-alanine or L-phenylalanine, with differences in sensitivity to beta-phenethylamine or PEA evident for each type of induction employed. The pH affected the inhibitor reaction at the initiation receptor site. The data indicated an allosteric receptor site might be responsible for initiation.

PAN inhibited initiation at lower concentrations than were necessary for beta-phenethylamine or PEA. It also arrested cell division, being more active against gram-negative species. At bacteriostatic concentrations, PAN was shown to have a preferential effect on DNA synthesis, as indicated by thymine incorporation.

Both PEA and PAN uncoupled oxidative phosphorylation, but were also inhibitory to cells growing anaerobically. Both inhibited transformation and thymineless death, as did the respiratory poisons, cyanide and DNP. The effect of PEA and PAN on aerobic energy flow may not be the sole mechanism, but should not be excluded as a factor in the inhibitory properties.

The observed interference with energy flow could be due to: (1) interference or inactivation of the enzymatic transfer of electrons, (2) inactivation of the soluble peptide responsible for energy coupling (Lam, Warshaw, and Sanidi, 1967; Ishikawa and Lehninger, 1962), or (3) an interruption of the electron transport flow sequence by alteration of the supporting membrane structure. The observed general toxicity of these compounds could account for either or a combination of these effects. It could be possible the observed phenomena associated with PEA's action, such as inhibition of DNA replication, RNA and DNA phage replication, transformation, conjugation, thymineless death, and anaerobic growth, is directly related to energy imbalance. The retarding effect of respiratory poisons on DNA replication is not unprecedented (Dr. J. Cairns, personal communication).

It was found that PEA and PAN inactivated NADH oxidase. This indicated that structures, instead of systems, may be directly involved. This type of action was further implicated when it was found that PEA and PAN interferred

with the physical reaggregation of cellular membrane subunits. The association of subunits has been proposed by Green, et al. (1967) and Lenard and Singer (1966) to involve protein-protein interaction. The hypothesis that the Micrococcus lysodeikticus membrane involves a "protein-protein continuum" (Grula, et al., 1967) would be consistent with the proposal that PEA and PAN affect reaggregation by altering the protein surface of the subunit. This does not contradict the action on the initiation receptor site, since the outer spore coat is also protein.

In light of this proposal it is possible to visualize that replication may be affected directly, by inactivation of the protein "initiator" (Lark and Lark, 1964), or indirectly, by altering the membrane surface to disallow genome attachment and subsequently inhibit replication (Jacob, Brenner, and Cuzin, 1963; Chai and Lark, 1967).

It is the contention of this thesis that PEA and PAN, and possibly related apolar compounds (eg., toluene), have a toxic action on protein surfaces or disrupt hydrophobically bonded tertiary structures. This type of action would be facilitated in apolar areas, such as the membrane.

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